Application Compendium

Clinical Research and Forensic Toxicology Application Compendium

- Clinical Research
- Drug Monitoring Research
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An LC-MS/MS Research Method for the Quantification of Mycophenolic Acid (MPA) in Plasma

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Key Words

TSQ Quantum Ultra, mycophenolic acid, MPA, plasma, quantitation

Goal

The goal of this work was to use LC-MS/MS to validate the MassTox® Mycophenolic Acid kit from ChromSystems® on the Thermo Scientific™ TSQ Quantum Ultra™ mass spectrometer for research purposes.

Introduction

This note describes a method developed to quantify mycophenolic acid (MPA) by LC-MS/MS with the ChromSystems MassTox Mycophenolic Acid kit. The method was analytically validated for research use using the following parameters:

- Both intraday and interday accuracy and precision for the quality controls
- Lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ)
- Carryover

Methods

The MassTox Mycophenolic Acid kit consists of:

- Four calibrators (1 blank and 3 calibrators)
- Two quality controls (Level I and Level II)
- Internal standard set consisting of an internal standard mix and a reconstitution buffer
- Precipitation reagent solution
- Extraction buffer solution
- Mobiles phases A and B

Calibrator and Quality Control (QC) Preparation

Lyophilized calibrators and quality controls were reconstituted with 1 mL of distilled water. They were left at room temperature for 15 minutes and shaken occasionally until the contents were homogeneous. Aliquots of 50 µL were stored in 1.5 mL vials at -20 °C for a maximum period of 3 months.

Internal Standard Preparation

Internal standards were reconstituted with 1 mL of reconstitution buffer. The vial was left for 5 minutes at room temperature. It was shaken periodically and gently until the contents were homogeneous. Next, 800 µL of this solution was added to 12 mL of precipitation reagent and the mixture was stored in the dark at 4 °C for 28 days.

Sample Preparation

A 25 µL measure of extraction buffer was added to 50 µL of each calibrator, control, and sample. The mixture was vortexed for 10 seconds and incubated for 2 minutes at room temperature. Then, 250 µL of reconstituted internal standard mix was added to the vial and vortexed for 30 seconds. It was then centrifuged at 14,000 rpm for 5 minutes. Finally, 10 µL of the supernatant was diluted 20 times in a mixture containing methanol and water (LC/MS grade) (50/50, v/v).

Calibration Curve

The concentrations of the calibrators were 0.97, 3.89, and 9.46 mg/L.

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Liquid Chromatography

Chromatographic separation was performed with a Thermo Scientific™ Accela™ 1250 pump and Accela Open autosampler. The analytical column was a MassTox TDM analytical column series A from ChromSystems. The column was maintained at room temperature. Mobile phases A and B were also provided by ChromSystems. Details of the LC gradient are shown in Table 1. The injection volume was 10 µL.

Table 1. LC gradient.

Time (min)	A (%)	B (%)	Flow rate (µL/min)
0	40	60	600
0.5	0	100	600
1	0	100	600
1.10	40	60	600
1.8	40	60	600

Mass Spectrometry

MS/MS was performed using a Thermo Scientific™ TSQ Quantum Ultra™ triple-stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe in positive mode. The MS conditions were as follows:

Spray voltage	2500 V
Vaporizer temperature	350 °C
Sheath gas pressure (arbitrary units)	30
Auxilliary gas pressure (arbitrary units)	15
Capillary temperature	250 °C

Data were acquired in selected-reaction monitoring (SRM) mode. SRM settings for the MPA and its internal standard are shown in Table 2.

Table 2. SRM settings for the analyte and its corresponding internal standard.

Analyte	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Collision Energy (V)	Tube Lens (V)
MPA	321.0	207.0	22	80
MPA-d_3	324.0	210.0	22	80

Results and Discussion

Intraday Precision and Accuracy

The intraday precision and accuracy were evaluated using 20 replicates of the two quality control samples at the following concentrations: Level 1 (1.94 mg/L) and Level 2 (5.5 mg/L). The precision was calculated as the coefficient of variation (CV, %) within a single run and the accuracy as the bias or percentage of deviation between nominal and measured concentration. Results are reported in Table 3.

Table 3. Intraday accuracy and precision results.

Quality Controls	Accuracy (%)	CV (%)
Level 1	113.2	2.7
Level 2	112.1	2.7

Interday Precision and Accuracy

The interday precision and accuracy were evaluated using 10 replicates of the two quality control samples at the following concentrations: Level 1 (1.94 mg/L) and Level 2 (5.5 mg/L). The precision was calculated as the coefficient of variation (CV, %) between different extractions and runs, and the accuracy as the bias or percentage of deviation between nominal and measured concentration. Results are reported in Table 4.

Table 4. Interday accuracy and precision result.

Quality Controls	Accuracy (%)	CV (%)
Level 1	103.1	7.0
Level 2	103.1	4.9

Lower Limit of Quantitation (LLOQ), Upper Limit of Quantitation (ULOQ), and Linear Range

As previously noted, the MassTox Mycophenolic Acid kit contains 3 calibrators at 0.97, 3.89, and 9.46 mg/L. Figure 1 shows SRM chromatograms of MPA at 0.97 mg/L (Calibrator 1).

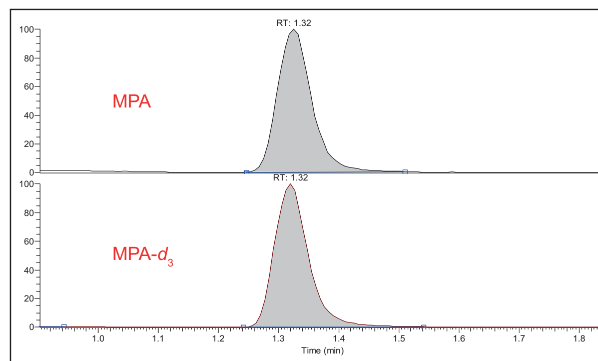

Figure 1. SRM chromatograms of MPA at 0.97 mg/L (Calibrator 1).

In some cases, real samples may present concentrations up to 30 mg/L. For this reason, it was decided to evaluate the upper limit of quantitation. A 50 mg/L solution of MPA was prepared in bovine serum albumin (Sigma-Aldrich). This concentration in the linear range gave accuracy within 100 ± 15% and a CV less than 15%.

To determine the best weighting factor, concentrations were back-calculated and the model with the lowest total bias across the concentration range was considered the best suited. Four-point calibration standard curves were calculated and fitted by linear models. To determine LLOQ, a ten-fold dilution with bovine serum albumin was made from Calibrator 1 to get a concentration of 0.097 mg/L. At this LLOQ, the accuracy and precision values were, respectively, 108% and 4.6% for 10 replicates.

Figure 2 shows a representative calibration curve of MPA.

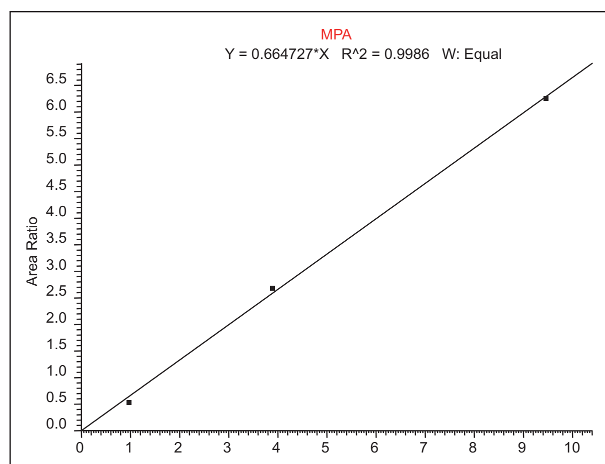

Figure 2. Calibration curve of MPA.

Carryover

Carryover was evaluated by injecting two levels of plasma in triplicate: a blank plasma (B) and a high-level plasma spiked at 20 mg/L (H). The injection sequence was three injections of the high level (H_1, H_2, H_3) followed by three injections of the low level (B_1, B_2, B_3). The sequence was repeated five times and the carryover was calculated using the following formula expressed as a percentage:

Carryover = (b1-b3) / (h-b3) x 100

b1: average concentration obtained for all B1 injections

b3: average concentration obtained for all B3 injections

h: average concentration obtained for all H injections (from H1 to H3)

Carryover was evaluated to be less than 1.6%.

Conclusion

A simple and fast LC-MS/MS method was analytically validated for the analysis of mycophenolic acid for research purposes. Intraday and interday accuracy and precision were successfully assessed in plasma-based samples.

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LC-MS/MS Analysis of Estradiol with Dansyl Derivatization

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Key Words

Estradiol, dansyl chloride, TSQ Quantiva, derivatization

Goal

To develop a high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the clinical research analysis of estradiol in human plasma with a limit of quantitation of 1 pg/mL.

Introduction

Estradiol is an endogenous steroid in the human body. The current goal of researchers is to determine estradiol concentrations at 1 pg/mL in plasma. Here a method for the analysis of estradiol in human plasma was evaluated for clinical research based on these requirements.

Experimental Methods

Sample Preparation

Samples were processed by liquid-liquid extraction (LLE) and subsequently derivatized. Charcoal stripped serum (CSS) was used as the matrix for the calibration curve. The calibration curve was prepared by spiking the CSS with known amounts of estradiol. A 500 µL aliquot of CSS was fortified with internal standard (estradiol-d_5) and extracted with 6 mL of methyl tert-butyl ether (MTBE). The samples were vortexed, centrifuged, and frozen. The resulting organic layer was decanted into a clean test tube and evaporated to dryness. The residue was reconstituted and derivatized with dansyl chloride dissolved in acetone and carbonate buffer. An aliquot was then injected into the HPLC-MS/MS.

Liquid Chromatography

Chromatographic separations were performed under gradient conditions using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system and UltiMate™ 3000 RS autosampler. The analytical column was a Thermo Scientific™ Hypersil GOLD™ column (50 x 2.1 mm, 1.9 µm particle size). The column was heated to 50 °C. The injection volume was 50 µL. Mobile phases A and B consisted of 0.1% formic acid in water and methanol, Fisher Chemical™ Optima™ grade solvents, respectively. The total run time was 9 minutes.

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific™ TSQ Quantiva™ triple quadrupole mass spectrometer equipped with a Thermo Scientific™ Ion Max NG source and heated electrospray ionization (HESI-III) probe. Two selected-reaction monitoring (SRM) transitions were monitored for estradiol and its deuterated internal standard to provide ion ratio confirmations (IRC). Mass spectrometer and SRM parameters are shown in Tables 1 and 2, respectively.

Table 1. Mass spectrometer parameters for estradiol

Parameter	Value
Spray Voltage	4500 V
Sheath Gas	30 Arb
Aux Gas	10 Arb
Sweep Gas	1 Arb
Ion Transfer Tube	380 °C
Vaporizer	400 °C
CID Gas	2.5 mTorr
Cycle Time	0.3 s
Divert Valve	5.0–7.5 min

Table 2. SRM parameters

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	CE	Lens
Estradiol	506.2	156.2	35	120
		171.2	35	120
Estradiol-d_5	511.2	170.2	35	120
		171.2	35	120

Data was acquired and processed with Thermo Scientific™ TraceFinder™ software.

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Method Performance

Precision and accuracy were determined by analyzing triplicate calibration curves. Method ruggedness and matrix effects were determined by performing a mixing test using a plasma sample analyzed at 1-fold, 2-fold, and 4-fold dilution with water.

Results and Discussion

Estradiol was linear in the range of 1 to 1000 pg/mL. Figure 1 shows a representative calibration curve for estradiol in human plasma. Table 3 shows the excellent precision and accuracies of the calibration curve using this method. In addition to the precision shown, accuracies for all calibrators across the method were within 14.3%. Figures 2 and 3 show chromatograms of the quantifier and confirming ions for estradiol at the lowest calibrator level of 1 pg/mL and 5 pg/mL, respectively. The method proved to be rugged with no matrix effects observed in the mixing test. All diluted plasma samples showed good recovery when compared to an undiluted sample (Table 4). Figure 4 shows a chromatogram of a donor plasma sample.

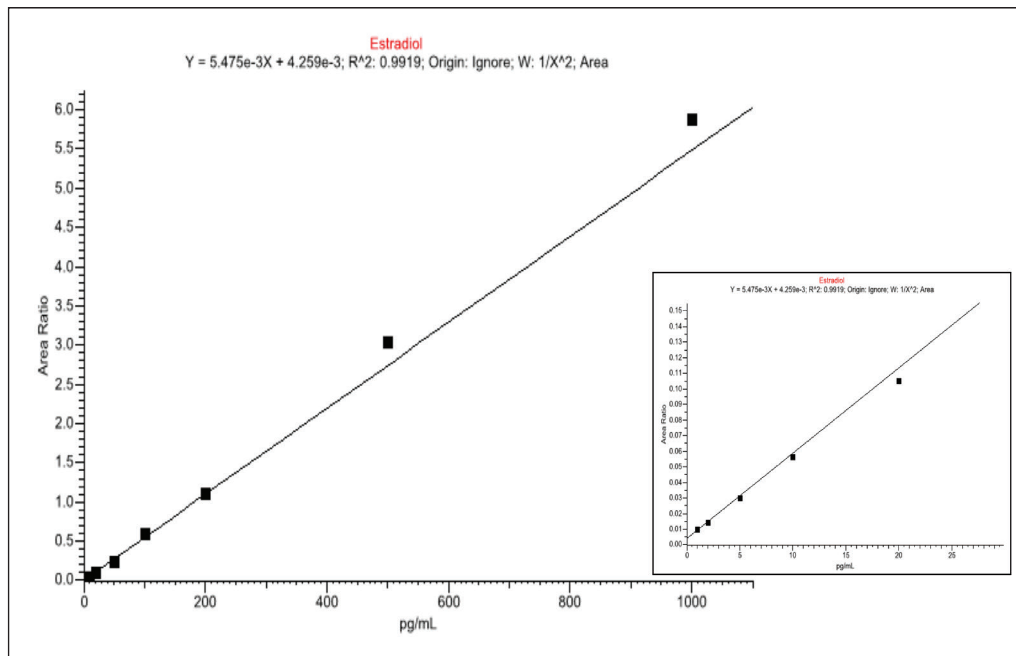

Figure 1. Representative calibration curves for estradiol in human CSS

Table 3. Precision and accuracies of replicate injections of calibrators

Concentration	% RSD (n = 3)	% Diff
1 pg/mL	6.86	6.70
2 pg/mL	8.09	-9.55
5 pg/mL	1.74	-5.48
10 pg/mL	2.06	-4.50
20 pg/mL	1.96	-7.62
50 pg/mL	1.51	-8.96
100 pg/mL	0.122	9.45
200 pg/mL	1.76	1.38
500 pg/mL	0.886	11.0
1000 pg/mL	0.807	7.52

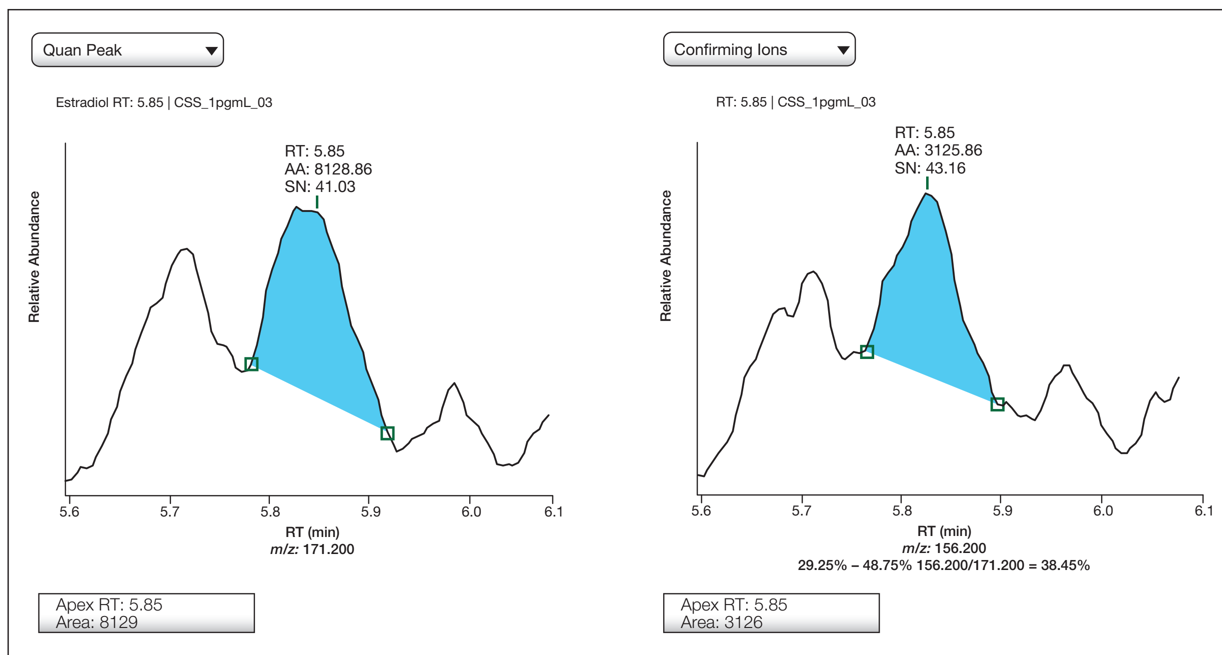

Figure 2. Chromatogram of 1 pg/mL calibrator for estradiol in CSS showing quantifier and confirming ion with passing ion ratio

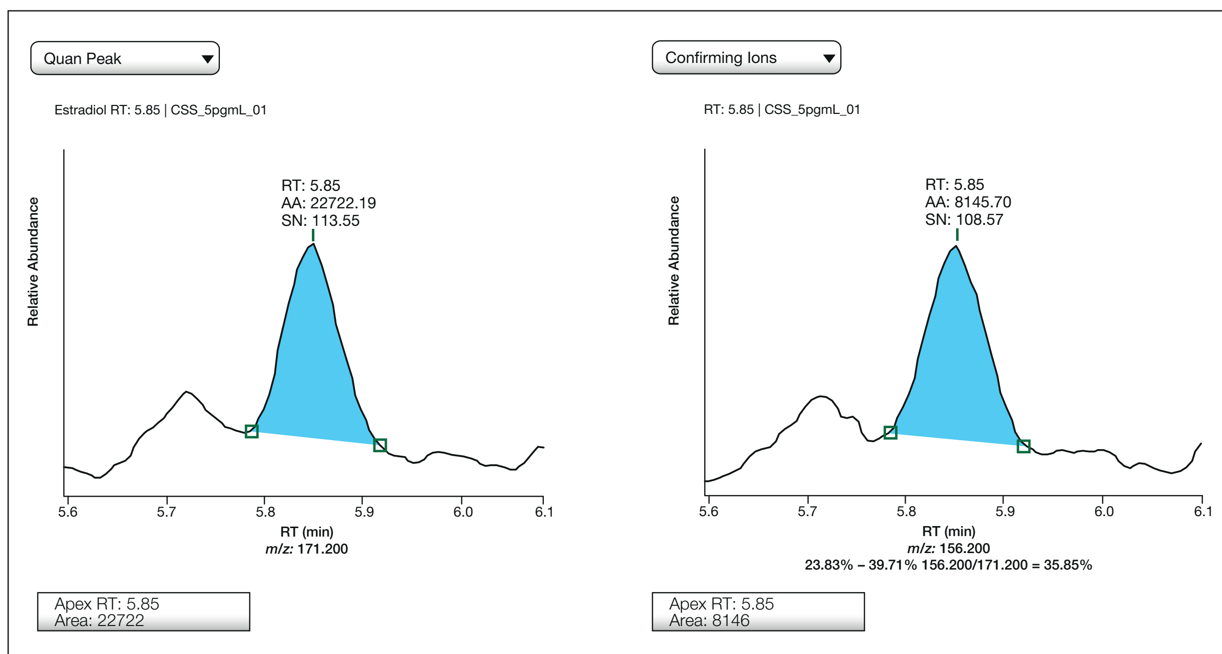

Figure 3. Chromatogram of 5 pg/mL calibrator for estradiol in CSS showing quantifier and confirming ion with passing ion ratio

Table 4. Results of mixing test showing recovery of estradiol in a sample of human plasma diluted 1-, 2-, and 4-fold

Sample	Mean Conc (pg/mL)	% RSD	Mean % Rec
Plasma	20.6	1.1	–
2-fold dilution	9.67	1.7	94.0
4-fold dilution	4.34	3.7	84.4

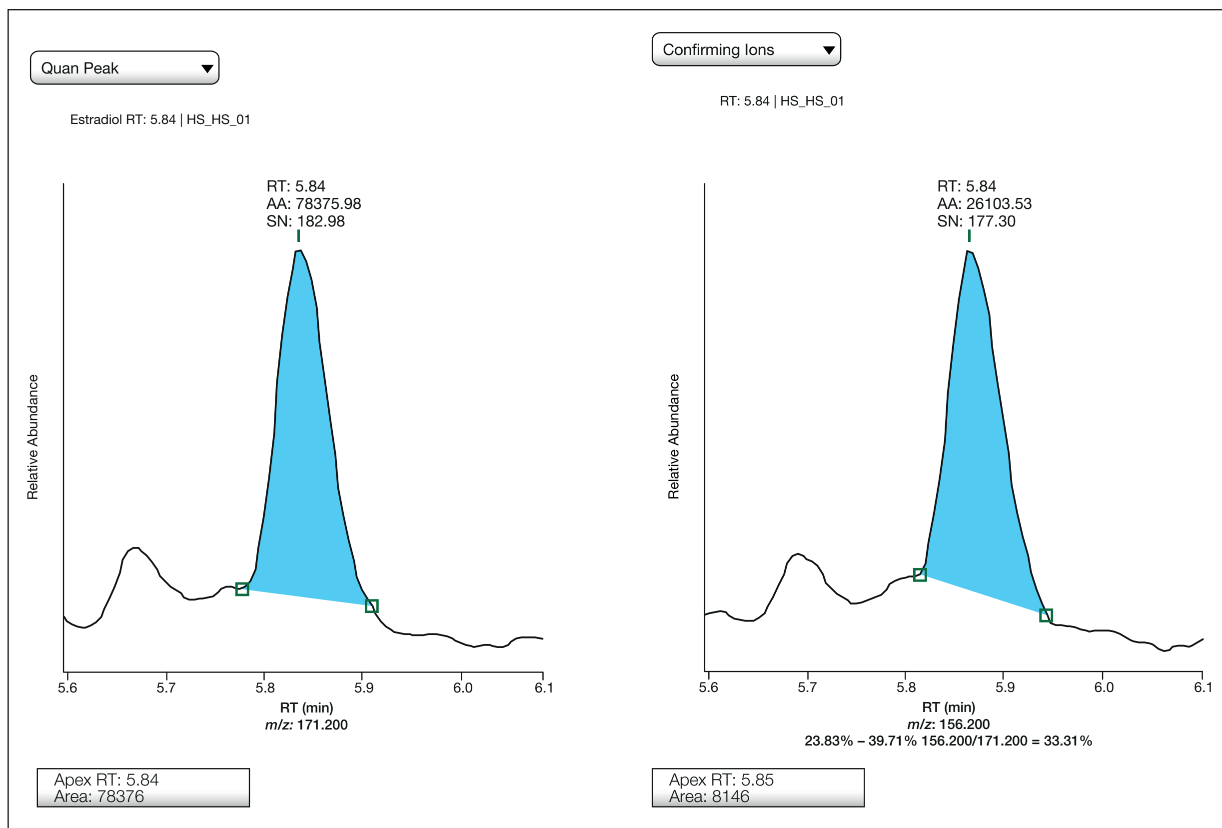

Figure 4. Chromatogram of estradiol in donor plasma at 22.6 pg/mL showing quantifier and confirming ion with passing ion ratio

Conclusion

- This clinical research method was able to reach the desired limit of quantitation of 1 pg/mL in human plasma.
- The method shows excellent precision, accuracy, and ion ratio confirmation over the entire calibration range of 1 to 1000 pg/mL.
- No matrix effects were observed.

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Quantitative Analysis of Low Testosterone Concentrations in Plasma Using the TSQ Quantiva Triple-Stage Quadrupole MS

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Key Words
Testosterone, liquid chromatography, tandem mass spectrometry

Goal
To develop an LC/MS method meeting requirements for testosterone research analysis in female and juvenile plasma.

Figure 1. TSQ Quantiva triple-stage quadrupole mass spectrometer

Introduction
Analysis of testosterone in female and juvenile plasma samples for research requires an analytically sensitive method with a limit of quantitation of at least 10 pg/mL. Liquid chromatography coupled with mass spectrometry (LC/MS), an analytically sensitive and selective technique, is widely accepted for testosterone analysis in complex matrices such as human serum or plasma.

Experimental
Sample Preparation
Plasma and serum samples were spiked with an internal standard (testosterone-D_3) and subjected to liquid-liquid extraction method using methyl tert-butyl ether. The resulting organic layer was evaporated, and the residue was reconstituted in Fisher Chemical™ methanol/water (1:1). A 10 µL aliquot of processed sample was analyzed with the following LC/MS method:

HPLC

Pump:	Thermo Scientific™ Accela™ 1250 pump
Autosampler:	Accela AS
HPLC column:	Thermo Scientific™ Accucore™ aQ 100 x 2.1 mm, 2.6 µm, ambient temperature
Mobile phase A:	5 mM ammonium acetate in water/methanol (95:5 v/v) (Fisher Chemical)
Mobile phase B:	5 mM ammonium acetate in methanol (Fisher Chemical)
Mobile phase C:	Acetonitrile/isopropyl alcohol/acetone (45:45:10 v/v/v) (Fisher Chemical)
LC gradient:	Refer to Table 1

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Table 1. LC gradient

Time (min)	A (%)	B (%)	C (%)	Flow Rate (µL/min)
0.00	95	5	0	400
0.10	60	40	0	400
3.60	20	80	0	400
3.61	0	100	0	400
4.60	0	100	0	400
4.61	0	0	100	800
5.00	0	0	100	800
5.01	95	5	0	600
6.50	95	5	0	600

Mass Spectrometry

MS analysis was performed on a Thermo Scientific™ TSQ Quantiva™ triple-stage quadrupole mass spectrometer (Figure 1). The MS conditions were as follows:

Ionization:	Heated electrospray ionization (HESI)
Vaporizer temp (°C):	500
Capillary temp (°C):	375
Spray Voltage (V):	800
Sheath gas (AU):	55
Auxiliary gas (AU):	25
Data acquisition mode:	Selected reaction monitoring (SRM)
Chrom filter peak width (s):	3
Collision gas pressure (mTorr):	2
Cycle time (s):	0.2
Q1 (FWMH):	0.7
Q3 (FWMH):	0.7
SRM parameters:	Refer to Table 2

Table 2. Optimized SRM parameters

Analyte	Q1 (*m/z*)	Q3 (*m/z*)	CE (V)
Testosterone	289.1	97.1	30
		109.1	30
Testosterone-D_3	292.1	97.1	30
		109.1	30

Method Evaluation

Calibration standards were prepared in charcoal stripped serum (CSS) (Bioreclamation, LLC) at concentrations of 5, 10, 20, 40, 100, 200, and 500 pg/mL. QC samples were prepared in CSS at 10 and 50 pg/mL. Intra-assay precision was obtained by processing and analyzing a standard curve along with three replicates of each QC sample. Inter-assay precision was obtained by processing and analyzing a standard curve along with three replicates of each QC samples on three different days. Matrix effects were evaluated by comparing peak areas of a 25 pg/mL sample prepared in CSS to a sample prepared in reconstitution solution. Matrix effects in different lots of plasma were evaluated by comparing the internal standard signal in donor plasma samples to the internal standard signal in solvent matrix.

Data Processing

Data was processed with Thermo Scientific™ TraceFinder™ software version 3.1. The target ion ratio was calculated by averaging the values obtained for calibrators and applying a tolerance of 20% for QC and donor samples.

Results and Discussion

The limit of quantitation (LOQ) was 5 pg/mL, equivalent to 100 fg on column, with excellent signal-to-noise. The LOQ was limited by the presence of endogenous testosterone in CSS (about 1 pg/mL). Figure 2 shows chromatograms for testosterone quantifier and qualifier ions at a concentration of 5 pg/mL in CSS. The calibration range is 5–500 pg/mL. Figure 3 shows a representative calibration curve. Intra-assay precision was better than 3.4% RSD for the 10 pg/mL QC and 2.0% RSD for the 50 pg/mL QC (Table 3). Inter-assay precision was 2.4% and 4.6% RSD for the 10 and 50 pg/mL QCs, respectively. Matrix effects in CSS were not observed. The average percentage recovery calculated against the spiked solvent was 94.8%. Limited matrix effects were observed in donor plasma. Internal standard signal in donor plasma was about 30% lower when compared to signal in solvent samples. Ion ratios passed for all calibration standards, QCs, and donor samples. Figures 4 and 5 present a TraceFinder chromatogram and calculated ion ratio for selected donor samples obtained in separate analytical runs.

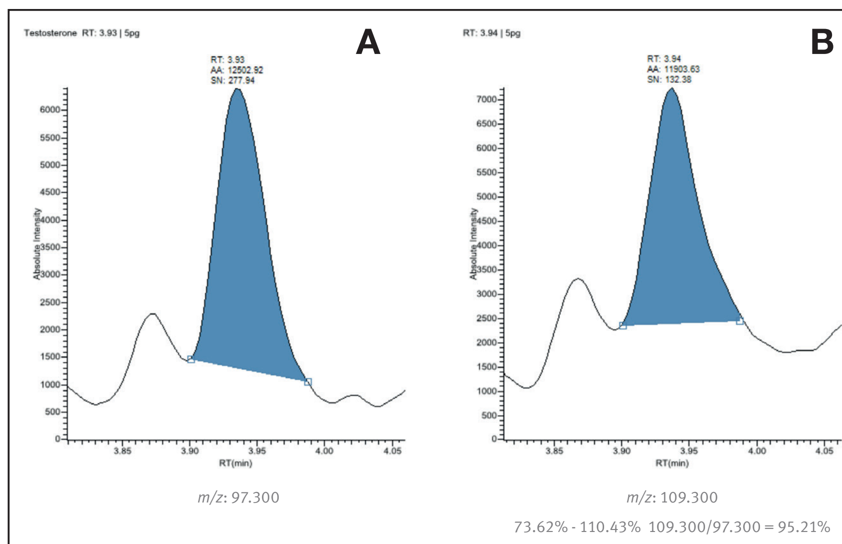
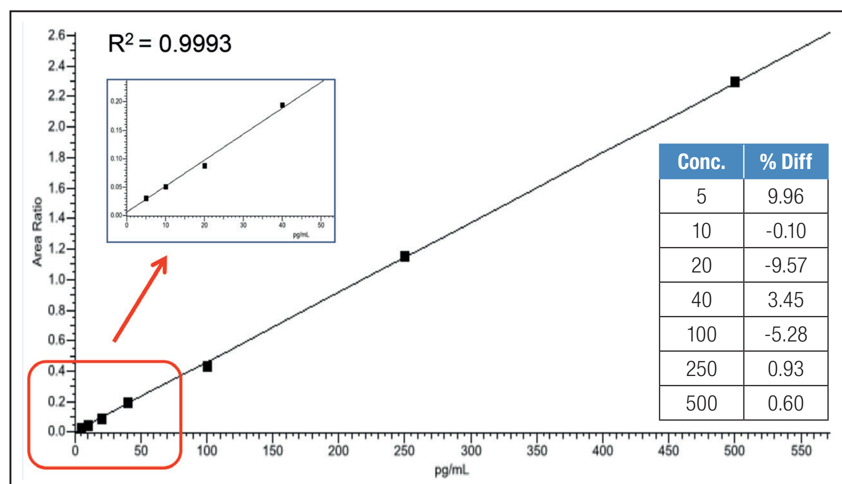


Figure 2. Chromatogram of the lowest calibration standard 5 pg/mL in CSS: (A) quantifier and (B) qualifier peaks



Conc.	% Diff
5	9.96
10	-0.10
20	-9.57
40	3.45
100	-5.28
250	0.93
500	0.60

Figure 3. Representative calibration curve for testosterone

Table 3. Intra-assay precision

	Concentration (pg/mL)		
QC1- 10 pg/mL	Batch #1	Batch #2	Batch #3
QC1-1	10.6	11.1	11.0
QC1-2	11.4	11.4	11.1
QC1-3	11.0	11.4	10.9
% RSD	**3.4**	**1.5**	**0.58**

	Concentration (pg/mL)		
QC2- 50 pg/mL	Batch #1	Batch #2	Batch #3
QC2-1	46.7	49.3	44.3
QC2-2	47.4	50.1	43.8
QC2-3	46.6	48.1	44.9
% RSD	**0.89**	**2.0**	**1.2**

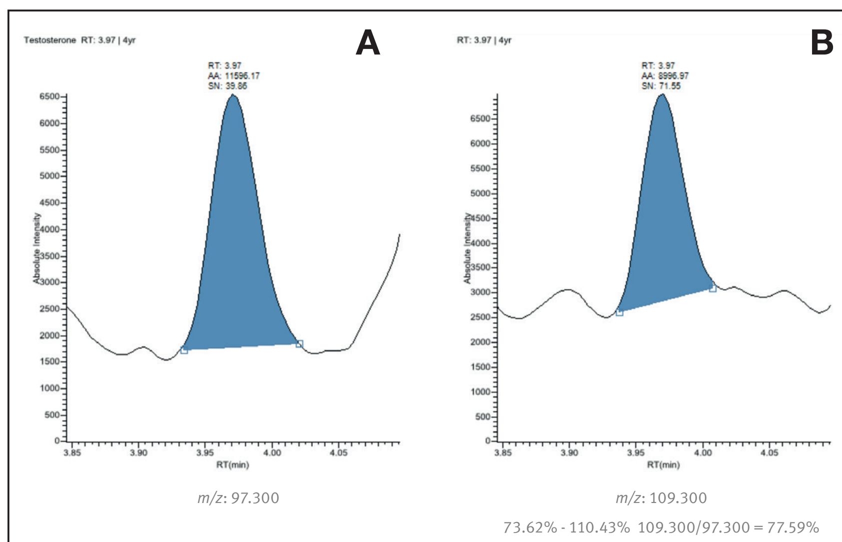


Figure 4. Chromatogram of testosterone (A) quantifier and (B) qualifier peaks in sample from a 4-year-old donor at concentration of 6.29 pg/mL

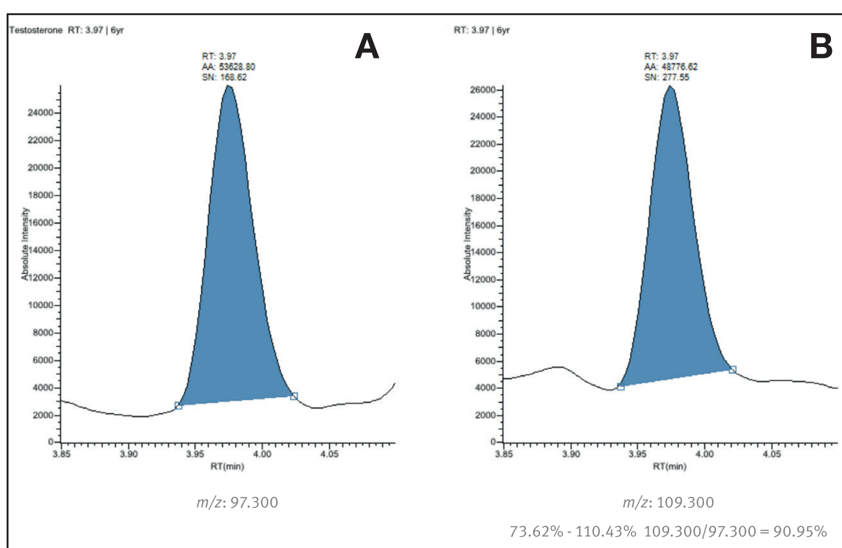


Figure 5. Chromatogram of testosterone (A) quantifier and (B) qualifier peaks in sample from a 6-year-old donor at concentration of 33.2 pg/mL

Conclusion

Using the TSQ Quantiva mass spectrometer, an analytically sensitive and robust method was evaluated for the research analysis of low testosterone concentrations in human plasma. The LOQ of 5 pg/mL is lower than that reported with other research methods. Precision was better than 5% RSD, and the ion ratios easily met industry standard criteria. The data show the TSQ Quantiva mass spectrometer has excellent analytical sensitivity and robustness to facilitate research.

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Quantitation of Mycophenolic Acid in Plasma for Research by TurboFlow HPLC-MS/MS

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Application Note 612

Key Words

Mycophenolic acid, TurboFlow online extraction method, mass spectrometry, APCI, therapeutic drug monitoring in research, MPA, immunosuppressants, organ transplantation

Goal

To present a precise and accurate research method for the quantitation of total mycophenolic acid (MPA) in plasma using Thermo Scientific™ TurboFlow™ technology.

Introduction

Mycophenolic acid (MPA) is an immunosuppressive drug commonly used in solid organ transplantation. MPA has been available since the early 1990s, but the merits of monitoring MPA levels in blood plasma have still not been finalized.[1] Many research laboratories utilize plasma concentrations from commercial assays instead of generating their own analytical methods. Some published methods show MPA analyzed along with cyclosporine, tacrolimus, sirolimus, and everolimus. However, combining the analytes into one method is not advisable because MPA is measured in plasma and the others are measured in whole blood, which would require separate, matrix-matched calibrations. It is therefore better to have a simple and fast method for MPA analysis.

To accurately measure MPA with high throughput and minimal sample preparation, a method using LC-MS/MS and TurboFlow technology has been developed. All validation was performed in compliance with current guidelines.[2,3]

Experimental

Reagents

Three mobile phases were required for the TurboFlow method (all Fisher Chemical brand reagents):

Mobile phase A:	100% ultrapure water + 10 mM ammonium acetate (Must be made fresh daily, unless an antimicrobial reagent such as 2% acetonitrile or 5% methanol is added.)
Mobile phase B:	100% methanol + 10 mM ammonium acetate
Mobile phase C:	Acetonitrile/2-propanol /acetone (40:40:20 v/v/v) (used for cleaning the TurboFlow column and a wash solution for the autosampler)
Autosampler wash 1:	100% water (no modifier)
Autosampler wash 2:	Mobile phase C
Extracting reagent:	Zinc sulphate in methanol

Sample Preparation

Plasma samples were separated from EDTA-anticoagulated whole blood within 24 hours of venipuncture to prevent potential degradation of mycophenolic acid glucuronide (MPAG) to MPA. Plasma samples were stored at 4–8 °C while awaiting analysis. Plasma samples, calibrators (Chromsystems Instruments & Chemicals GmbH, Germany), and quality controls (More Diagnostics, Inc, CA, USA) were extracted by adding 100 µL sample, 100 µL internal standard solution (indomethacin), and 800 µL extracting solution. This was vortex-mixed and centrifuged for 5 minutes at >10,000*g*. Supernatants were transferred to a microtitre plate and covered with a silicon sealing mat before being loaded into the autosampler thermostatted at 10 °C while awaiting analysis.

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Instrument Parameters

A Thermo Scientific™ Transcend™ TLX-II LC system powered by TurboFlow technology was used to run the LC-MS/MS method. The TurboFlow online extraction column was a Thermo Scientific™ TurboFlow™ C18XL column (50 x 0.5 mm) and the analytical column was a Thermo Scientific™ Hypersil GOLD™ column (1.9 µm 50 x 2.1 mm). Chromatography gradient information is provided in Table 1 for two-dimensional TurboFlow chromatography.

Table 1. HPLC gradient table for 2D TurboFlow chromatography

			Loading Pump								Eluting Pump					
Step	Start	Sec	Flow mL/min	Gradient	%A	%B	%C	%D	Tee	Loop	Flow mL/min	Gradient	%A	%B	%C	%D
1	00:00	40	2.00	Step	100	-	-	-	-	Out	0.60	Step	90	10	-	-
2	00:40	80	0.20	Step	100	-	-	-	T	In	0.40	Step	90	10	-	-
3	02:00	120	2.00	Step	-	-	100	-	-	In	0.60	Ramp	-	100	-	-
4	04:00	20	2.00	Step	10	90	-	-	-	In	0.60	Step	-	100	-	-
5	04:20	60	2.00	Step	100	-	-	-	-	Out	0.60	Step	90	10	-	-

A Thermo Scientific™ TSQ Quantum Ultra™ triple quadrupole mass spectrometer was operated in positive ion mode with an atmospheric pressure chemical ionization (APCI) source. The details are provided in Table 2. Selected-reaction monitoring (SRM) transitions are listed in Table 3. Data were acquired using Thermo Scientific™ Xcalibur™ software with Thermo Scientific™ Aria™ system control. Data analysis was performed using Thermo Scientific™ LCQuan™ software version 2.5.

Table 2. Source parameters for the MPA analysis in APCI mode

Parameter	Value
Discharge current	6.0 µA
Sheath gas	50 arbitrary units
Aux gas	5 arbitrary units
Vaporizer temperature	400 °C
Capillary temperature	275 °C

Table 3. SRM transitions for MPA, MPAG, and internal standard (indomethacin)

	Precursor Ion *m/z*	Product Ion *m/z*	Collision Energy	Tube Lens
MPA	321.1	159, 207, 303	35, 23, 12	103
MPAG	514.0	321	18	103
Internal standard	357.9	111, 129, 138	43, 33, 22	108

Results and Discussion

Measurement of MPA by LC-MS/MS is not without its challenges, as it is possible to observe interferences in samples that are not observed in commercial or blank plasma spiked with MPA. For example, interference from the ac0079l glucuronide metabolite of MPA, MPAG, should be considered since it breaks down in the ionization source to MPA. This occurs in both electrospray ionization (ESI) and APCI. Therefore, it is essential to ensure that MPA and MPAG are chromatographically separated.

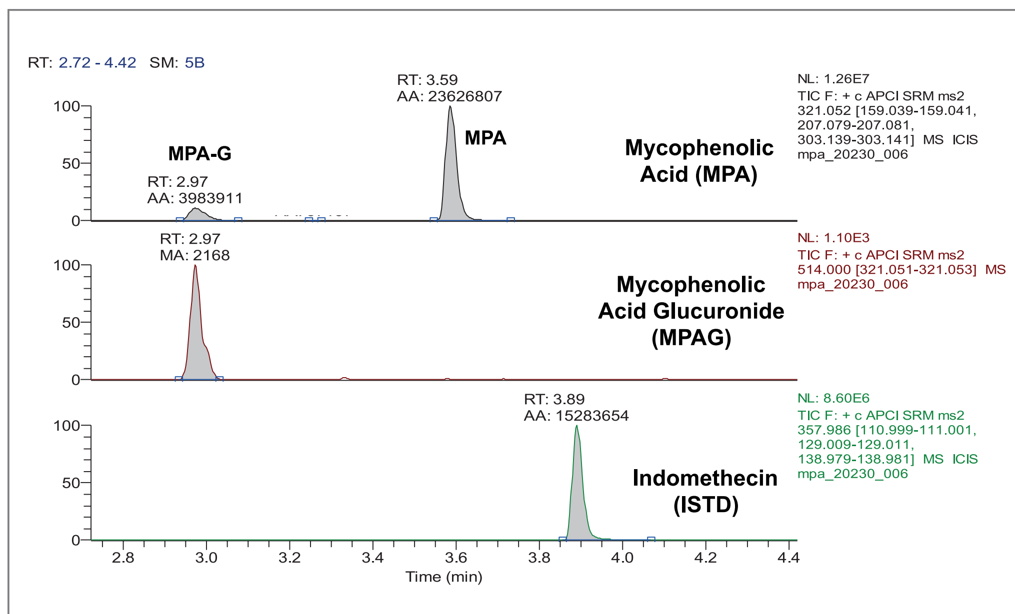

Figure 1. Extracted ion chromatograms for MPA (top), MPAG (middle), and internal standard (bottom)

Figure 1 presents a chromatogram showing the three SRM scans. MPA and MPAG peaks are separated by a retention time (RT) difference of approximately 0.6 minutes on the scan for the $[M+H]^+$ ion of MPA. For quantitative analysis of the metabolite, maximum conversion of MPAG to MPA is driven by optimizing the severity of the ionization process.

Table 4. Interday precision study for LC-MS/MS analysis of MPA. Four levels of quality control material, 10 replicates per level, repeated over 5 consecutive days.

	QC1	QC2	QC3	QC4
MEAN mg/L	1.7	6.9	11.3	33.5
STD DEV	0.1	0.4	0.7	1.9
CV%	6.4	6.3	6.2	5.8

The calibration curve was linear from 0.2 mg/L to 40 mg/L. Precision data is presented in Table 4 showing CVs of 5.8–6.4% across the calibration range of the assay.

Conclusion

The research method developed using TurboFlow technology allowed an accurate detection and quantification of MPA. It is also fast, requires minimal manual sample preparation, and conforms to current guidelines.

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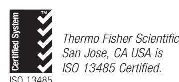

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Quantification of 17 Antiepileptics and Their Metabolites in Human Plasma by LC-MS/MS for Research

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Key Words

Antiepileptic drugs, antiepileptics, AEDs, anticonvulsants, antiseizure drugs, liquid chromatography, triple quadrupole mass spectrometry, TSQ Quantum Access MAX

Goal

Implement an LC-MS/MS method for the quantification of 17 antiepileptics and their metabolites in human plasma.

Introduction

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is a valuable tool that can help clinical researchers to monitor that antiepileptic drugs remain within the desired range. Here, an LC-MS/MS method on the Thermo Scientific™ TSQ Quantum Access MAX™ triple quadrupole mass spectrometer was used with the MassTox® TDM Series A kit for antiepileptics from Chromsystems™ to quantify a panel of 17 antiepileptics and their metabolites in human plasma. The MassTox TDM Series A kit includes levetiracetam, theophylline, felbamate, lacosamide, rufinamide, carbamazepine, oxcarbazepine, carbamazepine diol, carbamazepine-10,11-epoxide, 10-hydroxycarbamazepine, phenylethylmalonamide (PEMA), primidone, phenytoin, stiripentol, zonisamide, phenobarbital, valproic acid, and 12 internal standards.

Experimental

Sample Preparation

The MassTox TDM Series A kit for antiepileptics was used. The 17 evaluated analytes were divided into three groups. Each group required a different extraction procedure and analytical method. The kit included dried calibrators at three different concentration levels and dried controls at two different levels. Concentrations of calibrators and controls are reported in Table 1.

The kit also included an extraction buffer, a precipitating agent containing all the internal standards (IS), and two different dilution buffers (dilution buffer 1 and dilution buffer 2).

Dry calibrators and controls were resuspended using 1 mL of distilled water and let rest for 15 minutes at room temperature. Blanks, calibrators, controls, and samples were protein precipitated as follows:

- 100 µL of blank, calibrator, control, or sample
- 50 µL of extraction buffer
- 500 µL of precipitating agent containing the internal standards

Calibrators and controls were extracted in duplicate. Precipitated samples were vortex-mixed and centrifuged for 10 minutes at 4 °C at 3200*g*. Supernatant was diluted using different dilution schemes depending on the group prior to injection onto the LC-MS/MS system:

- Group 1: dilution 1:10 (20 µL + 180 µL) with dilution buffer 1 / dilution buffer 2, 50:50 (v/v)
- Group 2: dilution 1:5 (100 µL + 400 µL) with dilution buffer 1
- Group 3: no dilution

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Table 1. Concentrations (ng/mL) of calibrators and controls

Group	Analyte	CAL 1	CAL 2	CAL 3	CTRL1	CTRL2
1	Carbamazepine	1.62	8.77	15.1	3.25	10.6
	Oxcarbazepine	0.14	1.89	3.64	0.46	2.75
	Carbamazepine diol	0.16	5.77	11.5	1.11	8.21
	Carbamazepine-10,11-epoxide	0.16	5.61	11.0	1.07	8.1
	10-hydroxycarbamazepine	3.83	26.6	48.3	8.48	36.1
	Felbamate	14.3	70.8	130	27.2	92.9
	Lacosamide	0.78	6.13	11.4	1.91	8.5
	Levetiracetam	4.90	28.5	84.0	16.0	62.8
	Rufinamide	3.98	21.0	35.3	7.54	28.0
	Theophylline	6.11	15.9	24.6	9.78	18.9
2	Phenytoin	3.49	13.3	23.3	5.86	16.9
	Primidone	3.07	10.7	18.3	5.11	13.6
	Phenylethylmalonamide (PEMA)	1.07	6.68	12.7	2.21	9.03
	Stiripentol	2.62	14.3	27.3	5.01	20.3
3	Phenobarbital	7.38	34.2	60.1	13.8	44.7
	Valproic Acid	31.6	79.1	125	47.2	97.7
	Zonisamide	3.97	27.4	50.4	9.04	36.8

Liquid Chromatography

Liquid chromatography analysis was performed using a Thermo Scientific™ Transcend™ TLX-1 system. The LC conditions were as follows:

LC column	Provided with the kit
Mobile phase A	Provided with the kit
Mobile phase B	Provided with the kit
Injection volume	Group 1 – 20 µL
	Group 2 – 100 µL
	Group 3 – 30 µL
LC gradient	See Table 2

Table 2. LC gradient

Group	Time (min)	Flow Rate (mL/min)	A (%)	B (%)
1	0.0	0.8	100	0
	0.1	0.8	100	0
	0.5	0.8	60	40
	2.5	0.8	60	40
	3.0	1.0	0	100
	4.0	1.0	0	100
	4.1	0.8	100	0
2	0.0	1.0	100	0
	0.1	1.0	100	0
	1.0	1.0	0	100
	3.0	1.0	0	100
	3.1	1.0	100	0
3	0.0	1.0	100	0
	0.1	1.0	100	0
	1.0	1.0	0	100
	3.0	1.0	0	100
	3.1	1.0	100	0

Mass Spectrometry

The LC system was connected to a TSQ Quantum Access MAX triple quadrupole mass spectrometer. Acquisition time ranges were used for each analyte and the following MS conditions were used:

Source type	Heated electrospray ionization (HESI)
Vaporizer temp	350 °C
Capillary temp	350 °C
Spray voltage	3500 V
Sheath gas	70 AU
Sweep gas	0 AU
Auxiliary gas	40 AU
Data acquisition mode	Selected-reaction monitoring (SRM)
Chrom filter peak width	5.0 s
Collision gas pressure	1.5 mTorr
Cycle time	0.300 s
Q1 mass resolution (FWMH)	0.7
Q3 mass resolution (FWMH)	0.7
SRM settings	See Tables 3, 4 and 5

Table 3. SRM settings – Group 1

Analyte	Start Time (min)	Stop Time (min)	Ionization Mode	Precursor Ion Mass (*m/z*)	Product Ion Mass (*m/z*)	Collision Energy (V)	Tube Lens (V)
Levetiracetam	0.9	1.8	+	171.1	69.3	28	70
				171.1	126.2	13	
				171.1	154.1	5	
IS7	0.9	1.8	+	174.1	69.3	29	70
Theophylline	0.9	1.9	+	181.0	69.3	26	110
				181.0	96.2	24	
				181.0	124.1	17	
IS8	0.9	1.9	+	187.0	127.1	19	110
Felbamate	1.3	2.3	+	239.0	91.2	34	100
				239.0	117.1	16	
				239.0	178.0	5	
Rufinamide	1.3	2.3	+	239.0	127.1	23	100
				239.0	211.0	5	
				239.0	222.0	11	
IS5	1.3	2.3	+	243.0	182.1	5	100
Lacosamide	1.3	2.3	+	251.1	91.2	27	100
				251.1	108.2	7	
				251.1	116.1	13	
IS6	1.3	2.3	+	254.1	108.2	6	100
Carbamazepine diol	1.7	2.7	+	271.0	180.0	27	100
				271.0	236.0	11	
				271.0	253.1	5	
Carbamazepine-10,11-epoxide	1.9	2.8	+	253.0	180.1	29	100
				253.0	210.0	13	
				253.0	236.0	12	
10-hydroxycarbamazepine	1.9	2.9	+	255.0	179.0	36	100
				255.0	194.0	19	
				255.0	237.0	5	
IS3	1.9	2.9	+	259.0	198.0	19	100
IS2	1.9	2.9	+	263.1	190.1	25	100
IS4	2.4	3.4	+	257.0	184.0	25	100
				257.0	212.0	19	
				257.0	240.0	13	
Oxcarbazepine	2.5	3.5	+	253.0	180.0	25	100
				253.0	208.0	19	
				253.0	236.0	11	
Carbamazepine	2.9	3.9	+	237.0	165.0	42	100
				237.0	179.1	32	
				237.0	194.0	19	
IS1	2.9	3.9	+	247.1	204.1	20	100

Table 4. SRM settings – Group 2

Analyte	Start Time (min)	Stop Time (min)	Ionization Mode	Precursor Ion Mass (*m/z*)	Product Ion Mass (*m/z*)	Collision Energy (V)	Tube Lens (V)
PEMA	0.6	1.6	+	207.1	91.2	25	70
				207.1	119.2	15	
				207.1	162.1	10	
Primidone	0.8	1.8	+	219.0	91.2	25	90
				219.0	119.2	15	
				219.0	162.1	10	
IS13	0.8	1.8	+	224.0	167.1	20	90
IS5	0.8	1.8	+	243.0	182.1	5	100
Phenytoin	1.1	2.1	+	253.0	104.1	20	100
				253.0	182.0	15	
				253.0	225.0	10	
Stiripentol	1.3	2.3	+	217.0	145.1	16	55
				217.0	159.1	13	
				217.0	187.1	10	

Table 5. SRM settings – Group 3

Analyte	Start Time (min)	Stop Time (min)	Ionization Mode	Precursor Ion Mass (*m/z*)	Product Ion Mass (*m/z*)	Collision Energy (V)	Tube Lens (V)
Zonisamide	0.7	1.7	-	211.1	119.1	18	70
				211.1	147.1	12	
IS18	0.7	1.7	-	216.0	123.2	15	70
Phenobarbital	0.9	1.9	-	231.0	85.3	15	70
				231.0	144.2	15	
				231.0	188.0	10	
IS16	0.9	1.9	-	236.0	193.1	10	70
Valproic Acid	1.2	2.2	-	143.1	143.1	10	70
IS17	1.2	2.2	-	147.1	147.1	10	70

Data Acquisition and Processing

Data were quantitated using a linear regression, and 1/x weighting was used to build the calibration curves. Maximum percentage bias between nominal and calculated concentration of 15% and 20% was set as acceptance criterion for calibrators and controls, respectively.

Results and Discussion

Linear calibration curves were obtained for all the analytes in the evaluated concentration ranges, and correlation factors (R^2) were always above 0.99. The percentage bias between nominal and experimental concentration for all calibrators and controls was always within the set acceptance criteria (15% for calibrators and 20% for controls). A summary of calibration range, intercept, slope, and correlation factor (R^2) for each analyte is reported in Table 6.

Table 6. Calibration range, intercept, slope, and correlation factor (R^2)

Analyte	Calibration Range (ng/mL)	Intercept	Slope	R^2
Carbamazepine	1.62 – 15.1	-0.068	0.610	0.998
Oxcarbazepine	0.14 – 3.64	-0.054	1.585	0.998
Carbamazepine diol	0.16 – 11.5	0.000	0.115	0.996
Carbamazepine-10,11-epoxide	0.16 – 11.0	-0.056	7.099	0.995
10-hydroxycarbamazepine	3.83 – 48.3	0.072	0.347	0.999
Felbamate	14.3 – 130	-0.119	0.484	0.995
Lacosamide	0.78 – 11.4	-0.064	1.145	0.996
Levetiracetam	4.90 – 84.0	-0.451	0.954	0.995
Rufinamide	3.98 – 35.3	-0.449	1.026	0.996
Theophylline	6.11 – 24.6	0.117	0.198	0.991
Phenytoin	3.49 – 23.3	-2.185	0.667	0.996
Primidone	3.07 – 18.3	2.570	1.375	0.997
PEMA	1.07 – 12.7	-1.964	6.885	0.996
Stiripentol	2.62 – 27.3	-2.298	1.922	0.994
Phenobarbital	7.38 – 60.1	-0.027	0.083	0.998
Valproic Acid	31.6 – 125	-0.041	0.011	0.998
Zonisamide	3.97 – 50.4	-0.157	0.286	0.999

Representative calibration curves for carbamazepine and phenobarbital are shown in Figure 1. Representative chromatograms at the limit of quantitation (LOQ) for each analyte, including the internal standards, are shown in Figures 2, 3, and 4.

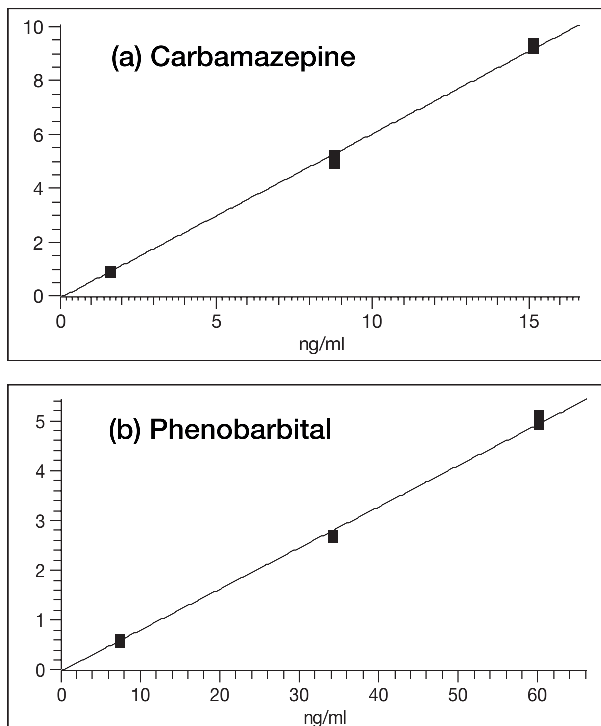

Figure 1. Calibration curves for (a) carbamazepine and (b) phenobarbital

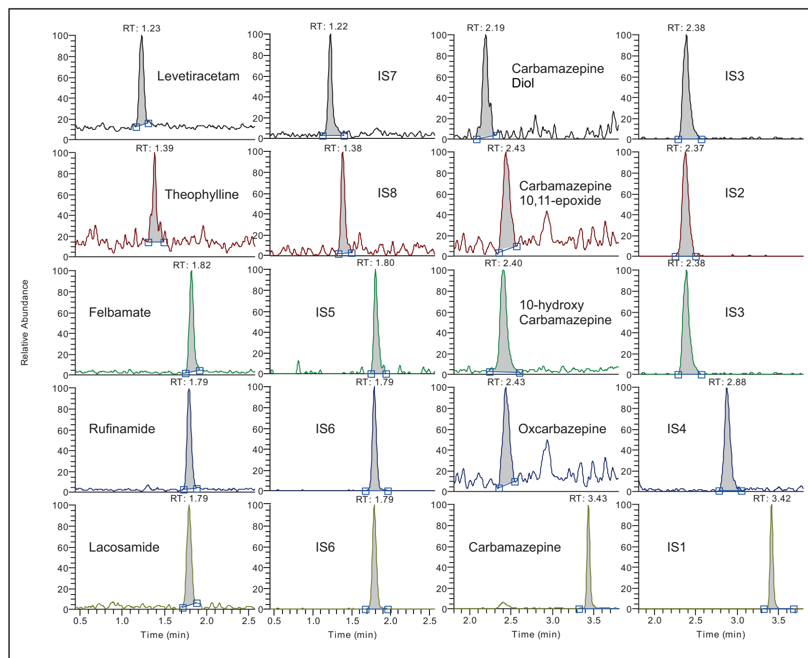

Figure 2. Representative chromatograms of each analyte at the LOQ and corresponding IS for Group 1

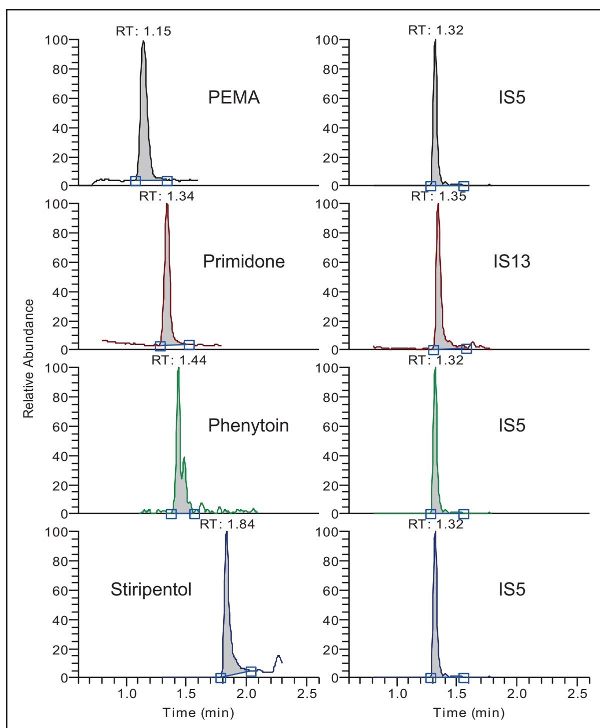

Figure 3. Representative chromatograms of each analyte at the LOQ and corresponding IS for Group 2

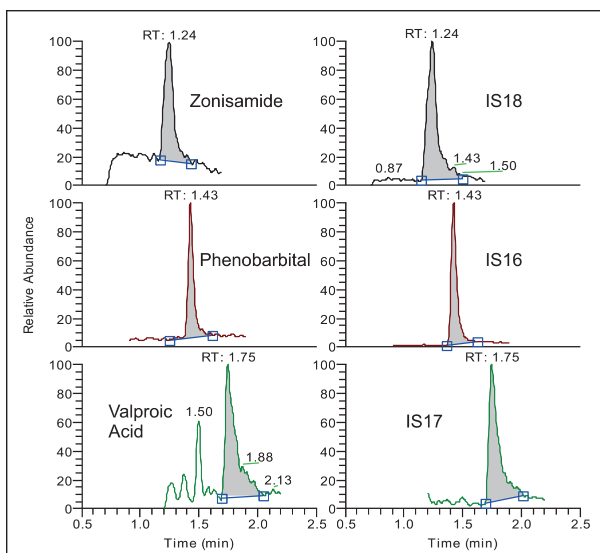

Figure 4. Representative chromatograms of each analyte at the LOQ and corresponding IS for Group 3

Conclusion

The quantification of a panel of antiepileptic drugs in human plasma has been implemented and analytically validated on a TSQ Quantum Access MAX mass spectrometer using the MassTox TDM Series A kit for antiepileptics from Chromsystems. The TSQ Quantum Access MAX mass spectrometer proved to have the proper sensitivity, accuracy, and precision for the application of this analytical method to clinical research.

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Selected-Reaction Monitoring–Mass Spectrometric Immunoassay Analysis of Parathyroid Hormone and Related Variants

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Key Words
LTQ Orbitrap XL, TSQ Vantage, Pinpoint Software, parathyroid hormone, PTH, biomarkers, MSIA

Goal
To develop a highly sensitive and selective selected-reaction monitoring–mass spectrometric immunoassay analysis (SRM-MSIA)-based method for the concurrent detection and quantification of full-length parathyroid hormone (PTH) [amino acid (aa)1–84] and two N-terminal variants [aa7–84 and aa34–84] for clinical research use.

Introduction

Parathyroid hormone is produced in the parathyroid glands through the two-step conversion of prepro-PTH (115 amino acids) to pro-PTH (90 amino acids) to the 84 amino acid peptide (PTH1–84). Conventional PTH measurements typically rely on two-antibody recognition systems coupled to a variety of detection modalities.[1] The most specific modalities are able to differentiate between different truncated forms of PTH and are referred to as second- and third-generation PTH assays.[2] The key to the application of these later-generation assays is the ability to selectively detect and quantify various PTH forms. In particular, two variants are the subject of increased research investigation: full-length PTH1–84 and PTH missing the 6 N-terminal amino acids (PTH7–84). Because of the inability of existing tests to detect microheterogeneity,[3] these variants were historically considered as a single PTH value (by the first-generation assays). The classification of each variant as its own molecular entity, and the analysis of each independently, suggest an antagonistic relationship between the two different forms in regard to calcium homeostasis.[4] In fact, there is mounting research showing that the ratio between PTH1–84 and PTH7–84 could have future clinical relevance for distinguishing between hyper-parathyroid bone turnover and adynamic bone disease.[5-7]

The ratio of PTH1–84 to PTH7–84 is an example of the potential utility of the microheterogeneity within the PTH protein. Another PTH variant, PTH1–34, has been identified as exhibiting biochemical activity comparable to the full-length protein. There are indications that the microheterogeneity of PTH has yet to be fully characterized, challenging researchers' efforts to determine the utility and/or confounding effects on present-day methods. Accurate examination of known PTH variants and the simultaneous evaluation of other possible variants requires a degree of analytical freedom that universally escapes conventional methods. This work describes mass spectrometric immunoassays that, although specifically designed for the detection of PTH1–84 and PTH7–84, also facilitate the simultaneous discovery and evaluation of further microheterogeneity in PTH.

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Experimental

Approach

In addition to the well-characterized truncated PTH variants, PTH1–84 and PTH7–84, four other molecular versions have been reported in the literature as present in human biofluids (primarily plasma or serum). Aligning these fragments to the sequence of PTH1–84 produced a variant map revealing forms stemming predominantly from N-terminal truncations (Figure 1). A conserved region (among several variants) was evident between residues 48 and 84. This region was suitable for immunoaffinity targeting to capture ragged N-terminal variants (for example, PTH1–84 and PTH7–84). Postcapture digestion of retained PTH (and variants) created the basis for SRM-MSIA,[8-11] for which surrogate peptides representative of the different PTH variants were selected for analysis.

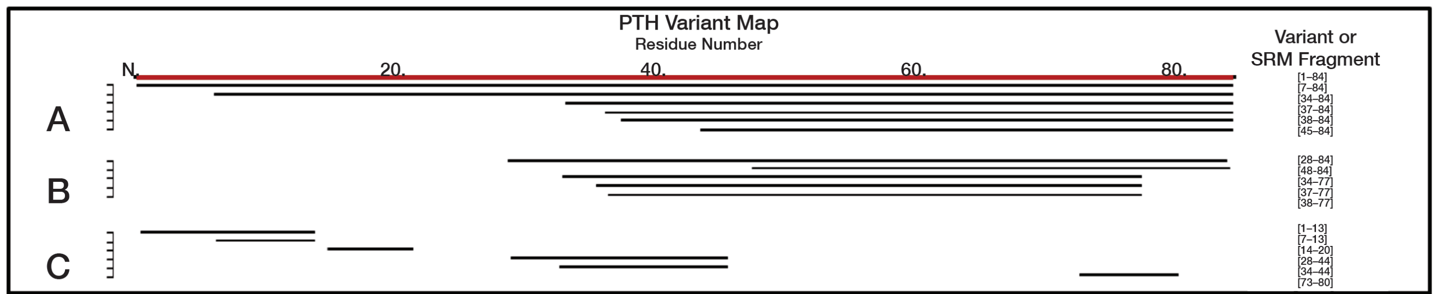


Figure 1. PTH variant map. (A) N-terminally truncated PTH variants identified previously. [7, 12] (B) Variants added to map by top-down MS analysis. (C) Conserved and truncated tryptic fragments chosen for SRM-MSIA.

Reagents

Goat polyclonal anti-PTH39–84 antibody was purchased from Immutopics International. Recombinant human PTH (rhPTH) was obtained from Bachem. Premade 0.01 M HEPES-buffered saline with 3 mM EDTA and 0.05% (vol/vol) surfactant P20 (HBS-EP) was purchased from Biacore. Thermo Scientific™ Pierce™ premixed 2-[morpholino]ethanesulfonic acid–buffered saline powder packets and Thermo Scientific synthetic heavy-labeled peptides were used. High purity solvents from Fisher Chemical brand were used.

Samples

A total of 24 plasma samples were used in the research study: 12 from individuals with previously diagnosed severe renal impairment or end-stage renal disease (ten males and two females; mean age 66.7 years) and 12 from healthy individuals (ten males and two females; mean age 65 years). Among the individuals with renal failure, three were Hispanic, two were Asian, two were African American, and six were Caucasian. The ethnicity information for the healthy sample donors was not available.

Calibration Curves Samples

Samples for creation of calibration curves were prepared from pooled human plasma by step-wise, 2-fold serial dilution of an initial sample containing rhPTH at a concentration of 1000 ng/L (eight steps, range 1000–7.8 ng/L). Samples were frozen at -80 °C until use.

Sample Preparation and Immunocapture

Purification and concentration of the PTH was accomplished by immunoaffinity capture. Extraction of PTH from plasma was carried out with proprietary Thermo Scientific™ Mass Spectrometric Immunoassay (MSIA™) pipette tips derivatized with the PTH antibodies via 1,1 -carbonyldiimidazole chemistry.[13-17] After extraction, PTH was digested, separated by liquid chromatography, and analyzed by high-resolution MS/MS on an ion trap-Orbitrap™ hybrid mass spectrometer and by SRM on a triple quadrupole mass spectrometer as described below.

Sample Elution and Trypsin Digestion

Bound proteins were eluted from the tips into a 96 well plate by pipetting 100 µL of 30% acetonitrile/0.5% formic acid up and down for a total of 15 cycles. Samples were lyophilized to dryness and then resuspended in 30 µL of 30% n-propanol/100 mmol/L ammonium bicarbonate, pH 8.0, diluted with 100 µL of 25 M acetic acid containing 100 ng of trypsin. Samples were allowed to digest for 4 hours at 37 °C. After digestion, samples were lyophilized and resuspended in 30 µL of 3% (vol/vol) acetonitrile/0.2% (vol/vol) formic acid/glucagon/PTH heavy peptides.

High-Resolution LC-MS/MS

High-resolution LC-MS/MS analysis was carried out using a Thermo Scientific™ EASY-nLC™ system and Thermo Scientific™ LTQ Orbitrap XL™ hybrid ion trap-Orbitrap mass spectrometer. Samples in 5% (vol/vol) acetonitrile/0.1% (vol/vol) formic acid were injected into a Thermo Scientific™ Hypersil GOLD™ aQ fused-silica capillary column (75 µm x 25 cm, 5 µm particle size) in a 250 µL/min gradient of 5% acetonitrile/0.1% formic acid to 30% acetonitrile/0.1% formic acid over the course of 180 minutes. The total run time was 240 minutes and the flow rate was 285 nL/min. The LTQ Orbitrap XL MS was operated at 60,000 resolution (FWHM at *m/z* 400) for a full scan for data-dependent Top 5 MS/MS experiments (CID or HCD). The top 5 signals were selected with monoisotopic precursor selection enabled, and +1 and unassigned charge states rejected. Analyses were carried out in the ion trap or the Orbitrap analyzer. The experiments were performed using collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD) fragmentation modes.

SRM Methods

SRM methods were developed on a Thermo Scientific™ TSQ Vantage™ triple stage quadrupole mass spectrometer with a Thermo Scientific™ Accela™ pump, a CTC PAL® autosampler (Leap Technologies), and a Thermo Scientific™ Ion Max™ source equipped with a high-flow metal needle. A mass window of 0.7 full width at half maximum (FWHM, unit resolution) was used in the SRM assays because the immunoenriched samples had a very high signal-to-noise ratios. Narrower windows were necessary when the matrix background was significant and caused interferences that reduced signal-to-noise in the SRM channels. Reversed-phase separations were carried out on a Hypersil GOLD column (1 mm x 100 mm, 1.9 µm particle size) with a flow rate of 160 µL/min. Solvent A was 0.2% formic acid in LC-MS-grade water, and solvent B was 0.2% formic acid in Fisher Scientific™ Optima™-grade acetonitrile.

Software

Thermo Scientific™ Pinpoint™ software was used for targeted protein quantification, automating the prediction of candidate peptides and the choice of multiple fragment ions for SRM assay design. Pinpoint software was also used for peptide identity confirmation and quantitative data processing. The intact PTH sequence was imported into the software and digested with trypsin *in silico*. Then, transitions for each peptide were predicted and tested with recombinant PTH digest to determine those peptides and transitions delivering optimal signal. After several iterations, a subset of six peptides with multiple transitions was chosen.

Further tests were conducted with this optimized method. After the target peptides were identified, heavy arginine or lysine versions were synthesized to be used as internal quantitative standards. Target peptides were subsequently identified and quantified by coeluting light- and heavy-labeled transitions in the chromatographic separation. Time alignment and relative quantification of the transitions were performed with Pinpoint software. All samples were assayed in triplicate.

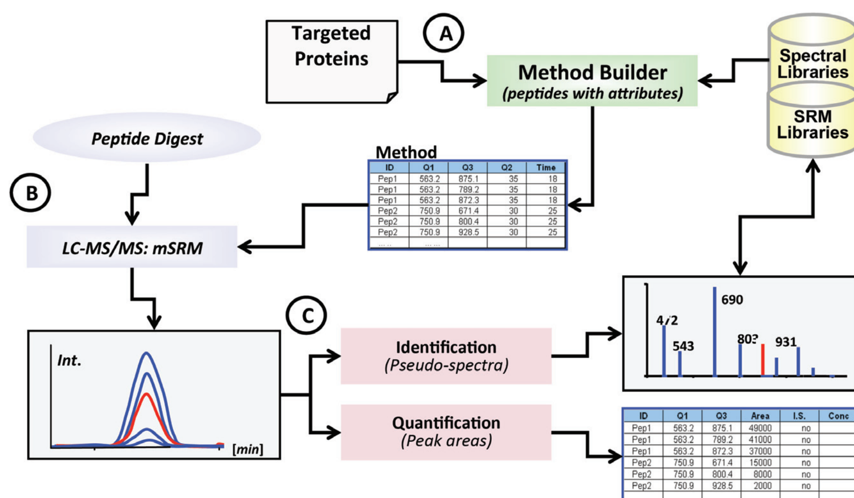
Results and Discussion

Top-Down Analysis and Discovery of Novel Variants

The approach described herein coupled targeting a common region of PTH by use of a polyclonal antibody (raised to the C-terminal end of the protein) with subsequent detection by use of SRM MS. Numerous PTH variants were simultaneously extracted with a single, high-affinity polyclonal antibody, and the selection of the epitope was directed by the target of interest (i.e., intact and N-terminal variants). The primary goal was to differentiate between intact PTH1–84 and N-terminal variant PTH7–84 while simultaneously identifying any additional N-terminal heterogeneity throughout the molecule. The results of these top-down experiments allowed the development of an initial standard profile for PTH. Clearly, this profile is not finite, and may be expanded to include additional variants found through literature search and/or complementary full-length studies. However, this standard profile provided an initial determination of target sequences for developing specific SRM assays.

Selection of Transitions for SRM

During LC-MS/MS analysis, multiple charge states and fragmentation ions were generated from each fragment, resulting in upwards of 1000 different precursor/product transitions possible for PTH digested with trypsin. Empirical investigation of each transition was not efficient. Therefore, a workflow incorporating predictive algorithms with iterative optimization was used to predict the optimal transitions for routine monitoring of tryptic fragments (Figure 2). The strategy facilitated the translation of peptide intensity and fragmentation behavior empirically obtained by high-resolution LC-MS/MS analyses to triple quadrupole SRM assays. Inherent to the success of the workflow was the similarity of peptide ion fragmentation behavior in these ion trap and triple quadrupole instruments.[12] Empirical data from such LC-MS/MS experiments were used in conjunction with computational methods (*in silico* tryptic digestions and prediction of SRM transitions) to enhance the design of effective SRM methods for selected PTH peptides.



ID	Q1	Q3	Q2	Time
Pep1	563.2	875.1	35	18
Pep1	563.2	789.2	35	18
Pep1	563.2	872.3	35	18
Pep2	750.9	671.4	30	25
Pep2	750.9	800.4	30	25
Pep2	750.9	928.5	30	25

ID	Q1	Q3	Area	I.S.	Conc
Pep1	563.2	875.1	49000	no	
Pep1	563.2	789.2	41000	no	
Pep1	563.2	872.3	37000	no	
Pep2	750.9	671.4	15000	no	
Pep2	750.9	800.4	8000	no	
Pep2	750.9	928.5	2000	no	

Figure 2. Pinpoint workflow for development of multiplexed SRM assays. [Q = quadrupole; mSRM = multiple SRM; Int. = intensity; I.S. = internal standard; Conc = concentration. Time measurements are in minutes (min).]

The initial list of transitions was queried empirically to produce an LC-MS/MS profile based on four tryptic peptides that collectively spanned >50% (45 of 84 amino acids) of the full PTH sequence. SVSEIQLMHNLGK [amino acid (aa)1–13] was monitored to represent PTH species with an intact N-terminus, such as PTH1–84. Other tryptic peptides, HLNSMER (aa14 –20), DQVHNFVALGAPLAPR (aa28–44), and ADVNVLTK (aa73–80) were included for monitoring across the PTH sequence. In addition, transitions for two truncated tryptic peptides, LMHNLGK (aa7–13) and FVALGAPLAPR (aa34–44), were added to the profile to monitor for truncated variants PTH7–84 and PTH34–84, respectively. In total, 32 SRM transitions tuned to these six peptides were used to monitor intact and variant forms of PTH (Figure 1).

Generation of Standard Curves and Limits of Detection and Quantification

rhPTH was spiked into stock human blood plasma to create calibration curves for all target tryptic peptides through serial dilution. As illustrated in Figure 3 for peptides LQDVHNFVALGAPLAPR (aa28–44) and SVSEIQLMHNLGK (aa1–13), SRM transitions for the four wild-type tryptic fragments exhibited linear responses (R^2 = 0.90–0.99) relative to rhPTH concentration, with limits of detection for intact PTH of 8 ng/L and limits of quantification for these peptides calculated at 31 and 16 ng/L, respectively. Standard error of analysis for all triplicate measurements in the curves ranged from 3% to 12% for all peptides, with <5% chromatographic drift between replicates. In addition, all experimental peptide measurements were calculated relative to heavy-labeled internal standards. CVs of integrated areas under the curve for 54 separate measurements (for each heavy peptide) ranged from 5% to 9%. Monitoring of variant SRM transitions showed no inflections relative to rhPTH concentration, owing to the absence of truncated variants in the stock rhPTH.

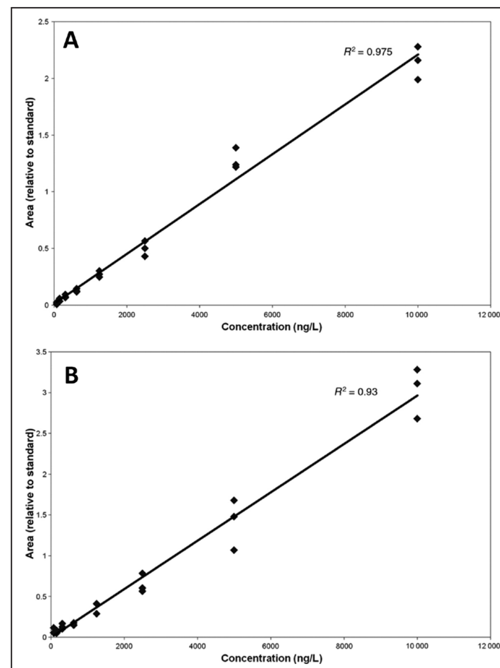


Figure 3. SRM calibration curves for PTH peptides.
(A) Peptide LQDVHNFVALGAPLAPR aa28–44.
(B) Peptide SVSEIQLMHNLGK aa1–13.

Evaluation of Research Study Samples

Initial SRM data were acquired from replicate plasma samples. The light and heavy peptides coeluted precisely in all samples. Further SRM experiments were carried out on the cohort of renal failure (n = 12) and normal (n = 12) samples. The most prominent PTH variant in the renal failure samples was PTH34–84. To quantify this observation with SRM, all samples were interrogated to determine the expression ratios of renal failure to normal for the various target peptides, including FVALGAPLAPR (aa34–84), which should be specific to the 34–84 variant. Chromatographic data from single renal-failure samples for peptides FVALGAPLAPR (aa34–44) and SVSEIQLMHNLGK (aa1–13) are shown in Figure 4. The peak integration area and individual coeluting fragment transitions for each peptide are illustrated. Similar chromatograms were obtained for peptides LQDVHNFVALGAPLAPR (aa28–44), HLNSMER (aa14–20), and ADVNVLTK (aa73–80) (data not shown). The sample variances and expression ratios of renal-failure samples to normal samples for each peptide are shown in Figure 5. The expression ratios for the peptides ranged from 4.4 for FVALGAPLAPR (aa34–44) to 12.3 for SVSEIQLMHNLGK (aa1–13). Notable quantities of peptide LMHNLGK (aa 7–13) were not detected in these samples. Sample variances illustrated in the scatter plots in Figure 5 demonstrate that the renal failure and normal samples groups were clearly segregated by the five target peptides.

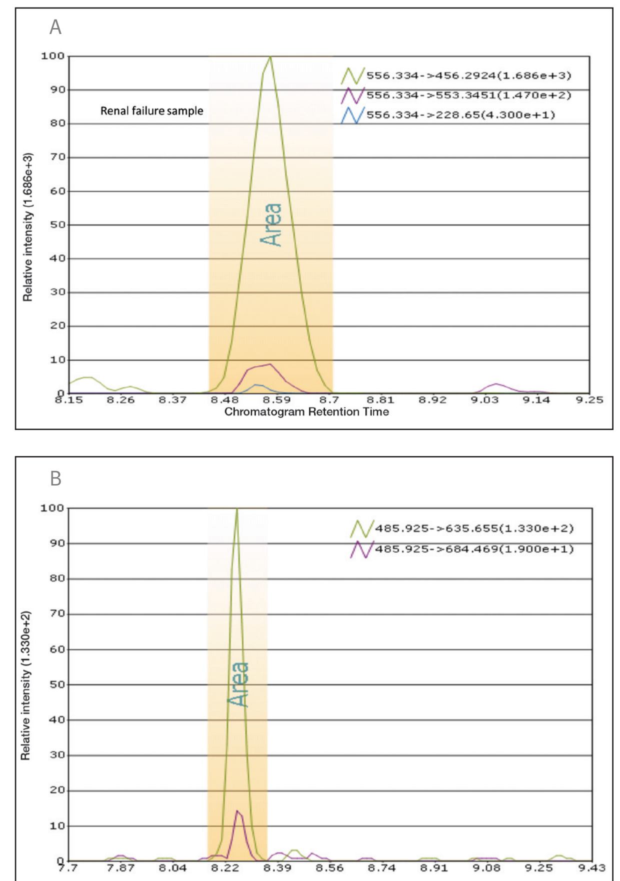
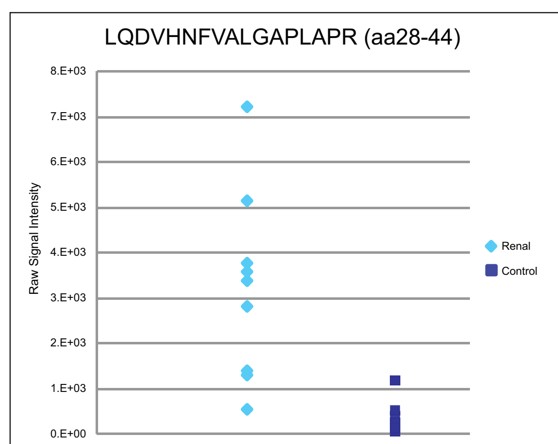
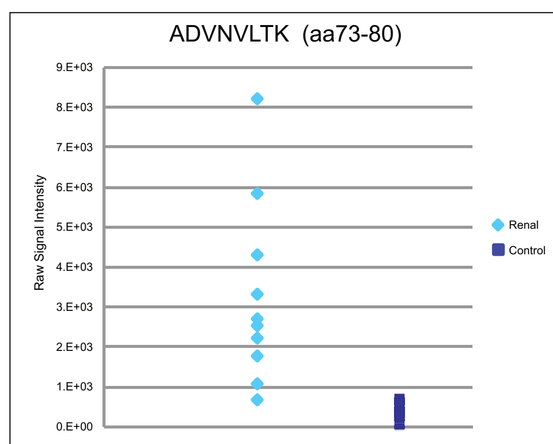
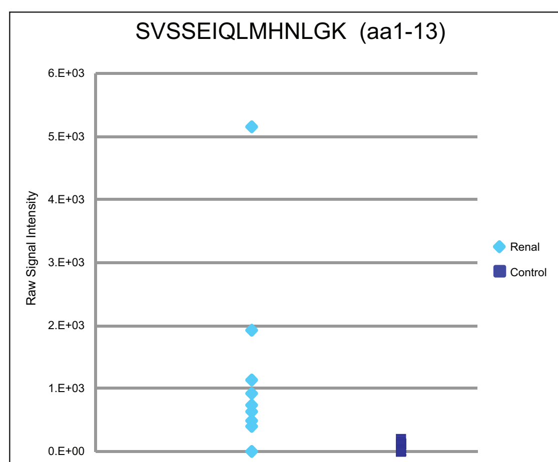
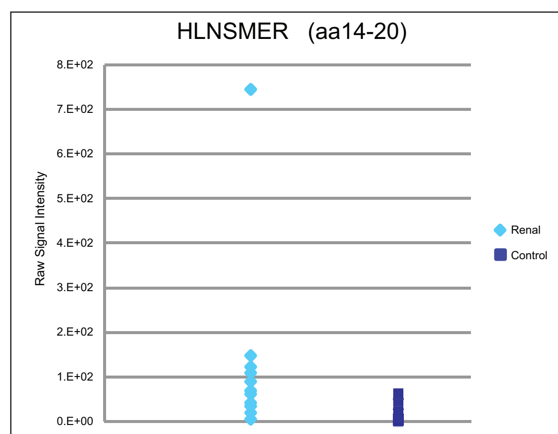


Figure 4. Pinpoint software SRM data from samples of normal and renal failure patients. Chromatographic data illustrate peak integration area and individual fragment transitions for peptides from single renal failure samples. (A) Semitryptic peptide FVALGAPLAPR (aa34–44), specific to the 34–84 variant (see Figure 1). (B) Tryptic peptide SVSEIQLMHNLGK (aa1–13).









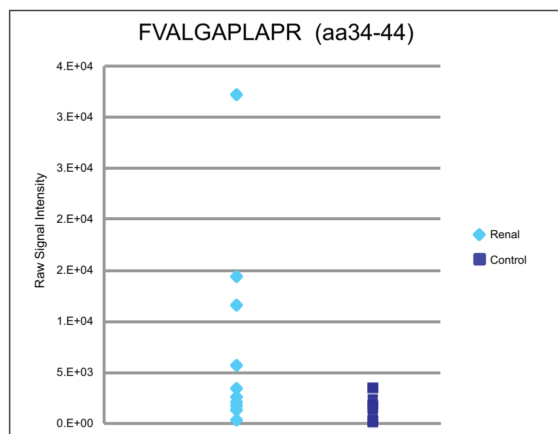


Figure 5. SRM quantitative ratios and sample variances of PTH peptides in samples from renal failure patients (Renal) and healthy controls. Ratios refer to the average value of the renal cohort divided by the average value of the healthy control cohort.

Conclusion

An SRM-MSIA-based analysis method was developed capable of simultaneously monitoring full-length PTH and truncated variants with analytical metrics suitable for clinical research use. Using a workflow incorporating postcapture tryptic digestion, surrogate peptides representative of PTH1–84 and PTH7–84 were generated and then monitored using SRM. In addition, tryptic fragments spanning other regions of PTH were incorporated into the analysis. Relative ion signals for these species confirmed that the clinical research method was functional and created the basis for a standard PTH profile. This standard profile was expanded to include a peptide representative of a novel variant, PTH34–84, clipped at the N-terminus. In total, 32 SRM transitions were analyzed in a multiplexed method to monitor nonvariant PTH sequences with >50% sequence coverage, as well as the two truncated variants. Peptides exhibited linear responses (R^2 = 0.90–0.99) relative to the limit of detection for an intact recombinant human PTH concentration of 8 ng/L. Limits of quantification were 16–31 ng/L, depending on the peptide. Standard error of analysis for all triplicate measurements was 3%–12% for all peptides, with <5% chromatographic drift between replicates. The CVs of integrated areas under the curve for 54 separate measurements of heavy peptides were 5%–9%.

Pinpoint software was used to develop and implement “intelligent SRM” data acquisition strategies, increasing instrument efficiency by avoiding the need to monitor all of the specified transitions at all times. Use of these techniques may be particularly advantageous for clinical research laboratories in methods where a large number of PTH variants are monitored, or where the analyzed sample contains a complex mixture of PTH-derived peptides and components produced by digestion of compounds in the sample matrix.

Acknowledgements

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Automated, High-Throughput LC-MS/MS Workflow for the Analysis of 25-Hydroxyvitamin $D_{2/3}$ and 3-*epi*-25-Hydroxyvitamin D_3

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Application Note 584

Key Words

Vitamin D, 25-hydroxyvitamin $D_{2/3}$, 3-*epi*-25-hydroxyvitamin D_3, TurboFlow method, Transcend, TSQ Vantage, Versette, LC-MS/MS

Goal

Develop an automated, high-throughput LC-MS/MS workflow for the analysis of 25-hydroxyvitamin $D_{2/3}$ and 3-*epi*-25-hydroxyvitamin D_3 in human serum for research laboratories.

Introduction

Analysis of total serum 25-hydroxyvitamin D_2/D_3 (25OHD, shown in Figure 1) is performed routinely in many research laboratories. Demand for this analysis continues to grow, and liquid chromatography with tandem mass spectrometry (LC-MS/MS) is increasingly used for this purpose.[1]

Compared with other sample preparation techniques, Thermo Scientific™ TurboFlow™ technology has been shown to significantly improve the removal of matrix components prior to LC-MS/MS analysis of 25OHD.[2] However, there are additional pre-analytical steps that must be performed, the most important of which are the complete removal of the analytes from the endogenous vitamin D binding protein and the addition of isotopically-labeled internal standards for quantitation. When performed manually, these steps can increase the total analysis time by approximately two hours for a batch of 96 samples. This application note presents a workflow that uses an automated liquid handling system to reduce the time required to prepare a 96 well plate for analysis to less than 20 minutes.

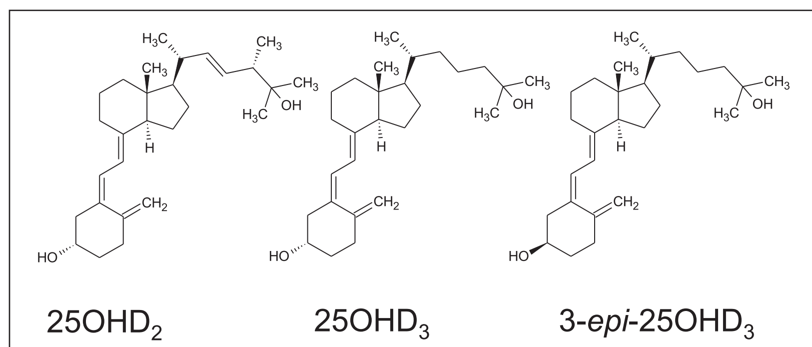

Figure 1. Structures of 25-hydroxyvitamin D_2/D_3

Further, MS/MS alone is an achiral technique. This can be problematic for some isobaric 25OHD metabolites, notably 3-*epi*-25-hydroxyvitamin D_3 (shown in Figure 1 as 3-*epi*-25OHD$_3$). For accurate LC-MS/MS analysis of 25OHD$_3$, LC-MS/MS extended chromatographic analysis times are needed to resolve 3-*epi*-25OHD$_3$. In this application note, multiplexing technology is used to maximize throughput of the chromatographic method used to resolve interfering 3-*epi*-25OHD$_3$.

Experimental

Sample Preparation

Human serum samples from the international Vitamin D External Quality Assessment Scheme (DEQAS, samples 404 and 405) were used for the analysis.

All liquid handling was carried out using a Thermo Scientific™ Versette™ automated liquid handling system. An overview of the liquid handling procedure is shown in Figure 2. The Versette system was fitted with a 96 channel pipetting head and Thermo Scientific™ D.A.R.T.'S™ 300 µL extended-tip disposable pipette tips. Calibration standards and quality controls (both from the Chromsystems MassChrom® 25-hydroxyvitamin $D_{2/3}$ kit) and samples (100 µL) were transferred from decapped 1 mL Thermo Scientific™ Nunc™ Cryobank storage vials to a 96 well filter plate. Internal standard solution (25 µL, $^2H_6$-25OHD$_3$) and precipitation reagent (200 µL), both from Chromsystems, were then added separately from the reagent reservoirs. The filter plates were covered and mixed on a plate shaker (600 rpm, 10 min). Supernatents were collected into a microtitre plate by centrifugation (200 *g*, 3 min). The plate was sealed with an adhesive plate seal and transferred to a Thermo Scientific™ Transcend™ TLX-2 system for analysis. All of the consumables utilized in the process are listed in Table 1.

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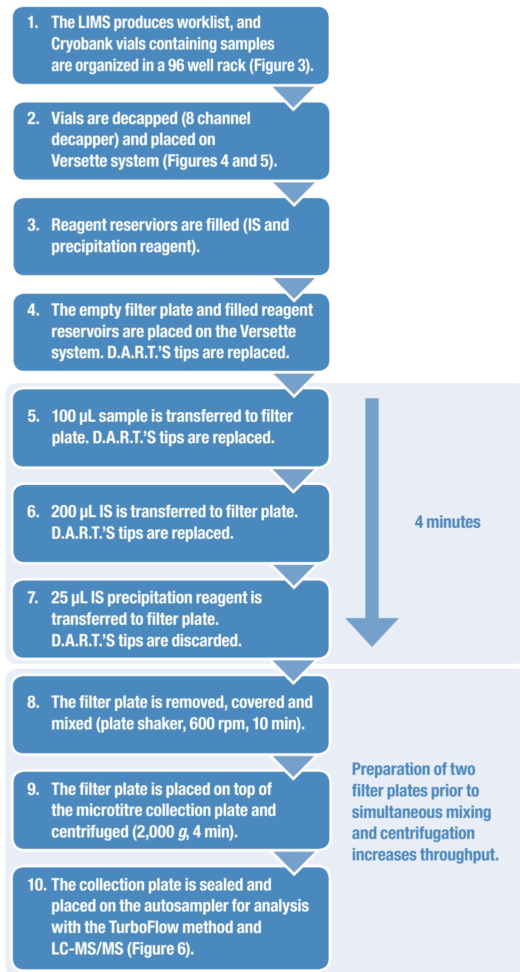

Figure 2. Liquid handling procedure

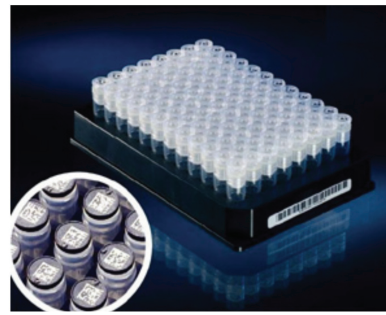

Figure 3. Cryobank vials

Figure 4. 8 Channel handheld screw cap capper/decapper

Figure 5. Versette liquid handling system and one rack of D.A.R.T.'S tips

Figure 6. Transcend system and TSQ Vantage mass spectrometer

Table 1. Parts and consumables required for specific steps in the liquid handling procedure

Step Number	Description	Part Number
1	1 mL Cryobank vials (96 tubes per rack)	374088
2	8 channel handheld screw cap capper/decapper with stand for Thermo Scientific Nunc-style tubes	4105NUN
3	Thermo Scientific™ Matrix™ 96 deep-well automation reservoir	1064-15-6
4	Filter plate	Chromsystems
2–8	Thermo Scientific Versette system	
	- Base unit	650-01-BS
	- 96 and 384 channel pipetting module	650-02-NTC
	- 6 position stage	650-03-SPS
	- 96 channel air disp. pipetting head, volume 5–300 µL	650-06-96300
4–7	Tips – 300 µL extended-length D.A.R.T.'S	5536
8	Titer plate shaker	4625-1CEQ
9	Thermo Scientific™ Heraeus™ Labofuge™ 400 with microplate rotor package	75008371
10	Nunc pierceable 96 well cap mats	276002
	Nunc polypropylene microplate - 96 well (1.0 mL)	260252

Liquid Chromatography

Sample supernatants (100 µL) were injected onto a TurboFlow XL C18 column (50 x 0.5 mm i.d.) under turbulent flow conditions (2 mL/min). Retained analytes were back-flushed from the TurboFlow column using elution solvent stored in a holding loop and focused onto a Thermo Scientific™ Accucore™ PFP analytical column (2.6 µm particle size, 50 x 2.1 mm i.d.) maintained at 40 °C. During isocratic elution (0.40 mL/min) from the analytical column, the TurboFlow column was back-flushed with eluent C and the elution solvent loop refilled. Eluent flow was diverted to waste for 8 minutes following each injection onto the TurboFlow columns. The system was then re-equilibrated prior to the next injection.

System eluents (Fisher Chemical™ brand) were as follows:

Loading and Eluting Pumps A:	0.1 % (v/v) aqueous formic acid
Loading and Eluting Pumps B:	0.1 % (v/v) formic acid in methanol
Loading Pump C:	Acetone/2-propanol/acetonitrile (1:2:2 v/v/v)

Mass Spectrometry

Mass spectrometry was carried out in positive ionization mode using atmospheric pressure chemical ionization (APCI) on a Thermo Scientific™ TSQ Vantage™ triple-stage quadrupole LC-MS/MS system. Selected-reaction monitoring (SRM) transitions did not include water-loss fragmentations.[3] MS/MS data were acquired for 5 minutes per analysis to allow multiplexing. Total LC time was 14 minutes, as shown in Figure 7, which means the TLX-2 system could do one analysis every 7 minutes).

Results and Discussion

As shown in Figure 7, retention times were 10.94, 11.47, and 11.82 minutes for 25OHD$_3$, 3-*epi*-25OHD$_3$, and 25OHD$_2$, respectively. Total analysis time was 14 minutes, including column re-equilibration. Sample 405 was correctly found to contain 3-*epi*-25OHD$_3$. This compound would have been misidentified as additional 25OHD$_3$ if a C18 analytical column, which does not resolve the epimer well, if at all, had been used as part of the LC-MS/MS method.

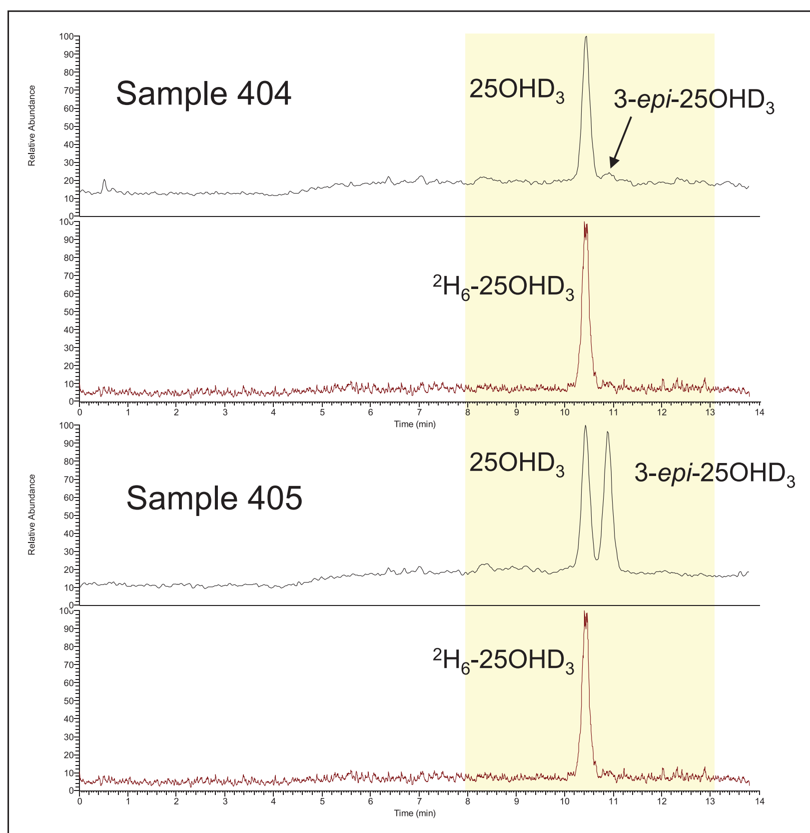

Figure 7. Chromatograms showing resolution of 3-*epi*-25OHD$_3$ from 25OHD$_3$ in DEQAS samples 404 and 405. 25OHD$_2$ chromatograms not shown. Sample 405 was prepared by addition of 3-*epi*-25OHD$_3$ to sample 404.[4] Yellow highlighted area corresponds to data window for multiplexing.

Conclusion

The Versette automated liquid handling system reduced the time required to prepare 96 samples from two hours to less than 20 minutes – a dramatic reduction of 80%. In addition, the Versette system reduced the number of manual pipetting steps from as many as 864 to none. The workflow minimized manual errors, increased method precision, and reduced the risk of repetitive strain injury in research laboratories.

Using multiplexing on the Transcend TLX-2 and an analytical column using a pentafluorophenyl stationary phase, the method resolved 3-*epi*-25OHD$_3$, an interferent in most LC-MS/MS 25OHD$_3$ methods, without significantly decreasing chromatographic throughput.

The same basic workflow employing automated liquid handling and automated online sample preparation can be used by research laboratories for the analysis of other compounds including mass spectrometric immunoassay-selective (MSIA) assays to measure parathyroid hormone (PTH) and vitamin D-binding protein.

Acknowledgements

Thanks to Graham Carter (DEQAS) for permission to quote DEQAS data.

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Analysis of 25-Hydroxyvitamin D in Serum Using an Automated Online Sample Preparation Technique with a High-Resolution Benchtop Orbitrap Mass Spectrometer

Application Note 567

Matthew Berube, Joe DiBussolo, Catherine Lafontaine, Yang Shi
Thermo Fisher Scientific, Franklin, MA

Key Words
Transcend TLX-1 System, TurboFlow Technology, Exactive Plus, Vitamin D

Goal

To demonstrate the effectiveness of a clinical research method for the quantitation of 25-hydroxyvitamin D using online sample preparation and high-resolution, accurate mass (HR/AM) quantitation with a Thermo Scientific Exactive Plus Orbitrap mass spectrometer.

Introduction

Blood levels of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 are commonly tested by clinical researchers to assess vitamin D sufficiency. In the last decade, liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MS/MS) has become a popular technique for such measurements. Due to their higher resolving power relative to triple-stage quadrupole mass spectrometers, Orbitrap™-based mass spectrometers are better able to resolve analytes from sample matrices. In addition, the ease of initial method set up and daily use provides an advantage over triple-stage quadrupole mass spectrometers for clinical research.

A method has been created that allows precipitated serum to be injected into an HPLC system with minimal sample preparation and analyzed by an Exactive™ Plus benchtop Orbitrap mass spectrometer. Total method time is 7.75 minutes on a Thermo Scientific Transcend TLX-1 system utilizing TurboFlow technology. Throughput can be increased to a sample every 3.7 minutes by using a Transcend™ TLX-2 multiplexed UHPLC system or 1.9 minutes with a Transcend TLX-4 system.

Experimental

Standard solutions of 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, and deuterated 25-hydroxyvitamin D_3 internal standard were obtained from Cerilliant, Inc. (Figure 1). Six calibrators at 2, 5, 10, 25, 50 and 100 ng/mL and three QCs at 5, 40 and 80 ng/mL were prepared by fortifying bovine serum albumin diluent with 200 ng/mL 25-hydryoxyvitamin D_2 and D_3 standard mix. Precipitating reagent was prepared by adding deuterated D_6-25-hydroxyvitamin D_3 to acetonitrile for a final concentration of 75 ng/mL. In addition, pooled human serum samples were crashed 2 to 1 with acetonitrile and spiked with analytes for a final concentration of 20 ng/mL for 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3, and 50 ng/mL of D_6 25-hydroxyvitamin D_3 internal standard.

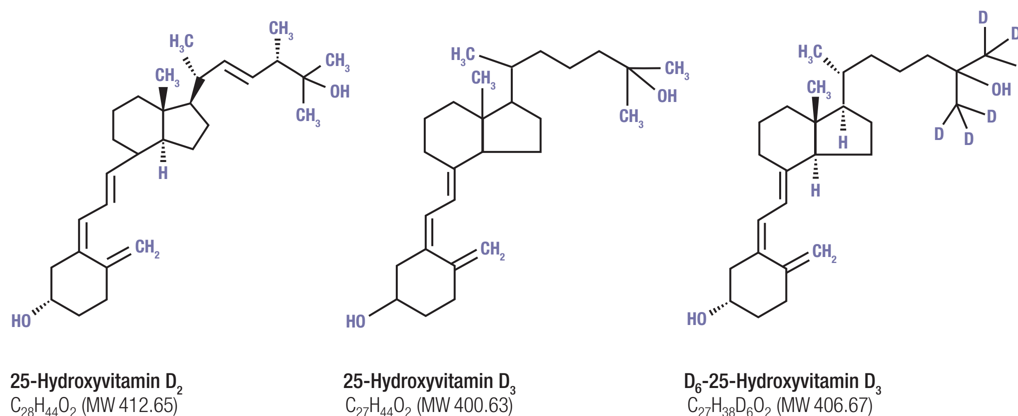

Figure 1. Analytes

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Samples were prepared by adding 200 µL of precipitating reagent containing internal standard to each centrifuge tube containing 100 µL of calibrants and controls. Tubes were vortexed for 30 seconds and then centrifuged at 5,000 RCF for 10 minutes. Supernatants were then aliquoted into autosampler vials for analysis. Calibration curves and QCs were run in triplicate each day across four days. In addition, 800 pooled serum sample replicates containing 20 ng/mL 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 and 50 ng/mL of D_6-25-hydroxy-vitamin D_3 internal standard were injected to test robustness of the method. Thermo Scientific Xcalibur software was used to collect data and analyze the results. The Exactive Plus mass spectrometer was used with an APCI source in positive ionization mode. Full-scan data was collected from *m/z* 350 to 425.

LC/MS Conditions

TurboFlow Method Parameters (see also Figure 2)	
Plumbing mode:	Focus Mode
Column:	Thermo Scientific TurboFlow XL C-18P 0.5 x 50 mm
Injection volume:	50 µL
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in methanol
Solvent C:	40:40:20 acetonitrile: isopropyl alcohol: acetone (v:v:v)
Analysis time:	7.75 minutes
Cycle time when multiplexed 4x:	1.9 minutes

HPLC Method Parameters	
Analytical column:	Thermo Scientific Accucore C18 3 x 50 mm 2.6 µm
Solvent A:	0.1% formic acid in water
Solvent B:	0.1% formic acid in methanol

Mass Spectrometer Parameters	
Scan mode:	Full
Scan range:	*m/z* 350 – 425
Fragmentation:	None
Polarity:	Positive
Microscans:	1
Resolution:	70,000
AGC target:	3×10^6
Maximum inject time:	200

Ion Source Parameters	
Ion source:	APCI
Discharge current:	3.5 uA
Vaporizer temperature:	500 °C
Sheath gas pressure:	30 units
Ion sweep gas pressure:	1 unit
Aux gas pressure:	5 units
Capillary temperature:	250 °C
S-Lens RF level:	60

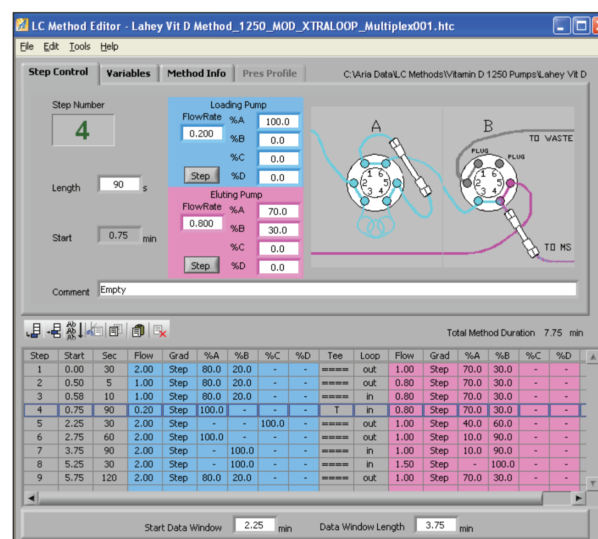

Figure 2. TurboFlow method details

Results and Discussion

The lower limit of quantitation (LLOQ) was determined to be 2 ng/mL for both analytes in BSA as indicated in Figure 3. Limits of quantitation (LOQs) were estimated from the triplicate injections of the standard solutions. The signal-to-noise ratio was greater than 10 and the coefficient of variation (CV) values were less than 10% at the LLOQ of 2 ng/mL for both 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 (Table 1). The correlation coefficients obtained using 1/X weighted linear regression analysis of the standard curves were greater than 0.99 for both analytes (Figures 4 and 5). A relative standard deviation (%RSD) test was performed in pooled human serum fortified with analytes at 20 ng/mL and crashed with internal standard solution for a total internal standard concentration of 50 ng/mL. The RSDs of ten replicate injections were less than 10% for both analytes (Table 2). A recovery study was also performed using a neat standard of 20 ng/mL 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 with 50 ng/mL D_6-25-hydroxy-vitamin D_3. The standard was injected ten times on the TurboFlow™ column and analytical column, and ten times on the analytical column only, and area counts were compared. The relative recoveries were 97% and 99% for 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3, respectively.

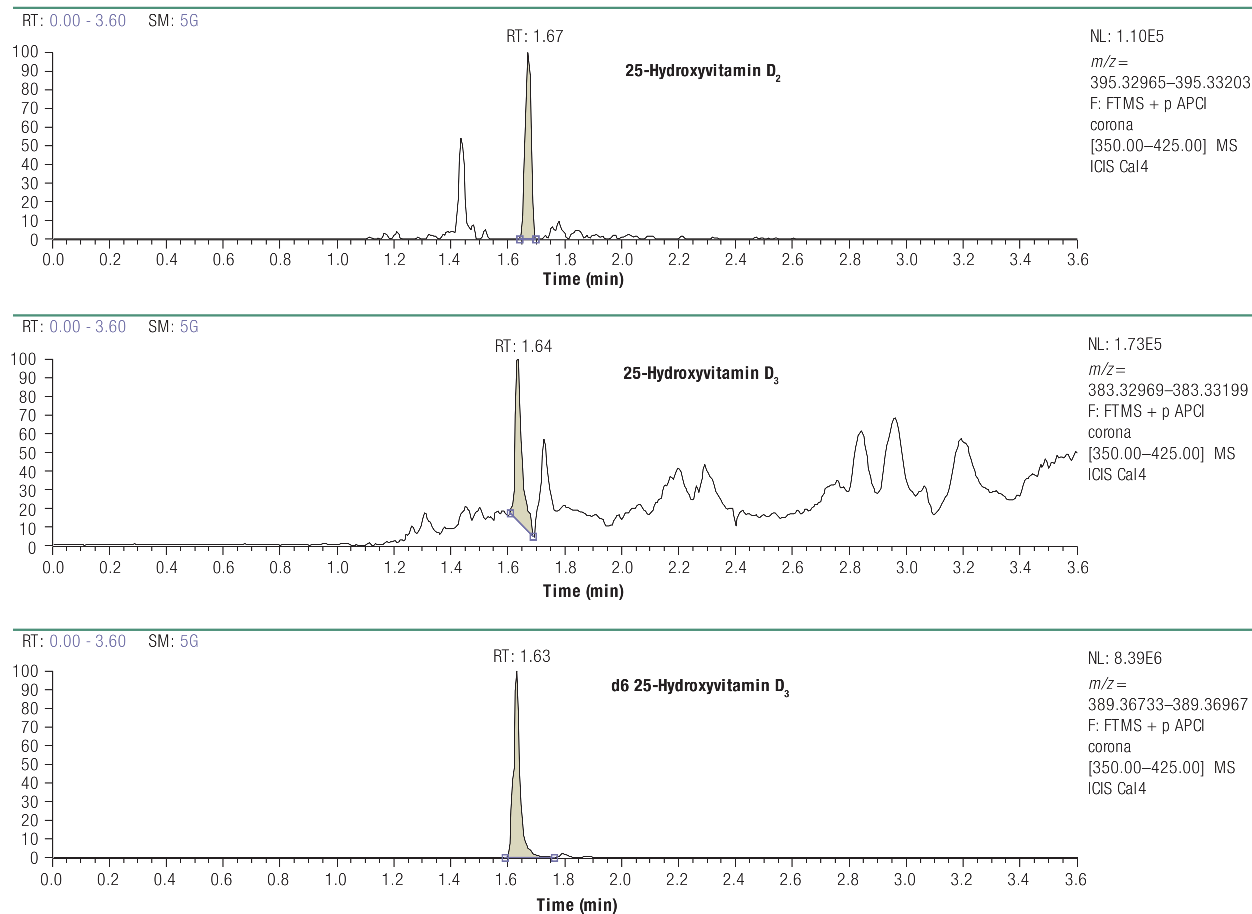

Figure 3. Chromatograms at LLOQ of 2 ng/mL with 50 ng/mL internal standard

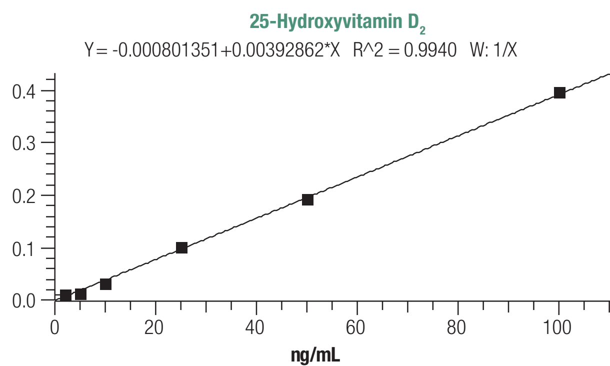

Figure 4. Calibration curve of 25-hydroxyvitamin D_2 in BSA

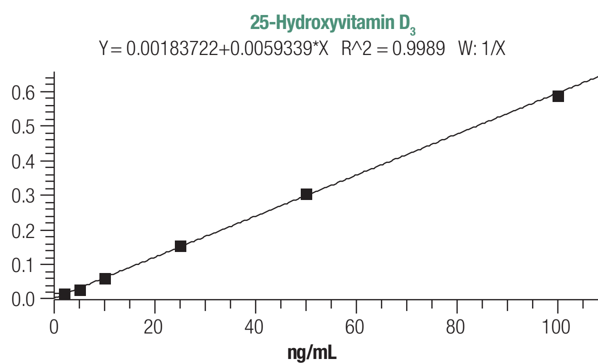

Figure 5. Calibration curve of 25-hydroxyvitamin D_3 in BSA

Table 1. 2 ng/mL replicate LLOQ injections

25-hydroxyvitamin D_2	2 ng Area
Replicate 1	134195
Replicate 2	162585
Replicate 3	148309
Mean	148363
SD	14195.1
%CV	9.6

25-hydroxyvitamin D_3	2 ng Area
Replicate 1	201766
Replicate 2	242186
Replicate 3	212094
Mean	218682
SD	20999.9
%CV	9.6

Table 2. 20 ng/mL serum injection replicates

D_2 20 ng Serum	Area
Replicate 1	4464244
Replicate 2	3757594
Replicate 3	4544819
Replicate 4	4332109
Replicate 5	3857037
Replicate 6	4581097
Replicate 7	5148234
Replicate 8	4704084
Replicate 9	4319873
Replicate 10	4175023
Mean	4388411
SD	405245.1
%CV	9.2

D_3 20 ng Serum	Area
Replicate 1	11759664
Replicate 2	10759647
Replicate 3	10886536
Replicate 4	10825748
Replicate 5	12543252
Replicate 6	12223745
Replicate 7	11278373
Replicate 8	11445949
Replicate 9	12537176
Replicate 10	11033701
Mean	11529379
SD	698829.3
%CV	6.1

Conclusion

An Exactive Plus high-resolution Orbitrap mass spectrometer with TurboFlow automated on-line sample extraction technology provides reliable detection for clinical researchers of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 in serum.

In addition, the Exactive Plus MS offers higher resolving power and easier initial method setup than triple quadrupole mass spectrometers. Throughput can be increased to a sample every 3.7 minutes by using a Transcend TLX-2 multiplexed UHPLC system or a sample every 1.9 minutes with a Transcend TLX-4 system.

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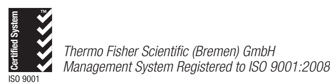

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Plasma Free Metanephrines Quantitation with Automated Online Sample Preparation and Liquid Chromatography—Tandem Mass Spectrometry

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Key Words

TSQ Vantage, Clinical Research, TurboFlow Technology, Metanephrine, MN, Normetanephrine, NMN, Pmets, Pheochromocytoma

Goal

To develop an automated method to quantitate plasma free metanephrines reducing method time while maintaining analytical performance compared to the original offline SPE method.

Introduction

Plasma free metanephrine (MN) and normetanephrine (NMN), collectively known as Pmets, are preferred biomarkers for pheochromocytoma for clinical research. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become widely used to measure Pmets because of its high analytical specificity.

Recently, we reported an LC-MS/MS method for measuring Pmets using ion-pairing solid phase extraction (IP-SPE) and porous graphitic carbon (PGC) column chromatography[1, 2]. Although the method is fast and analytically sensitive, it can be further improved by automating the offline sample preparation with online sample preparation technology, which is more time- and cost-effective.

Thermo Scientific TurboFlow technology is an automated online sample preparation technology that has been coupled to LC-MS/MS for the quantitative analysis of a variety of biological samples.

To date, its use has been reported in clinical research, pharmaceutical analysis, bioanalysis, environmental testing, food safety, and forensic toxicology.

Methods

Sample Preparation

The 0.5-mL samples of human plasma and of charcoal stripped serum (CSS) were spiked with internal standards (IS) and then mixed with 0.25 mL of 10% tricholoacetic acid (w/v) in water. The mixtures were vortexed and stored at –30 °C for 30 minutes. Then, the mixtures were centrifuged at 16,000 g for 10 minutes, and 100 µL of the supernatants were injected for LC-MS/MS analysis.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Thermo Scientific Transcend TLX-1 system. The TurboFlow™ method with automated online sample preparation was performed with a TurboFlow Cyclone MCX-2 column. Perfluoroheptanoic acid (PFHA) was used as the ion-pair during the sample preparation.

		Loading								Eluting					
Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B	%C	%D
00:00	30	2.00	Step	100.0	-	-	-	====	out	1.00	Step	98.0	2.0	-	-
00:30	30	2.00	Step	90.0	10.0	-	-	====	out	0.50	Step	90.0	-	-	10.0
01:00	90	0.10	Step	90.0	10.0	-	-	T	in	1.00	Step	90.0	-	-	10.0
02:30	1	0.10	Step	90.0	10.0	-	-	====	in	0.50	Step	98.0	2.0	-	-
02:31	300	0.50	Step	-	-	-	100.0	====	in	0.50	Ramp	60.0	40.0	-	-
07:31	60	2.00	Step	-	-	100.0	-	====	in	1.00	Step	-	100.0	-	-
08:31	60	2.00	Step	70.0	30.0	-	-	====	in	1.50	Step	-	-	100.0	-
09:31	150	2.00	Step	100.0	-	-	-	====	out	1.00	Step	98.0	2.0	-	-

Loading:
A: 0.1% PFHA in water
B: 60% ACN in water
C: Mixture of isopropanol, ACN and acetone (1:1:1 v/v/v) with 0.3% formic acid
D: 5 mM NH_4Ac and 50% ACN in water

Eluting:
A: 50 mM NH_4FA and 1% formic acid in water
B: 0.1% formic acid in ACN
C: Mixture of isopropanol, ACN and acetone (9:9:2 v/v/v)
D: 0.1% PFHA in water.

Eluting LC column temperature: 70 °C

Figure 1. TurboFlow and LC method

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Analytical separation was carried out on a Thermo Scientific Hypercarb column (50×3 mm, 5.0-µm particle size) at 70 °C. The total LC runtime was 12 minutes (Figure 1). The mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode. Data was acquired in selected-reaction monitoring (SRM) mode.

Validation

The validation procedure included tests for 1) recovery; 2) lower limit of quantitation (LLOQ), dynamic range, accuracy; 3) precision; 4) ion suppression; 5) carryover; and 6) interferences.

Results and Discussion

Charcoal stripped serum (CSS) was first evaluated by comparing it to human plasma using a generally adopted mixing study[3]. It was determined that CSS is an appropriate matrix to conduct the validation experiments.

Recovery

The extraction recovery was assessed by comparing the direct injection to the TurboFlow method injection of MN, NMN, MN-d3 and NMN-d3 spiked in mobile phase (n=2). The absolute recovery of MN, NMN and their IS ranged from 56.4% to 62.4%, and the relative recovery of MN and NMN was 90.9% and 97.8%, respectively (Table 1).

Determination of LLOQ, Linearity and Accuracy

CSS was spiked with MN and NMN to achieve final concentrations of 500 and 1000 pg/mL, respectively. A serial two-fold dilution with CSS was performed to make eight levels of linearity samples with concentration ranges of 500 to 3.9 pg/mL and 1000 to 7.8 pg/mL for MN and NMN, respectively. Linearity samples were analyzed in triplicate along with one set of calibrators. The calibration curve was constructed by plotting the analyte:IS peak area ratio vs. analyte concentration.

The linearity was determined to be 6.3 to 455.4 pg/mL for MN and 12.6 to 954.5 pg/mL for NMN. Within the linear range, the accuracy ranged from 80.6% to 93.5% for MN, and from 80.9% to 101.7% for NMN. The CV (n=3) from all linearity levels ranged from 3.1% to 13.7% for MN, and from 1.6% to 10.7% for NMN (Table 1 and Figures 2 and 3). The determined LLOQ was 6.3 pg/mL for MN and 12.6 pg/mL for NMN (Table 2).

Table 1. Recovery

	Online Extraction (mean ± CV)[b]	Direct Injection (mean ± CV)	Absolute Recovery (%)	Relative Recovery (%)
MN (500 pg/mL)[a]	60281 ± 2.7%	106866 ± 10.5%	56.4	90.9
NMN (250 pg/mL)[a]	32186 ± 5.6%	51878 ± 9.4%	62.0	97.8
MN-d3 (500 pg/mL)[a]	40716 ± 1.1%	66790 ± 11.4%	61.0	N/A
NMN-d3 (500 pg/mL)[a]	28983 ± 3.7%	46482 ± 11.8%	62.4	N/A

[a] MN, NMN, MN-d3 and NMN-d3 were spiked to mobile phase at specified concentration levels.

[b] Measured peak area with CV (n=2)

Table 2. LLOQ, dynamic range and accuracy

	MN				NMN			
Dilution factor	**Expected (pg/mL)**	**Measured (pg/mL)**	**CV of triplicates (%)**	**Accuracy (%)**	**Expected (pg/mL)**	**Measured (pg/mL)**	**CV of triplicates (%)**	**Accuracy (%)**
128	3.91	5.5	17.2	71.1	7.8	7.4	35.3	94.9
64	7.81	6.3	13.7	80.6	15.6	12.6	10.7	80.9
32	15.6	13.9	7.2	88.8	31.3	30.8	1.6	98.7
16	31.3	27.5	4.9	88.0	62.5	61.0	6.0	98.1
8	62.5	56.6	10.3	90.6	125.0	121.2	9.2	96.9
4	125.0	112.2	4.0	89.8	250.0	254.2	9.4	101.7
2	250.0	233.7	3.1	93.5	500.0	496.9	2.7	99.4
1	500.0	455.4	4.0	91.1	1000.0	954.5	3.3	95.5
Mean (%)				**88.9**				**95.9**
Stdev (%)				**4.1**				**6.9**

Precision

Precision was assessed with spiked CSS. Inter- and intra-assay CV values at low and high quality control concentrations of both analytes varied between 2.0% and 10.5% (Table 3).

Table 3. Precision data

	MN		NMN	
Charcoal Stripped Serum	31.3 pg/mL	250.0 pg/mL	62.5 pg/mL	500.0 pg/mL
Intra 1 (%) n=5	6.7	4.2	4.5	5.4
Intra 2 (%) n=5	4.9	3.0	10.5	4.2
Intra 3 (%) n=5	7.3	4.7	10.0	2.0
Inter-assay (%) n=15	8.4	7.7	8.9	4.8

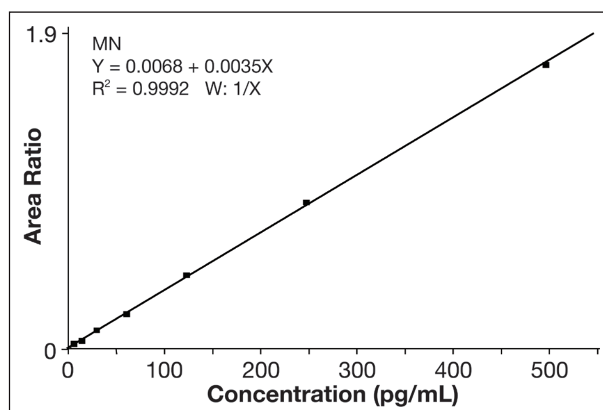

Figure 2. Calibration curve of MN in CSS

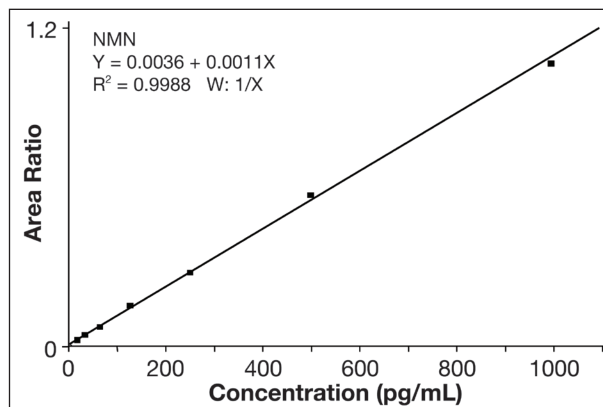

Figure 3. Calibration curve of NMN in CSS

Ion Suppression

The MS responses of MN-d3 and NMN-d3 in solvent (n=4) and individual human plasma samples (n=4) at the same concentrations (400 pg/mL for both MN-d3 and NMN-d3) were measured with LC-MS/MS analysis. The average MS responses (integrated area) of MN-d3 and NMN-d3 from solvent and real human plasma samples were calculated. The intensity ratios with standard deviations between human plasma (n=4) and solvent (n=4) were 113.3% ± 18.4% and 126.4% ± 18.0% for MN-d3 and NMN-d3, respectively. This indicated that this method has no obvious ionization suppression or enhancement.

Carryover

No carryover was observed.

Interferences

Epinephrine (EPI) and NMN share the same SRM transitions and could not be differentiated just by MS/MS analysis. Using the Hypercarb™ analytical column, the EPI peak was baseline resolved from the NMN peak (0.3 min apart, data not shown).

Data Examples of Clinical Research Samples

Figure 4 shows the SRM chromatograms of MN and NMN in an individual plasma sample. Figure 5 shows the SRM chromatograms of MN and NMN in a CSS sample.

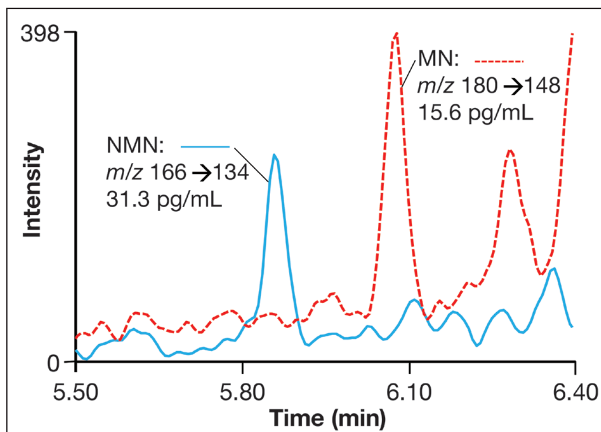

Figure 4. SRM chromatograms of MN and NMN in human plasma sample

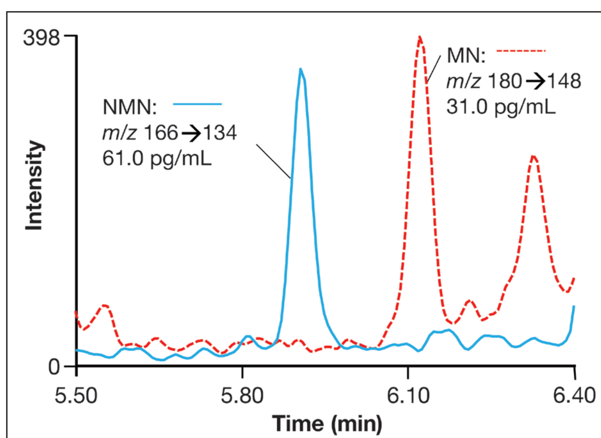

Figure 5. Representative SRM chromatograms of MN (31.0 pg/mL) and NMN (61.0 pg/mL) in CSS sample

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Conclusion

A fast, automated and analytically sensitive LC-MS/MS method was developed to quantify plasma metanephrines for clinical research purposes[4]. By using TurboFlow technology, the sample preparation procedure was significantly simplified compared to a previously reported offline IP-SPE method. The presence of PFHA during the online sample preparation was critical to the success of this method. A PGC column was used for chromatographic separation of metanephrines. The total online extraction and analytical LC runtime was 12 minutes. This method was linear from 6.3 to 455.4 pg/mL for metanephrine and 12.6 to 954.5 pg/mL for normetanephrine, with an accuracy of 80.6% to 93.5% and 80.9% to 101.7%, respectively. The lower limit of quantitation was 6.3 pg/mL for metanephrine and 12.6 pg/mL for normetanephrine. Inter-assay and intra-assay precision for metanephrine and normetane-phrine at low and high concentration level ranged from 2.0% to 10.5%.

Overall, the analytical performance achieved with this automated online TurboFlow method is consistent with the previously reported offline SPE method[2]. More importantly, the online method significantly saved sample preparation time by more than 50% and eliminated the expense of SPE cartridges with an offline approach.

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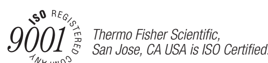

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Application Note: 558

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Quantitation of Estrone and Estradiol with Automated Online Sample Preparation and LC-MS/MS

Xiang He and Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Key Words

- **TSQ Vantage**
- **Transcend TLX System**
- **Accucore RP-MS Column**
- **Clinical Research**
- **TurboFlow Technology**

Introduction

Estrone (E1) and estradiol (E2) are two major biologically active estrogens. Quantitative measurements of these two estrogens are important in clinical research.

Quantitation of serum estrogens has been performed with immunoassay and gas chromatography-mass spectrometry (GC-MS). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is preferred over immunoassay and other analytical techniques because it is more analytically specific. Recently, we developed a simple, fast and analytically sensitive method for measuring underivatized E1 and E2 in serum or plasma by LC-MS/MS using atmospheric pressure chemical ionization (APCI).[1]

Thermo Scientific TurboFlow technology is an automated online sample preparation technology that has been coupled to LC-MS/MS for the quantitative analysis of a variety of biological samples. To date, its use has been reported in the fields of clinical research, pharmaceutical analysis, bioanalysis, environmental testing, food safety, and forensic toxicology.

Goal

To develop a fast and analytically sensitive LC-MS/MS method with automated online sample preparation for simultaneous quantitation of underivatized E1 and E2 in serum using TurboFlow™ technology.

Methods

Sample Preparation

Briefly, 0.5 mL of sample was mixed with 0.5 mL of working internal standard (E2-d5, IS) solution in methanol. The mixture was vortexed, kept at -30 °C for 30 min and then centrifuged at 16,000 g for 3 min at room temperature. This process was repeated once for complete protein precipitation. The supernatant (300 µL) was directly injected for TurboFlow LC-MS/MS analysis.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Thermo Scientific Transcend TLX-1 system equipped with Accela 1250 pumps. The online sample preparation was performed with TurboFlow Cyclone-P polymer-based columns. Analytical high-performance liquid chromatography (HPLC) was carried out on a Thermo Scientific Accucore RP-MS solid core column (100 × 3 mm, 2.6 µm particle size) at room temperature using water and methanol as mobile phases (Figure 1). The total runtime was 10 min. The mass spectrometer was operated with an APCI source in negative ion mode. Data was acquired in selected reaction monitoring (SRM) mode.

			Loading								Eluting					
Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B	%C	%D
1	0.00	45	2.00	Step	100.0	-	-	-	====	out	0.60	Step	100.0	-	-	-
2	0.75	60	0.10	Step	100.0	-	-	-	T	in	0.60	Step	100.0	-	-	-
3	1.75	60	2.00	Step	-	100.0	-	-	====	in	0.60	Ramp	40.0	60.0	-	-
4	2.75	120	2.00	Step	100.0	-	-	-	====	in	0.60	Ramp	20.0	80.0	-	-
5	4.75	60	2.00	Step	-	100.0	-	-	====	in	0.60	Step	-	100.0	-	-
6	5.75	90	2.00	Step	100.0	-	-	-	====	out	0.60	Step	100.0	-	-	-

Loading: A: water; B: methanol. **Eluting:** A: water; B: methanol.

Figure 1. TurboFlow and LC method

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Validation

The validation procedure included tests for 1) recovery of sample preparation; 2) lower limit of quantitation (LLOQ), dynamic range, accuracy; 3) precision; 4) ionization suppression; and 5) carryover.

Results and Discussion

Human plasma has endogenous E1 and E2 so it was not suitable for validation experiments except the precision study. Therefore, charcoal stripped serum (CSS) is used to conduct the validation experiments.

Recovery

The absolute recoveries of E1, E2 and IS from CSS samples compared to spiked neat solutions ranged from 61.2% to 65.6%. The relative recoveries of E1 and E2 against IS ranged from 99.0% to 107.1% at the two spiked concentration levels (20 and 100 pg/mL).

Determination of LLOQ, Linearity and Accuracy

A stock solution of E1 and E2 at 1000 pg/mL was prepared in CSS. A serial 2-fold dilution with blank CSS was performed to make 9 levels of linearity samples with concentrations from 1000 to 3.9 pg/mL for both E1 and E2. Linearity samples were analyzed in triplicate. The calibration curve was constructed by plotting the analyte:IS peak area ratio vs. expected analyte concentration.

The method was linear between 3.8 and 1000.9 pg/mL with accuracy (n=3) from 95.5% to 103.2% for E1, and between 3.7 and 993.1 pg/mL with accuracy (n=3) from 92.7% to 112.3% for E2 (Table 1, Figures 2 and 3). The LLOQ for E1 and E2 are 3.8 and 3.7 pg/mL, respectively (Table 1 and Figure 4).

Table 1. LLOQ, dynamic range and accuracy

		E1			E2		
Dilution Factor	**Expected (pg/mL)**	**Measured (mean, pg/mL)**	**CV (n=3 %)**	**Accuracy (n=3, %)**	**Measured (mean, pg/mL)**	**CV (n=3, %)**	**Accuracy (n=3, %)**
256	3.9	**3.8**	5.0	97.8	**3.7**	11.7	94.6
128	7.8	8.0	9.0	102.9	8.8	13.9	112.3
64	15.6	16.1	5.1	102.8	15.7	7.4	100.4
32	31.3	32.2	8.4	103.2	29.0	7.6	92.7
16	62.5	59.7	0.8	95.5	62.7	4.4	100.3
8	125.0	123.3	9.9	98.7	129.4	9.8	103.5
4	250.0	245.9	7.0	98.4	253.1	3.7	101.2
2	500.0	503.5	2.3	100.7	478.9	4.1	95.8
1	1000.0	**1000.9**	4.5	100.1	**993.1**	5.3	99.3
Mean				**100.0**			**100.0**

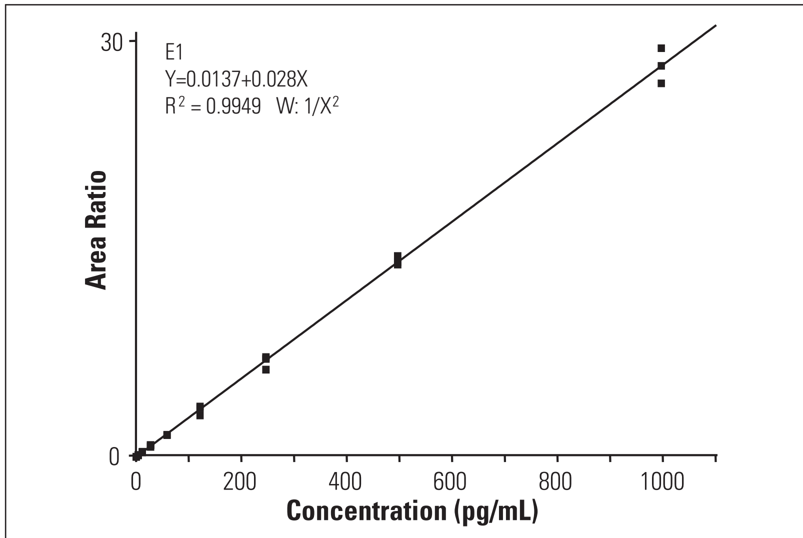

Figure 2. Calibration curve of E1 in CSS

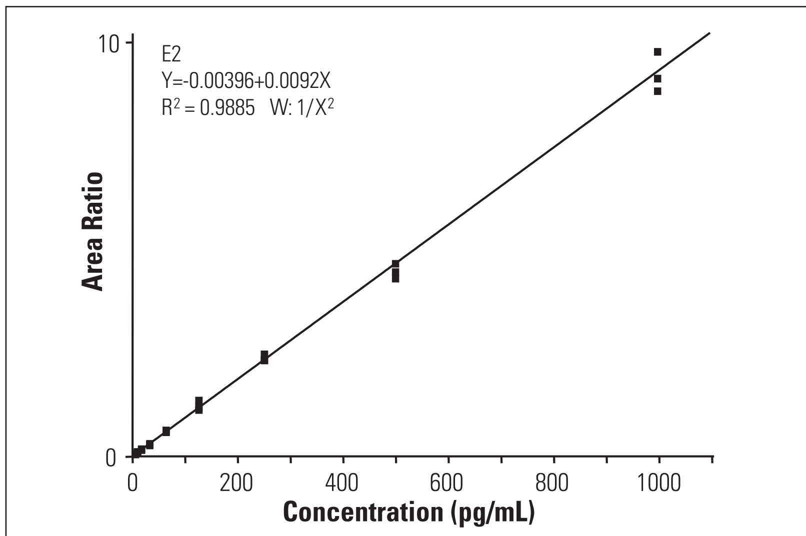

Figure 3. Calibration curve of E2 in CSS

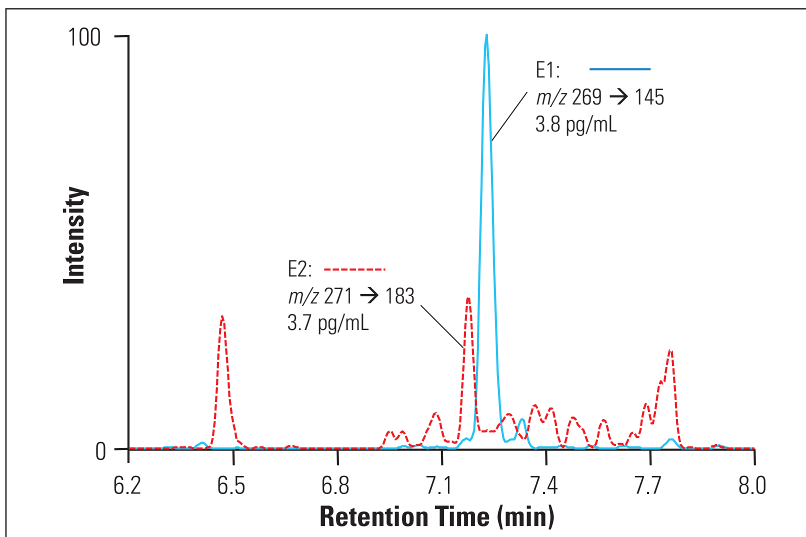

Figure 4. SRM chromatograms of E1 and E2 at their LLOQ in spiked CSS

Precision

Precision was assessed with spiked CSS and human plasma at low and high concentration levels. Inter- (n=15) and intra-batch (n=5) coefficient of variation (CV) values ranged between 3.5% and 18.0% (Table 2).

Table 2. Precision data

		E1		E2	
	Charcoal Stripped Serum	**Low (15 pg/mL)**	**High (364 pg/mL)**	**Low (15 pg/mL)**	**High (357 pg/mL)**
Batch 1	Intra-assay Precision (n=5, %)	9.9	9.6	13.5	11.5
Batch 2	Intra-assay Precision (n=5, %)	17.1	3.5	12.4	4.3
Batch 3	Intra-assay Precision (n=5, %)	14.6	7.2	17.2	4.8
Batch 1-3	Inter-assay Precision (n=15, %)	13.1	8.1	14.0	8.4
	Spiked Pooled Plasma	**Low (12 pg/mL)**	**High (239 pg/mL)**	**Low (11 pg/mL)**	**High (227 pg/mL)**
Batch 1	Intra-assay Precision (n=5, %)	5.3	5.8	18.0	7.9
Batch 2	Intra-assay Precision (n=5, %)	12.9	7.1	16.3	4.3
Batch 3	Intra-assay Precision (n=5, %)	10.0	6.8	12.3	9.0
Batch 1-3	Inter-assay Precision (n=15, %)	9.3	6.3	17.3	7.1

Ionization Suppression

In this test, a constant flow (5 µL/min) of E2-d5 (100 ng/mL) was infused post-column into the mobile phase using a T-junction while protein-crashed human plasma (without internal standards) or mobile phase buffer (blank) were injected. An SRM transition of the infused E2-d5 was monitored for the entire LC gradient. Compared to the solvent blank (60% methanol in water), no obvious ionization suppression was detected in the SRM chromatogram of infused E2-d5 (Figure 5).

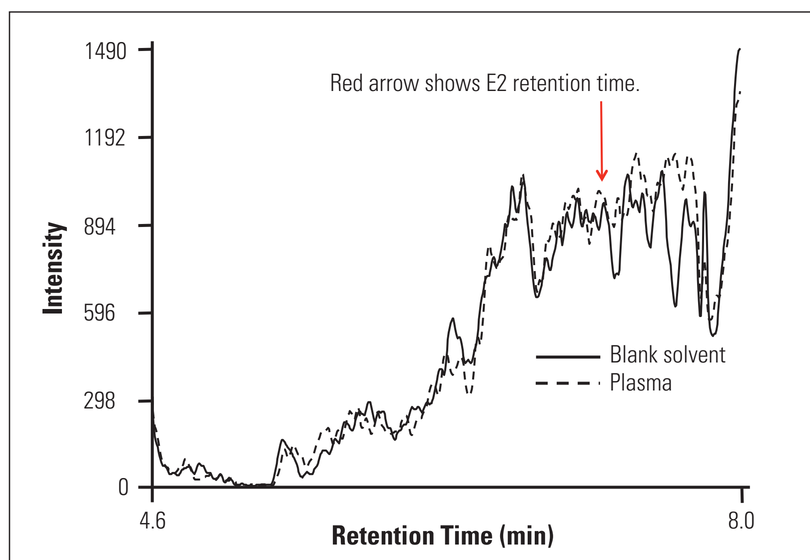

Figure 5. Ionization suppression test

Carryover

CSS was spiked with E1 and E2 to create a high-level sample (>500 pg/mL) and a low-level sample (8 pg/mL). The low-level sample was injected first (Low1) for LC-MS/MS analysis followed by the injection of the high-level sample (High). Immediately afterward, another low-level sample was injected (Low2). No carryover was observed by testing the spiked CSS samples with Low1 (9.9 pg/mL)-High (556.0 pg/mL)-Low2 (9.1 pg/mL) for E1 and Low1 (10.0 pg/mL)-High (582.5 pg/mL)-Low2 (8.9 pg/mL) for E2.

Data examples of clinical research samples

Figures 6 and 7 show the SRM chromatograms of E1 and E2 in two individual plasma samples.

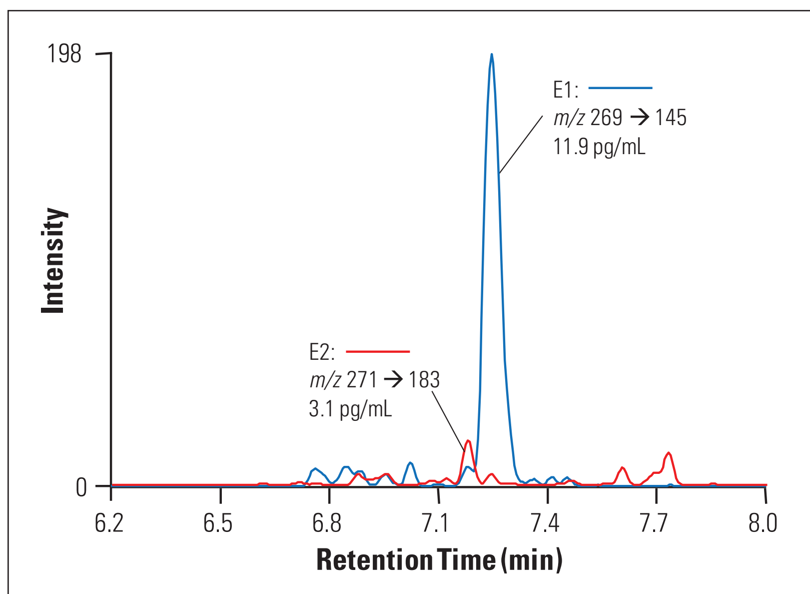

Figure 6. SRM chromatograms of E1 and E2 in human plasma sample 1 (female)

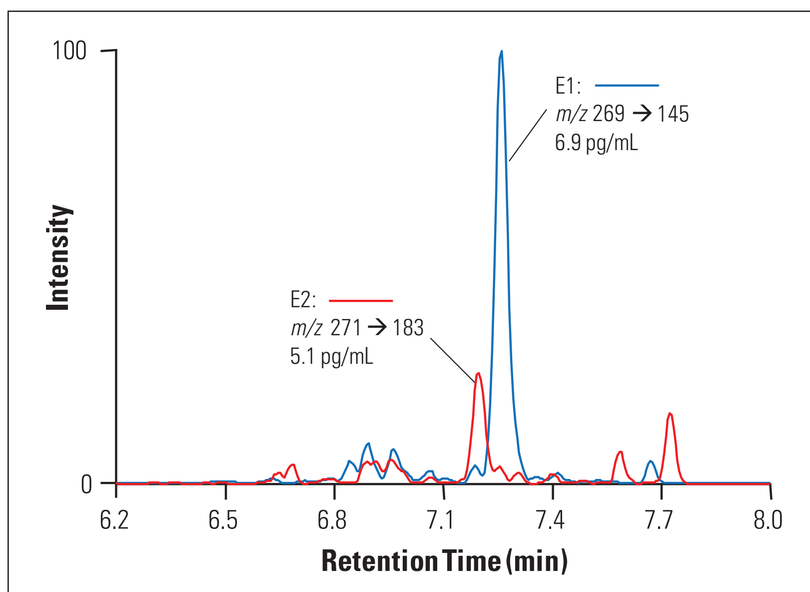

Figure 7. SRM chromatograms of E1 and E2 in human plasma sample 2 (male)

Conclusion

We have developed a novel 10-min LC-MS/MS method for quantitation of E1 and E2 in serum using TurboFlow technology for clinical research laboratories. This method is fast and analytically sensitive and sample preparation effort is significantly reduced. The Accucore HPLC column was used for analytical LC separation because of its superior performance. The lower limit of quantitation was 3.8 pg/mL for estrone and 3.7 pg/mL for estradiol. This method was linear from 3.8 to 1000.9 pg/mL for estrone and 3.7 to 993.1 pg/mL for estradiol with accuracy from 95.5% to 103.2% for estrone and from 92.7% to 112.3% for estradiol, respectively. Inter-assay and intra-assay CV for estrone and estradiol at low and high concentration levels in both spiked charcoal stripped serum and pooled human plasma ranged from 3.5% to 18.0%.

Reference

1. Xiang He and Marta Kozak, Fast and Sensitive LC-APCI-MS/MS Quantitative Analysis of Estrone and Estradiol in Serum without Chemical Derivatization, Thermo Fisher Scientific Application Note 530.

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Quantitative Analysis of Serum 1α,25-dihydroxyvitamin D by APPI-LC-MS/MS

Xiang He, Marta Kozak; Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- TSQ Vantage
- Clinical Research
- Endocrine Analysis

Introduction

Quantitation of 1α,25-dihydroxyvitamin D_2 and D_3 (1,25D) in serum is very important in clinical research but is challenging because of the low circulating serum concentration of 1,25D. Due to its high analytical specificity and sensitivity, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been used for quantitation of 1,25D.

We have previously reported the use of immunoextraction and atmospheric pressure chemical ionization (APCI) for LC-MS/MS analysis of 1,25D in human serum[1]. Immunoextraction greatly simplifies the sample preparation and efficiently removes interferences. In addition, while APCI is good for this analysis, atmospheric pressure photoionization (APPI) is a more specific ionization technique than APCI and, therefore, further improves the analytical sensitivity of 1,25D detection.

Goal

To develop a highly sensitive LC-MS/MS analytical method to quantitate 1,25D with APPI using immunoextraction that provides better sensitivity than an APCI method.[1]

Methods

Sample Preparation

Serum 1,25D was purified with an immunoextraction method using an ImmunoTube® immunoextraction tube (Immundiagnostik AG, Bensheim, Germany). Briefly, samples were mixed with immobilized 1,25D antibody slurry and incubated at room temperature for 1 hour before the 1,25D-antibody beads were washed with aqueous buffer. Then, 1,25D_2 and 1,25D_3 were eluted with ethanol, dried, and reconstituted for LC-MS/MS injection.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Thermo Scientific Accela UHPLC system. A Thermo Scientific Hypersil GOLD column (150 × 1 mm, 3 µm particle size) was used. The column temperature was maintained at 50 °C. Mobile phases were 70% methanol in water and methanol from Fisher Chemical brand. The LC method used a 10-minute gradient, and the LC flow was diverted to the mass spectrometer between 2 and 5 minutes.

The mass spectrometer was equipped with an APPI probe and operated in the positive ion mode. Selected reaction monitoring (SRM) transitions of 1,25D_2, 1,25D_3, d6-1,25D_2 and d6-1,25D_3 were monitored (see Table 1).

Table 1. SRM transitions

	Q1 *(m/z)*	Q3 *(m/z)*	CE (V)	S-Lens (V)
1,25D_2	411.3	135.0	19	87
		151.0	20	87
1,25D_3	399.2	135.0	21	90
		151.0	22	90
d6-1,25D_2	417.3	151.0	19	95
d6-1,25D_3	405.3	151.0	20	90

Validation

The validation procedure included tests for 1) recovery, linearity, and lower limit of quantitation (LLOQ) and 2) precision.

Results and Discussion

1. Sample Preparation

The immobilized 1,25D antibody used in this study was highly specific and had no cross-reactivity from other vitamin D derivatives. Serum samples processed with immunoextraction showed no matrix effects or ionization suppression.

2. Recovery, Linearity, and LLOQ

Two sets of calibrators were prepared in ethanol (solvent) and pooled human plasma sample. Human plasma contains endogenous 1,25D, so it is not an appropriate choice to be used as the matrix for calibrators. Different levels of 1,25D were spiked into both solvent and human plasma to evaluate the feasibility of using solvent as the calibrator matrix. Solvent calibrators were prepared without immunoextraction, but with drying and reconstituting steps. Endogenous concentrations of 1,25D in pooled plasma were determined with solvent calibrators first. The pooled human plasma samples were then spiked with increasing levels of 1,25D and processed with immunoextraction. Concentrations of total 1,25D (endogenous and spiked concentration) in plasma were determined against solvent

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calibrators and compared to expected concentrations to calculate recovery (Table 2).

Table 2. Recovery

1,25D_2			1,25D_3		
Expected (pg/mL)	Measured (pg/mL)	Recovery (%)	Expected (pg/mL)	Measured (pg/mL)	Recovery (%)
43.7	45.0	103.0	11.1	11.1	100.0
48.7	47.3	97.2	16.1	17.4	108.5
58.7	57.0	97.1	26.1	27.7	106.5
88.7	99.5	112.2	56.1	57.6	102.7
238.7	235.9	98.8	206.1	203.2	98.6

The slopes of the calibration curves of 1,25D_2 and D_3 in both solvent and pooled human plasma calibrators were compared and found to be nearly identical (Figures 1 and 2). This indicated that 1,25D originated from spiked solvent and 1,25D originated from human plasma behaved similarly relative to their corresponding IS during the whole process of immunoextraction and LC-MS/MS.

The method was linear between 5 and 200 pg/mL for both 1,25D_2 and 1,25D_3. The LLOQ was 5 pg/mL for both 1,25D_2 and D_3. Figure 3 shows the representative SRM chromatograms of 1,25D_2 and 1,25D_3 of the lowest calibrator in solvent and pooled human plasma.

3. Precision

Precision was determined with spiked charcoal stripped serum at both 10 and 20 pg/mL, which are close to the LLOQ (Table 3).

Table 3. Precision

1,25D_2	Measured (pg/mL)	Accuracy (%)	Precision (%)
10 pg/mL	9.1	90.8	8.4
20 pg/mL	19.8	99.2	7.4
1,25D_3			
10 pg/mL	9.9	98.8	12.5
20 pg/mL	20.9	104.4	11.1

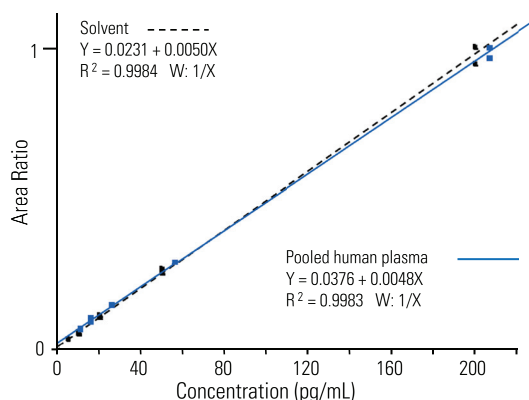

Figure 1. Calibration curves of 1,25D_2 in solvent (dotted line, black) and pooled human plasma (solid line, blue)

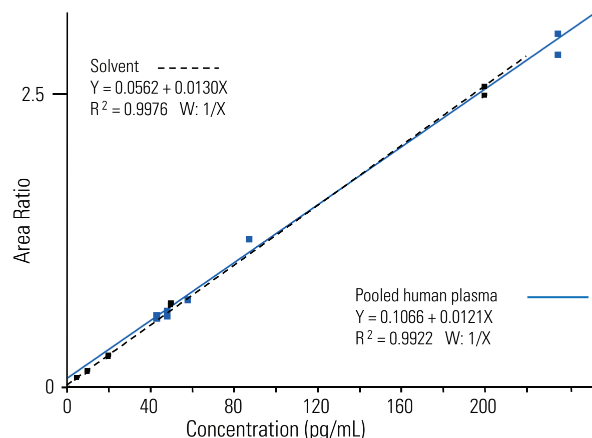

Figure 2. Calibration curves of 1,25D_3 in solvent (dotted line, black) and pooled human plasma (solid line, blue)

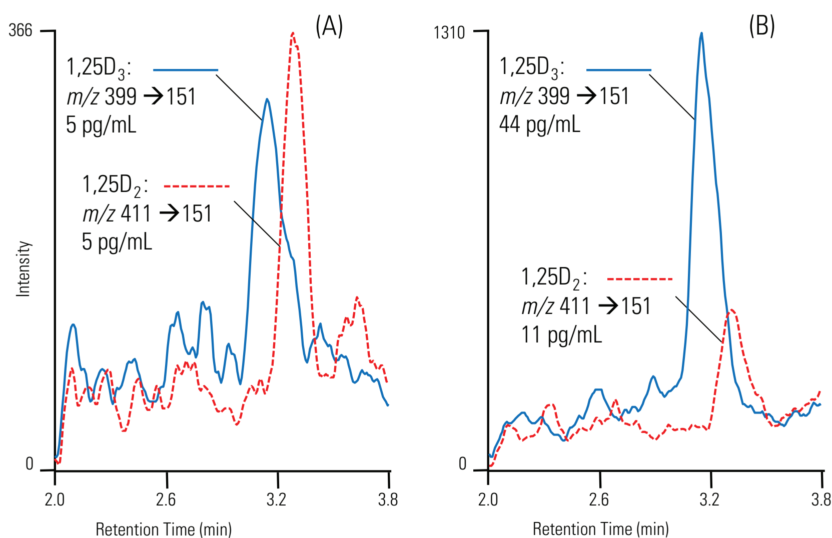

Figure 3. Representative SRM chromatograms of $1,25D_2$ and $1,25D_3$ of the lowest calibrator in solvent (A) and in pooled human plasma (B)

Conclusion

A fast and analytically sensitive LC-MS/MS method for quantitation of 1,25D in human plasma was developed for clinical research laboratories. Sample preparation was done with immunoextraction. APPI ionization was used for its ionization specificity and sensitivity. The LLOQ of this method was 5 pg/mL for both $1,25D_2$ and $1,25D_3$.

Reference

1. He, X; Damkroger, G; Kozak, M. *Quantitative Analysis of 1,25-dihydroxyvitamin D_2 and D_3 using Immunoaffinity Extraction with APCI-LC-MS/MS*, Thermo Fisher Scientific Application Note: 522.

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Quantitative Measurement of Plasma Free Metanephrines by Ion-Pairing Solid Phase Extraction and LC-MS/MS with Porous Graphitic Carbon Column

Xiang He, Marta Kozak; Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- TSQ Vantage
- Hypercarb HPLC column
- Clinical Research
- LC-MS/MS

Introduction

Plasma free metanephrine (MN) and normetanephrine (NMN), collectively known as Pmets, are important molecules for clinical research. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become widely used to measure Pmets because of its high analytical sensitivity and specificity.

Because Pmets are very polar, special solid phase extraction (SPE) and chromatographic methods have been developed for their analysis. Ion-paring (IP)-SPE, which has been used to purify a wide range of polar compounds, is well suited for the purification of Pmets.

Goal

To develop an LC-MS/MS method for measuring Pmets using IP-SPE and porous graphitic carbon (PGC) column chromatography.

Methods

Sample Preparation

Thermo Scientific HyperSep C-18 cartridges (1 mL) were preconditioned with acetonitrile and 0.1% perfluoroheptanoic acid (PFHA) before samples were loaded. After sample loading, cartridges were washed with 0.1% PFHA and eluted with 60% acetonitrile. The eluate was dried and reconstituted for LC-MS/MS analysis.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Thermo Scientific Accela UHPLC system. A Thermo Scientific Hypercarb column (50 × 2.1 mm, 5 µm particle size) was used. This PGC-based column is highly durable and ideal for retaining and resolving very polar and hydrophilic molecules. The column temperature was maintained at 70 °C. Mobile phases were 1% formic acid in water with ammonium formate, and 0.1% formic acid in acetonitrile. The LC gradient was 7 minutes long.[1]

The mass spectrometer was equipped with a heated electrospray ionization probe (HESI-II) and operated in the positive electrospray ionization mode. MN-d3 and NMN-d3 were used as the internal standards for MN and NMN.

Validation

The validation procedure included tests for 1) interference; 2) SPE recovery; 3) ion suppression; 4) lower limit of quantitation (LLOQ), dynamic range, accuracy; 5) precision; and 6) carryover.

Results and Discussion

1. Interference

Epinephrine (EPI) and NMN share the same selected reaction monitoring (SRM) transitions and could not be differentiated by MS/MS analysis alone. With Hypercarb™ column chromatography, the EPI-d3 peak was baseline resolved from the NMN-d3 peak (Figure 1).

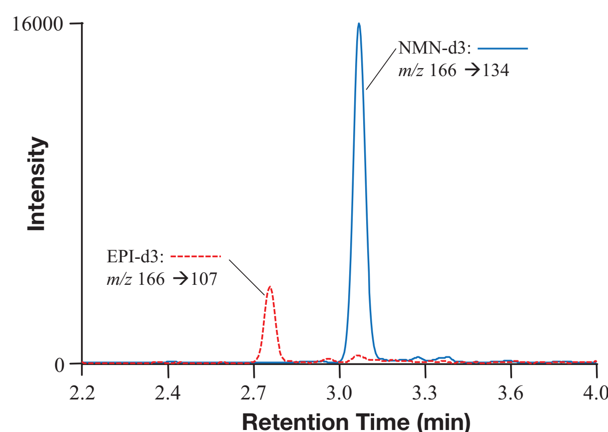

Figure 1. SRM chromatograms of EPI-d3 and NMN-d3 in a processed CSS sample

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2. SPE Recovery

Extraction efficiency was assessed in charcoal stripped serum (CSS, n=3). Absolute recovery of Pmets and IS ranged from 86.4% to 97.5%, and the relative recovery of MN and NMN was 97.7% and 113.5%, respectively (Table 1).

Table 1. SPE Recovery

In Charcoal Stripped Serum	Spiked before SPE[a] (mean ± CV)	Spiked after SPE[b] (mean ± CV)	Absolute Recovery (%)	Relative Recovery (%)
MN (n=3)	22865 ± 13.9%	25265 ± 9.3%	90.5	97.7
NMN (n=3)	11165 ± 11.1%	11453 ± 12.5%	97.5	113.5
MN-d3 (n=3)	27809 ± 7.2%	30140 ± 12.9%	92.3	n/a
NMN-d3 (n=3)	22627 ± 9.2%	26192 ± 4.5%	86.4	n/a

[a] *Measured peak area of charcoal stripped serum spiked with 100, 400, 400, and 1600 pg/mL of MN, NMN, MN-d3, and NMN-d3, respectively, before SPE*

[b] *Measured peak area when equivalent amounts of above compounds were spiked after SPE*

3. Ion Suppression

Results from the post-column infusion experiments are shown in Figure 2. Compared to injections of blanks, no obvious ion suppression was detected in the SRM chromatograms of MN-d3 and NMN-d3 using processed human plasma samples.

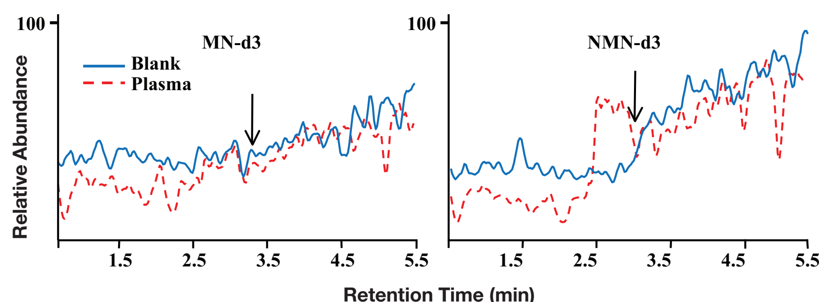

Figure 2. Representative SRM chromatograms of post-column infusion of 100 ng/mL MN-d3 (left) and NMN-d3 (right) after injections of buffer blanks (solid lines) and processed human plasma samples (dashed lines). No internal standards were added to human plasma samples. Arrows indicate retention times of MN and NMN.

4. LLOQ, Linearity and Accuracy

It was determined that CSS is a suitable matrix to conduct this part of validation (mixing study, data not shown). CSS samples with progressively lower concentrations of MN and NMN were prepared in triplicate along with one set of CSS calibrators.

The linearity range was determined to be 7.2 - 486.8 pg/mL for MN and 18.0 - 989.1 pg/mL for NMN (Figure 3). Accuracy ranged from 92.2% to 118.0% for MN, and from 92.1% to 115.0% for NMN. The determined LLOQ was 7.2 pg/mL for MN and 18.0 pg/mL for NMN.

Figures 3 and 4 show the calibration curves for MN and NMN. Figure 5 shows the representative SRM chromatograms of MN and NMN at their LLOQ in CSS.

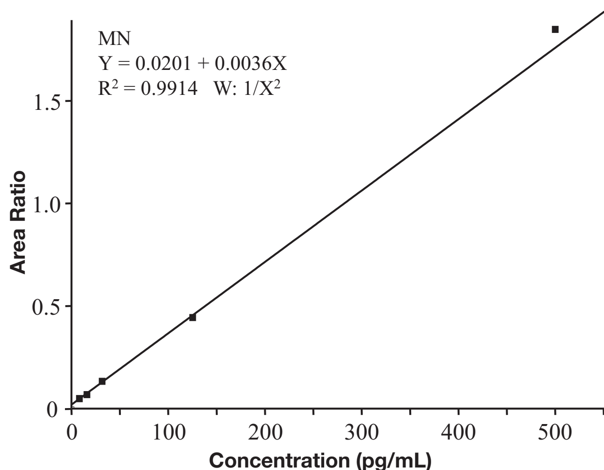

Figure 3. Calibration curve of MN in CSS

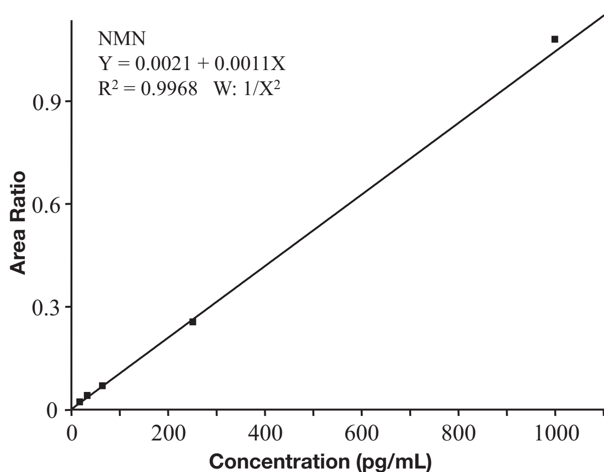

Figure 4. Calibration curve of NMN in CSS

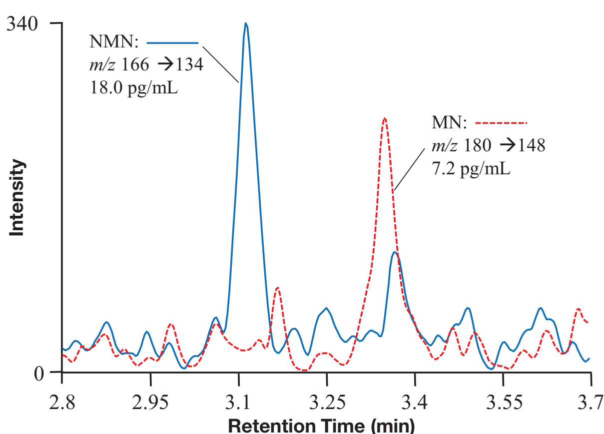

Figure 5. Representative SRM chromatograms of MN and NMN at their LLOQ in a spiked CSS sample.

5. Precision

Precision results are summarized in Table 2.

A) CSS samples: Precision was first assessed with spiked CSS at two concentration levels (25 and 250 pg/mL for MN, and 50 and 500 pg/mL for NMN). Inter- (n=15) and intra-batch (n=5) CV values ranged from 2.1% to 10.9%.

B) Pooled human plasma samples: Precision was also assessed with a spiked human plasma pool (35.6 pg/mL of MN and 53.1 pg/mL of NMN, n=5). The determined intra-assay CV (n=5) was 6.3% and 7.8% for MN and NMN, respectively.

Table 2. Precision Data in Spiked CSS

	MN		NMN	
	25 pg/mL	250 pg/mL	50 pg/mL	500 pg/mL
Intra-assay Precision (%) n=5	10.9	4.6	9.6	2.1
Accuracy (%)	98.9	96.9	110.2	90.9
Inter-assay Precision (%) n=15	10.3	6.5	10.6	5.6
Accuracy (%)	100.6	102.7	108.7	97.4

Figure 6 shows representative SRM chromatograms of MN and NMN using a processed human plasma sample.

6. Carryover

No carryover was observed up to 500 and 1000 ng/mL for MN and NMN, respectively.

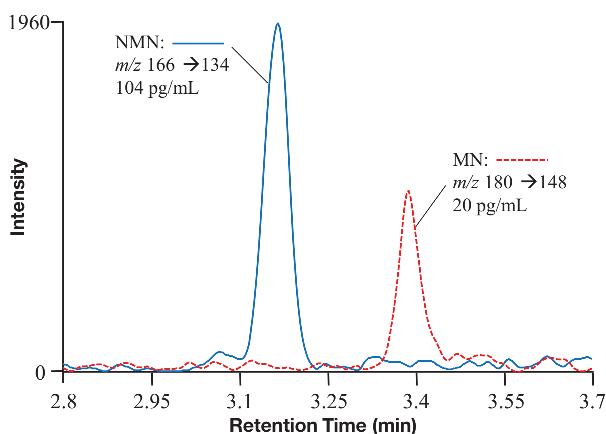

Figure 6. Representative SRM chromatograms of MN and NMN using a processed human plasma sample

Conclusion

A sensitive LC-MS/MS method was developed to quantify plasma free metanephrines in clinical research laboratories. This method has an LLOQ of 7.2 and 18.0 pg/mL for metanephrine and normetanephrine, respectively. Method precision ranged from 2.0% to 10.9%. Ion-pairing SPE was used for sample preparation, and a Hypercarb column was used for chromatographic separation of metanephrines.

Reference

1. He, X.; Gabler, J.; Yuan, C.; Wang, S.; Shi, Y.; Kozak, M. Quantitative Measurement of Plasma Free Metanephrines by Ion-pairing Solid Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry with Porous Graphitic Carbon Column *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2011**, 879(23), 2355-2359.

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Fast and Sensitive LC-APCI-MS/MS Quantitative Analysis of Estrone and Estradiol in Serum without Chemical Derivatization

Xiang He and Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TSQ Vantage
- Accela UHPLC
- Clinical Research

Introduction

In the clinical research setting, quantitative measurements of estrone (E1) and estradiol (E2) in serum typically have been done with immunoassay or liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS is preferred over immunoassay and other analytical techniques because of its high sensitivity.

E1 and E2 are usually chemically derivatized before they are detected by mass spectrometry for enhanced sensitivity. The derivatization step extends the sample preparation procedure and usually involves chemicals/reagents that might compromise the performance of the mass spectrometer in the long term.

Goal

To develop and validate a simple, fast and sensitive analytical method for measuring E1 and E2 in serum or plasma by LC-APCI-MS/MS.

Methods

Sample Preparation

Serum was spiked with internal standard (IS, deuterated E2) and underwent liquid-liquid extraction (LLE) with methyl tert-butyl ether (MTBE). After extraction, the MTBE layer was dried under nitrogen and re-suspended with 60% methanol. The reconstituted sample was centrifuged to remove particulates and the supernatant was injected for LC-MS/MS analysis.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Thermo Scientific Accela UHPLC system. UHPLC was carried out on a Thermo Scientific Hypersil GOLD column (150 × 2.1 mm, 3 µm) at room temperature using water and methanol as mobile phases. The total LC run time was 6 minutes. The mass spectrometer was operated with an atmospheric pressure chemical ionization (APCI) source in negative ion mode. Data was acquired in selected reaction monitoring (SRM) mode.

Validation

The validation procedure included tests for 1) recovery of sample preparation; 2) calibration range; 3) lower limit of quantitation (LLOQ), dynamic range, accuracy; 4) precision; 5) ion suppression; and 6) carryover.

Results and Discussion

Sample Preparation

LLE was used to extract E1 and E2 from serum/plasma and was found to be efficient. MTBE was selected as the extraction solvent for its excellent recovery and ease of handling.

Validation

1. Recovery for LLE Sample Preparation

The absolute recovery of E1, E2 and their internal standard from liquid-liquid extraction ranged from 70% – 115% (n=4).

2. Calibration Range

Calibration curves (Figures 1 and 2) using calibrators in charcoal stripped serum (CSS) showed excellent linearity ($R^2 > 0.998$) between 5 and 1000 pg/mL.

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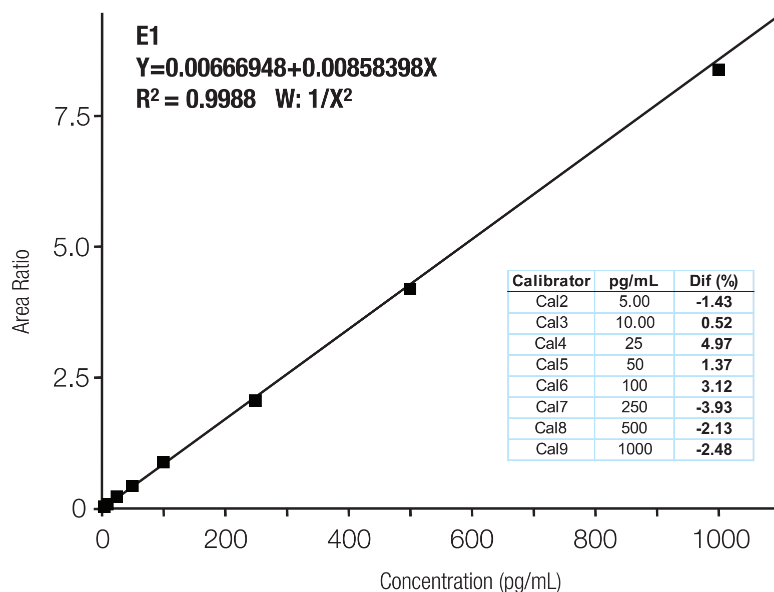
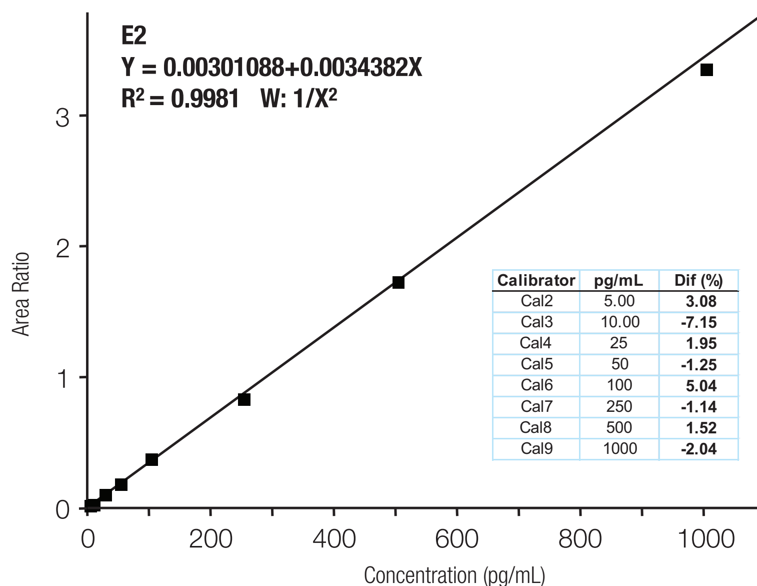


Calibrator	pg/mL	Dif (%)
Cal2	5.00	-1.43
Cal3	10.00	0.52
Cal4	25	4.97
Cal5	50	1.37
Cal6	100	3.12
Cal7	250	-3.93
Cal8	500	-2.13
Cal9	1000	-2.48

Figure 1: Calibration curve of E1 in CSS



Calibrator	pg/mL	Dif (%)
Cal2	5.00	3.08
Cal3	10.00	-7.15
Cal4	25	1.95
Cal5	50	-1.25
Cal6	100	5.04
Cal7	250	-1.14
Cal8	500	1.52
Cal9	1000	-2.04

Figure 2. Calibration curve of E2 in CSS

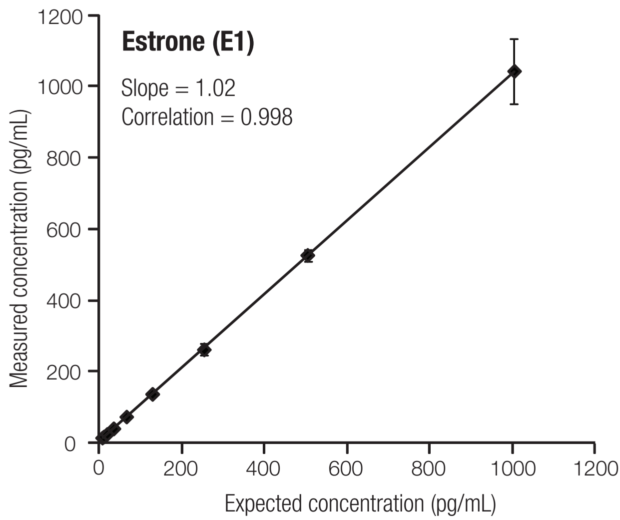
3. Determination of LLOQ, Linearity and Accuracy

CSS was first evaluated by comparing it to human plasma to determine if it was suitable. During this stage of the validation, CSS samples with progressively lower concentrations of E1 and E2 were prepared in triplicate along with one set of CSS calibrators.

The method was linear between 3.5 and 1019.3 pg/mL with accuracy (n=3) from 85.8% to 107.0% for E1, and between 4.4 and 1032.5 pg/mL with accuracy (n=3) from 92.9% to 112.8% for E2 (Table 1 and Figure 3). The LLOQ for E1 and E2 are 3.5 and 4.4 pg/mL, respectively (Table 1 and Figure 4).

Table 1. LLOQ, dynamic range and accuracy

		E1			E2		
Dilution factor	**Expected (pg/mL)**	**Measured (mean, pg/mL)**	**CV of Triplicates (%)**	**Accuracy (%)**	**Measured (mean, pg/mL)**	**CV of Triplicates (%)**	**Accuracy (%)**
256	3.91	3.5	18.8	90.5	4.4	7.1	112.8
128	7.81	8.4	4.5	107.0	8.0	9.0	102.2
64	15.63	15.8	9.4	101.2	18.0	5.1	115.2
32	31.25	28.7	0.6	92.0	31.0	8.8	99.1
16	62.50	56.7	4.8	90.7	60.8	6.7	97.2
8	125.00	107.2	3.9	85.8	116.1	6.8	92.9
4	250.00	224.2	7.4	89.7	242.2	4.4	96.9
2	500.00	484.2	3.5	96.8	492.2	2.4	98.4
1	1000.00	1019.3	8.9	101.9	1032.5	9.1	103.2
Mean				**95.1**			**102.0**



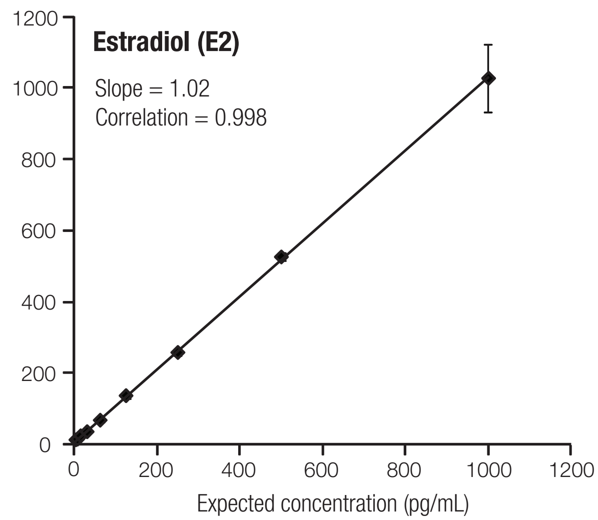


Figure 3: Linearity (Deming regression)

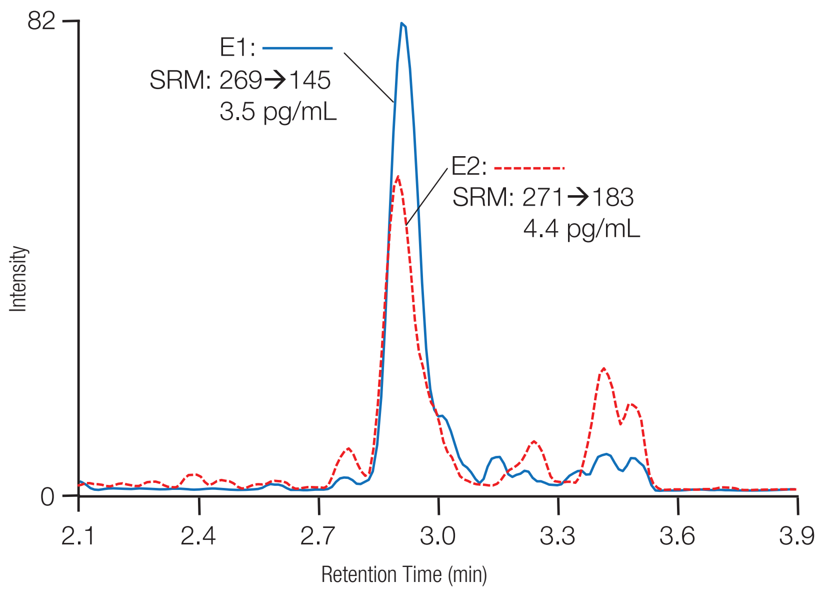


Figure 4. SRM chromatograms of E1 and E2 at their LLOQ in spiked CSS

4. Precision

A) CSS samples: Precision was first assessed with spiked CSS at two concentration levels (12 and 300 pg/mL). Inter- (n=15) and intra-batch (n=5) CV values ranged between 1.6% to 12.5% (Table 2).

B) Pooled human plasma samples: Precision was also assessed with a spiked human plasma pool (35.4 pg/mL of E1 and 18.1 pg/mL of E2, n=5) and the determined intra-batch CV was 2.2% and 3.6% for E1 and E2, respectively.

Table 2. Precision data

		E1		E2	
	Charcoal Stripped Serum	**Low (12 pg/mL)**	**High (300 pg/mL)**	**Low (12 pg/mL)**	**High (300 pg/mL)**
Batch 1	Intra-assay Precision (n=5, %)	7.1	6.9	9.4	6.7
Batch 2	Intra-assay Precision (n=5, %)	5.5	1.6	12.5	3.0
Batch 3	Intra-assay Precision (n=5, %)	7.2	4.9	8.0	3.1
Batch 1-3	Inter-assay Precision (n=15, %)	7.3	4.7	10.9	4.4

	Spiked Pooled Plasma	E1 (35.4 pg/mL)	E2 (18.1 pg/mL)
	Precision (n=5, %)	2.2	3.6

5. Ion Suppression

Results from the post-column infusion experiments are shown in Figure 5. Compared to solvent blank (60% methanol), no obvious ion suppression was detected in the SRM chromatograph of IS using a processed human plasma sample without IS. The red arrow indicates where E1 and E2 elute during the LC gradient.

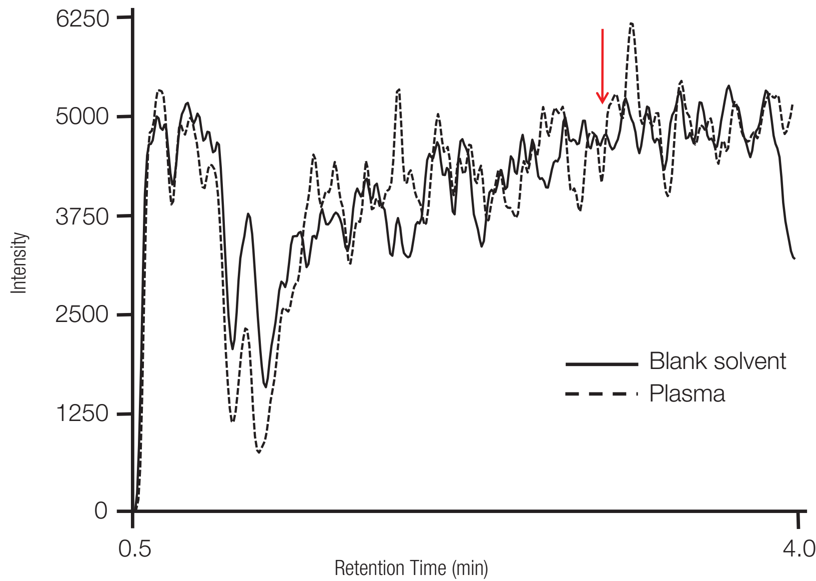


Figure 5: Ion suppression test

6. Carryover

No carryover was observed in the solvent blank injection that was right after a processed spiked CSS sample with E1 and E2 concentration at 300 pg/mL.

Figures 6 and 7 show the SRM chromatograms of E1 and E2 in two individual plasma samples.

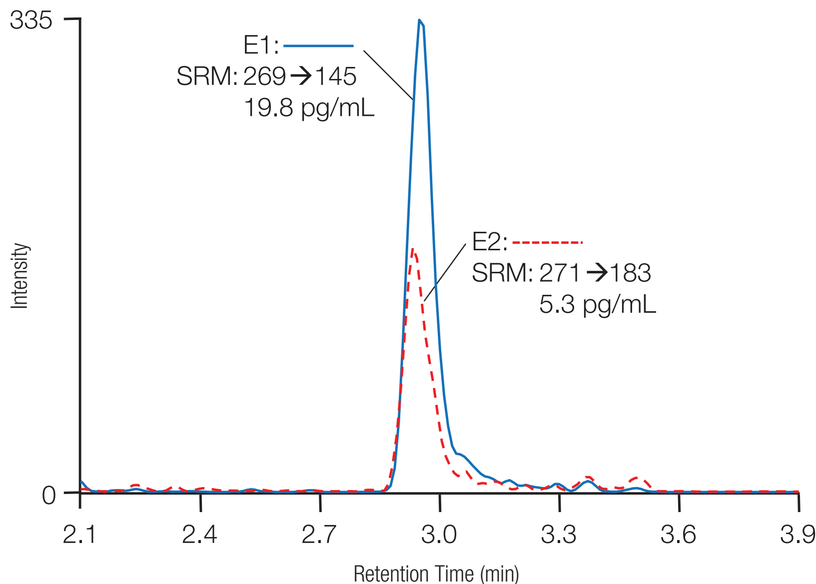


Figure 6: SRM chromatograms of E1 and E2 in human plasma sample 1 (male)

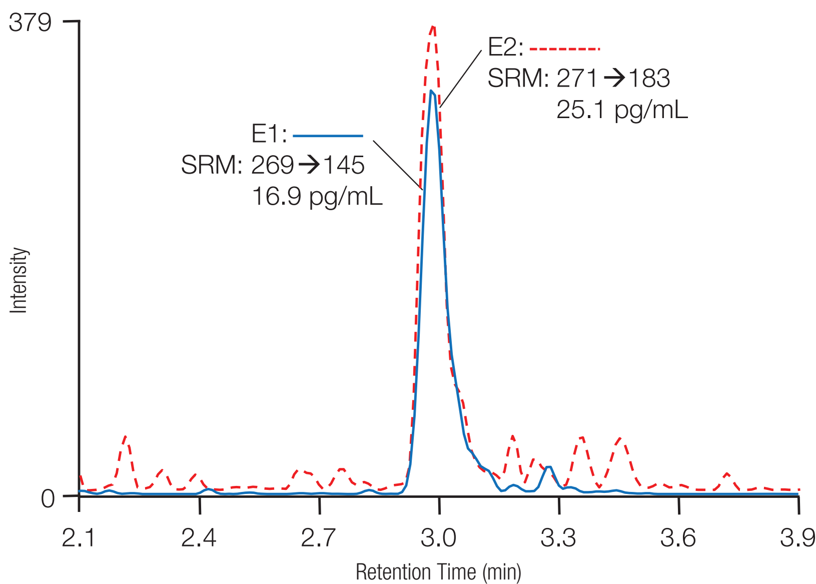


Figure 7: SRM chromatograms of E1 and E2 in human plasma sample 2 (female)

Conclusion

We have developed and fully validated a simple, fast and sensitive LC-APCI-MS/MS method for measurement of E1 and E2 in serum/plasma without derivatization. The LLOQ for E1 and E2 are 3.5 and 4.4 pg/mL, respectively. The method was linear between 3.5 and 1019.3 pg/mL for E1, and 4.4 and 1032.5 pg/mL for E2. No ion suppression or carryover was observed. In addition, for clinical research laboratories, this method offers high precision and recovery.

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Quantitative Analysis of Cortisol and Cortisone in Urine by LC-MS/MS

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Key Words

- Aria TLX-2 System
- TSQ Quantum Ultra
- Clinical Research

Introduction

Cortisol is a steroid-hormone synthesized from cholesterol by a multienzyme cascade in the adrenal glands. It is the main glucocorticoid in humans and acts as a gene-transcription factor influencing a multitude of cellular responses in virtually all tissues. Its production is under hypothalamic-pituitary feedback control.

Only a small percentage of circulating cortisol is biologically active (free), with the majority of cortisol inactive (protein bound). As plasma cortisol values increase, free cortisol (i.e., unconjugated cortisol and hydrocortisone) increases and is filtered through the glomerulus. Urinary free cortisol (UFC) in the urine correlates well with the concentration of plasma free cortisol. UFC represents excretion of the circulating, biologically active, free cortisol.

Goal

To develop a sensitive quantitative LC-MS/MS method for measuring cortisol and cortisone in urine for research applications.

Experimental Conditions/Methods

Chemicals and Reagents

Cortisol standard was purchased from the National Bureau of Reference Materials in the powder form and is stored at room temperature. Cortisone standard was purchased from Sigma in the powder form and is stored at room temperature. The internal standard, Cortisol 9,12,12-d_3, was purchased from Cambridge Isotope Laboratory in the powder form and is also stored at room temperature. Stripped urine was purchased from SeraCare Life Sciences and is stored at -20 °C.

Sample Preparation

0.050 mL deuterated stable isotope (d3-cortisol) is added to a 0.1 mL urine sample as internal standard. The cortisol, cortisone and internal standard are extracted by an online extraction utilizing high-throughput liquid chromatography (HTLC). This is followed by conventional liquid chromatography and analysis on a tandem mass spectrometer equipped with a heated nebulizer ion source.

Calibration Curve Standards Preparation

A standard stock solution of 1 mg/mL of cortisol and cortisone was prepared in methanol. Standard spiking solutions of cortisol and cortisone in methanol/water at concentrations of 5 µg/mL were prepared by dilution of the stock standard solution. The appropriate amount of standard spiking solution was added to 100 mL of stripped urine to prepare calibration standards at the following concentrations: 0.25 µg/dL, 1 µg/dL, 4 µg/dL, and 20 µg/dL. The standards were processed with the sample preparation procedure described above. The standard stock solution and the standard spiking solutions were stored at -20 °C.

HPLC

HPLC analysis was performed using the Thermo Scientific Aria TLX-2 System. The 0.1 mL samples were injected onto a Thermo Scientific 0.5 x 50 mm C18 HTLC Column that served as an extraction column. The analyte was directly transferred from the extraction column and focused onto the analytical column which was a C18, 30 x 4.6 mm, packed with 3 micron particles. Loading Mobile phase A was 95% water and 5% acetonitrile. Loading phase B was acetonitrile. Loading phase C was a solution containing 45% acetonitrile, 45% isopropanol, and 10% acetone. Loading phase D was water with 0.1% ammonium hydroxide. Eluting Mobile phase A was 90% acetonitrile and 10% water. Eluting Mobile phase B was 90% water and 10% acetonitrile. The appropriate gradients and flow rates are described in Table 1.

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Time (min)	Loading Flow (µL/min)	Loading A%	Loading B%	Loading C%	Loading D%	Eluting Flow (µL/min)	Eluting A%	Eluting B%
0.00	1.5	100				0.75		100
1.00	1.5	100				0.75		100
2.00	0.2	100				0.55		100
2.10	0.2	70	30			0.55		100
3.60	1.0			100		0.75	20	80
5.10	2.0				100	0.75	20	80
5.82	2.0			100		0.75	20	80
6.53	2.0				100	0.75	20	80
7.25	2.0			100		0.75	20	80
7.97	1.5				100	0.75	20	80
8.47	1.5	70	30			0.75	100	
9.47	1.5	100				0.75		100

Table 1: HPLC gradient

MS/MS

MS/MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer with an atmospheric pressure chemical ionization (APCI) probe.

The MS/MS conditions were as follows:

Ion Polarity:	Positive Ion Mode
Vaporizer Temperature:	475 °C
Capillary Temperature:	200 °C
Discharge Current:	5.0 µA
Sheath Gas Pressure:	60 units
Auxiliary Gas Pressure:	20 units
Scan Type:	Unit Resolution
Scan Time:	0.100 s

Analyte	Parent Ion (Q1)	Product Ion (Q3)	Collision Energy	Tube Lens
Cortisol	363.188	121.047	24	109
Cortisol	363.189	97.034	18	109
Cortisone	361.179	163.067	22	103
Cortisol IS	366.300	121.000	25	140

Table 2: SRM Transitions and their parameters

Results and Discussion

Representative-SRM chromatograms for cortisol and cortisone at 0.25 µg/dL and 20 µg/dL are shown in Figures 1 through 4. Clearly identifiable and quantifiable peaks were observed.

Figure 5 shows the linear fit calibration curve for cortisol. The calibration curve has an R^2 value greater than 0.99, which indicates an excellent linear fit over the dynamic range of 0.12 – 20 µg/dL. The LOQ value is 0.12 µg/dL with LOD values approximately 3 times lower.

Figure 6 shows the linear fit calibration curve for cortisone. The calibration curve has an R^2 value greater than 0.99, which indicates an excellent linear fit over the dynamic range of 0.20 – 20 µg/dL. The LOQ value is 0.20 µg/dL with LOD values approximately 3 times lower.

The method precision for cortisol was evaluated by analyzing urine cortisol pools at concentrations of 0.06, 0.15, 0.9, 4.1 and 10 µg/dL. For cortisone, precision was evaluated by analyzing urine cortisone pools at concentrations of 0.07, 0.29, 3.2, 5.1, and 12.1 µg/dL. Intra-assay variability was determined by processing and analyzing twenty replicates of one low urine pool and two quality control urine pools. Inter-assay variability was determined by processing and analyzing two replicates of the four urine quality pools in five different batches. Intra-assay and inter-assay precision results are displayed in Table 3 as % CV.

Conclusion:

A fast, sensitive and reliable LC-MS/MS SRM method has been developed for the determination of cortisol and cortisone in urine for use in clinical research. Sample analysis was performed with a runtime of 10 minutes with a quantification limit of 0.12 µg/dL for cortisol and a linearity range of 0.12 – 20 µg/dL for cortisol. The quantification limit for cortisone is 0.20 µg/dL and a linearity range of 0.20 – 20 µg/dL. The low intra-assay and inter-assay variability of the results demonstrates the reliability of the method.

References and Acknowledgements:

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2. Lin CL, Wu TJ, Machacek DA, Jiang NS, Kao PC. Urinary free cortisol and cortisone determined by High Performance Liquid Chromatography in the Diagnosis of Cushing's syndrome. J Clin Endo Metab, 1997;82:151-155.
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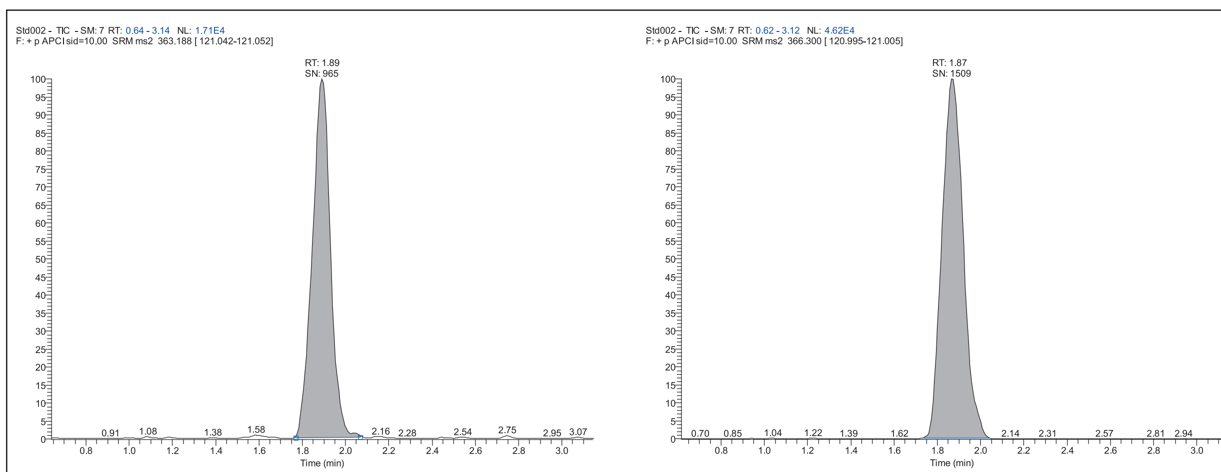

Figure 1: 0.25 µg/dL Cortisol Standard with deuterated internal standard (d3-cortisol)

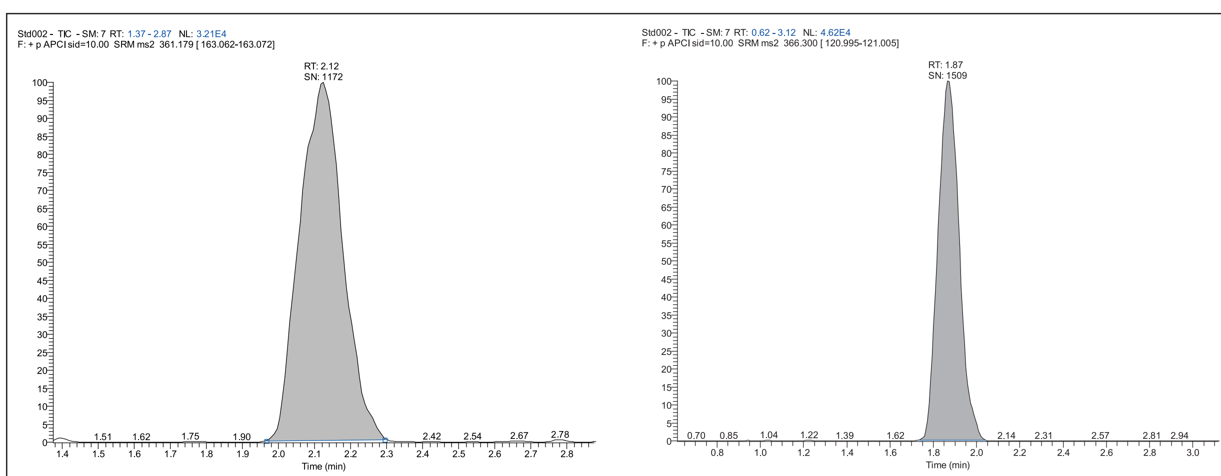

Figure 2: 0.25 µg/dL Cortisone Standard with deuterated internal standard (d3-cortisol)

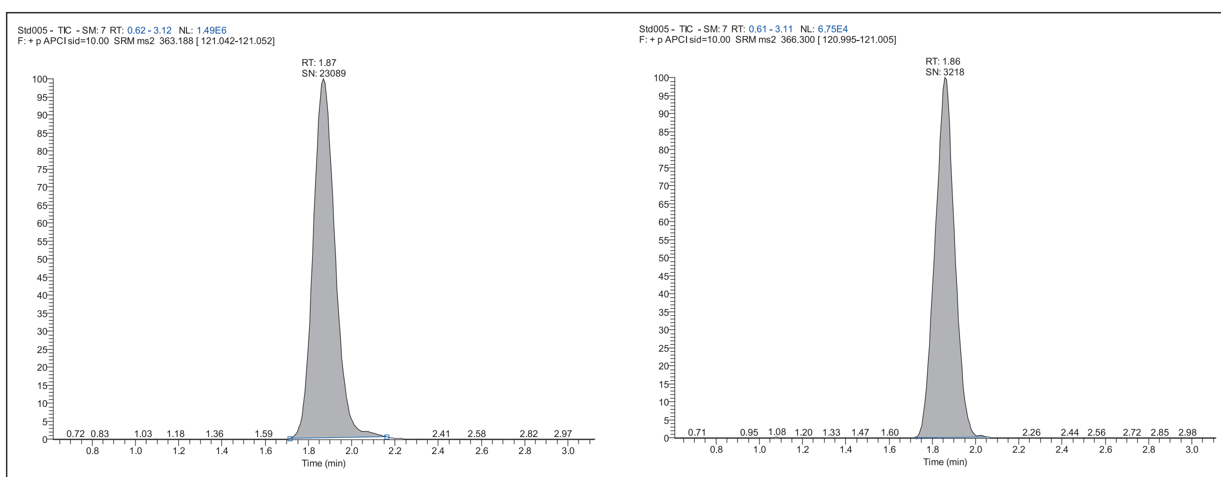

Figure 3: 20 µg/dL Cortisol Standard with deuterated internal standard (d3-cortisol)

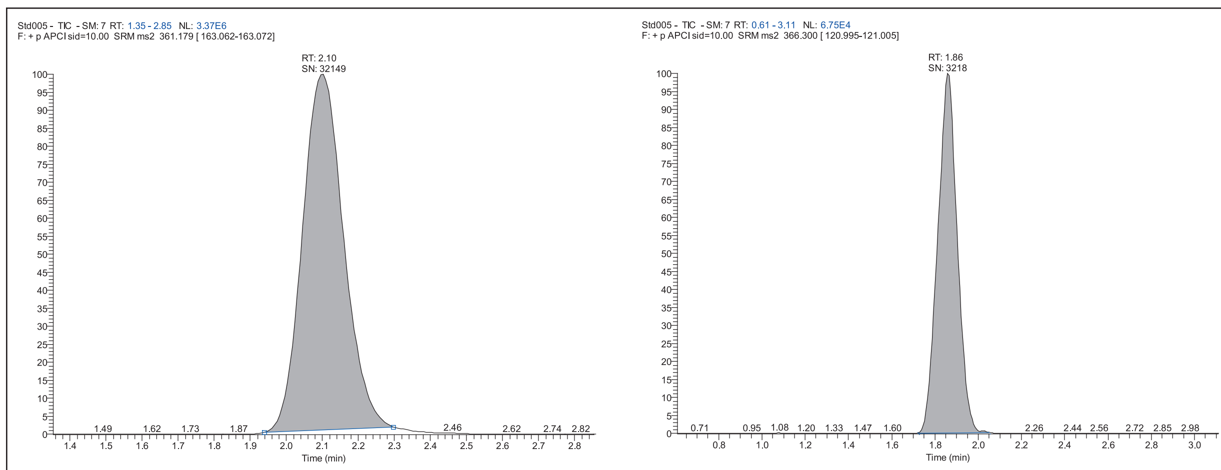

Figure 4: 20 µg/dL Cortisone Standard with deuterated internal standard (d3-cortisol)

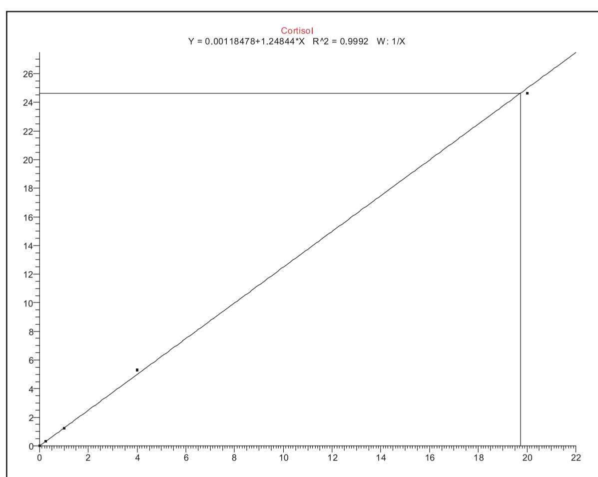

Figure 5: Urine cortisol calibration curve

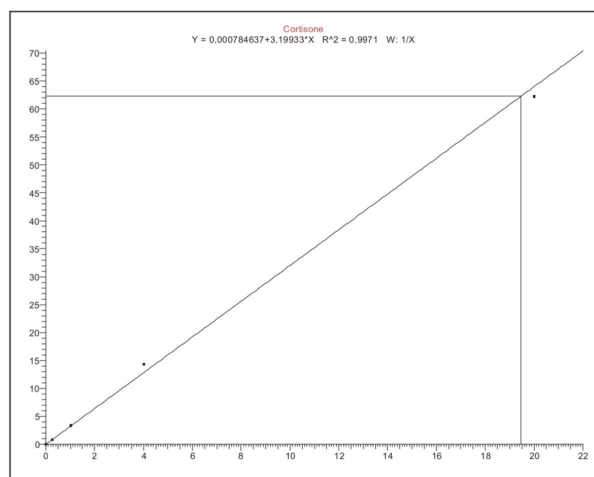

Figure 6: Urine cortisone calibration curve

	Cortisol		Cortisone	
	Intra-assay	**Inter-assay**	**Intra-assay**	**Inter-assay**
Urine Low Pool (n=20)	14.9%	N/A	6.9%	N/A
Urine QC Pool 2 (n=20)	8.6%	N/A	7.6%	N/A
Urine QC Pool 3 (n=20)	8.3%	N/A	7.1%	N/A
Urine QC Pool 1 (n=10)	N/A	20.2%	N/A	14.1%
Urine QC Pool 2 (n=10)	N/A	6.0%	N/A	8.7%
Urine QC Pool 3 (n=10)	N/A	7.5%	N/A	7.2%
Urine QC Pool 4 (n=10)	N/A	6.1%	N/A	6.2%

Table 3: Intra- and inter- assay precision

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Quantitative Analysis of 1,25-dihydroxyvitamin D_2 and D_3 using Immunoaffinity Extraction with APCI-LC-MS/MS

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Key Words

- **Endocrine Testing**
- **TSQ Vantage**
- **Accela U-HPLC**

Introduction

1,25-dihydroxyvitamin D (1,25D) tests are important in conducting clinical research in chronic renal failure and hypoparathyroidism. Circulating 1,25D levels are a thousand-fold less than 25-hydroxyvitamin D levels, making it a challenging test that benefits from immunoaffinity purification prior to analysis with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). In this work, both 1,25D_2 and 1,25D_3 were extracted from human plasma using immunoaffinity extraction and quantified with LC-MS/MS.

Goal

To validate a very sensitive LC-MS/MS method to quantify 1,25-dihydroxyvitamin D by combining immunoaffinity extraction and highly selective atmospheric pressure chemical ionization (APCI).

Materials

ImmunoTube® kits (KM1000) were purchased from Immundiagnostik AG (Bensheim, Germany). Immunoextraction tubes, washing and eluting buffers, and calibrators (CAL1 and CAL2) and controls (CTRL1 and CTRL 2) were provided in the KM1000 kit. The concentrations of the calibrators and controls are specified in Table 1.

Table 1: Calibrators and controls in KM1000 kit

Standards	1,25D_2 (pg/mL)	1,25D_3 (pg/mL)
CAL1	33	26
CAL2	350	250
CTRL1	63-105	49-81
CTRL2	203-348	146-244

Sample Preparation

Five hundred (500) µL of plasma were spiked with deuterated 1,25D_3 and processed with the ImmunoTube kit. The immunoaffinity method for processing plasma was provided in the kit.

Instrument Method

A Thermo Scientific Accela UHPLC pump and Accela autosampler were used as the front end system. The detector was a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer run in selected reaction monitoring (SRM) mode and equipped with an APCI probe. The LC gradient consisted of a fast, 5-minute method at a flow rate of 500 µL/min.

Results and Discussion

Figures 1 and 2 display the data collected for 1,25D_2 and 1,25D_3 using the calibrators and controls provided in the ImmunoTube kit. Calibration curves were plotted without weighting and set to include the origin of the coordinate (x, y = 0,0).

Conclusion

In this research, ImmunoTube immunoaffinity extraction was used to prepare human plasma prior to LC-MS/MS to quantify 1,25D_2 and 1,25D_3. Immunoaffinity extraction allows for the efficient extraction of target compounds from biological samples and almost completely eliminates matrix effects and interferences in LC-MS/MS analysis. The sample preparation is fast, simple, and does not require chemical derivatization. These features make it an ideal method in clinical research for the quantitation of 1,25D_2 and 1,25D_3. APCI was used for the method validation with an ImmunoTube kit, and the lowest concentrations tested for 1,25D_2 and 1,25D_3 in the kit were 26 and 33 pg/mL, respectively. Based on the S/N ratios at these concentrations, the limit of quantitation (LOQ) of this method was estimated to be around 15 pg/mL.

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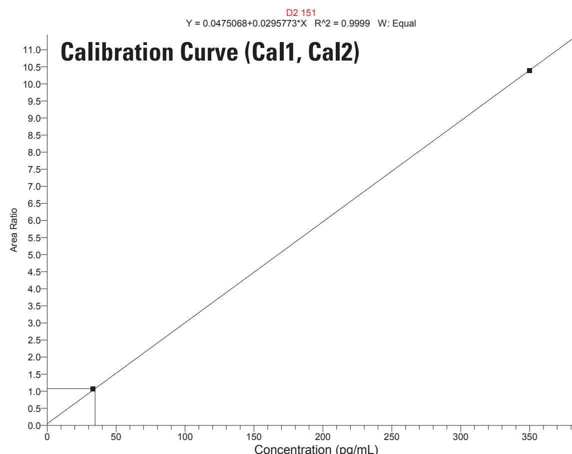
$1{,}25D_2$	Measured (pg/mL)	Specified (pg/mL)
Cal1	34.8	33
Cal2	349.8	350
Ctrl1	94.3	63-105
Ctrl2	311.6	203-348



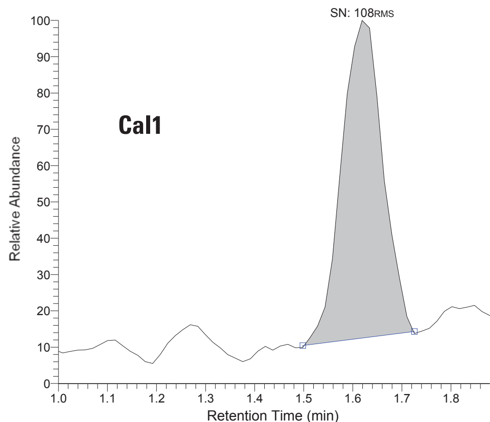
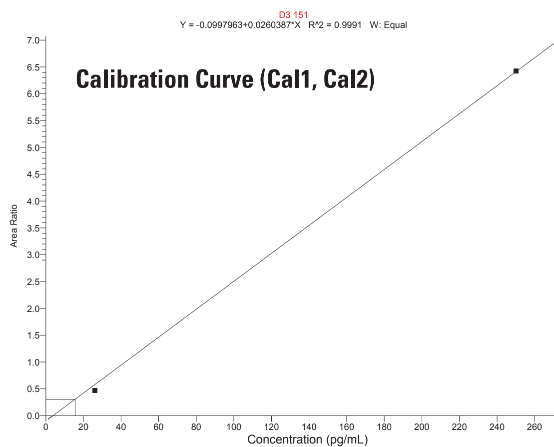


Figure 1: Data for $1{,}25D_2$ (Transition 411⟶151)

$1{,}25D_3$	Measured (pg/mL)	Specified (pg/mL)
Cal1	21.7	26
Cal2	250.4	250
Ctrl1	59.9	49-81
Ctrl2	200.8	146-244



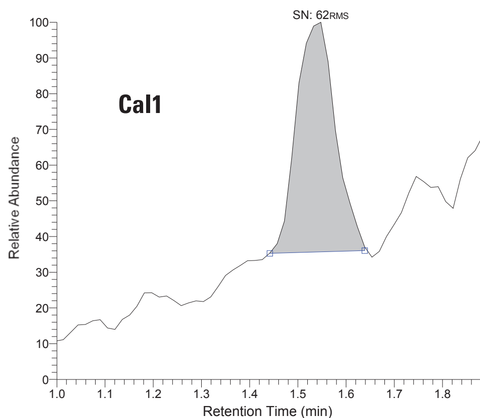


Figure 2: Data for $1{,}25D_3$ (Transition 399⟶151)

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Quantitative LC-MS/MS Analysis of 25-OH Vitamin D_3/D_2 Comparing 1D Chromatography, 2D Chromatography and Automated Online Sample Preparation

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Key Words

- Transcend TLX-1
- TurboFlow Technology
- TSQ Quantum Ultra
- Endocrinology

Introduction

High performance liquid chromatography – tandem mass spectrometry (HPLC-MS/MS) is now widely accepted for measurement of vitamin D metabolites. Many clinical research laboratories use 1-dimensional (1D) chromatography (for example, a single HPLC pump and chromatography column) with a triple stage quadrupole mass spectrometer. Various sample cleanup protocols, such as solid phase extraction (SPE), liquid-liquid extraction (LLE), and protein precipitation (PPT), have been applied in these analyses. Frequently, interfering peaks are seen in 25-OH vitamin D3 chromatograms, adversely affecting peak integration and leading to poor accuracy and reproducibility. Here we investigate the use of 2-dimensional chromatography using TurboFlow technology to remove all interfering peaks and significantly improve data quality.

Goal

Compare three methods for the quantitative analysis of 25-OH vitamin D_3/D_2: a validated, online TurboFlow™ method; a commercially available 2D-SPE-LC-MS/MS kit method (Chromsystems MassChrom® 25-OH Vitamin D_3/D_2); and a 1D chromatography method.

Experimental Conditions

Sample Preparation

A 100 µL sample of plasma was mixed with 200 µL internal standard (IS) in acetonitrile, vortexed, and centrifuged. For analysis, 50 µL of supernatant was injected onto the column. Details of the commercial calibrator and QC values (Chromsystems) used in each assay are provided in Tables 1 and 2. (Please note that the control product has since been reformulated to validate borderline D_3 insufficiency and normal levels.) These commercial products were validated against in-house calibration and control material over a wider dynamic range.

HPLC

HPLC analysis was performed using the Thermo Scientific Transcend TLX-1 system powered by TurboFlow™ technology. For analysis, a TurboFlow XL C18 extraction column (50 x 0.5 mm) and a Thermo Scientific Hypersil GOLD analytical column (50 x 2.1 mm, 1.9 µm) were used. For 1D analysis, the analytical column alone was used. For the commercial 2D set up, columns provided within the 2D-SPE-LC-MS/MS kit were used. Eluents for the TurboFlow method were 0.1% formic acid, methanol + 0.1% formic acid, and acetonitrile/IPA/acetone blend (wash solution).

Table 1. Calibrator levels.

Calibrator	Cal 1 (nmol/L)	Cal 2 (nmol/L)	Cal 3 (nmol/L)	Cal 4 (nmol/L)
25-OH Vitamin D_3	9.9	47.8	86.2	174.0
25-OH Vitamin D_2	0.0	37.5	72.3	146.0

Table 2. Quality control levels.

	Mean 25-OH vitamin D_3 (nmol/L)	Mean 25-OH vitamin D_2 (nmol/L)
QC1	77.1	72.7
QC2	167	150

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Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer. Atmospheric pressure chemical ionization (APCI) was used to generate the $[M-H_2O]+$ ion for 25-OH vitamin D_3, D_2 and the IS.

Results and Discussion

Example calibration lines for the D_3 and D_2 metabolites analyzed by the TurboFlow LC-MS/MS method are presented in Figures 1A and 1B.

Examples of a plasma sample analyzed by the 1D LC-MS/MS method and by the TurboFlow method are provided in Figures 2A and 2B, respectively. There is an interference peak observed in the LC-MS/MS 25-OH-D_3 selected reaction monitoring (SRM) extracted ion chromatogram (XIC). This is commonly observed in analyses where only 1D LC-MS/MS is utilized. When using the TurboFlow method, the interference is removed and larger peak areas with better signal-to-noise ratios are achieved.

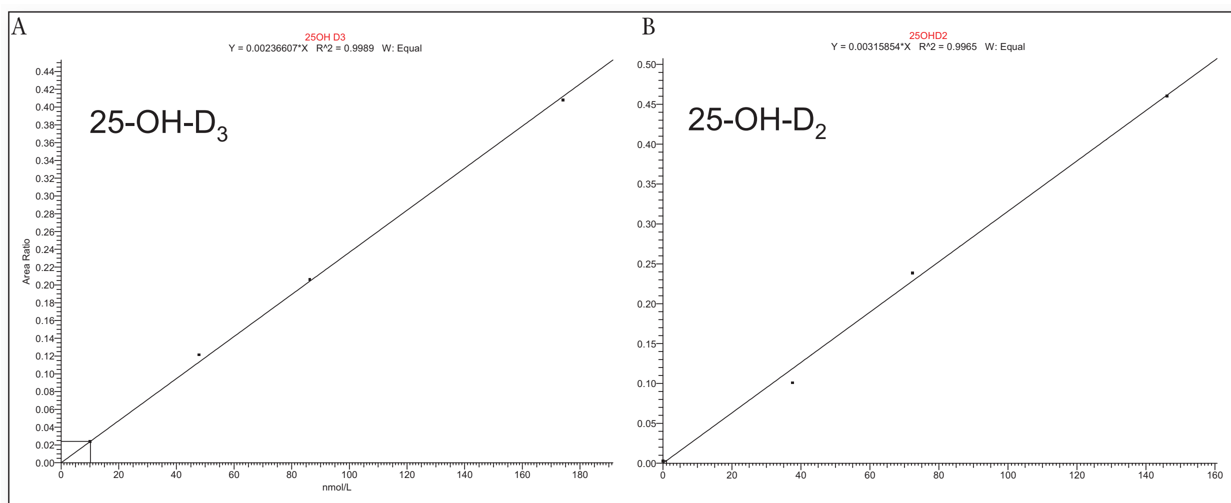


Figure 1. Calibration curves for 25-OH vitamin D_3 (A) and 25-OH vitamin D_2 (B) by TurboFlow LC-MS/MS.

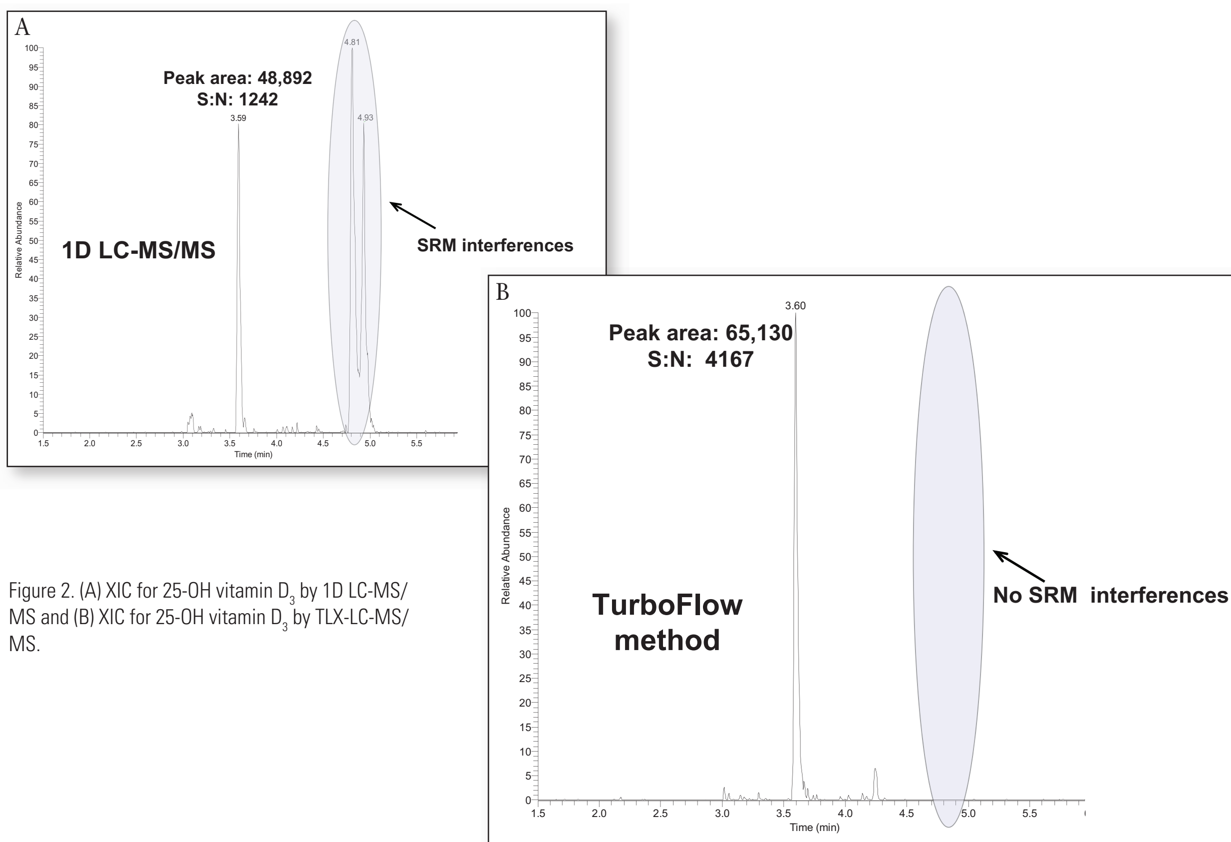


Figure 2. (A) XIC for 25-OH vitamin D_3 by 1D LC-MS/MS and (B) XIC for 25-OH vitamin D_3 by TLX-LC-MS/MS.

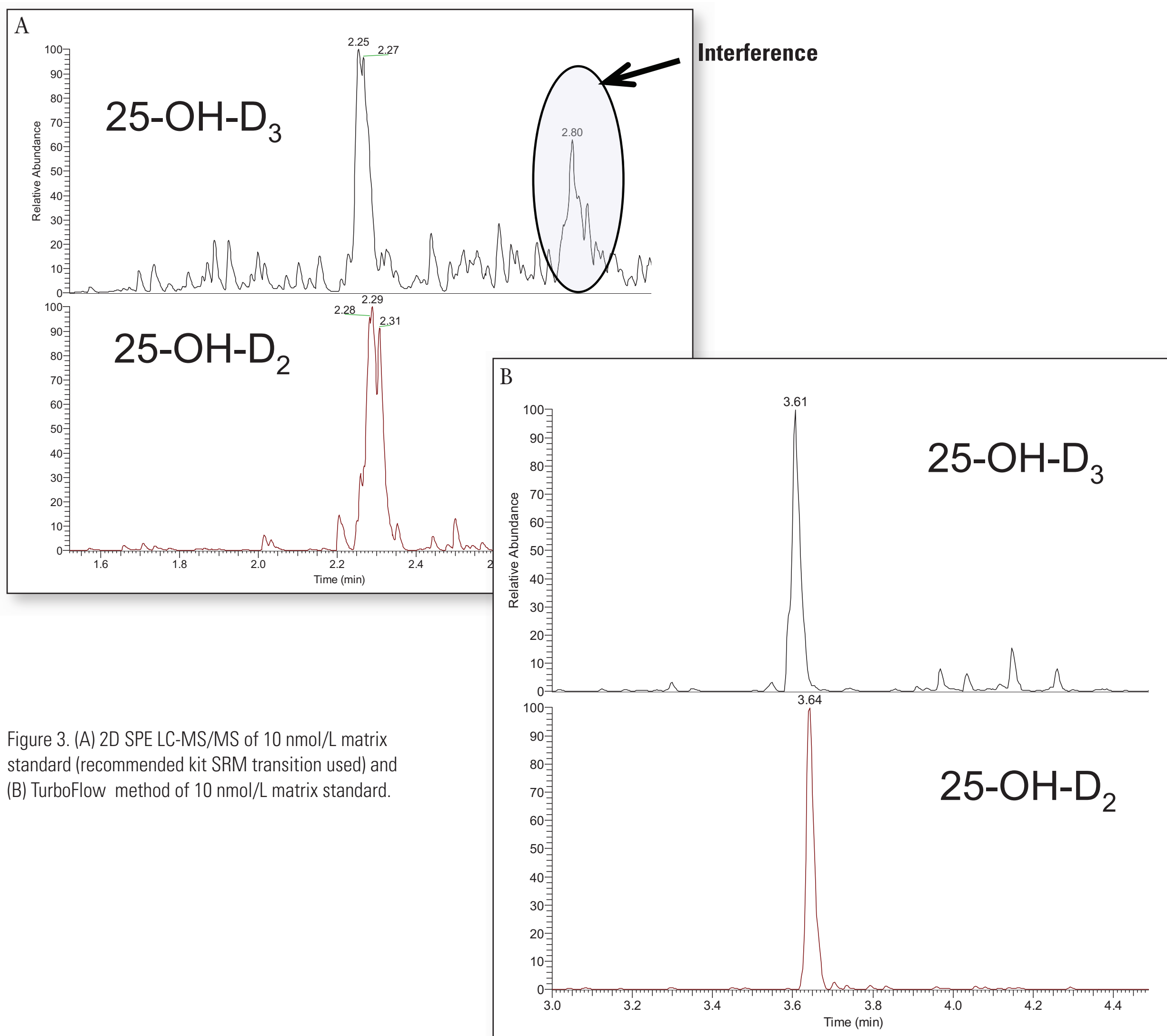


Figure 3. (A) 2D SPE LC-MS/MS of 10 nmol/L matrix standard (recommended kit SRM transition used) and (B) TurboFlow method of 10 nmol/L matrix standard.

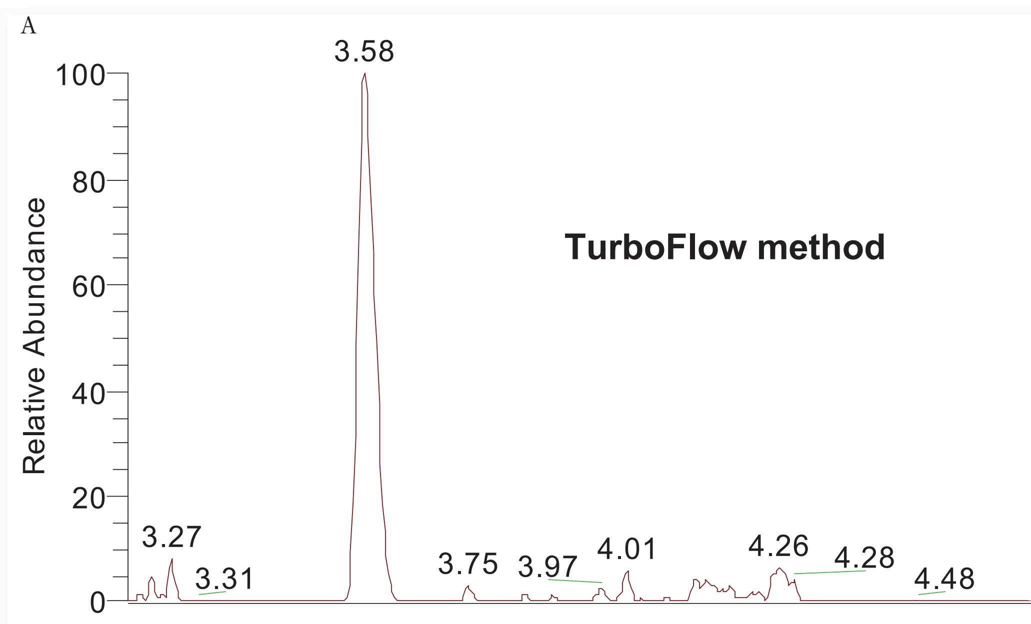
Although cleanup is improved when using other 2D LC-MS/MS methods, interferences are still observed in the 25-OH-D_3 XIC (Figure 3A). Furthermore, at the bottom of the range for 25-OH-D_3 (~10 nmol/L), is detected with greater analytical sensitivity and less noise when analyzed using the TurboFlow method versus a 2D SPE cleanup procedure (Figure 3B).

The 2D-LC-MS/MS approach reduces SRM interferences in the 25-OH-D_3 XICs because the integration of the analyte peak is easier and more accurate. An example of the impact of these interferences on peak integration is shown in Figures 4A and 4B. Here, the result for an individual with normal levels of 25-OH-D_3 would be reported incorrectly due to the high level of interference merging with the analyte peak, and thus, affecting the peak integration.

Conclusion

The TurboFlow method described here has been developed and validated to industry recommended guidelines for clinical laboratories.

Isobaric interferences observed with a 1D LC-MS/MS method at low 25-OH D_3 metabolite concentrations were much reduced by using a 2D-LC-MS/MS approach, and even further improved by using TurboFlow technology. The Transcend™ TLX-1 LC-MS/MS with TurboFlow technology improved the sensitivity and the signal-to-noise ratio.



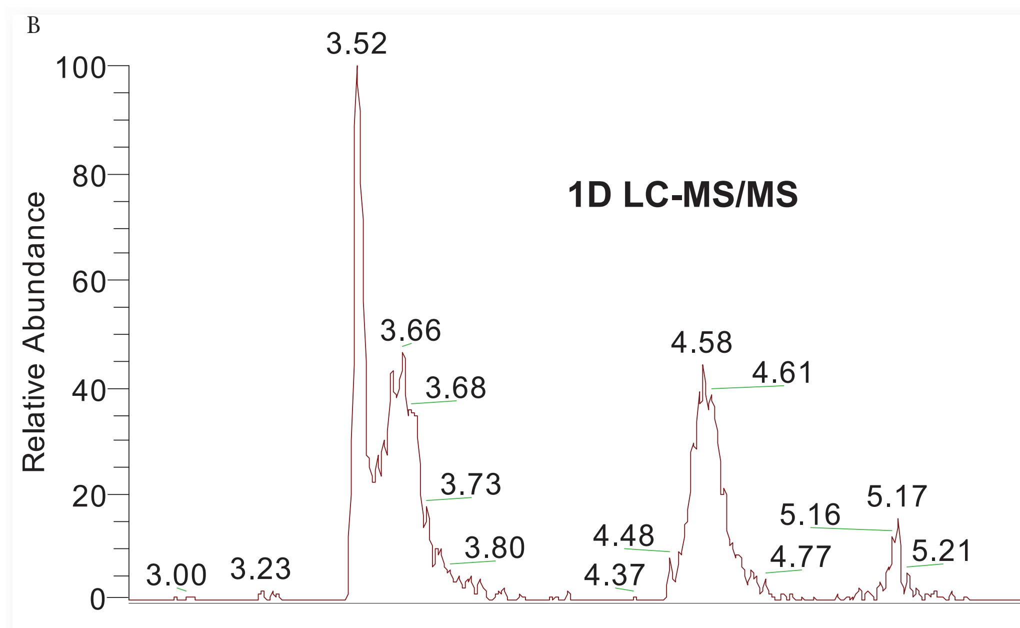


Figure 4. XICs for 25-OH vitamin D_3 by (A) TurboFlow method and (B) 1D LC-MS/MS analysis of a sample at normal levels of analyte (83 nmol/L).

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PN64229: The Use of A New Meta-calculation Software for Automated Data Processing of Tandem MS for Inborn Error Metabolism Research

PN64227: Multiplexing Multiple Methods to Maximize Workflow Efficiency in LC-MS Laboratories

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The Use of A New Meta-calculation Software for Automated Data Processing of Tandem MS for Inborn Error Metabolism Research

Jia Wang,[1] Jason Lai,[1] Brad Hart,[1] Claudio De Nardi,[2] Marta Kozak,[1] Kristine Van Natta,[1] Bénédicte Duretz,[3] David C. Kasper,[4] Chengya Liang[1]

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Overview

Purpose: Streamline tedious and multiple steps of manual calculations; remove transcription errors in post-analytical phase of testing processing; improve turn-around-time of data analysis.

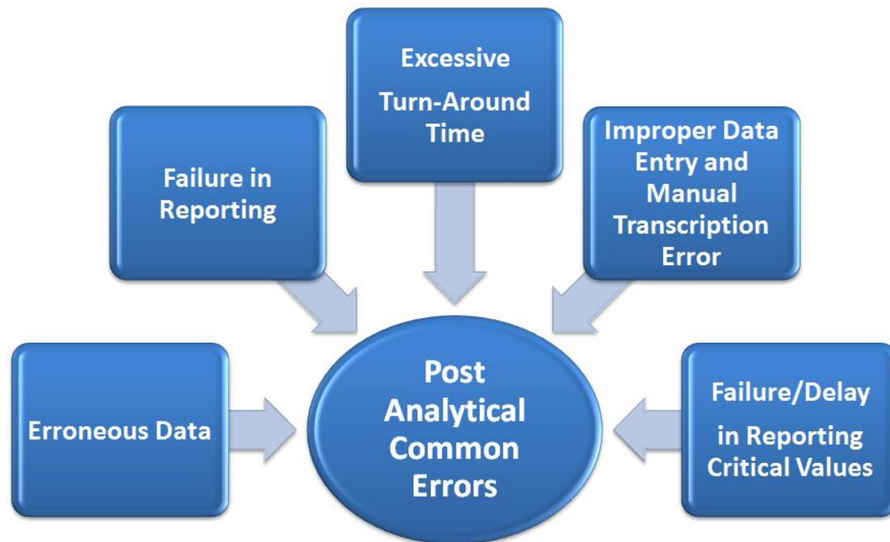
Methods: Use a new software for research to automatically process raw data files generated from flow injection tandem MS analysis of amino acids and acylcarnitines in dried blood spot cards.

Results: A total of 3200 calculations from 100 donor samples were compared between single step software processing with multiple-steps manual calculations, including 1,900 analyte peak areas, 1,000 analyte concentrations and 300 user defined formulas. An agreement of results was demonstrated, and processing time reduced from hours to minutes.

Introduction

The use of Tandem MS for inborn error metabolism research started in early 1990 [1]. With advancement of Tandem MS technology, more compounds can be detected and quantified using a simple sample introduction method such as flow injection with isotopic internal standards. A major challenge is to process a large quantity of generated data efficiently without transcription errors [2] (Figure 1).

FIGURE 1. Common errors in post analytical phase of testing



Methods

Sample Preparation

Samples were extracted from dried blood spot cards; the internal standards were added during the extraction procedure and extracted samples were derivatized prior to injection onto an LC-Tandem MS system. Quality Control (QC) samples were added to the batch.

Liquid Chromatography Tandem Mass Spectrometry

The flow injection was conducted using a LC with open-tube providing an automated sample introduction to a Tandem MS (Thermo Fisher Scientific, San Jose, CA) without chromatographic separation. The Tandem MS used selected reaction monitoring (SRM) scanning for the detection of amino acids and acylcarnitines. Transitions used in this study are listed in Table 1.

TABLE 1. SRMs Monitored for Amino Acids and Acylcaritines

Analyte	Precursor (m/z)	Product (m/z)	Analyte	Precursor (m/z)	Product (m/z)
Cit	232.10	113.10	**C0**	218.25	85.00
Cit IS	234.10	115.10	**C0 IS**	227.25	85.00
Met	206.15	104.10	**C8**	344.25	85.00
Met IS	209.20	107.10	**C8 IS**	347.25	85.00
Orn	189.20	70.20	**C14**	428.35	85.00
Orn IS	191.20	72.20	**C14:1**	426.35	85.00
Phe	222.10	120.10	**C14 IS**	437.35	85.00
Phe IS	228.20	125.90	**C16**	456.35	85.00
Tyr	238.10	136.10	**C16 IS**	459.35	85.00
Tyr IS	244.10	142.10			

Meta Calculation Software

A new meta calculation software, iRC PRO (2Next srl, Prato, Italy) was used for offline automated calculation of raw data files generated from Tandem MS in SRM scanning mode. This software is designed specifically for Thermo Scientific™ TSQ Tandem MS. This beta version of software is developed for an automatic calculation of mass ion ratio and user defined formulas.

Data Analysis

Manual Calculation: Manual calculation was performed by creating a processing method to extract chromatograms and calculate peak areas for each analyte and IS using Thermo Scientific™Xcalibur™ software.

Peak areas were exported in Excel (Microsoft Co.) format and copied and pasted into an Excel worksheet setup to calculate analyte concentrations and values based on the same formulas used by the meta-calculation software.

Software Calculation: The SRM transitions for each analyte and internal standard are entered in the software for data analysis; IS concentration and analyte/IS relative response factor are also entered to calculate analyte concentration.

User defined formulas can be created to perform calculations using peak areas or analyte concentrations.

Upper and lower concentration limits can be set for each analyte; different values can be used for unknown and quality control samples; the software will flag samples outside these acceptance ranges. The same applies to user defined formulas.

A processing method is created by selecting the peak areas, analyte concentrations and user defined formula results that will be displayed by the software.   Results can be exported in Excel or text format.

As depicted below, Figures 2 shows the workflow of software, and Figure 3 shows workflow comparison between software and manual process.

FIGURE 2. Intuitive Workflow – icon based User Interface

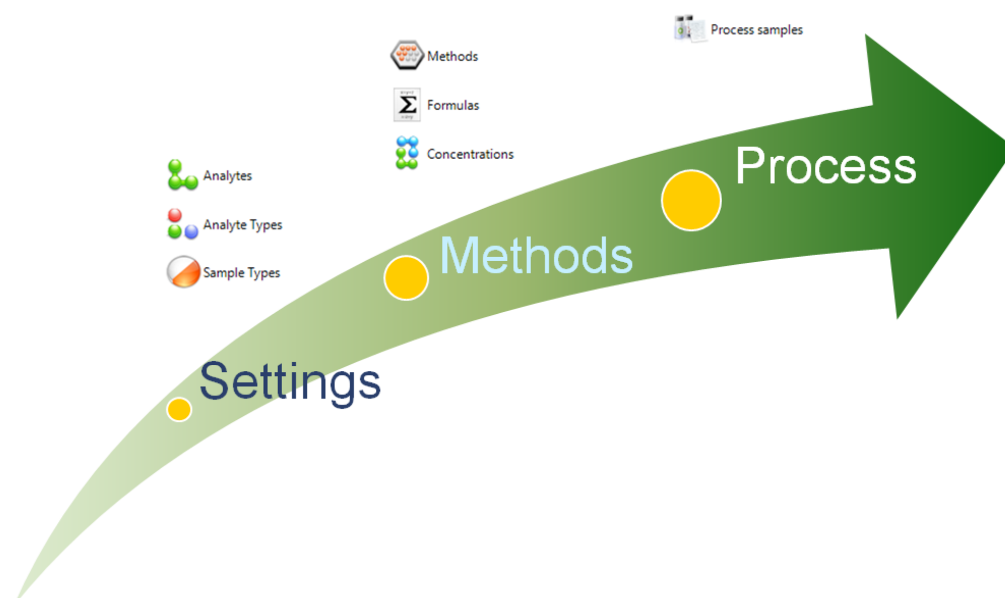
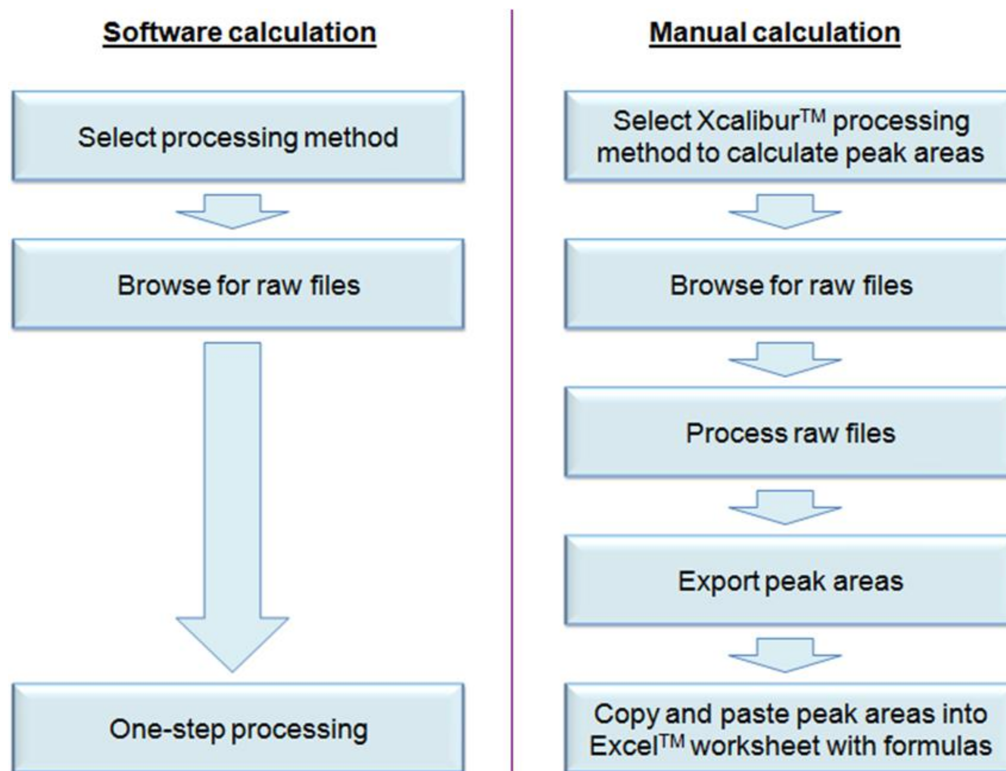


FIGURE 3. Workflow Comparison between Software and Manual Approach



Results

Over 96% of calculations of analyte peak area and concentration (Analytes and Formulas) are within 10% of bias. Over 82% of Formulas Ratios are within 10% of bias. Table 2 below shows comparison between software calculations and manual calculations.

TABLE 2. Comparison between software and manual calculations

Type	Analyte/ Formula	Number (N)	Bias%	Value Range
Analyte Peak Area	19 Analytes	1,900	< 48%	17,400 – 174,827,146
		1898	< 30%	
		1894	< 20%	
		1842	< 10%	
		1672	< 5%	
	R2 = 0.999438 Y = - 7879 + 0.999719X			
Analyte Concentration	Cit, Met, Orn, Phe, Tyr, C0, C8, C14, C14:1, C16	1,000	< 45%	0.62 – 431.51
		998	< 30%	
		993	< 20%	
		958	< 10%	
		845	< 5%	
	R2 = 0.997733 Y = - 0.19774 + 0.998866X			
Formula Concentration (User Defined)	F1=C0+C14:1	100	< 5%	20.83 – 386.55
	R2 = 0.999544 Y = 0.174589 + 0.999772X			
Formula Peak Area (User Defined)	F2=(Orn-Phe)/Tyr F3=(C8+C14:1-C16)/(Orn+Tyr)	200	< 30%	-1.4403 – 3.534176
		192	< 20%	
		165	< 10%	
		113	< 5%	
	R2 = 0.991617 Y = 0.004626 + 0.995799X			

Figures 4 and 5 show additional statistics for the comparison.

FIGURE 4. Residual plot of 1900 calculations of analyte peak area from 100 donor Samples

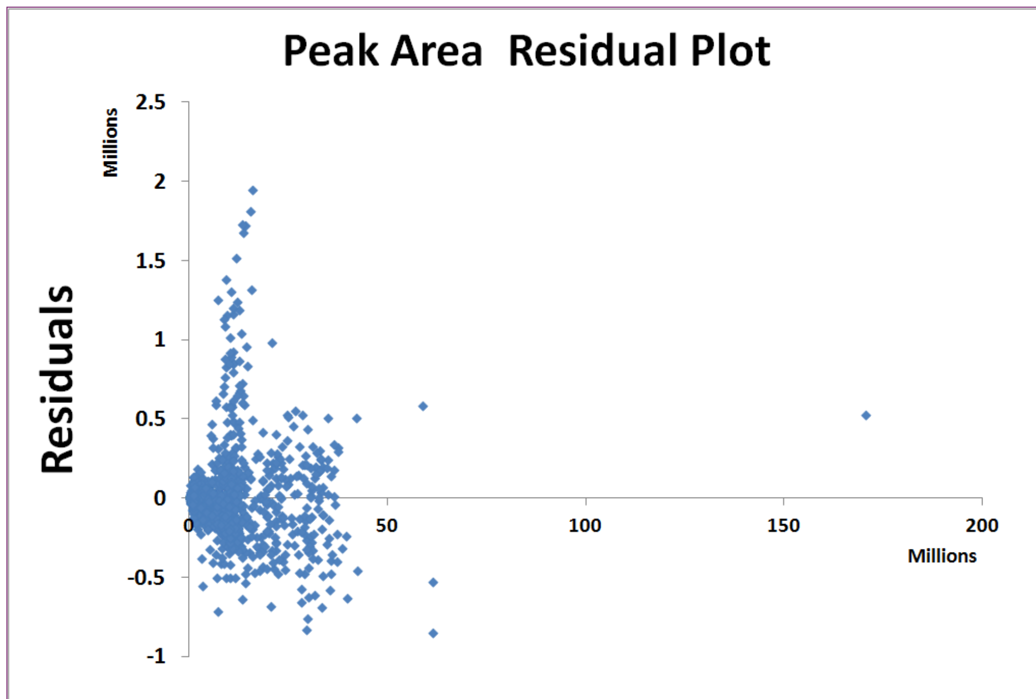
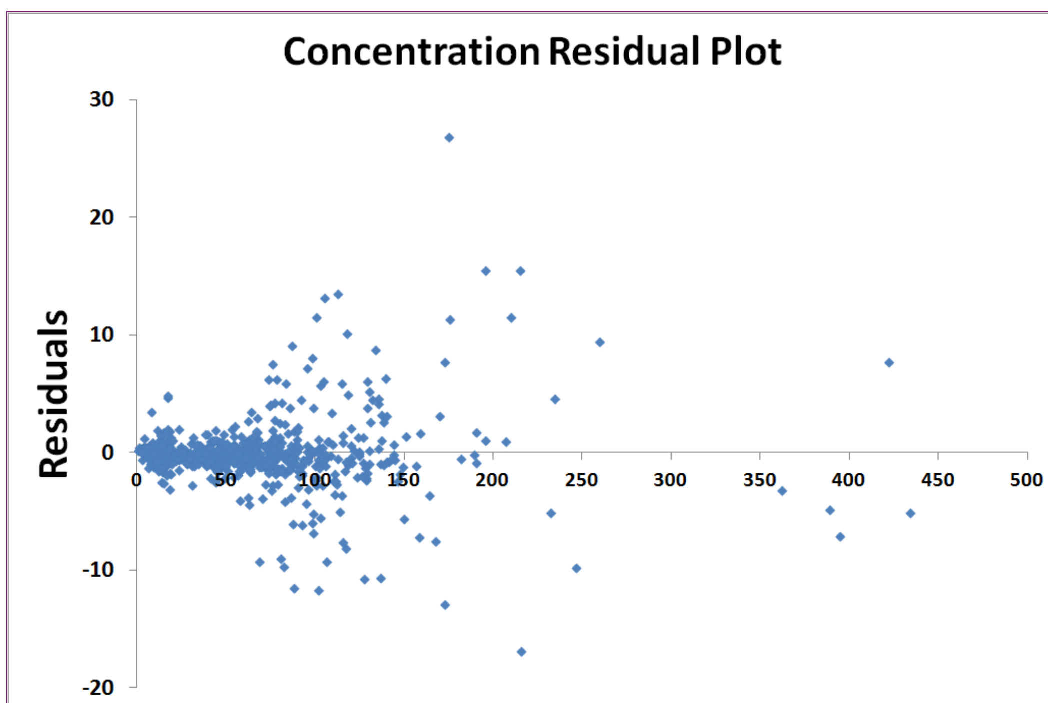


FIGURE 5. Residual plot of 1000 calculations of analyte concentrations from 100 donor Samples



Conclusion

This off-line automated data processing tool shows a good agreement with the manual calculation process, and it can process peak area, concentration and user defined formulas.

This meta calculation software for research improves time effectiveness by eliminating the manual calculation process and removing transcription errors in the post-analytical phase. The processing time is reduced from hours to minutes.

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Acknowledgements

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Multiplexing Multiple Methods to Maximize Workflow Efficiency in LC-MS Laboratories

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Overview

Purpose: To demonstrate the performance of research and forensic methods running simultaneously across most or all channels of a multichannel ultra-high performance liquid chromatography (UHPLC) system interfaced to a tandem mass spectrometer (MS/MS) in order to maximize sample throughput and workflow efficiency.

Methods: Reversed-phase liquid chromatography of analytes with corresponding stable-isotope internal standards eluting from up to four UHPLC channels into a common ion source of a triple-quadrupole mass spectrometer were used to measure blood serum levels of the following compounds for research purposes:

> ***25-OH-Vitamins D2 & D3*** after protein precipitation and
> ***Methylmalonic Acid*** after protein precipitation and butylation, eluting to an atmospheric-pressure chemical ionization (APCI) source;

or to measure urine levels of the following forensic compounds:

> ***Buprenorphine & Norbuprenorphine*** after hydrolysis and
> ***Ethyl-Glucuronide & Ethyl Sulfate*** after dilution, eluting into a heated electro-spray ionization (HESI) source.

Results: Desired quantitation ranges, accuracy and repeatability criteria were achieved for each application when various specimen batches ran on any of the channels of the 4-channel UHPLC system. Typically, internal standard (IS) peak area counts showed less than 20% coefficient of variability (CV) among calibrators, QCs and specimens (n = 20) on any and across all 4 channels. Retention time variations through these batches were less than 3% CV. Calculated amounts were within +/- 15% of theoretical amounts.

Introduction

Many laboratories run several different LC-MS methods in series on a single channel LC-MS system. If the methods involve different ion sources, columns and mobile phases, the changeover is time consuming, labor intensive and increases the risk of mistakes and contamination. A four-channel UHPLC system multiplexed into one mass spectrometer permits parallel batches of up to four different methods utilizing a common ion source and unique columns and mobile phases to be completed in a fraction of the time and effort.

Methods

Sample Preparation:

"Neat" specimens were prepared in HPLC-grade solvents - acetonitrile, methanol, water - using standards purchased from Cerilliant (Round Rock, TX).

Blood serum specimens and corresponding calibrators and quality controls (QCs) were subjected to protein precipitation by mixing 1:2 with acetonitrile containing internal standard (IS) - 25-OH-VitD3-d6 or d3-methylmalonic acid. After centrifugation, 50 uL of supernatants from the 25-OH-Vitamin D (VitD) batches were injected directly into the UHPLC system. From the methylmalonic acid (MMA) batches, 100 uL of supernatants were evaporated to dryness by heated nitrogen flow. The residues were derivatized by 100 uL of 10% acetyl chloride in butanol for 15 minutes. After evaporation to dryness and reconstitution with 100 uL of 50% methanol in water, 10 uL injections of each sample preparation were made into the UHPLC system.

Urine specimens and corresponding calibrators and QCs to be analyzed for buprenorphine & norbuprenorphine (Bup/Norbup) were hydrolyzed by incubating a mixture of 150 uL of β-glucuronidase solution (10,000 U/mL, pH 5) with 200 uL of specimen, and 50 uL of IS solution containing - buprenorphine-d3 & norbuprenorphine-d4 for 1.5 hours at 60°C. Each preparation was then mixed with 200 uL of cold methanol and refrigerated for 10 minutes before centrifugation. 20 uL injections of supernatants from each preparation were made into the UHPLC system.

Urine specimens and corresponding calibrators and QCs to be analyzed for ethyl-glucuronide & ethyl-sulfate (EtG/EtS) were diluted 1:10 with water and then spiked with 50 uL of IS solution containing EtG-d3 & EtS-d3 before making 20 uL injections into the UHPLC system.

Note: β-glucuronidase powder was purchased from Sigma-Aldrich. All other reagents and consumables were from Thermo Fisher Scientific.

Liquid Chromatography: The UHPLC system was a Thermo Scientific™ Transcend™ II LX4 equipped with binary-solvent pumps and a dual-arm autosampler configuration. The columns and mobile phase conditions for each method are described with the results.

Mass Spectrometry

The Thermo Scientific™ TSQ Endura™ triple-quadrupole mass spectrometer was used with APCI when multiplexing VitDs with MMA batches or HESI when multiplexing Bup/Norbup with EtG/EtS batches. Ion source and MS/MS conditions are described with the results.

System Control & Data Analysis

Thermo Scientific™ TraceFinder™ with Aria™ MX software was used to control the Transcend II LX4 and Endura MS/MS systems, submit batches to desired channels as well as for analyzing data and reporting results.

Results

Multi-channeling batches of 25-OH-VitDs and MMA

FIGURE 1. Common APCI Source Conditions

Ion Source Type: **APCI**
Current LC Flow (µL/min): **0**
Sheath Gas (Arb): **20**
Aux Gas (Arb): **5**
Sweep Gas (Arb): **0**
Ion Transfer Tube Temp (°C): **300**
Vaporizer Temp (°C): **400**
Pos Ion Discharge Current (µA): **4**

Cycle Time (secs): **.25**
Use Calibrated RF Lens: **False**
RF Lens (V): **70**
Q1 Resolution (FWHM): **0.7**
Q3 Resolution (FWHM): **0.7**
CID Gas (mTorr): **1.5**
Source Fragmentation (V): **10**
Chrom Filter (secs): **5**

FIGURE 2. MS/MS & LC Conditions for 25-OH-VitDs

SRM Table

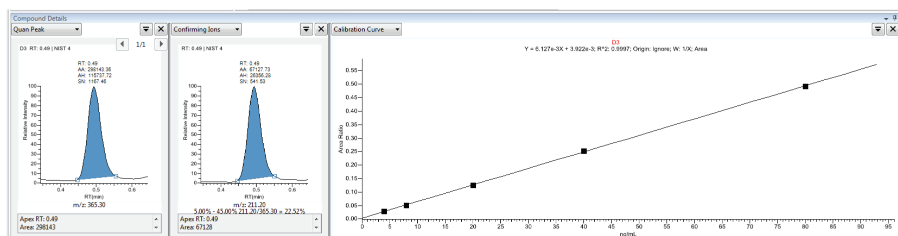
Compound	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
D3	0	1	Positive	383.35	211.15	30	112
D3	0	1	Positive	383.35	365.3	20	112
IS	0	1	Positive	389.4	263.25	23	115
IS	0	1	Positive	389.4	371.35	20	115
D2	0	1	Positive	395.35	269.1	25	115
D2	0	1	Positive	395.35	377.4	20	115

Start data 1.1 min   Data window: 1.0 min   Total run time: 4.0 min

Column 1 Accucore RP-MS, 2.6um, 50x2.1mm
Loading Pump: Binary
A: Water + 0.1% Formic Acid
B: Methanol + 0.1% Formic Acid

Step	Start	Sec	Flow	Grad	%A	%B	Comments
1	0.00	15	0.50	Step	25.0	75.0	Load sample
2	0.25	45	0.50	Ramp	5.0	95.0	Separate analytes
3	1.00	60	0.50	Step	5.0	95.0	Elute analytes
4	2.00	5	0.50	Ramp	25.0	75.0	Ramp to initial conditions
5	2.08	115	0.50	Step	25.0	75.0	Equilibrate column

FIGURE 3. Typical results for 25-OH-VitDs
Desired quantitation range from 4 to 80 ng/mL achieved



25-OH-VitDs batches submitted to one or two channels have throughputs of 15 or 28 injections per hour, respectively. Large batches submitted across 4 channels typically have throughputs around 58 injections per hour.

FIGURE 4. Multi-channeling 25-OH-VitDs

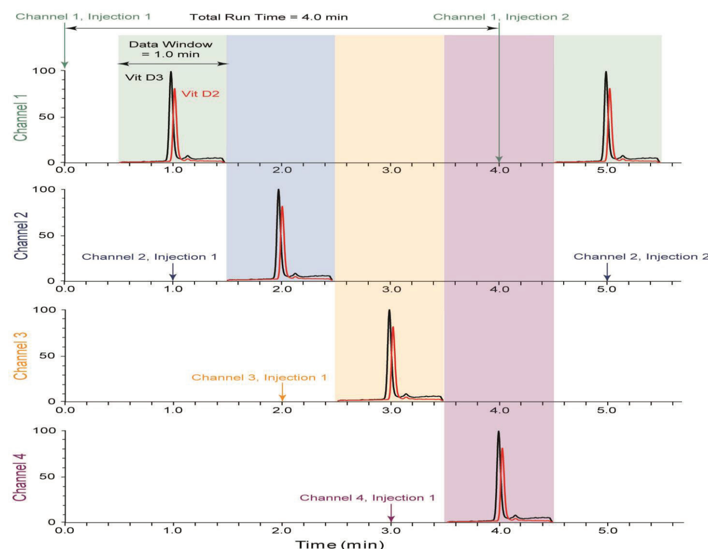


FIGURE 5. MS/MS & LC Conditions for Butyl-MMA

SRM Table

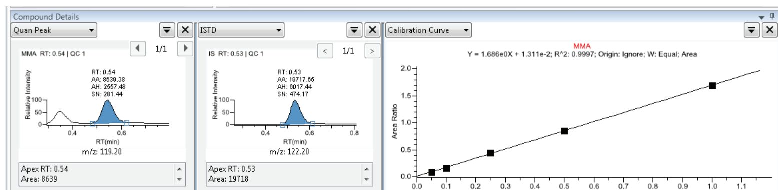
Compound	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)
Butyl-MMA	0	1	Positive	231.15	101.15	23
Butyl-MMA	0	1	Positive	231.15	119.3	16
IS	0	1	Positive	234.2	104	23
IS	0	1	Positive	234.2	122.2	16

Start data 1.75 min Data window: 1.0 min Total run time: 5.0 min

Column 1	Accucore C8, 2.6um, 50x2.1mm
Loading Pump	Binary
A	Water + 0.1% Formic Acid
B	Methanol + 0.1% Formic Acid

Step	Start	Sec	Flow	Grad	%A	%B	Comments
1	0.00	10	0.50	Step	45.0	55.0	Load sample
2	0.17	30	0.50	Ramp	35.0	65.0	Separate analytes
3	0.67	90	0.50	Ramp	30.0	70.0	Elute analytes
4	2.17	60	0.50	Step	-	100.0	Wash column
5	3.17	110	0.50	Step	45.0	55.0	Equilibrate column

FIGURE 6. Typical results for MMA (butylated) Desired quantitation range from 0.05 to 1.00 uM achieved



MMA batches submitted to one or two channels have throughputs of 12 or 23 injections per hour, respectively. Since demand for MMA is much less than for 25-OH-VitDs, one channel is used while the other three are used for the VitDs. Thus, 8 injections from MMA and 36 injections of 25-OH-VitDs are completed in one hour.

Internal standard peak areas among blood serum specimens varied greatly due to ion suppression by co-eluting interferences or by sample matrix components that interfere with the butylation reaction. The cause(s) of this variability is being investigated. However, the calculated amounts of MMA concentrations in the QCs and specimens measured agreed with theoretical values within +/- 15%.

Multi-channeling batches of Bup/Norbub and EtG/EtS

FIGURE 7. Common HESI Source Conditions

Ion Source Type: **HESI**
Spray Voltage:
Positive Ion (V): **3500**
Negative Ion (V): **1000**
Current LC Flow (μL/min): **0**
Sheath Gas (Arb): **50**
Aux Gas (Arb): **15**
Sweep Gas (Arb): **2**
Ion Transfer Tube Temp (°C): **350**
Vaporizer Temp (°C): **400**

Cycle Time (secs): **.5**
Use Calibrated RF Lens: **False**
Q1 Resolution (FWHM): **0.7**
Q3 Resolution (FWHM): **0.7**
CID Gas (mTorr): **2**
Source Fragmentation (V): **10**
Chrom Filter (secs): **3**

FIGURE 8. MS/MS & LC Conditions for Bup/Norbup

SRM Table

Compound	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
NorBup	0	1.5	Positive	414.3	243.1	30	150
NorBup	0	1.5	Positive	414.3	340.2	30	150
NorBup-d3	0	1.5	Positive	417.3	246.1	30	150
NorBup-d3	0	1.5	Positive	417.3	343.2	30	150
Bup	0	1.5	Positive	468.35	396.3	40	170
Bup	0	1.5	Positive	468.35	414.3	35	170
Bup-d4	0	1.5	Positive	472.35	243.05	40	170
Bup-d4	0	1.5	Positive	472.35	400.2	40	170

Start data 0.5 min   Data window: 1.5 min   Total run time: 4.0 min

Column 1 Accucore RP-MS, 2.6um, 50x2.1mm
Loading Pump: Binary
A: Water + 0.1% Formic Acid
B: Methanol + 0.1% Formic Acid

Step	Start	Sec	Flow	Grad	%A	%B	Comments
1	0.00	60	0.50	Step	60.0	40.0	Load sample
2	1.00	60	0.50	Ramp	-	100.0	Elute analytes
3	2.00	30	0.50	Step	-	100.0	Wash column
4	2.50	90	0.50	Step	60.0	40.0	Equilibrate column

FIGURE 9. Typical results for Bup/NorBup Desired quantitation range from 5 to 500 ng/mL achieved.

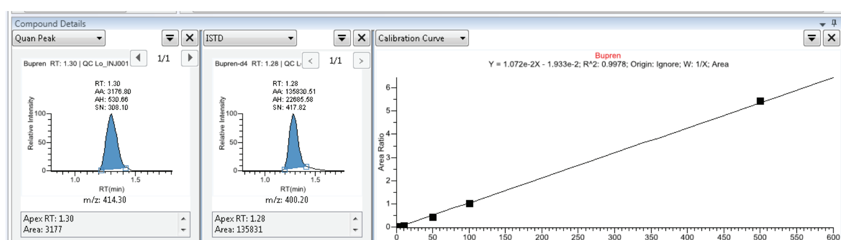


FIGURE 10. MS/MS & LC Conditions for EtG/EtS

SRM Table

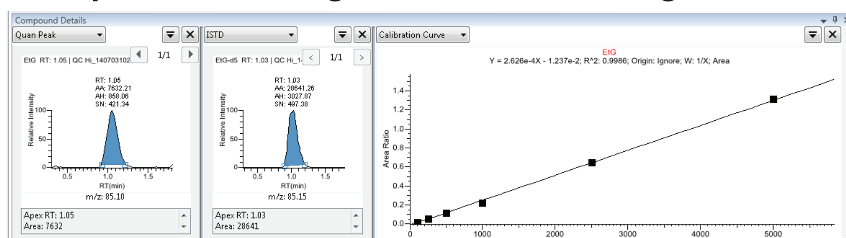
Compound	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
EtS	0	1.5	Negative	125.1	80.1	35	60
EtS	0	1.5	Negative	125.1	97.05	15	60
EtS-d5	0	1.5	Negative	130.1	80.1	35	65
EtS-d5	0	1.5	Negative	130.1	98.05	15	65
EtG	0	1.5	Negative	221.1	75.2	15	75
EtG	0	1.5	Negative	221.1	85.1	15	75
EtG-d5	0	1.5	Negative	226.1	75.2	15	75
EtG-d5	0	1.5	Negative	226.1	85.1	15	75

Start data 0.1 min   Data window: 1.5 min   Total run time: 4.0 min

Column 1	Syncronis aQ 5um, 50x3mm
Loading Pump	Binary
A	Water + 0.1% Formic Acid
B	Methanol

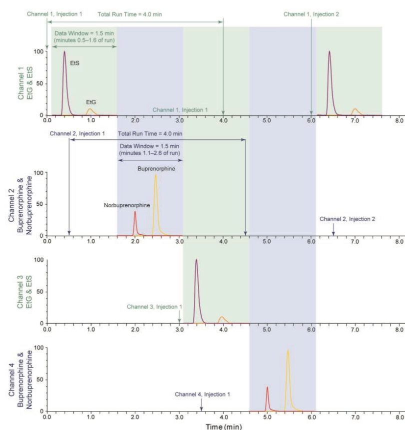
Step	Start	Sec	Flow	Grad	%A	%B	Comments
1	0.00	60	0.70	Step	100.0	-	Load sample
2	1.00	30	0.70	Ramp	-	100.0	Elute analytes
3	1.50	60	0.70	Step	-	100.0	Wash column
4	2.50	90	0.70	Step	100.0	-	Equilibrate column

FIGURE 11. Typical results for EtG/EtS
Desired quantitation range from 100 to 5000 ng/mL achieved.



Bup/Norbup batches and EtG/EtS batches submitted to one or two channels have throughputs of 15 or 28 injections per hour.

FIGURE 12. Multi-channeling Bup/Norbup & EtG/EtS Batches



Multi-channeling Bup/Norbup across 2 channels while EtG/EtS runs on one allows 26 Bup/Norbup and 13 EtG/EtS injections/hour.

Using 2 channels for each does not increase throughput but ensures completion of all batches in case one channel stops because of leakage or over-pressurization.

Conclusion

- Multi-channeling LC-MS research and forensic methods improves efficiency and throughput
- Multi-channeling also increases the cost-effectiveness of your mass spectrometer

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We thank Dr. Hashim Othman of BioReference Laboratories (Elmwood Park, NJ) for supplying QCs and specimens for our tests and advice on desired quantitation ranges, accuracy and repeatability criteria.

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Full Optimization of LC/MS Methods to Increase Robustness of Complicated Matrix Containing Samples Using Active Flow Management Chromatography

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Overview

Purpose: This poster demonstrates the proof of concept of using active flow management (AFM) or curtain flow (CF) chromatography for LC/MS research and forensic methods to increase sensitivity and robustness especially when complex matrices are used.

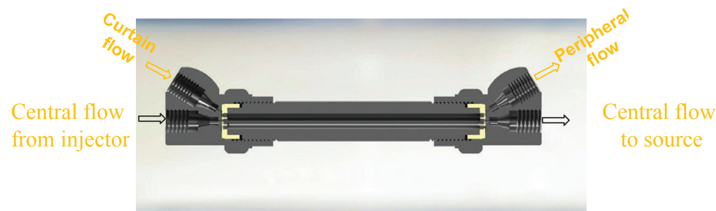
Methods: To investigate improvements in sensitivity and reproducibility, a model 50 x 4.6 mm i.d. AFM column was compared with a conventional 50 x 4.6 mm i.d. column and a conventional 50 x 2.1 mm i.d. column using a series of standard steroids. Comparisons were made of peak heights and areas, S/N, %RSD, and %Diff (precision and accuracy) between column dimensions. Mobile phase flow rates were adjusted to supply an equal flow to the MS source. Detection limits were also determined using alprazolam (2 fg to 2 pg alprazolam on column) in crashed plasma. Column robustness was investigated through repeated injections (20 µL) of a 5 fg/µL alprazolam solution in (1:1 v) synthetic urine. All analyses were performed on the Thermo Scientific™ TSQ Quantiva™ triple quadrupole mass spectrometer.

Results: Comparison between a conventional 50 x 4.6 mm i.d. column with a post-column split (PCS), a 50 x 2.1 mm i.d. column, and a CF 50 x 4.6 mm i.d. column showed increases in peak areas, heights, and S/N with the CF column over both the conventional 4.6 mm i.d. (on average, 2.5 times increase) and conventional 2.1 mm i.d. (on average, 3.5 times increase), while increasing both precision and accuracy of the analysis. Analysis of alprazolam in 30% crashed plasma showed increased sensitivity, reducing the LOQ of 6 fg on column and an LOD below 2 fg on column. Further robustness studies showed excellent peak shape retention following more than 220 injections of 100 fg on column of alprazolam in synthetic urine.

Introduction

Small-bore (2 mm i.d.) columns are a very popular choice for LC/MS separations, primarily due to their optimum flow rate facilitating both electrospray and atmospheric pressure chemical ionization methods. One disadvantage of small-bore HPLC columns is their increased wall effects that decrease chromatographic efficiency. A study by Gritti and Guiochon[1] showed that the loss in optimal performance in a 2.1 mm i.d. column format compared to a 4.6 mm i.d. format (based on the reduced plate height, *h*) for superficially porous particles, with particle size diameters of 1.7 to 2.7 µm was between 13% and 42%, depending on particle size, column length, and manufacturer. The use of narrow-bore columns may also limit sensitivity in LC/MS research and forensic methods due to the reduced mass loading capability of the column, which is essentially proportional to the square of the column radius. One approach to remove the influence of wall effects on column efficiency which is presented here uses curtain flow chromatography. In curtain flow columns, the mobile phase is managed at both the inlet and outlet of the column to create a virtual column inside the analytical column (Figure 1).[2] The virtual column has a narrower internal diameter; the dimensions are related to the volumetric ratio of flow exiting the column through the central zone relative to the flow exiting the peripheral zones. Using an active flow management chromatography column with an overall larger inner diameter (4.6 mm) adds an additional benefit of increased loading capacity (facilitating larger injection volumes) and increasing robustness to matrix containing samples.

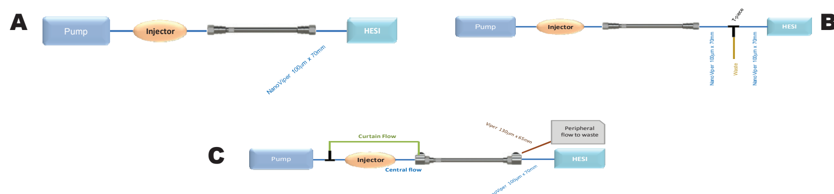
FIGURE 1. Illustration of curtain flow column. Mobile phase is split prior to the sample injector. Sample is injected to the central flow region. Mobile phase flow is managed at both the inlet and outlet ends of the column. Virtual column diameter is related to the ratio of flow between the central flow region and the peripheral flow region.



Methods

Columns were connected in the manner shown in Figure 2. For the conventional 2.1 mm i.d. column, a 1 mL/min direct flow to source was used with a 2 µL injection volume to maintain concentration consistency. The conventional 4.6 mm i.d. column included a 70% post-column split to waste for consistent comparison of sample introduction to the MS source. All columns were packed with Thermo Scientific™ Accucore™ C18 2.6 µm stationary phase.

FIGURE 2. Illustrations of column installation. The length and inner diameter of the connective tubing was adjusted to provide similar backpressures (linear velocities and split flow ratios). A) Conventional 50 x 2.1 mm i.d. column connected directly to injector and MS source. B) Conventional 50 x 4.6 mm i.d. column connected directly to injector but including a 70% split to waste post column. C) Curtain flow 50 x 4.6 mm i.d. column installation. Flow split from injector to peripheral inlet. Peripheral outlet flow to waste. Central flow zone from injector to MS source.



The LC system was a Thermo Scientific™ UltiMate™ 3000 RS system. For curtain flow column installation, the flow was split immediately prior to the injection valve in the autosampler. Mobile phase composition for the column performance study was A: 0.1% formic acid (aq), B: methanol (MeOH) + 0.1% formic acid in a 65:35 ratio isocratically. Gradient elution was used for all other studies with mobile phase A: 2 mM ammonium acetate (NH_4OAc) (aq) and B: acetonitrile (ACN) (sensitivity and linearity) and 2 mM NH_4OAc (aq) and B: MeOH + 2 mM NH_4OAc (urine study). Flow rates were measured volumetrically to determine the actual flow entering the MS source (with the exception of the 2.1 mm i.d. column). Samples were analyzed by selected-reaction monitoring (SRM) with a TSQ Quantiva MS in heated-electrospray ionization (HESI) mode

Results

Column Performance

Column performance was measured using a mixture of steroids – ketotestosterone, nortestosterone, testosterone and epitestosterone – in concentrations from 1 to 100 pg/µL with six replicate injections per sample. Figure 3 shows the TIC chromatograms of the test compounds (1 pg/µL). The mean of the performance parameters of peak area, height, S/N, and RSD% (area and height) were graphed and compared. Figure 4 shows peak area comparison (A) and area RSD% (B), peak height comparison (C), and %RSD for peak height

FIGURE 3. TIC Chromatograms (SRM) of ketotestosterone, nortestosterone, testosterone and epitestosterone on A) Conventional 4.6 mm i.d. column with PCS, B) Conventional 2.1 mm i.d. column and C) CF 4.6 mm i.d. column. Intensity was normalized to most intense base peak.

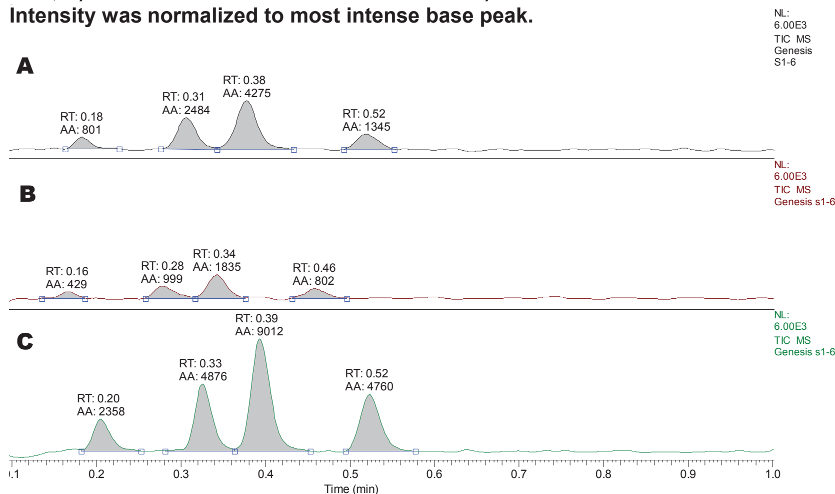
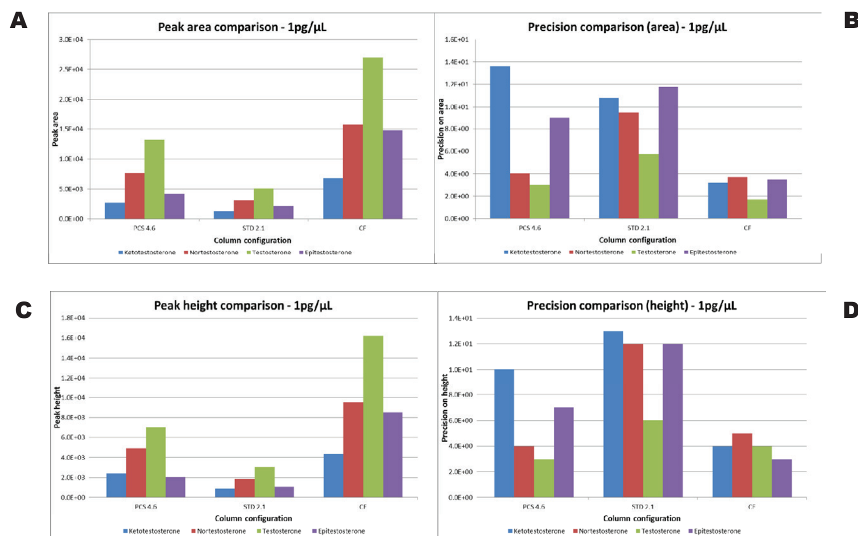


FIGURE 4. A) Comparison of peak areas for conventional 4.6 mm i.d. column with PCS, conventional 2.1 mm i.d. column, and CF 4.6 mm i.d. column. B) %RSD of peak area for six replicate injections. C) Peak height comparison. D) %RSD peak height. Individual components are coded in the graph legend.



For all test compounds, the curtain flow 4.6 mm i.d. column showed increased performance in peak area and peak height, as well as an increase in precision of those performance measurements.

Calibration curves were generated on the performance test mixture at concentrations from 1 pg/µL to 100 pg/µL. RSD% were calculated using six replicate injections. Figure 5 shows the CF 4.6 mm i.d. column maintains excellent linearity compared to a conventional 4.6 mm i.d. column with PCS and a conventional 2.1 mm i.d. column. Table 1 lists RSD% on the lowest concentration sample (1 pg/µL). The CF 4.6 mm i.d. column provides better reproducibility at the lower concentration samples then either the conventional 4.6 mm i.d. or the 2.1 mm i.d. column.

FIGURE 5. Calibration curves for 11-ketotestosterone generated on A) conventional 4.6 mm i.d. column with PCS, B) conventional 2.1 mm i.d. column, and C) CF 4.6 mm i.d. column

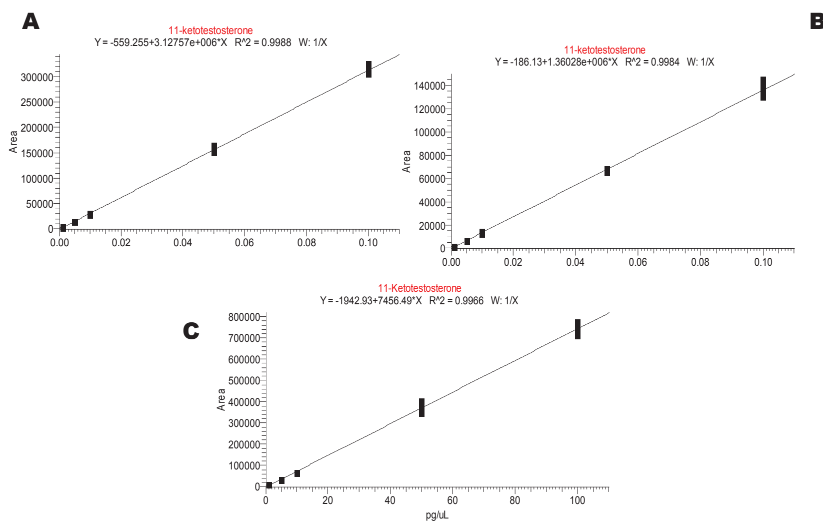


TABLE 1. %RSD (precision) for 1 pg/µL (6 replicates) sample of 11-ketotestosterone (Cmpd 1), nortestosterone (Cmpd 2), testosterone (Cmpd 3) and epitestosterone (Cmpd 4) analyzed on a conventional 4.6 mm i.d. column with PCS, a conventional 2.1 mm i.d. column, and a curtain flow 4.6 mm i.d. column

Column	Cmpd 1	Cmpd 2	Cmpd 3	Cmpd 4
4.6 mm PCS	13.58	4.17	2.76	7.01
2.1 mm	10.79	9.54	5.73	11.79
4.6 mm CF	3.2	3.67	1.75	3.48

A sensitivity study was also performed using the CF 4.6 mm i.d. column by generating a calibration curve for alprazolam from 2 fg to 2000 fg on column in 30% crashed plasma (30% ACN added to plasma and centrifuged to remove solids). Samples were analyzed in three replicates by gradient elution (mobile phase A = 2 mM NH_4OAc (aq), B = ACN) using the gradient conditions in Table 2. The pump flow rate setting was 3 mL/min, which resulted in a central zone flow of 900 µL/min. Samples were detected using a TSQ Quantiva MS by SRM (+), monitoring two transitions, *m/z* 309 → 281 and *m/z* 311 → 283 (Cl37) with 30 V collision energy and collision gas pressure of 2.5 mTorr. A calibration curve was generated with 1/x weighting and is shown in Figure 6. Both alprazolam (309 → 281) and the naturally occurring ^{37}Cl parent ion (311 → 283) were monitored and both show excellent linearity. %RSD indicates an LOQ of 6 fg on column with an LOD of 2 fg on column. Figure 7 shows TIC chromatograms at both LOQs and peak shape reproducibility at the LOQ of 6 fg on column.

TABLE 2. Gradient elution conditions

Time (min)	% Mobile Phase B
0	40
0.2	40
2	70
2.01	90
2.5	90
2.51	40
3	40

TABLE 3. MS Acquisition conditions

Source Conditions	
Ionization	HESI +
Source Voltage	600 V
Cap Temp	350 °C
Vap Temp	450 °C
Q1 FWHM	0.7
Q3 FWHM	0.7
Cycle Time	0.3 min
Chrom Filter	3 s

FIGURE 6. Calibration curves of alprazolam in crashed plasma. Top - alprazolam; bottom - alprazolam – ^{37}Cl (naturally occurring)

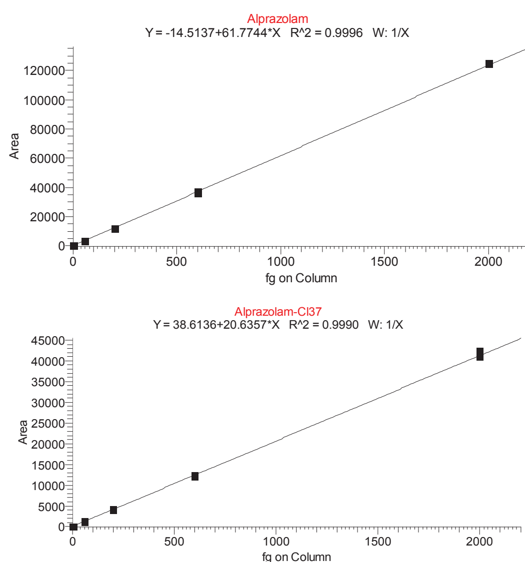
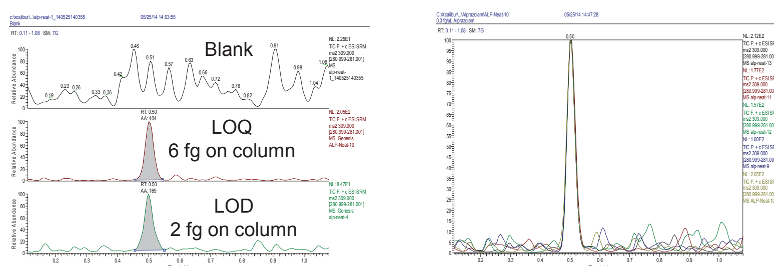


TABLE 4. RSD% for alprazolam. ^{37}Cl species was not detected in all five replicate samples and reported as not detected but was included in calibration curve.

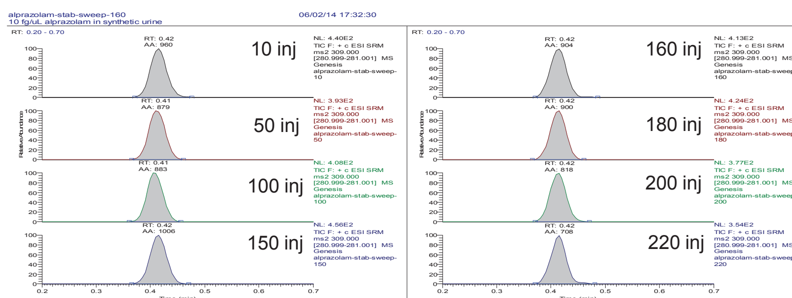
Concentration (fg on column)	Alprazolam	Alprazolam–^{37}Cl	^{37}Cl/^{35}Cl (peak area)
2	22.8	*	NA
6	14.7	15.9	0.266
60	3.4	9.2	0.261
200	0.9	3.2	0.251
600	1.2	1.3	0.248
2000	0.3	1.5	0.252

FIGURE 7. TIC chromatograms of LOQ and LOD of alprazolam by curtain flow chromatography. Also shown are the five replicate injections at the LOQ of 6 fg on column showing excellent reproducibility in crashed plasma..



To determine column robustness, 100 fg (in a 1:1 synthetic urine) was repeatedly injected and peak areas were reported. Figure 8 shows peak shape and areas at various stages of this analysis.

FIGURE 8. TIC chromatograms of various injections of 100 fg on column alprazolam in 1:1 v/v synthetic urine, 20 µL injection



Conclusion

- Curtain flow chromatography columns offers significant advantages in sensitivity and robustness over small-bore HPLC columns by eliminating wall effects that lead to decreased column performance.
- Curtain flow chromatography columns improve LC/MS research and forensic methods especially where larger injection volumes are required and samples contain complex matrices.
- Future work requires reducing the actual inner diameter of the column to allow virtual column inner diameters < 1mm, reducing overall flow rates and solvent consumption. This can potentially show a large efficiency improvement where micro-bore columns are more susceptible to wall effects than 2.1 mm i.d. columns.

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Targeted Quantitation of Insulin and Its Therapeutic Analogs for Research

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Overview

Purpose: To perform simultaneous qualitative and quantitative analyses of endogenous insulin and/or therapeutic analogs at biological levels for research.

Methods: We used a pan-anti insulin antibody in Thermo Scientific™ Mass Spectrometric Immunoassay (MSIA) D.A.R.T.'S™ pipette tips for highly-selective affinity purification of all insulin analogs. Analogs were detected, verified, and quantified using high-resolution, accurate-mass (HRAM) MS and MS/MS data from a Thermo Scientific™ Q Exactive™ mass spectrometer.

Results: We achieved a lower-limit-of-detection (LLOD) of 15 pM in plasma for all variants used with linear regressions of 0.99 or better. Further, we demonstrate inter- and intra-day CV's of < 3% and spike and recovery resulted in recoveries of 96–100%.

Introduction

The measurement of insulin is a paramount metric in clinical research, therapeutic research, forensic, and sports doping applications. Conventional insulin analytical methods are plagued by the inability to differentiate endogenous insulin from exogenous insulin analogs. The use of LC/MS can overcome this shortcoming[1]; however, the LC/MS methods to date lack the analytical sensitivity demanded by the field. Therefore, a highly selective sample interrogation workflow is required to address the complexity of plasma samples and, ultimately, for accurate and sensitive LC/MS detection and quantification. To meet these requirements, a MSIA research workflow was developed for the high-throughput, analytically sensitive quantification of insulin and its analogs from human donor plasma.

Methods

Sample Preparation

For spike and recovery studies, both neat and donor plasma samples containing a mix of insulin and its analogs were prepared. Insulin was added at three different amounts that spanned the dynamic range to the donor plasma. Up to four analogs were prepared in a single sample. For the limit-of-detection and limit-of-quantification studies, 1.5 pM to 960 pM insulin was added to bovine serum albumin in phosphate buffered saline. Additionally, either 0.05 nM of a heavy version of insulin or porcine insulin was added as an internal reference standard to each well of 500 µL plasma.

Samples were then addressed for the first stage in the MSIA workflow. Targeted selection was achieved using insulin MSIA Disposable Automated Research Tip's (D.A.R.T.'S) (Figure 1). The affinity purification step in the MSIA workflow was automated by the Thermo Scientific™ Versette™ automated liquid handler. Following extraction, intact insulin analogs were eluted with 75 µL 70:30 water/acetonitrile with 0.2% formic acid with 15 µg/mL ACTH 1-24. The final concentration was adjusted to 75:25 water/acetonitrile with 0.2% formic acid for LC/MS analysis.

Liquid Chromatography

A Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system was used for all experiments. 100 µL of each sample was separated on a 100 x 1 mm Thermo Scientific™ ProSwift™ column using a linear gradient (10–50% in 10 min) comprised of A) 0.1% formic acid in water and B) 0.1% formic acid in acetonitrile. The column was heated to 50 ºC.

Mass Spectrometry

All data was acquired using a Q Exactive Orbitrap mass spectrometer operated in data-dependent mode with dynamic exclusion enabled. Full scan MS data was acquired with a resolution setting of 70,000 (at *m/z* 200) and using a mass range of 800–2000 Da. A targeted inclusion list was used to trigger MS/MS events and MS/MS was acquired with a resolution setting of 17,500 (at *m/z* 200).

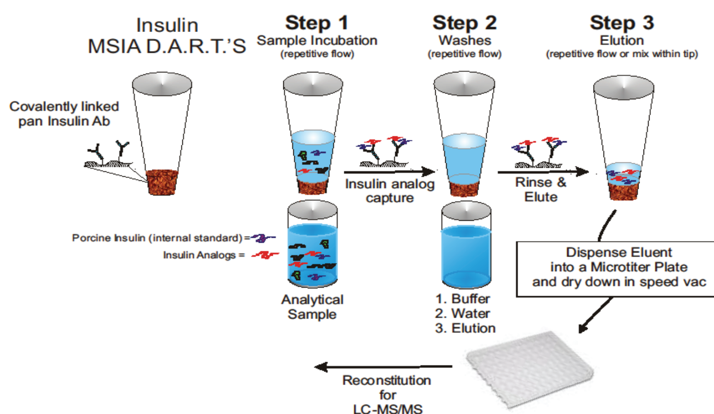
Data Analysis

Thermo Scientific™ Pinpoint™ software version 1.3 was used to analyze all LC/MS data. HRAM measurements were used for qualitative and quantitative measurement of insulin and its analogs.

The three most abundant precursor charge states per analog and the six most abundant isotopes per charge state provided qualitative validation for insulin and its analogs. Qualitative scoring was based on mass error, precursor charge state distribution, isotopic overlap, and corresponding LC elution peak profiles. Product ion data was used for sequence verification.

For quantification, a mass tolerance of ±5 ppm was used for all data extraction. Amounts of each insulin analog were determined by converting area-under-the-curve (AUC) values, normalized to the AUC of the internal reference, which was calculated from standard curve data.

FIGURE 1. Targeted selection using insulin MSIA D.A.R.T.'S.. First, insulin and its analogs are selectively bound. Then, a wash step removes background compounds. Lastly, the insulin and insulin variants are eluted into a new plate, which is ready for LC/MS analysis.



Results

Qualitative Validation of Insulin and Its Analogs

One of the primary limitations of current insulin analytical methods is the inability to distinguish between endogenous and exogenous insulin analogs. The immobilized insulin pan-antibody in the MSIA D.A.R.T.'S recognizes a common epitope region in the β-chain that is conserved across all of the analyzed variants. This allows the capture and detection of all variants from the sample as long as the β-chain epitope region remains conserved. Further, utilizing full scan MS mode in the analysis stage of the MSIA workflow enables simultaneous detection of multiple insulin analogs and the ability to screen for unsuspected insulin analogs post-acquisition.

LC/MS detection using HRAM MS data provided the analytical selectivity to distinguish insulin variants from the background signal using the accurate mass of multiple precursor charge states and isotopes. Figure 2 demonstrates the HRAM data analysis approach. Figure 3 shows simultaneous LC/MS detection of insulin variants. Further, fragmentation patterns from data-dependent MS/MS acquisition can also be used to confirm the identity of insulin variants (data not shown).

FIGURE 2. HRAM MS data analysis in Pinpoint software version 1.3. Extracted ion chromatograms for each targeted insulin variant were created using the isotopic *m/z* values from three precursor charge states. Integrated AUC values from each isotope were then co-added to generate the reported values. Additionally, each insulin variant was qualitatively scored based on 2a) comparative peak profiles (peak start and stop, apex, and tailing factors) as well as 2b) isotopic distribution overlap.

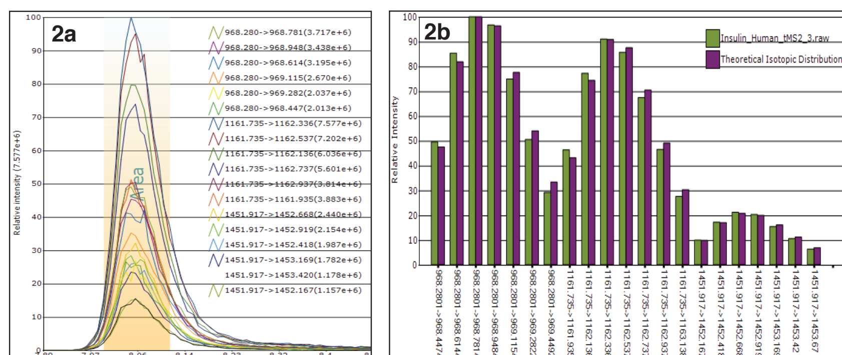
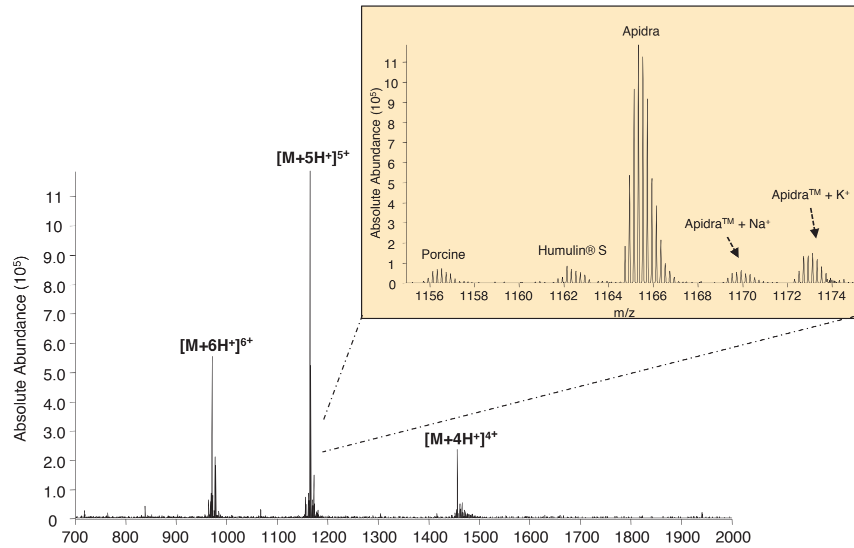


FIGURE 3. Simultaneous LC/MS detection of four insulin variants. Apidra™ (0.48 nM), Humulin® S (0.06 nM), Lantus™ (0.48 nM) , and porcine as the internal standard were processed from the same sample and detected simultaneously. The inset shows an enlargement of the 5+ charge state, and shows all three variants. Lantus elutes 0.5 minutes prior to the three displayed insulin variants.

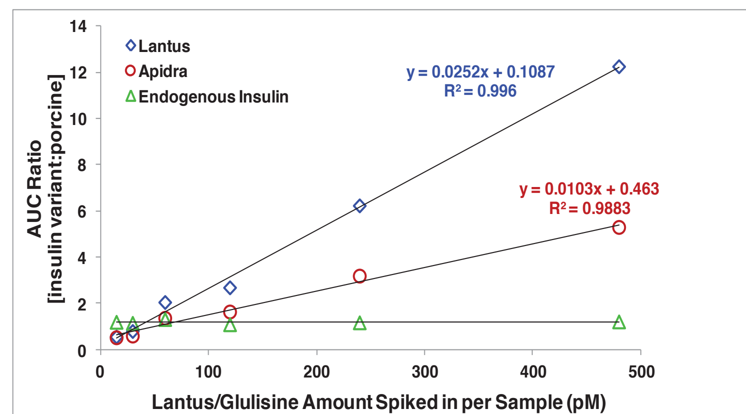


Quantitative Measurement of Insulin and Its Analogs

Additional limitations to high-throughput targeted quantification of insulin and its analogs in research are inefficient sample preparation protocols that result in their lack of analytical sensitivity and robustness. Using the insulin MSIA workflow described above, we achieved an LLOQ and LOD of 15 pM (87 pg/mL) for the intact variants in plasma. Quantification curves for Lantus and Apidra are shown in Figure 4. Tables 1 and 2 display LOQ and LOD.

Further, reproducibility studies demonstrated inter- and intra-day CVs of < 3% (Tables 3 and 4) and spike and recovery resulted in recoveries of 96–100% (Table 5). In addition to the improved sensitivity, the MSIA workflow significantly reduces the background matrix. The reduced complexity affords shorter LC gradients, and, therefore, shorter LC/MS analysis times.

FIGURE 4. Quantification curves for Lantus and Apidra. Lantus and Apidra were spiked into donor plasma at different concentrations. The endogenous insulin from the donor plasma is also plotted. Since the same amount of donor plasma was used for each sample, the level of endogenous insulin remains static. All AUC values were normalized to the porcine AUC response.



Method Characteristics for the MSIA Insulin Research Workflow

The LLOQ for the insulin MSIA research workflow is 15 pM (highlighted in red in Table 1), which was determined as the lowest concentration where we could achieve a %CV of <20% and an accuracy within ±20%.

An LOD of 15 pM (highlighted in red in Table 2) was also achieved for the insulin MSIA workflow. The LLOD was determined as the lowest concentration where the mean total area was greater than four standard deviations of the background signal added to the mean total area for the blank.

TABLE 1. Limit of quantification

STD Conc. (pM)	Mean (5 Curves)	StDev	%CV	Accuracy
0	7.42	1.02		
7.5	10.56	0.95	9.04%	40.80%
15	16.78	1.42	8.46%	11.87%
30	28.96	1.12	3.85%	-3.46%
60	58.41	1.61	2.75%	-2.66%
120	115.93	1.96	1.69%	-3.39%
240	232.65	2.80	1.20%	-3.06%
480	473.25	14.41	3.04%	-1.41%
960	963.31	6.47	0.67%	0.34%

TABLE 2. Limit of detection

STD Conc. (pM)	Mean Total File Area	4 × StDev	Plus 4 × StDev
0	2.37E+05	2.20E+05	4.57E+05
7.5	2.80E+05		
15	4.79E+05		
30	8.93E+05		

TABLE 3. Intra-day repeatability

STD Conc. (pM)	Mean (3 Controls x 5 Curves)	StDevp	%CV	Accuracy
50.00	51.21	1.33	3	2.43%

TABLE 4. Inter-day repeatability

STD Conc. (pM)	Mean (3 Controls x 5 Curves)	StDevp	%CV	Accuracy
50.00	51.07	0.81	2	2.15%

Sample	Spike Conc. (pM)	Exp. Conc. (pM)	Average (pM)	Exp Recovery Conc. (pM)	% Yield
Neat_1	0.00	43.79	44.59		
Neat_2		45.59			
Neat_3		44.38			
Low_1	19.50	65.08	64.11	19.52	100.12%
Low_2		63.65			
Low_3		63.61			
Medium_1	199.50	241.19	237.56	192.97	96.73%
Medium_2		239.80			
Medium_3		231.70			
High_1	919.50	960.91	928.63	884.05	96.14%
High_2		905.35			
High_3		919.64			

Conclusions

- Automated sample extraction is amenable to high-throughput analysis, thus decreasing sample preparation times.
- Insulin MSIA D.A.R.T.'S equivalently extract multiple insulin variants present at different concentrations for simultaneous detection and quantification for research.
- HRAM MS affords qualitative confirmation and quantification of the insulin variants present in one LC/MS run.
- Pinpoint software version 1.3 provides automated data extraction, confirmation, and quantification for all insulin analogs.
- Reduced complexity affords shorter LC gradients, and, therefore, shorter LC/MS analysis times.
- An LLOD < 15 pM and an LLOQ of 15 pM (87 pg/mL) in 0.5 mL of plasma were achieved.
- Intra- and inter-day repeatabilities were < 3%, thus making the insulin MSIA workflow highly reproducible.

References

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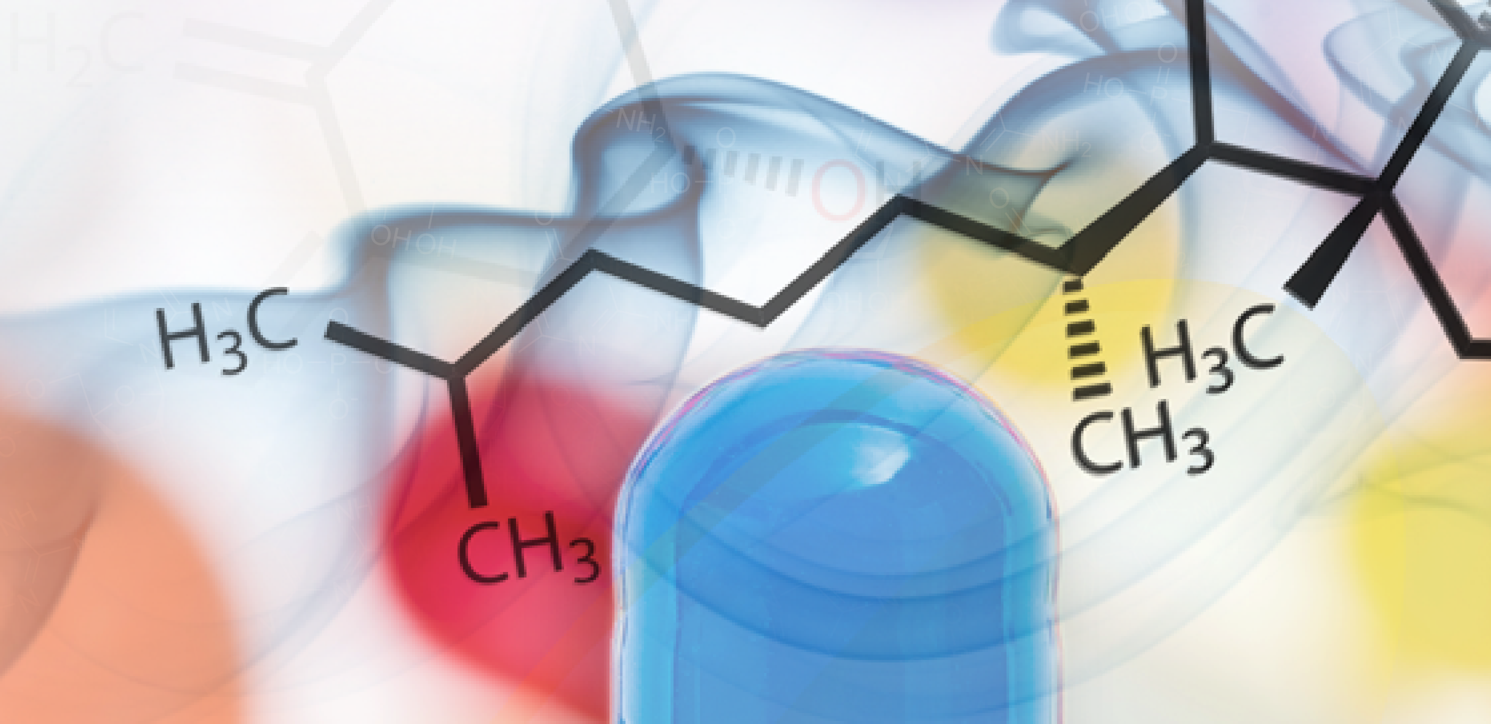

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Drug Monitoring Research

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AN618: An LC-MS/MS Research Method for the Quantification of Mycophenolic Acid (MPA) in Plasma

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AN603: Quantitative Analysis of Immunosupressants in Dried Blood Spots Using the TSQ Endura Triple Quadrupole MS for Research

AN472: Research Analysis of Clozapine and Norclozapine in Plasma Using Automated Sample Preparation and LC-MS/MS

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An LC-MS/MS Research Method for the Quantification of Mycophenolic Acid (MPA) in Plasma

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Key Words

TSQ Quantum Ultra, mycophenolic acid, MPA, plasma, quantitation

Goal

The goal of this work was to use LC-MS/MS to validate the MassTox® Mycophenolic Acid kit from ChromSystems® on the Thermo Scientific™ TSQ Quantum Ultra™ mass spectrometer for research purposes.

Introduction

This note describes a method developed to quantify mycophenolic acid (MPA) by LC-MS/MS with the ChromSystems MassTox Mycophenolic Acid kit. The method was analytically validated for research use using the following parameters:

- Both intraday and interday accuracy and precision for the quality controls
- Lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ)
- Carryover

Methods

The MassTox Mycophenolic Acid kit consists of:

- Four calibrators (1 blank and 3 calibrators)
- Two quality controls (Level I and Level II)
- Internal standard set consisting of an internal standard mix and a reconstitution buffer
- Precipitation reagent solution
- Extraction buffer solution
- Mobiles phases A and B

Calibrator and Quality Control (QC) Preparation

Lyophilized calibrators and quality controls were reconstituted with 1 mL of distilled water. They were left at room temperature for 15 minutes and shaken occasionally until the contents were homogeneous. Aliquots of 50 µL were stored in 1.5 mL vials at -20 °C for a maximum period of 3 months.

Internal Standard Preparation

Internal standards were reconstituted with 1 mL of reconstitution buffer. The vial was left for 5 minutes at room temperature. It was shaken periodically and gently until the contents were homogeneous. Next, 800 µL of this solution was added to 12 mL of precipitation reagent and the mixture was stored in the dark at 4 °C for 28 days.

Sample Preparation

A 25 µL measure of extraction buffer was added to 50 µL of each calibrator, control, and sample. The mixture was vortexed for 10 seconds and incubated for 2 minutes at room temperature. Then, 250 µL of reconstituted internal standard mix was added to the vial and vortexed for 30 seconds. It was then centrifuged at 14,000 rpm for 5 minutes. Finally, 10 µL of the supernatant was diluted 20 times in a mixture containing methanol and water (LC/MS grade) (50/50, v/v).

Calibration Curve

The concentrations of the calibrators were 0.97, 3.89, and 9.46 mg/L.

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Liquid Chromatography

Chromatographic separation was performed with a Thermo Scientific™ Accela™ 1250 pump and Accela Open autosampler. The analytical column was a MassTox TDM analytical column series A from ChromSystems. The column was maintained at room temperature. Mobile phases A and B were also provided by ChromSystems. Details of the LC gradient are shown in Table 1. The injection volume was 10 µL.

Table 1. LC gradient.

Time (min)	A (%)	B (%)	Flow rate (µL/min)
0	40	60	600
0.5	0	100	600
1	0	100	600
1.10	40	60	600
1.8	40	60	600

Mass Spectrometry

MS/MS was performed using a Thermo Scientific™ TSQ Quantum Ultra™ triple-stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe in positive mode. The MS conditions were as follows:

Spray voltage	2500 V
Vaporizer temperature	350 ˚C
Sheath gas pressure (arbitrary units)	30
Auxilliary gas pressure (arbitrary units)	15
Capillary temperature	250 ˚C

Data were acquired in selected-reaction monitoring (SRM) mode. SRM settings for the MPA and its internal standard are shown in Table 2.

Table 2. SRM settings for the analyte and its corresponding internal standard.

Analyte	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Collision Energy (V)	Tube Lens (V)
MPA	321.0	207.0	22	80
MPA-d_3	324.0	210.0	22	80

Results and Discussion

Intraday Precision and Accuracy

The intraday precision and accuracy were evaluated using 20 replicates of the two quality control samples at the following concentrations: Level 1 (1.94 mg/L) and Level 2 (5.5 mg/L). The precision was calculated as the coefficient of variation (CV, %) within a single run and the accuracy as the bias or percentage of deviation between nominal and measured concentration. Results are reported in Table 3.

Table 3. Intraday accuracy and precision results.

Quality Controls	Accuracy (%)	CV (%)
Level 1	113.2	2.7
Level 2	112.1	2.7

Interday Precision and Accuracy

The interday precision and accuracy were evaluated using 10 replicates of the two quality control samples at the following concentrations: Level 1 (1.94 mg/L) and Level 2 (5.5 mg/L). The precision was calculated as the coefficient of variation (CV, %) between different extractions and runs, and the accuracy as the bias or percentage of deviation between nominal and measured concentration. Results are reported in Table 4.

Table 4. Interday accuracy and precision result.

Quality Controls	Accuracy (%)	CV (%)
Level 1	103.1	7.0
Level 2	103.1	4.9

Lower Limit of Quantitation (LLOQ), Upper Limit of Quantitation (ULOQ), and Linear Range

As previously noted, the MassTox Mycophenolic Acid kit contains 3 calibrators at 0.97, 3.89, and 9.46 mg/L. Figure 1 shows SRM chromatograms of MPA at 0.97 mg/L (Calibrator 1).

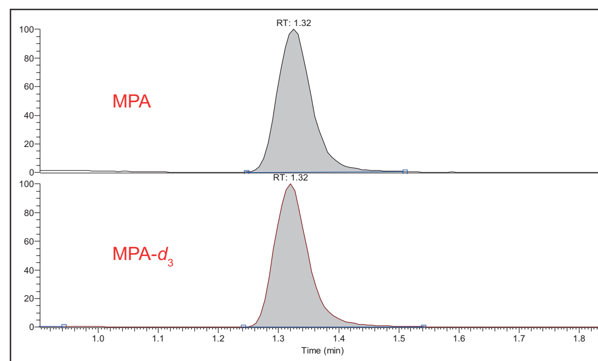

Figure 1. SRM chromatograms of MPA at 0.97 mg/L (Calibrator 1).

In some cases, real samples may present concentrations up to 30 mg/L. For this reason, it was decided to evaluate the upper limit of quantitation. A 50 mg/L solution of MPA was prepared in bovine serum albumin (Sigma-Aldrich). This concentration in the linear range gave accuracy within 100 ± 15% and a CV less than 15%.

To determine the best weighting factor, concentrations were back-calculated and the model with the lowest total bias across the concentration range was considered the best suited. Four-point calibration standard curves were calculated and fitted by linear models. To determine LLOQ, a ten-fold dilution with bovine serum albumin was made from Calibrator 1 to get a concentration of 0.097 mg/L. At this LLOQ, the accuracy and precision values were, respectively, 108% and 4.6% for 10 replicates.

Figure 2 shows a representative calibration curve of MPA.

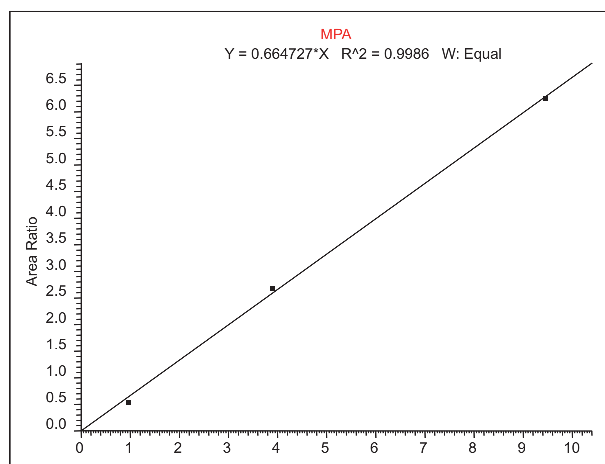

Figure 2. Calibration curve of MPA.

Carryover

Carryover was evaluated by injecting two levels of plasma in triplicate: a blank plasma (B) and a high-level plasma spiked at 20 mg/L (H). The injection sequence was three injections of the high level (H_1, H_2, H_3) followed by three injections of the low level (B_1, B_2, B_3). The sequence was repeated five times and the carryover was calculated using the following formula expressed as a percentage:

Carryover = (b1-b3) / (h-b3) x 100

b1: average concentration obtained for all B1 injections
b3: average concentration obtained for all B3 injections
h: average concentration obtained for all H injections (from H1 to H3)

Carryover was evaluated to be less than 1.6%.

Conclusion

A simple and fast LC-MS/MS method was analytically validated for the analysis of mycophenolic acid for research purposes. Intraday and interday accuracy and precision were successfully assessed in plasma-based samples.

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Quantitation of Immunosuppressant Drugs in Whole Blood Using the Prelude-SPLC System and TSQ Endura Mass Spectrometer for Research

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Key Words
Immunosuppressant drugs, Prelude SPLC, TSQ Endura

Goal
To develop a rapid, sensitive, selective, and robust LC-MS/MS method to determine the concentrations of cyclosporine A, tacrolimus, sirolimus, and everolimus in whole blood.

Introduction
LC-MS/MS-based methods have advantages due to their selectivity and low cost compared to traditional immunoassay-based methods. In addition, unlike immunoassays, LC-MS/MS-based methods are ideally suited to the analysis of multiple compounds in a single analytical run. In this report, a single analytical run was used to precisely and accurately measure the levels of four immunosuppressant drugs in blood for research. This was accomplished by using a sample preparation and liquid chromatography (SPLC)-MS/MS system, which combines online sample extraction powered by Thermo Scientific™ TurboFlow™ technology with chromatographic separation. The Thermo Scientific™ Prelude SPLC™ system features two independent channels of sample preparation and liquid chromatography. Thus, the chromatographic methods on the Prelude SPLC system can be executed in parallel, either with a different method on each channel or the same method on both channels. Two channel multichannel operation on the Prelude SPLC system is automatically optimized into one mass spectrometer for serial detection, which improves mass spectrometer utilization time, increases throughput, and reduces analysis cost. The Prelude SPLC syringe pumps and high pressure, low-volume gradient mixing provide enhanced HPLC performance with improved peak shape and resolution as well as stable retention times, compared to the dual piston reciprocating pumps.

Methods
Sample Preparation
A 200 µL aliquot of whole blood sample was mixed with 300 µL of zinc sulfate solution (0.1 M) in a 1.5 mL centrifuge tube and vortexed for 30 seconds. The mixture was further processed by adding 500 µL of methanol (Fisher Chemical brand) containing internal standards (40 ng/mL D_{12}-cyclosporine A and 4 ng/mL $^{13}CD_2$tacrolimus). The sample was immediately vortexed for another 30 seconds. The entire mixture was centrifuged at 4000 RCF for 10 minutes. A 40 µL sample was analyzed.

Liquid Chromatography
SPLC-MS/MS analysis was conducted using a Prelude SPLC powered by TurboFlow technology coupled to a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer. The processed sample was directly injected onto a Thermo Scientific™ Cyclone-P™ column (0.5 x 50 mm, Part Number: CH-953289) for online sample cleanup. This step was followed by chromatographic separation on a Thermo Scientific™ Accucore™ C8 column (3 x 30 mm, 2.6 µm particle size, Part Number: 17226-033030). The Cyclone-P TurboFlow column was maintained at room temperature while the Accucore C8 column was maintained at 70 °C. The total run time was 5 minutes and the total solvent consumption was 8.1 mL per sample, including online sample extraction and chromatographic separation. Figure 1 shows the SPLC method profile.

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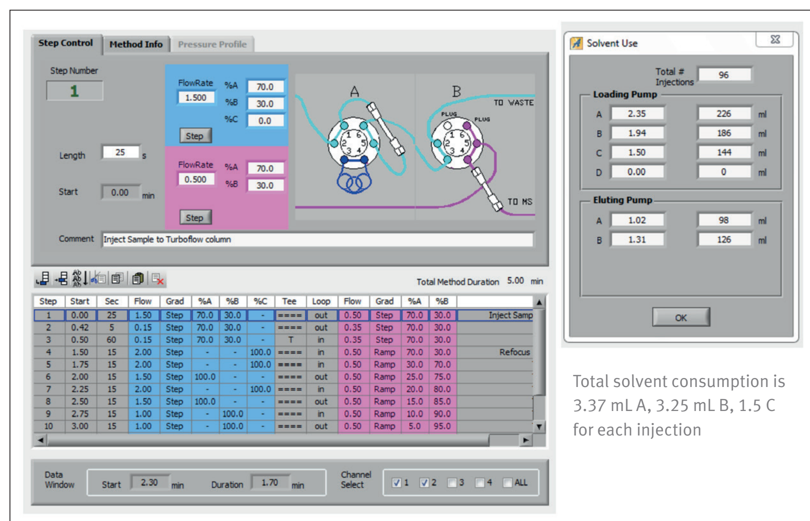


Total solvent consumption is 3.37 mL A, 3.25 mL B, 1.5 C for each injection

Figure 1. SPLC method profile

MS Method

Analyte detection was performed on a TSQ Endura MS equipped with a heated electrospray ionization (HESI) source. Table 1 shows MS conditions and Table 2 shows the selected-reaction monitoring (SRM) transitions for all four drugs and the two internal standards.

Table 1. MS conditions

Ionization	Heated electrospray ionization
Vaporizer temp	450 °C
Capillary temp	200 °C
Spray voltage	3500 V
Sheath gas	52 AU
Auxiliary gas	20 AU
Data acquisition mode	Selected-reaction monitoring (SRM)
Chrom filter peak width	3 s
Collision gas pressure	2 mTorr
Cycle time	0.2 s
Q1 (FWMH)	0.7
Q3 (FWMH)	0.7
SRM parameters	Refer to Table 2

Table 2. SRM Parameters

Compound Name	Q1 (*m/z*)	Q3 (*m/z*)	RF Lens	Collision Energy
Tacrolimus	821.6	768.5	224	24
$^{13}CD_2$-Tacrolimus	824.6	771.6	224	24
Cyclosporine A	1202.8	425.4	250	58
D_{12}-Cyclosporine A	1214.8	437.4	250	58
Sirolimus	931.7	864.5	250	23
Everolimus	975.5	908.5	224	23

Calibrators and Controls

Whole blood calibrators for the immunosuppressant drugs and quality control (QC) samples were purchased from ChromSystems Instruments & Chemicals GmbH. Vendor instructions were followed to reconstitute the lyophilized calibrators and controls.

Results and Discussion

Data were acquired and processed with Thermo Scientific™ TraceFinder™ software version 3.1. Figure 2 shows a representative chromatogram of the calibration standard at the lowest level. All calibration curves were linear with R^2 values greater than 0.9943. All of the QC samples were within 20% of the manufacturer-specified concentrations (Table 3). Table 4 shows the linearity range and R^2 values, and Figure 3 shows representative calibration curves for all four drugs. Figure 4 shows the extracted ion chromatogram along with the calculated concentrations for tacrolimus and sirolimus from different donor samples.

Table 3. Accuracy of QC samples

Sirolimus	Theoretical Amount	Calculated Amount	Difference (%)
QC1	2.90	2.66	-8.41
QC2	10.1	10.8	6.78
QC3	20.4	22.4	9.63
QC4	38.5	35.8	-7.09
Cyclosporin A	**Theoretical Amount**	**Calculated Amount**	**Difference (%)**
QC1	53.0	56.9	7.37
QC2	276	320	15.8
QC3	514	500	-2.75
QC4	1110	1190	6.77
Everolimus	**Theoretical Amount**	**Calculated Amount**	**Difference (%)**
QC1	2.30	2.21	-3.83
QC2	4.40	3.80	-13.6
QC3	8.50	9.42	10.8
QC4	28.8	27.1	-5.89
Tacrolimus	**Theoretical Amount**	**Calculated Amount**	**Difference (%)**
QC1	2.60	3.04	16.8
QC2	7.30	7.66	4.86
QC3	16.7	17.4	4.08
QC4	34.2	32.3	-5.60

Table 4. Linearity ranges

Compound Name	Linear Range (ng/mL)	R^2
Tacrolimus	2.1–38.5	0.9971
Cyclosporine A	23.3–919	0.9951
Sirolimus	2.3–46.1	0.9943
Everolimus	2.2–41.1	0.9973

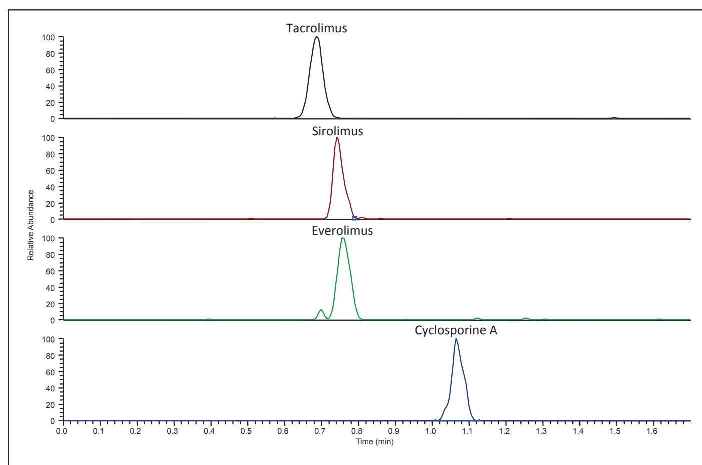
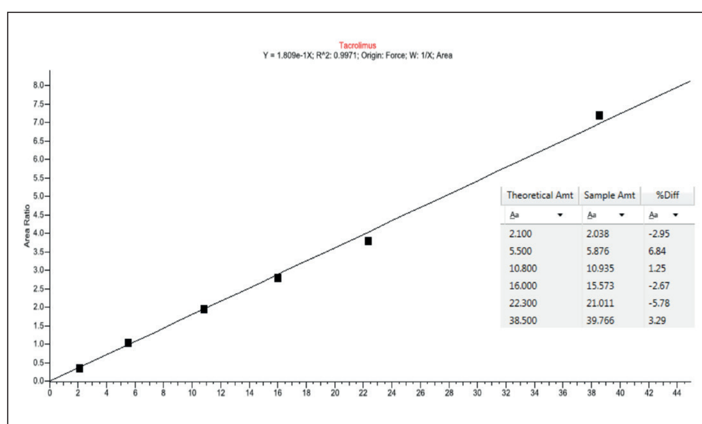
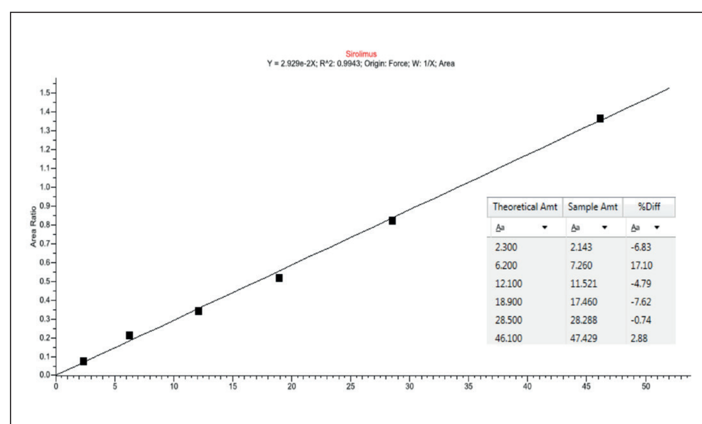


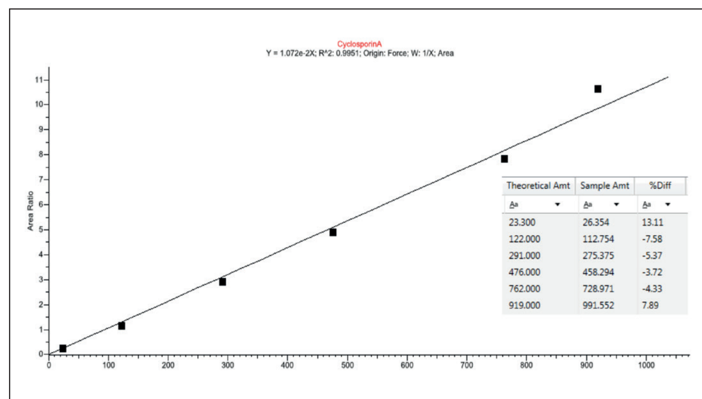
Figure 2. Chromatograms of the calibration standard at the lowest level



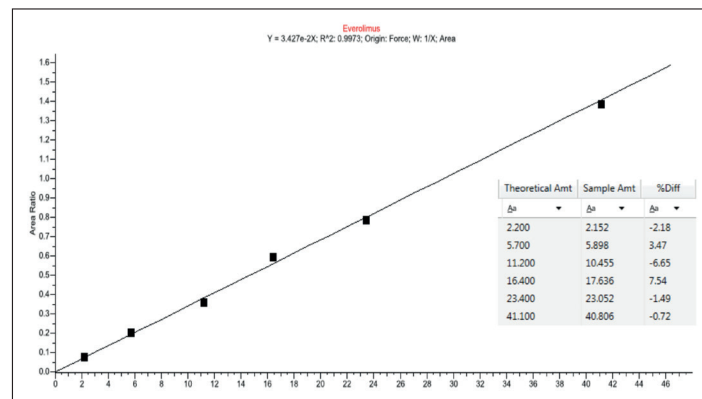
Theoretical Amt	Sample Amt	%Diff
2.100	2.038	-2.95
5.500	5.876	6.84
10.800	10.935	1.25
16.000	15.573	-2.67
22.300	21.011	-5.78
38.500	39.766	3.29



Theoretical Amt	Sample Amt	%Diff
2.300	2.143	-6.83
6.200	7.260	17.10
12.100	11.521	-4.79
18.900	17.460	-7.62
28.500	28.288	-0.74
46.100	47.429	2.88



Theoretical Amt	Sample Amt	%Diff
23.300	26.354	13.11
122.000	112.754	-7.58
291.000	275.375	-5.37
476.000	458.294	-3.72
762.000	728.971	-4.33
919.000	991.552	7.89



Theoretical Amt	Sample Amt	%Diff
2.200	2.152	-2.18
5.700	5.898	3.47
11.200	10.455	-6.65
16.400	17.636	7.54
23.400	23.052	-1.49
41.100	40.806	-0.72

Figure 3. Calibration curves of all four ISD drugs

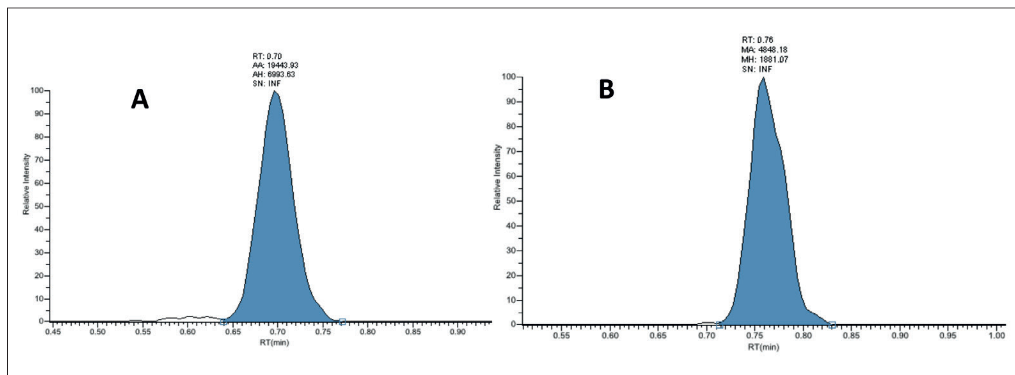


Figure 4. A: Chromatogram of tacrolimus quantifying peak from a donor at a calculated concentration of 13.6 ng/mL in whole blood (15.1 ng/mL reported from immunoassay); B: Chromatogram of sirolimus quantifying peak from a donor at a calculated concentration of 22.3 ng/mL in whole blood (22.4 ng/mL reported from immunoassay).

Conclusion

Using the Prelude SPLC system, a high-throughput and robust method was developed for the precise and accurate measurement of immunosuppressant drugs in blood for research. This method met analytical laboratory precision and accuracy criteria. Prelude SPLC system provides automated online sample cleanup and two-channel operation, thus minimizing the sample preparation steps and increasing the sample throughput. The total length of the SPLC run is 5 minutes with a data acquisition window of 1.75 minutes. With the two multiplexing channels on the Prelude SPLC, analytical throughput is 576 samples in 24 hours.

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Quantitative Analysis of Immunosuppressants in Dried Blood Spots Using the TSQ Endura Triple Quadrupole MS for Research

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Key Words
Immunosuppressant drugs, dried blood spots, TSQ Endura

Goal
To develop a rapid, sensitive, selective, and cost effective LC-MS/MS research method to determine the concentrations of cyclosporine A, tacrolimus, and sirolimus in dried blood spots down to 3 mm size.

Introduction
Immunosuppressants (IMS) have narrow therapeutic margins and thus have to be monitored routinely. Dried blood spots (DBS) on paper become a desirable method of sample collection because they can be collected in the field and shipped for analysis with minimal transportation safety requirements. Normally, 8 mm dried blood spots are used; however, reducing their size to 3 mm offers advantages in both minimizing sample volume sevenfold and automating sample preparation because standard size office paper punchers can be used to cut the dried blood spots. Sample reduction inevitably leads to a need for sensitive LC-MS/MS assays. In this application note, IMS in dried blood spots were analyzed using the Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer.

Methods
Sample Preparation
A stock internal standard (IS) solution in acetonitrile was prepared by spiking ascomycin (AsC), sirolimus-d_3 (d_3-SrL), and cyclosporin D (CsD) to a final concentration of 6 ng/mL (AsC and CsD) and 30 ng/mL (d_3-SrL).

A working IS solution was obtained by mixing two parts of the stock IS solution and one part of 0.01 M zinc sulfate in water to a final concentration of AsC, CsD, and d_3-SrL of 4, 4, and 20 ng/mL, respectively. Working IS solution was stored at 4 °C for 3 months.

Discs were punched from the DBS cards with an 8 mm punch into 2 mL microcentrifuge tubes. Then, 150 µL of working IS solution containing 0.01 M $ZnSO_4$ was added, ensuring that the entire spot was completely saturated. Tubes were vortex mixed gently for 3 sec and centrifuged at 15,700 rcf for 3 min. The sample was then mixed for 20 min. The supernatant was immediately transferred to autosampler vials, further diluted sevenfold with 66% acentonitrile in water to emulate 3 mm DBS, and 20 µL were injected into the LC-MS/MS system.

Liquid Chromatography

System:	Thermo Scientific™ Dionex™ UltiMate™ HPG3400-RS pump, UltiMate WPS-3000 autosampler, UltiMate TDS-3000 column compartment
Column:	Proprietary
Mobile phase A:	10 mM ammonium formate/0.1% formic acid in water (Fisher Chemical™ brand)
Mobile phase B:	10 mM ammonium formate/0.1% formic acid in methanol (Fisher Chemical brand)
LC gradient:	Refer to Table 1

Table 1. Chromatographic gradient

	Retention time (min)	Flow (mL/min)	% B
1	0.00	0.500	30
2	0.25	0.500	30
3	0.50	0.500	100
4	1.50	0.500	100
5	1.51	0.750	30
6	2.00	0.750	30

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MS Method

MS analysis was performed on a TSQ Endura triple quadrupole mass spectrometer (Figure 1). The MS conditions were as follows:

Ionization:	Heated electrospray ionization (HESI)
Vaporizer temp:	400 °C
Capillary temp:	250 °C
Spray Voltage:	1000 V
Sheath gas:	45 AU
Auxiliary gas:	5 AU
Sweep gas:	1 AU
Data acquisition mode:	Selected-reaction monitoring (SRM)
Chrom filter peak width:	3 s
Collision gas pressure:	2 mTorr
Cycle time:	0.5 s
Q1 (FWMH):	0.7
Q3 (FWMH):	0.7
SRM parameters:	Refer to Table 2

Table 2. SRM transitions

Compound	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)	RF Lens (V)
Ascomycin	809.75	756.4	21	203
Tacrolimus	821.6	768.45	20	187
Sirolimus	931.85	864.5	17	191
Sirolimus-d3	934.85	864.5	17	191
Cyclosporin A	1220	1202.8	17	224
Cyclosporin D	1234	1216.85	17	200

Results and Discussion

All data were acquired and processed with Thermo Scientific™ TraceFinder™ software version 3.1. The high selectivity of SRM detection using the TSQ Endura triple quadrupole mass spectrometer makes it possible the use of rapid chromatographic separation (2 min) on a short, 10 mm column achieving chromatographic peaks with excellent shape (Figure 1). Internal calibration curves were built for each analyte (Figures 2–4). QC and donor samples were analyzed in triplicate resulting with good correlation between spiked and measured results (Table 3).

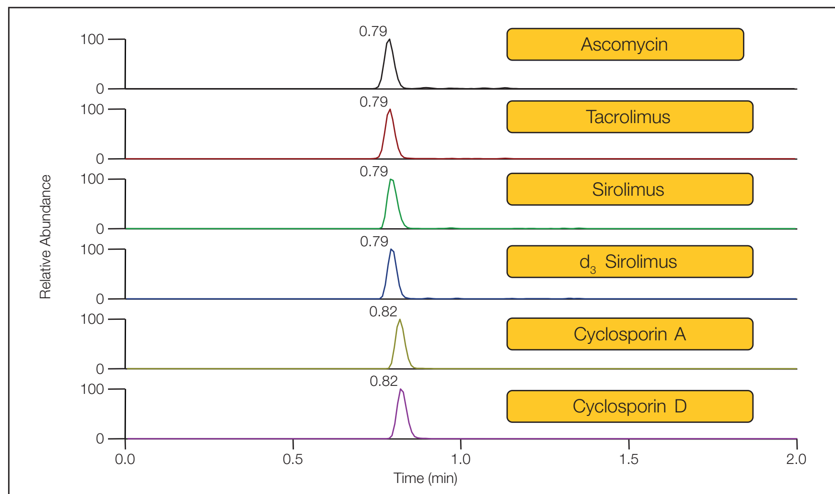
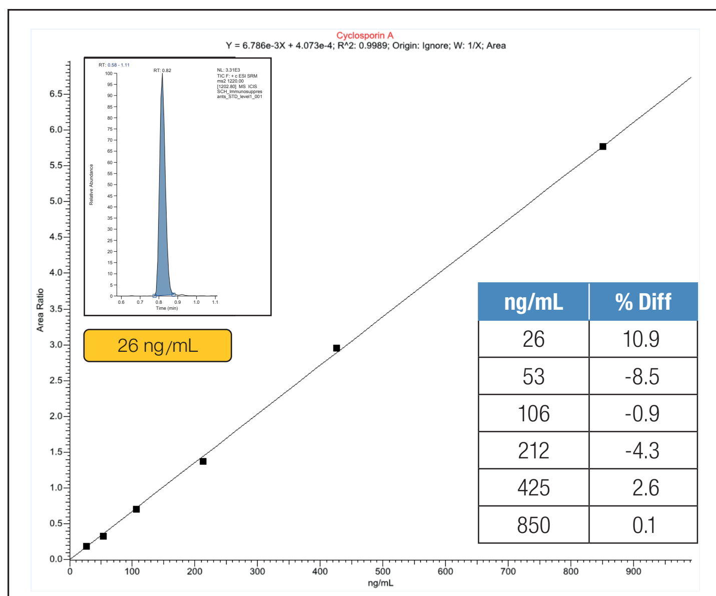
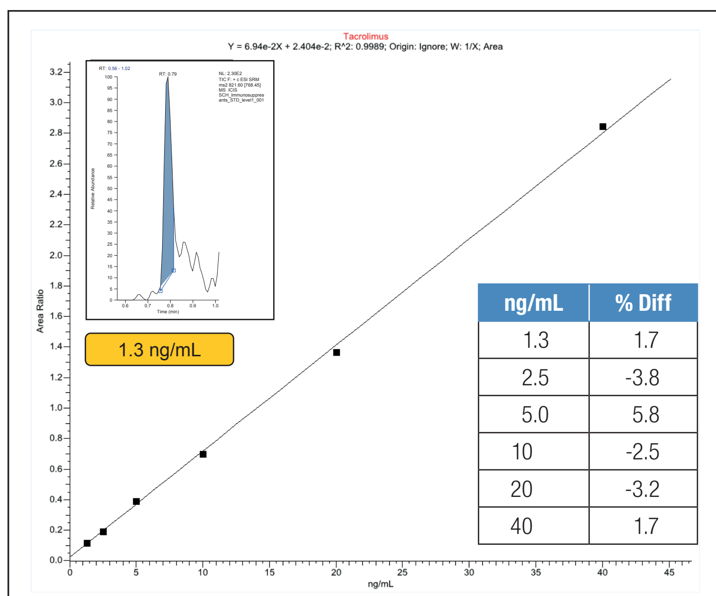


Figure 1. Chromatography



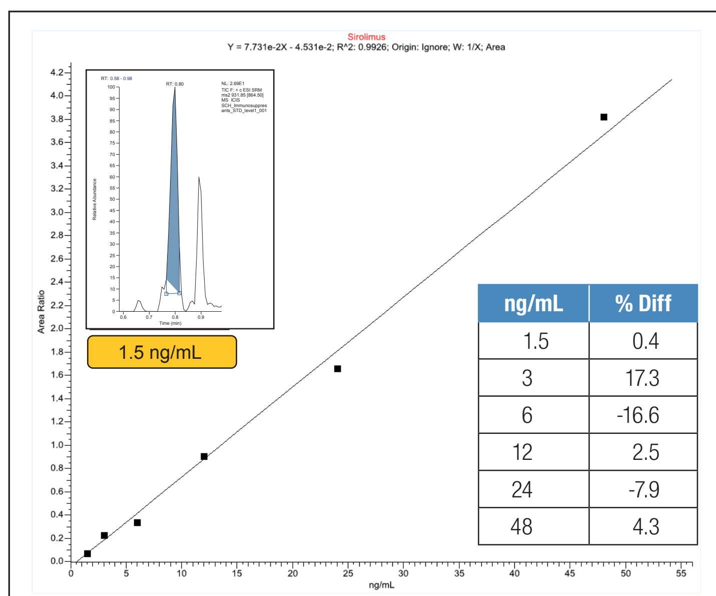
ng/mL	% Diff
26	10.9
53	-8.5
106	-0.9
212	-4.3
425	2.6
850	0.1

Figure 2. Cyclosporin A



ng/mL	% Diff
1.3	1.7
2.5	-3.8
5.0	5.8
10	-2.5
20	-3.2
40	1.7

Figure 3. Tacrolimus



ng/mL	% Diff
1.5	0.4
3	17.3
6	-16.6
12	2.5
24	-7.9
48	4.3

Figure 4. Sirolimus

Table 3. QC and unknowns

	Tacrolimus		Sirolimus		Cyclosporin A	
	Spiked (ng/mL)	Measured [average ± st dev] (ng/mL)	Spiked (ng/mL)	Measured [average ± st dev] (ng/mL)	Spiked (ng/mL)	Measured [average ± st dev] (ng/mL)
Low Control	6.0	6.2 ± 0.2	3.6	3.2 ± 0.3	76	74 ± 2
Mid Control	12	13.1 ± 0.5	9.7	11.0 ± 0.4	199	175 ± 3
High Control	22	21.2 ± 2.8	17.4	17.6 ± 4.2	311	276 ± 9
Subject 1	10	11.0 ± 1.0	5.3	6.2 ± 0.5	0	< LOQ
Subject 2	4.2	4.9 ± 0.4	0	< LOQ	0	< LOQ
Subject 3	0	< LOQ	2.1	3.0 ± 0.9	59	57 ± 3

Conclusion

A high-throughput, cost-efficient research method was developed for the precise and accurate measurement of immunosuppressant drugs in dried blood spots using a TSQ Endura triple quadrupole mass spectrometer. This method met analytical laboratory precision and accuracy criteria for 3 mm dried blood spots.

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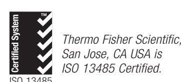

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Research Analysis of Clozapine and Norclozapine in Plasma Using Automated Sample Preparation and LC-MS/MS

Phillip Morgan, Kings College Hospital NHS Foundation Trust, London, UK
Shane McDonnell, Sarah Robinson, Thermo Fisher Scientific, Hemel Hempstead, UK

Key Words

- TurboFlow Technology
- TSQ Quantum Ultra
- Clinical Research

Introduction

Clozapine (Figure 1) is a tricyclic dibenzodiazepine drug used in the treatment of schizophrenia. It is uniquely effective in patients resistant to therapy with other antipsychotics. In addition to mandatory hematological monitoring to minimize the risk of agranulocytosis, there are large variations (50-fold) among patients' clozapine dose requirements. Moreover, changes in smoking habits can have a large effect on the clozapine dose requirement (on average, the clozapine dose for non-smokers is half that required for smokers) due to the induction of cytochrome P450 (CYP) enzymes in smokers.[1] Studies have indicated that accurate quantification of clozapine levels may help researchers better understand, and conduct analysis of, issues related to dose optimization and adherence.[2]

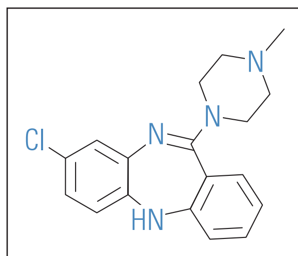

Figure 1: Structure of clozapine

Clozapine is metabolized via *N*-demethylation, *N*-oxidation, and aromatic hydroxylation, amongst other pathways. A few drugs, notably fluvoxamine, block all four CYP enzymes that can metabolise clozapine. Measurement of *N*-desmethylclozapine (norclozapine), which accumulates in plasma to concentrations similar to that of clozapine, can give useful information regarding adherence with medication, sample timing in relation to the last dose of clozapine and drug-drug interactions, such as that with fluvoxamine.

Current research methodology in our laboratory for clozapine and norclozapine involves off-line liquid-liquid extraction with manual transfer to a high pressure liquid chromatography-ultra violet (HPLC-UV) system. The Thermo Scientific Aria TLX-1 System powered by TurboFlow™ automated sample preparation technology is being investigated to simplify sample preparation, reduce the risk of operator error, improve sample throughput, and gain further selectivity by utilizing tandem mass spectrometry.

Goal

To assess Thermo Scientific TurboFlow automated sample preparation technology with tandem mass spectrometry for the research analysis of clozapine and norclozapine levels in plasma samples.

Experimental

Sample Preparation

Calibration standards (n=6) were prepared in the range 0.05 mg/L to 2 mg/L by addition of clozapine and norclozapine to newborn calf serum. Similarly, both analytes were added to drug-free human plasma to give internal quality control (IQC) solutions at 0.15, 0.40, and 1.20 mg/L. After centrifugation at 11,000 *g* for 2 min, 10 µL plasma was injected directly onto the Aria™ TLX-1 system.

The eluent gradients for both pumps are displayed in Table 1.

TurboFlow LC

Column:	TurboFlow Cyclone 50 x 0.5 mm
Mobile phase A:	0.05% (v/v) aqueous formic acid
Mobile phase B:	0.05% (v/v) formic acid in methanol
Mobile phase D:	45/45/10 Propan-2-ol/acetonitrile/acetone

Analytical LC

Column:	Thermo Scientific Hypersil GOLD C18 50 x 2.1 mm, 3 µm
Mobile phase A:	0.05% (v/v) aqueous formic acid
Mobile phase B:	0.05% (v/v) formic acid in methanol

			TurboFlow Method								Analytical			
Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	00:00	30	1.50	Step	100	-	-	-	====	out	0.50	Step	100	0
2	00:30	60	0.25	Step	100	-	-	-	T	in	0.25	Step	100	0
3	01:30	60	1.50	Step	-	-	-	100	====	in	0.50	Ramp	5	95
4	02:30	60	1.50	Step	70	30	-	-	====	in	0.50	Step	5	95
5	03:30	60	1.50	Step	100	-	-	-	====	out	0.50	Step	100	0

Table 1: Gradient programs for both TurboFlow and analytical methods (flow rate is mL/min)

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Mass Spectrometry

Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer	
Ion Source & Polarity:	APCI, positive ion mode
Discharge Current:	4.0 μA
Vaporizer Temperature:	325 °C
Sheath Gas:	60 units
Ion Sweep Gas:	0 units
Auxillary Gas:	10 units
Capillary Temperature:	275 °C
Collision Gas Pressure:	1.5 mTorr

The selective reaction monitoring (SRM) transitions used are presented in Table 2.

Analyte	Parent	Product	Scan Time	Collision Energy	Tube Lens
Clozapine	327.20	192 270	25 ms 25 ms	60 21	47 47
Norclozapine	313.20	164 192	25 ms 25 ms	67 41	113 113

Table 2: SRM transitions monitored in the experiment

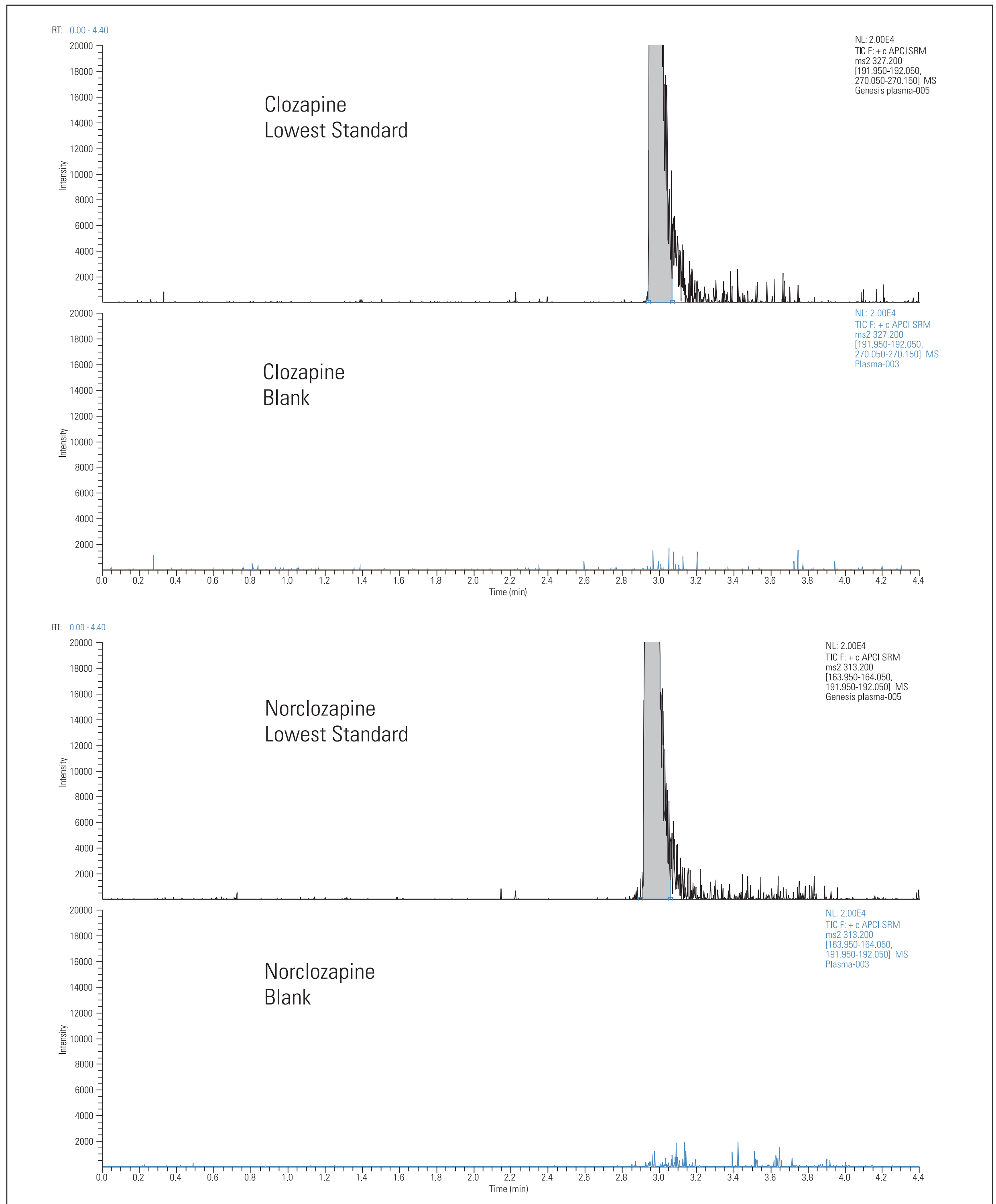

Figure 2: Extracted ion chromatogram of the plasma blank

Results and Discussion

Plasma was centrifuged prior to analysis. Calibration standards were analyzed from low to high concentration followed by IQCs. An injection of solvent after the highest concentration IQC was used for evaluation of carry-over. The volume of plasma injected was 10 µL, and all plasma analyses were in triplicate.

The extracted ion chromatograms of the plasma blank and lowest and highest concentration calibrators are presented in Figures 2, 3, and 4, respectively. The calibration curves for clozapine and norclozapine covered the range 0.05 - 2.00 mg/L (Figure 5 and 6). No internal standard was used, and thus, this work demonstrates the reproducibility of the system using external standard calibration.

Reproducibility and variance of the calibrators are shown in Figure 7.

Carry-over was calculated by comparing the response for clozapine and for norclozapine with that of a solvent blank injected immediately after a 1.2 mg/L IQC sample. This was shown to be ~0.1% for both clozapine and norclozapine. Additional clozapine metabolites were not investigated as part of this evaluation.

Conclusion

The research use of TurboFlow technology for automated sample preparation and tandem MS detection allowed the selective analysis of clozapine and norclozapine in plasma. The only sample preparation was the centrifugation of plasma. The sample volume required was one-tenth that used by the existing method – liquid-liquid extraction (LLE) followed by HPLC-UV – and provided lower limits of detection and quantitation. The calibration curves for all analytes were linear over the concentration range and carry-over was minimal. Use of the automated TurboFlow method has effectively eliminated two hours of sample preparation time for a 100-sample batch.

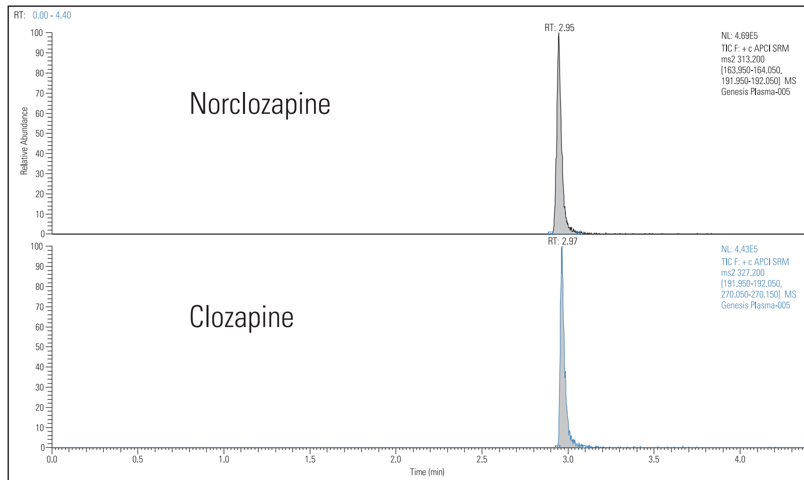

Figure 3: Clozapine and Norcloxapine lowest calibration from plasma, 0.05 mg/L

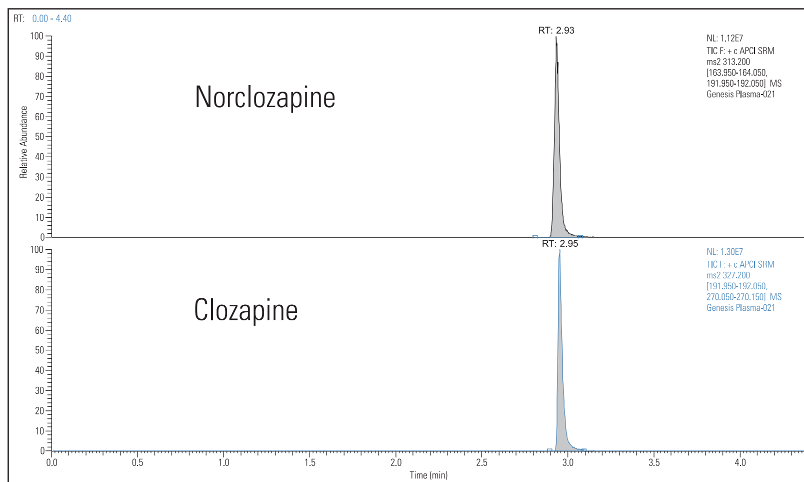

Figure 4: Clozapine and norcloxapine lowest calibration from plasma, 2 mg/L

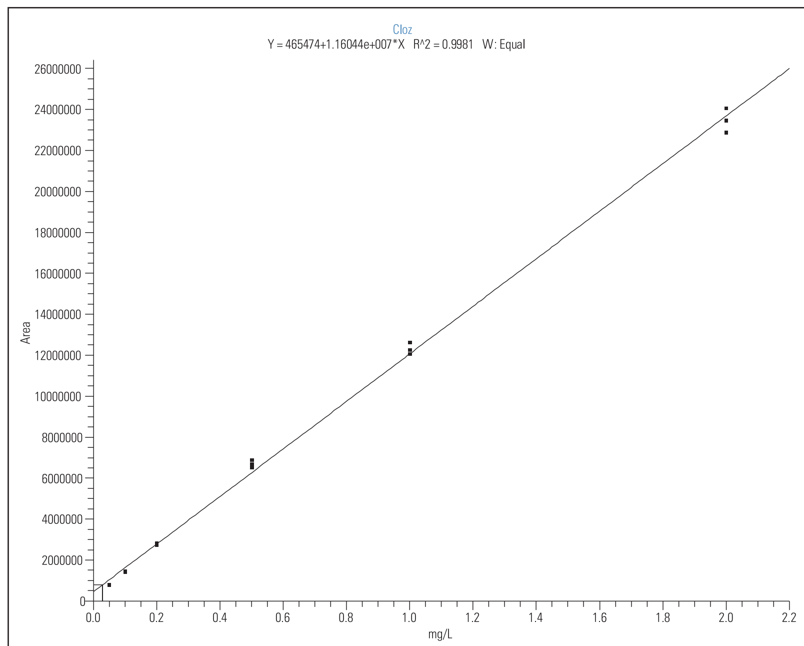

Figure 5: Clozapine calibration curve, 0.05 – 2 mg/L

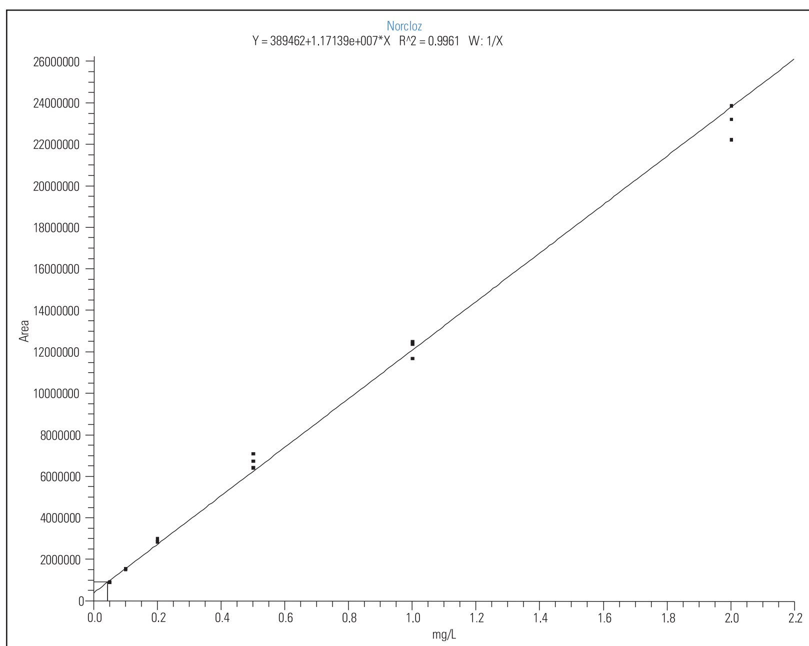

Figure 6: Norclozapine calibration curve, 0.05 – 2 mg/L

References

1. Rostami-Hodjegan A, Amin AM, Spencer EP, Lennard MS, Tucker GT, Flanagan RJ, *J Clin Psychopharmacol* 2004; 24 (1): 1-9

2. Flanagan RJ, *CPD Clin Biochem* 2006; 7 (1): 3-18

Clozapine					Norclozapine				
Concentration	Response	Specified Conc	Calculated Conc	% CV	Concentration	Response	Specified Conc	Calculated Conc	% CV
0.05 mg/L	784733	0.05	0.03	1.2	0.05 mg/L	905801	0.05	0.04	0.4
0.05 mg/L	797712	0.05	0.03	1.2	0.05 mg/L	897792	0.05	0.04	0.4
0.05 mg/L	780137	0.05	0.03	1.2	0.05 mg/L	900825	0.05	0.04	0.4
0.10 mg/L	1415271	0.10	0.08	1.7	0.10 mg/L	1555897	0.10	0.10	1.1
0.10 mg/L	1456027	0.10	0.09	1.7	0.10 mg/L	1554377	0.10	0.10	1.1
0.10 mg/L	1411624	0.10	0.08	1.7	0.10 mg/L	1525338	0.10	0.10	1.1
0.20 mg/L	2745962	0.20	0.20	1.8	0.20 mg/L	2847998	0.20	0.21	3.1
0.20 mg/L	2743289	0.20	0.20	1.8	0.20 mg/L	2859029	0.20	0.21	3.1
0.20 mg/L	2832044	0.20	0.20	1.8	0.20 mg/L	3006773	0.20	0.22	3.1
0.50 mg/L	6889405	0.50	0.55	2.6	0.50 mg/L	7099512	0.50	0.57	5.0
0.50 mg/L	6682781	0.50	0.54	2.6	0.50 mg/L	6741516	0.50	0.54	5.0
0.50 mg/L	6549395	0.50	0.52	2.6	0.50 mg/L	6420812	0.50	0.51	5.0
1.00 mg/L	12624439	1.00	1.05	2.3	1.00 mg/L	12521697	1.00	1.04	3.6
1.00 mg/L	12261014	1.00	1.02	2.3	1.00 mg/L	12383684	1.00	1.02	3.6
1.00 mg/L	12054848	1.00	1.00	2.3	1.00 mg/L	11695815	1.00	0.97	3.6
2.00 mg/L	24055429	2.00	2.03	2.5	2.00 mg/L	23888229	2.00	2.01	3.5
2.00 mg/L	22868295	2.00	1.93	2.5	2.00 mg/L	22259134	2.00	1.87	3.5
2.00 mg/L	23457123	2.00	1.98	2.5	2.00 mg/L	23241437	2.00	1.95	3.5

Figure 7: Clozapine/Norclozapine reproducibility and variance

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Bioanalytical Assay for Neurotransmitters in Whole Blood by LC-MS/MS

Yang Shi, Catherine Lafontaine, Francois A. Espourteille, Thermo Fisher Scientific, Franklin, MA, USA

Key Words

- **Aria TLX-1**
- **TSQ Quantum Ultra**
- **TurboFlow Technology**
- **Parkinson's Disease**

Introduction

Taken orally in conjunction with Levodopa (L-DOPA), Carbidopa (C-DOPA) inhibits the metabolism of L-DOPA before it reaches the brain so that more is available to be converted into dopamine in the brain. 3-methoxy-L-tyrosine (3-OMD) is an important metabolite produced after L-DOPA administration. The following LC-MS/MS method using TurboFlow™ technology for on-line sample extraction using a Thermo Scientific Aria™ TLX-1 system coupled with Thermo Scientific TSQ Quantum Ultra™ triple quadrupole mass spectrometer demonstrates its suitability as a research method for these compounds in human whole blood.

Goal

To develop a quantitative, fast, automated LC-MS/MS method for analysis of neurotransmitters in human whole blood.

Method Information

These analytes were extracted on-line from crashed human whole blood. Calibration curves were analyzed using an Aria TLX-1 LC system coupled with a TSQ Quantum Ultra with heated electrospray ionization (H-ESI) source. Internal standards used were 4-chloro-L-phenylalanine and L-DOPA-d_3.

Experimental Conditions

Sample Preparation

A standard stock solution of 50 µg/mL L-Dopa, C-Dopa and 3-OMD in methanol was prepared. Methanol-quenched human whole blood (K_2 EDTA) was centrifuged at 10,000 RPM for 10 minutes. Calibrators were prepared in the supernatant. Analyte concentration ratio of spiking solution was 4 to 1 of L-DOPA and 3-OMD to C-DOPA. Final internal standard concentrations were 90 ng/mL for 4-chloro-L-phenylalanine and 225 ng/mL for L-DOPA-d_3, respectively. Injection volumes were 0.010 mL.

Aria TLX-1 System Parameters

Two 0.5 x 50 mm Thermo Scientific Cyclone™ MAX TurboFlow columns with a C18 HPLC column (4.6 x 150 mm, 5 µm particle size).

LC Method Mobile Phases

Loading Pump

Mobile Phase A:	10 mM Ammonium Acetate with 0.2% Ammonium Hydroxide (aq)
Mobile Phase B:	0.1% Formic Acid (aq)
Mobile Phase C:	50 mM Ammonium Acetate with 10% Formic Acid (aq)
Mobile Phase D:	50 mM Ammonium Acetate with 10% Formic Acid in Methanol

Elution Pump

Mobile Phase A:	0.1% Formic Acid (aq)
Mobile Phase B:	0.1% Formic Acid in Acetonitrile

Mass Spectrometer Parameters

Ion Polarity:	Positive ion mode
Vaporizer Temperature:	400 °C
Capillary Temperature:	300 °C
Sheath Gas Pressure (N_2):	60 units
Auxiliary Gas Pressure (N_2):	55 units
Scan Type:	Highly-selective reaction monitoring (H-SRM)
Scan Time:	0.050 s
Q1 (FWHM):	0.7
Q3 (FWHM):	0.7

Positive single reaction mode (+SRM) transitions and other MS parameters for test compounds are shown in Table 1. The whole experiment was controlled by Aria software.

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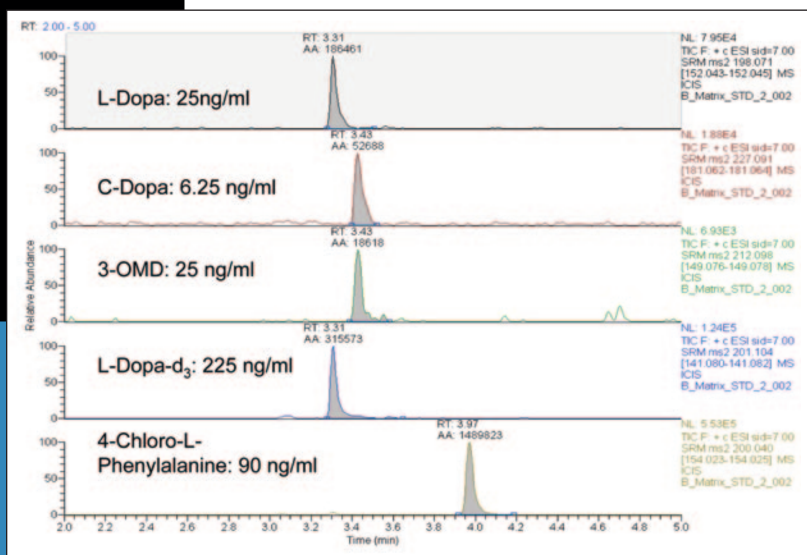

Figure 1: The representative chromatogram for the assay at the low end of the calibration curve

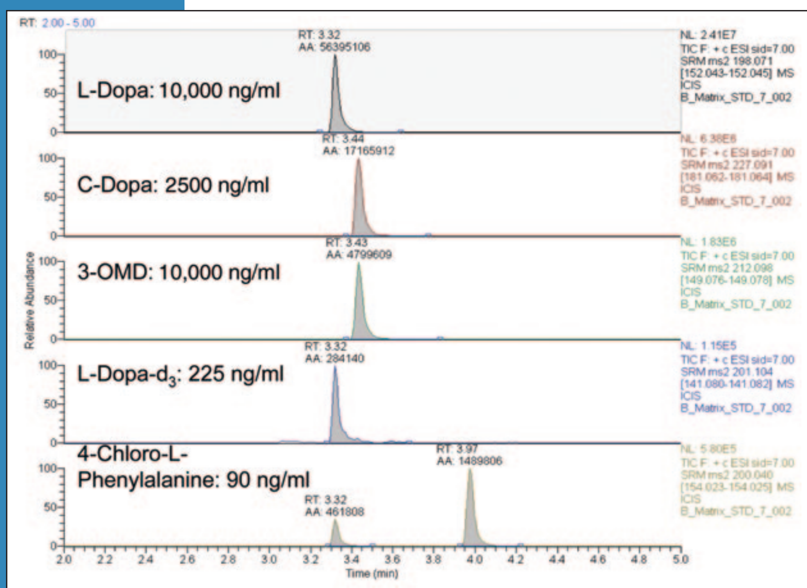

Figure 2: The representative chromatogram for the assay at the high end of the calibration curve

Results

Figures 1 shows a representative chromatogram for the assay at the low end of the curve. Figure 2 shows a representative chromatogram for the assay at the high end of the curve. Linearity of the calibration curves (N=3) ranged from 0.9942 to 0.9989 (with 1/x weighting). Figure 3 shows the representative linear calibration curves for all three test compounds. The excellent linear fits were over the range of 100-10000 ng/mL for L-Dopa and 3-OMD and 25-2500 ng/mL for C-Dopa. The limit of detection (LOD) levels were five-times lower for all compounds. The % CV values were less than 20% deviation for LLOQ and less than 15% deviation for all the other points on the calibration curve. Carryover was determined to be much less than 20% of lower limit of quantitation (LLOQ). A minimum of 85% recovery was achieved. The variability was determined by processing and analyzing five replicates of each of four QC samples. The test was repeated in three batches, Table 2. The results show that the %RSDs were well below the validation guideline of 15%.[1]

Table 1: Positive single reaction mode (+SRM) transitions and other MS parameters for test compounds

Compound	Parent Ion	Fragment Ion	Collision Energy (eV)	Tube Lens Offset
L-DOPA	198.071	152.044	14	72
C-DOPA	227.091	181.063	12	77
3-OMD	212.098	149.077	15	75
L-DOPA-d_3	201.104	141.081	16	87
4-Chloro-L-Phenyl-Alanine	200.040	154.024	14	61

Table 2: Low internal standard variability demonstrated the reliability of the method

L-Dopa-d_3 in QC Samples

	Batch #1	Batch #2	Batch #3
Number of Samples	20	20	20
RSD (%)	6.2	6.6	4.7

4-Chloro-L-Phenylalanine in QC Samples

	Batch #1	Batch #2	Batch #3
Number of Samples	20	20	20
RSD (%)	2.0	1.6	2.3

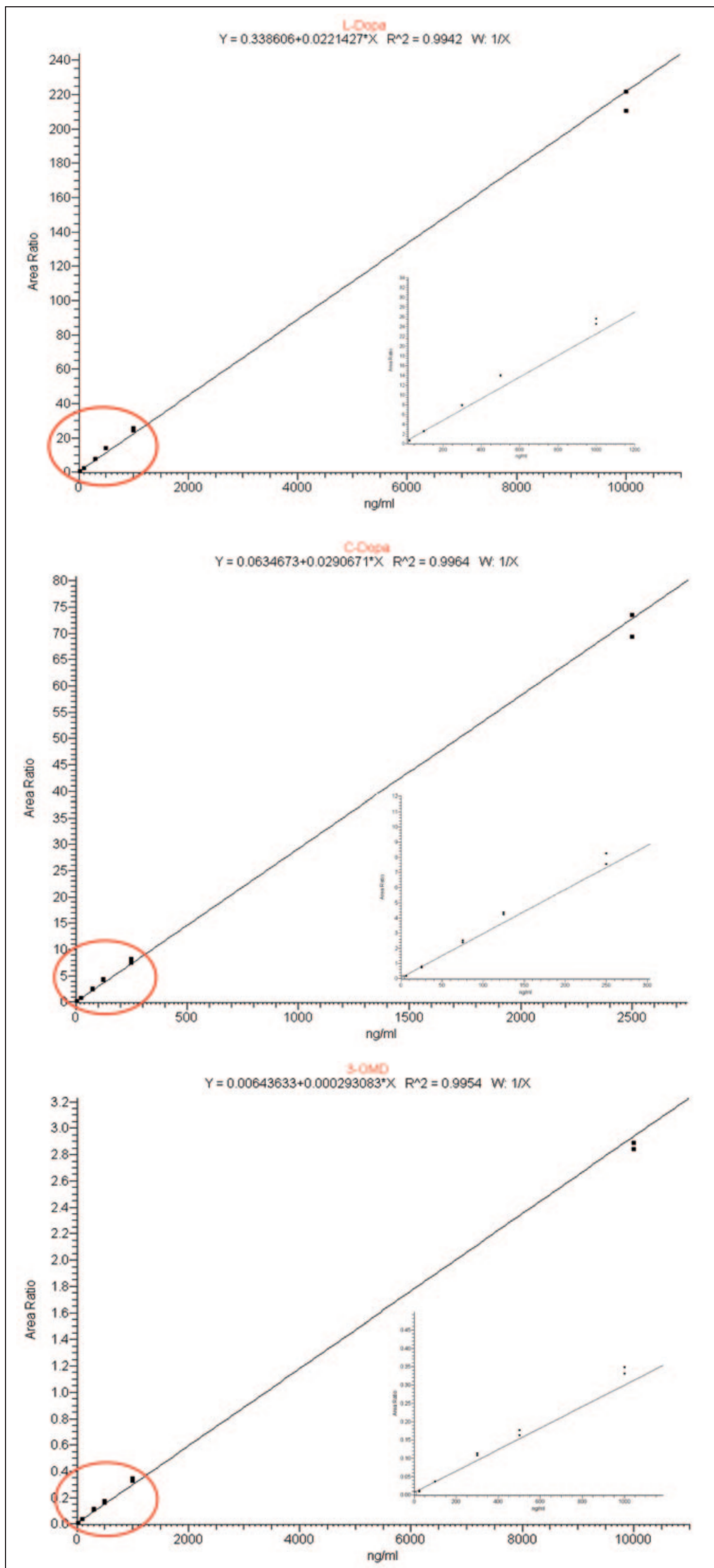

Figure 3: Representative linear calibration curves for all three test compounds

Conclusion

TurboFlow technology is a powerful technique for the direct analysis of drugs in biological fluids without the need for an extensive number of sample preparation steps. In this study, the use of an Aria TLX-1 LC system in front of a TSQ Quantum Ultra allows for low levels of detection (6.25 ng/mL for C-Dopa; 25 ng/mL for L-Dopa and 3-OMD) of each of these neurotransmitter compounds in human whole blood extract and yields results in less than 10 minutes per sample. With the Aria TLX-4 multiplexed system, the results will be available about every 2.5 minutes using only one mass spectrometer. The low variability of the results demonstrates the reliability of this research method.

Reference

1. Guidance for Industry Bioanalytical Method Validation, Food and Drug Administration, May 2001.

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Determination of Digoxin in Serum by Liquid Chromatography–Tandem Mass Spectrometry

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[2]Laboratory of Pharmacology, Faculty of Medicine, University of Limoges, France.

Key Words

- TSQ Quantum
- Clinical Research
- Toxicology

Introduction

Digoxin is a cardiac glycoside that can be used at very low concentrations. Identification and quantitation of this compound necessitate a sensitive and specific method. This study aims to describe a method using liquid chromatography/ tandem mass spectrometry and permitting to quantify digoxin at low concentrations for research applications.

Goal

The goal of this study was to identify and quantify digoxin in serum. This report demonstrates the use of the TSQ Quantum for this application.

Experimental Conditions/Methods

Chemicals and Reagents

Digoxin and 3-aminophenylsulfone (internal standard) were purchased from Sigma. Ammonium formate and formic acid (>99 % pure) were also purchased from Sigma. All reagents and solvents used in the extraction procedures were of analytical grade.

Sample preparation

To 1 mL of serum were added 50 µL of a 2.5 µg/mL aqueous solution of 3-aminophenylsulfone (Internal Standard), 1 mL of a solution of pH 9.50 carbonate buffer and 8 mL of Ether-Dichloromethane-Isopropanol (30:40:30 by volume). The tubes were vortex-mixed and shaken on an oscillatory mixer. After centrifugation at 3,400 g for 5 min, the organic phase was poured in a conical glass tube and evaporated under a stream of nitrogen at 37°C. The dried extracts were reconstituted in 50 µL of acetonitrile : pH 3.0, 2 mmol/L ammonium formate (30:70 by volume) and 10 µL were injected into the chromatographic system.

Instrumentation Methods

HPLC Conditions

The chromatographic system consisted of a CTC HTS PAL Autosampler kept at 6°C and a binary high-pressure pump. A C18, 5 µm (50×2.1 mm) column, maintained at 25°C, was used with a linear gradient of mobile phase A (pH 3.0, 2 mmol/L ammonium formate) and mobile phase B (acetonitrile:pH 3.0, 2 mmol/L ammonium formate (90:10; v/v)), flow rate of 200 µL/min, programmed as follows: 0-1.2 min, 20% B; 1.2–8.2 min, 20 to 80% B; 8.2–10.2 min, 80% B; 10.2–10.7 min, decrease from 80 to 20% B; 10.7–13 min, equilibration with 20% B.

MS Conditions

Mass Spectrometer: Thermo Scientific TSQ Quantum
Source: ESI mode
Ion Polarity: Positive
Spray Voltage: 3800 V
Sheath/Auxiliary gas: Nitrogen
Sheath gas pressure: 30 (arbitrary units)
Auxiliary gas pressure: 30 (arbitrary units)
Ion transfer tube temperature: 250°C
Scan type: SRM
Collision gas: Argon
Collision gas pressure: 1.5 mTorr

SRM Conditions

Settings were optimized by infusing at 5 µL/min a 1 µg/L solution containing the studied compound in acetonitrile: pH 3.0, 2 mmol/L ammonium formate (30:70, by volume). The structure of these compounds is shown in Figure 1.

Compounds	Quantification transition	Collision energy	Confirmation transition	Tube lens voltage
Digoxin	798.5/651.4	20	798.5/781.5	84
3-aminophenylsulfone	249.1/93.2	24		126

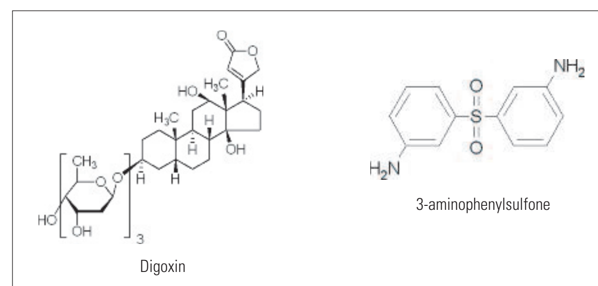

Figure 1: Structures of the investigated compounds

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Results and Discussion

The LC-ESI/SRM chromatograms for 3-aminophenylsulfone and digoxin for a blank serum sample and a blank serum sample spiked at 0.5 ng/mL are shown in Figures 2A and 2B respectively. Identification of digoxin was achieved with two characteristic SRM transitions and their relative retention time.

Linearity

Calibration curve obtained for digoxin spiked in serum samples is presented in Figure 3. Concentration range was comprised between 0.5 ng/mL and 100 ng/mL.

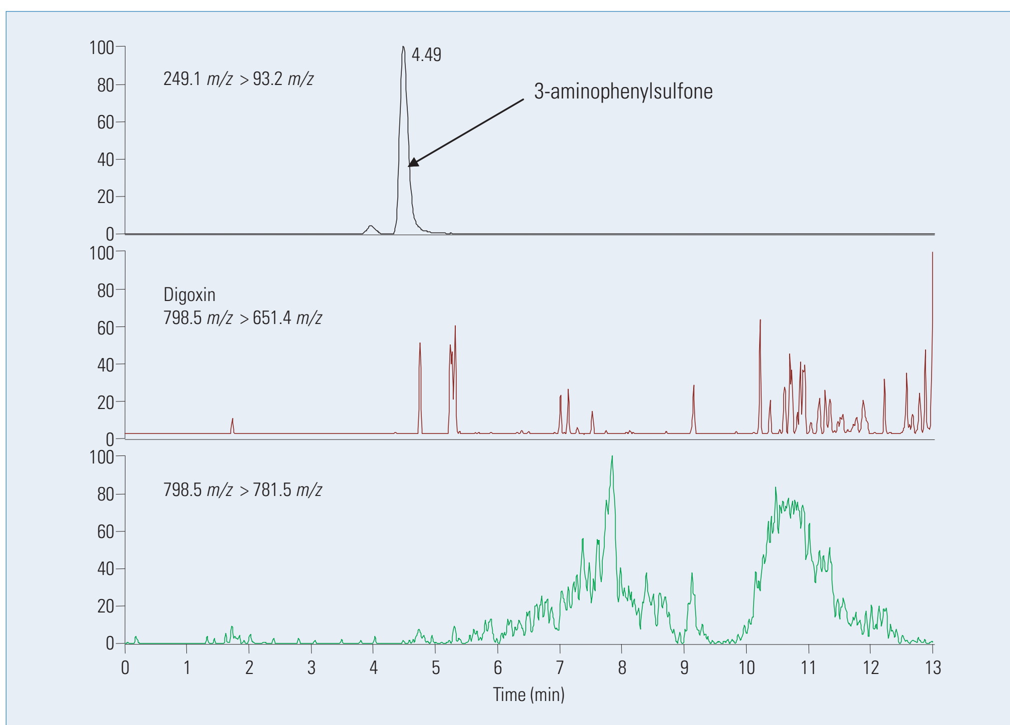

Figure 2A: Chromatogram of a blank serum

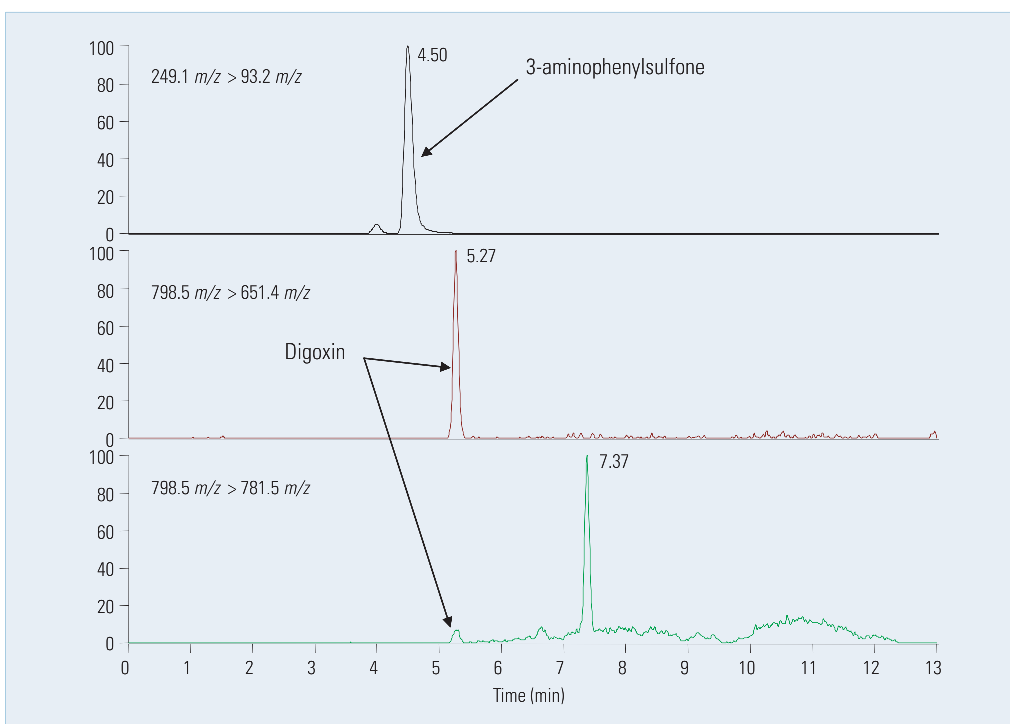

Figure 2B: Chromatogram of a blank serum spiked at 0.5 ng/mL

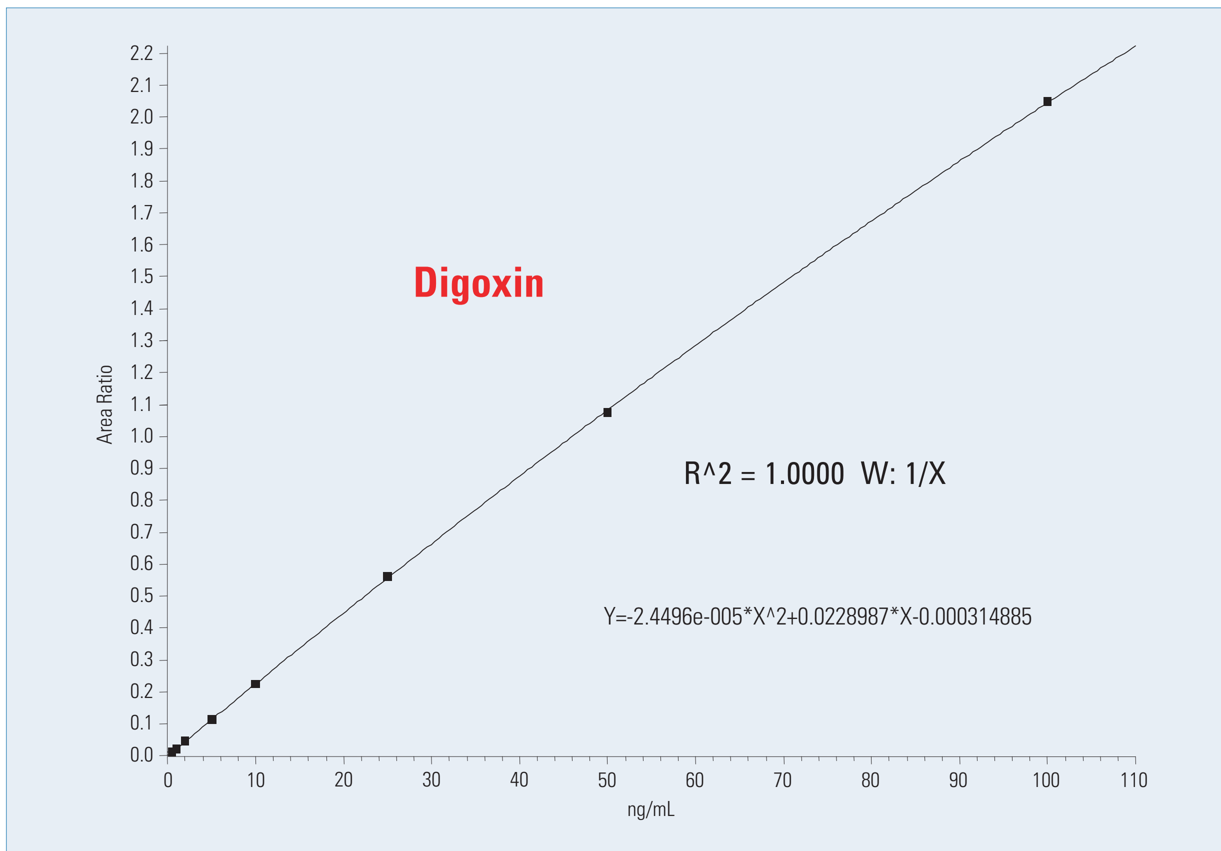

Figure 3: Representative calibration curve from standards spiked in serum

Corresponding Results of Calibration Standards

	Quadratic 1/x	
Specified Concentration (ng/mL)	Calculated Amount (ng/mL)	% Diff
0.5	0.503	0.53
1	.0972	-2.79
2	2.041	2.07
5	5.001	0.02
10	9.985	-0.15
25	25.239	0.95
50	49.614	-0.77
100	100.149	0.15

Intra-assay Accuracy and Precision (n=6)

	Quadratic 1/x	
Specified Concentration (ng/mL)	Calculated Amount (ng/mL)	% Diff
0.5	0.514	2.76
0.5	0.496	-0.75
0.5	0.546	9.10
0.5	0.558	11.62
0.5	0.510	1.97
0.5	0.563	12.62

Accuracy and precision

Intra-assay accuracy and precision (n=6) have been studied at the lowest concentration (0.5 ng/mL). Relative Standard Deviation was equal to 5.28% and Mean Relative Error to 6.23%.

Conclusion

This application note describes a sensitive and specific method developed for the quantitation of digoxin in serum for research applications.

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Application Note: 505

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Quantitative Analysis of Mevalonate in Plasma Using LC-MS/MS

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[2]Thermo Fisher Scientific, Rodano, Italy

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Key Words

- LTQ Ion Trap
- Clinical Research
- Cholesterol Synthesis

Introduction

Cholesterol is synthesized *in vivo* through a multiple step pathway. Because mevalonate is the key intermediate of this process, its plasmatic levels are an indirect measure of *in vivo* cholesterol synthesis and, therefore, facilitate clinical research into pharmacological activity of anti-hypercholesterolemic drugs such as statins.

Goal

To develop a reliable and fast analytical method for the quantitative determination of mevalonate in plasma using a Thermo Scientific LTQ linear ion trap mass spectrometer.

Experimental

Sample Preparation

The plasma sample (500 µL) was spiked with 20 ng of Mevalonate-D_7. Samples were acidified with hydrochloric acid allowing the conversion of mevalonate to mevalonolactone (Figure 1). After purification through solid phase extraction (SPE), samples were dried and dissolved in 400 µL of 0.2% ammonium hydroxide to restore the non-lactonic form. Then 10 µL were injected.

Quantitative analysis was performed on the basis of calibration curves, ranging from 2.5 to 250 ng/mL.

HPLC Conditions

High performance liquid chromatography (HPLC) analysis was performed using a Thermo Scientific Surveyor autosampler and pump. The 10 µL sample was injected directly on a Thermo Scientific BioBasic AX column (150 × 2.1 mm, 5 µm). A gradient LC method used mobile phases A (10 mM ammonium formate, pH 8) and B (acetonitrile) at a flow rate of 200 µL/min.

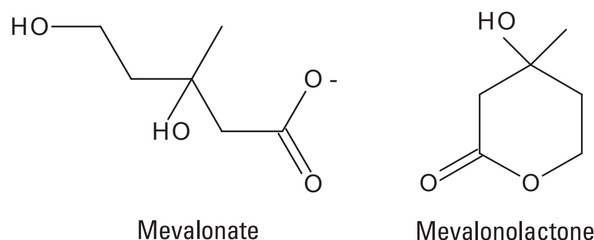

Figure 1. Structure of mevalonate and mevalonolactone

Mass Spectrometry

MS analysis was carried out on a LTQ™ linear ion trap mass spectrometer equipped with a Thermo Scientific Ion Max source with an electrospray ionization (ESI) probe.

Ion polarity:	Negative
Spray voltage:	2 kV
Sheath/Auxiliary gas:	Nitrogen
Sheath gas pressure:	40 (arbitrary units)
Auxiliary gas pressure:	10 (arbitrary units)
Sweep gas pressure:	5 (arbitrary units)
Ion transfer tube temperature:	300 °C
Scan type:	Full Scan MS/MS
Collision gas:	Helium
Collision energy:	30%
Divert valve:	3.0-6.5 min to source
Selected ions for quantification:	*m/z* 147→ 59 for mevalonate *m/z* 154→ 59 for mevalonate-D_7

Results and Discussion

Figure 2 shows the ion chromatograms of a lower sample of the calibration curve. Excellent linearity (r^2 = 0.999) fits for the calibration curve were observed over the range of 2.5 - 250 ng/mL plasma (Figures 3 and 4). The intraday CV% (n=3) was in the range 0.5% - 4%. The limit of detection (LOD) was 2 pg, and the limit of quantification (LOQ) was 2.5 ng/mL.

Figure 5 reports an ion chromatogram of a plasma sample of a healthy volunteer (24 ng/mL plasma), extracted and analyzed as described.

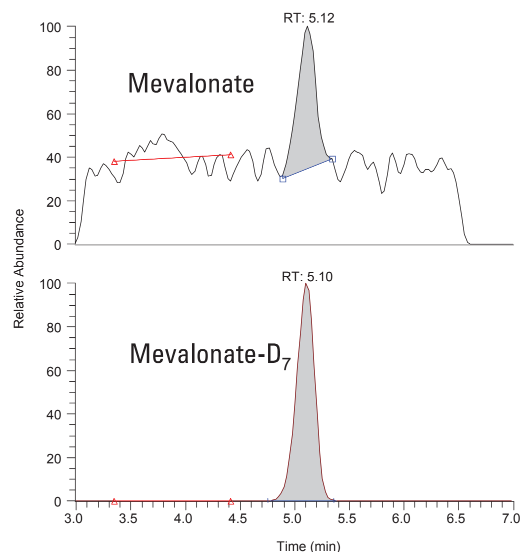

Figure 2. Ion chromatograms of 2.5 ng/mL calibration standard

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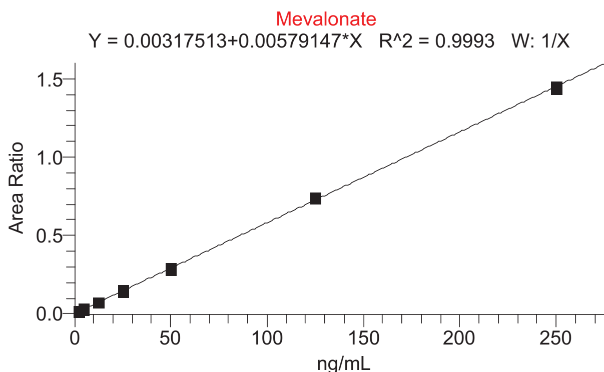

Figure 3. Calibration curve of mevalonate

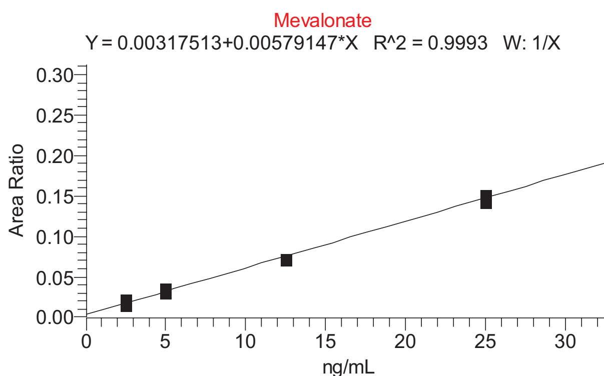

Figure 4. Zoom on low calibration points

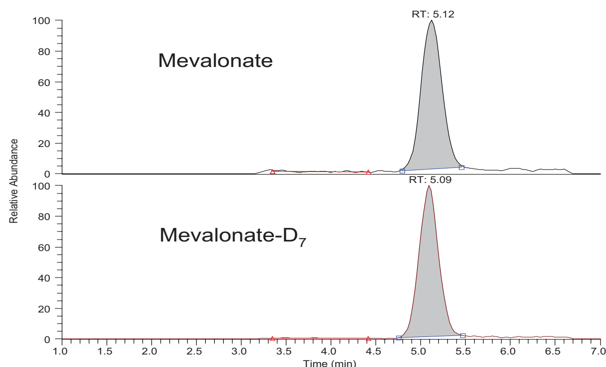

Figure 5. Ion chromatograms of plasma sample containing 24 ng/mL

Conclusion

A robust 10-minute method for the quantification of mevalonate with a dynamic range of 2.5 - 250 ng/mL plasma has been developed for clinical research using fast SPE purification and the LTQ linear ion trap mass spectrometer.

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Application Note: 513

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Validated LC-MS/MS Method for the Analysis of Immunosuppressant Drugs in Whole Blood Using the RECIPE ClinMass® Complete Kit

Samuele Scurati[1], Anke Trebstein[2], Lea Bonnington[2], Norbert Dirsch[2], Glenn Damkroeger[3], Miriam Drayss[3]; [1]Thermo Fisher Scientific, Rodano, Italy; [2]RECIPE Chemicals + Instruments GmbH, Munich, Germany; [3]Thermo Fisher Scientific, Dreieich, Germany

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Key Words

- TSQ Vantage
- Clinical Research
- Therapeutic Drugs

Introduction

Immunosuppressant drugs inhibit the immune system and are used in organ transplant patients to prevent organ rejection. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a widely accepted technique for the determination of immunosuppressant drugs in whole blood by clinical research laboratories. Tools providing reagents for sample extraction, calibrators, and QCs for analysis of these molecules are useful in facilitating analysis and increasing throughput.

Goal

To set up and validate an LC-MS/MS method for the analysis of Tacrolimus, Sirolimus, Everolimus, and Cyclosporin A in whole blood for clinical research laboratories by using the RECIPE ClinMass® Complete Kit with the Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer.

Experimental

This method has been developed using the RECIPE ClinMass® Complete Kit for the determination of immunosuppressants in whole blood according to the instruction manual.

Sample preparation

In a sample preparation vial, 200 µL of precipitation reagent, 20 µL of internal standard, and 100 µL of whole blood sample were combined. The sample was mixed for 30 seconds and incubated at ambient temperature for 5 minutes. The sample was mixed again for 10 seconds and centrifuged. Then, 50 µL of the supernatant was injected into the LC-MS/MS system.

HPLC

High performance liquid chromatography (HPLC) analysis was performed online by use of a 6-port, 3-channel, automatic switching valve and two Thermo Scientific Accela HPLC pumps working in isocratic mode. The sample was injected onto the solid phase extraction (SPE) column (with the switching valve in the “load” position), which extracted the analytes selectively from the sample matrix. The matrix components passed the SPE column widely unhindered and were eluted to waste. Meanwhile, the analytical column was re-equilibrated from the previous injection cycle. When the automatic switching valve switched to the “inject” position, the extracted analytes were eluted from the SPE column in backflush mode and transferred to the analytical column. After elution of the analytes, the automatic switching valve returned to the “load” position. Both columns (SPE and analytical) were re-equilibrated for the next injection. The effective run time was two minutes.

MS

Mass spectrometry analysis was performed using a TSQ Vantage™ triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization source (H-ESI II). The parameters are summarized in Table 1. MS analysis was performed in positive selected reaction monitoring (SRM) data acquisition mode. SRM parameters for all of the analytes and internal standards are shown in Table 2.

Table 1. Optimized ion source parameters

Ion Source	H-ESI II, positive
Resolution Q1 and Q3	0.7 amu
Spray Voltage	3500 V
Vaporizer Temp	300 °C
Sheath Gas Pressure	40
Ion Sweep Gas Pressure	2.0
Aux Gas Pressure	15
Capillary Temp	200 °C
Declustering Voltage	-2 V
Collision Pressure	1.5 mTorr

Table 2. SRM parameters used for the analysis

Compound	Precursor Ion	Product Ion	Scan Time [msec]	Collision Energy
Tacrolimus	821.6	768.4	50	18
Ascomycin	809.5	756.6	50	18
Sirolimus	931.7	864.6	75	15
Everolimus	975.7	908.8	75	16
d_4-Everolimus	979.7	912.6	75	16
Cyclosporin A	1220.0	1203.3	50	17
Cyclosporin D	1234.0	1217.0	50	17

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Results and Discussion

Figure 1 displays the representative lower limit of quantification (LLOQ) chromatograms for Tacrolimus, Sirolimus, Everolimus, Cyclosporin A, and the internal standards.

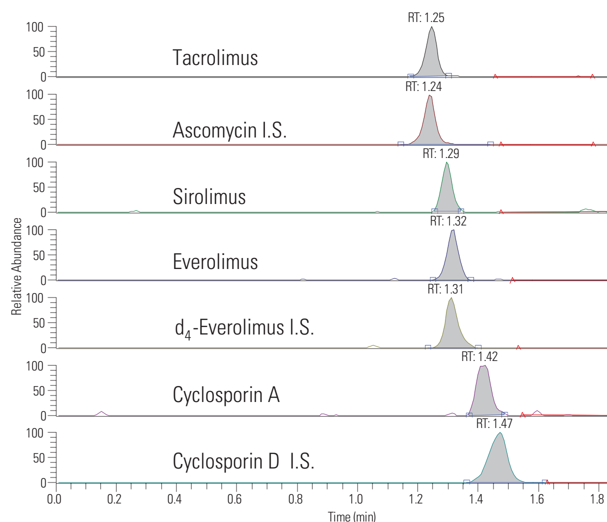

Figure 1: Chromatograms of the lowest calibration standard

In Table 3, the LLOQ and the linearity range for each analyte are reported and compared to the therapeutic range.

As shown in Tables 4 and 5, the intra- and inter-day variabilities were excellent as well as accurate. For each analyte, intra-day variability and accuracy were determined by performing two different extractions of each QC sample and analyzing them two times. Inter-day variability and accuracy were determined by repeating the intra-day procedure on three different days. Sample extractions were performed by different people.

Conclusion

A fast and reliable LC-MS/MS method for the quantification of Tacrolimus, Sirolimus, Everolimus, and Cyclosporin A in whole blood was validated using the RECIPE ClinMass® Complete Kit.

This method fulfills accuracy, precision, and dynamic range requirements of a routine method for clinical research.

Table 3. Summary of assay performance and therapeutic range

	Therapeutic Range [ng/mL]	LOQ [ng/mL]	Linearity Range [ng/mL]	I.S.
Tacrolimus	2 - 15	0.13	1.3 - 46.7	Ascomycin
Sirolimus	5 - 15	0.13	1.3 - 46.9	d_4-Everolimus
Everolimus	6 - 8	0.13	1.3 - 47.4	d_4-Everolimus
Cyclosporin A	100 - 350	24.90	24.90 - 1264.0	Cyclosporin D

Table 4. Intra-day variability (%RSD) and accuracy

	QC 1			QC 2			QC 3		
	Value	%RSD	%Accuracy	Value	%RSD	%Accuracy	Value	%RSD	%Accuracy
Tacrolimus	3.28	6.7	90.1	6.67	2.9	96.3	13.3	5.5	99.4
Sirolimus	3.64	2.7	81.7	11.20	3.8	93.6	18.9	5.2	101.8
Everolimus	3.34	7.2	90.1	10.60	7.1	97.4	18.2	7.2	101.5
Cyclosporin A	62.50	11.4	101.7	258.00	6.2	102.9	1341.0	2.8	94.6

Table 5. Inter-day variability (%RSD) and accuracy

	QC 1			QC 2			QC 3		
	Value	%RSD	%Accuracy	Value	%RSD	%Accuracy	Value	%RSD	%Accuracy
Tacrolimus	3.28	4.7	92.5	6.67	2.1	97.4	13.3	3.3	99.4
Sirolimus	3.64	8.4	89.6	11.20	4.6	95.7	18.9	5.1	102.8
Everolimus	3.34	7.6	96.7	10.60	5.1	96.5	18.2	4.7	100.9
Cyclosporin A	62.50	15.6	103.4	258.00	6.7	99.0	1341.0	12.0	102.9

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Simultaneous Quantitative Analysis of Four Immunosuppressive Drugs Using High Resolution Accurate Mass LC-MS

Neil Leaver[1], Bevean Chihoho[1], Helen Welchman[2], Sarah Robinson[2]
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[2]Thermo Fisher Scientific, Hemel Hempstead, UK

Key Words

- Exactive
- Accela U-HPLC
- Therapeutic Drug Monitoring
- Clinical Research

Introduction

Immunosuppressive drugs have been quantitatively analyzed by selected reaction monitoring (SRM) analysis using tandem mass spectrometry for over 10 years in the clinical research setting. High resolution accurate mass (HRAM) mass spectrometry offers the same quantitative performance characteristics with the added benefit of significantly faster method development. The HRAM method development time depends only on the sample preparation and chromatography conditions. In addition, mass analysis methods can be established rapidly because there is no requirement to tune SRM transitions, collision energies, or transfer lens voltages.

Goal

In this preliminary evaluation a set of calibrators, clinical samples, and QCs are investigated with the analysis of multiple replicates over the course of 7 days. The current in-house validated liquid chromatography – tandem mass spectrometry (LC-MS/MS) method data is directly compared against the use of HRAM LC-MS data.

Experimental Conditions

Sample Preparation

Commercial calibration standards in frozen stabilized whole blood were sourced from Chromsystems (München, Germany). Commercial quality control material in stabilized whole blood was sourced from More Diagnostics (Los Osos, CA, USA). All calibrators, QCs, and whole blood samples were extracted using a plate-based solid phase extraction (SPE) procedure.

HPLC

Chromatographic separation was accomplished using a Thermo Scientific Accela U-HPLC system. A Thermo Scientific AQUASIL C18 column (150 x 2.1 mm, 5 µm) heated to 50 °C, was used with an isocratic gradient of 90% MeCN + ammonium acetate (2 mM). For each sample, 20 µL was injected.

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific Exactive high performance benchtop mass spectrometer powered by Orbitrap™ technology. Atmospheric pressure chemical ionization (APCI) was used to generate the $[M+NH_3]^+$ ions for tacrolimus, sirolimus, and everolimus, and the $[M+H]^+$ ions for cyclosporin, as well as two internal standards: ascomycin (for cyclosporin and tacrolimus) and desmethoxyrapamycin (for sirolimus and everolimus).

The Exactive™ mass spectrometer was set to scan at 50 K resolution over the range *m/z* 700 – 1300 and was calibrated once at the start of the 7-day analysis. Data acquisition and analysis were carried out with Thermo Scientific LCQUAN software.

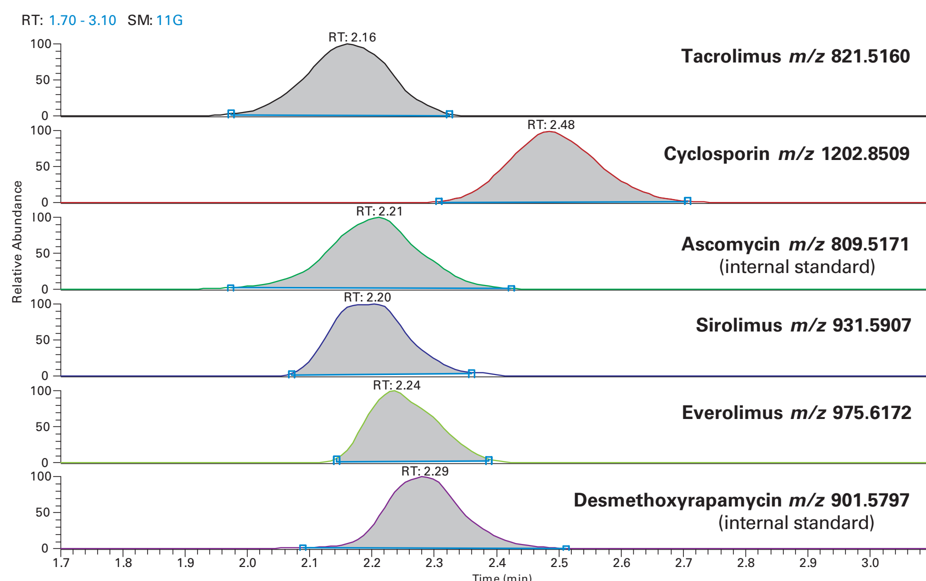

Figure 1. XIC of lowest calibration standard

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Results and Discussion

An accurate mass extracted ion chromatogram of the lowest calibration standard for each compound is presented in Figure 1. An example calibration line for each of the analytes is presented in Figure 2 A, B, C and D.

Inter-assay variability was determined by processing 30 replicates of each quality control over multiple batches. The precision data for inter-assay validation are presented in Table 1. The limit of quantitation (LOQ) has been set at 1 ng/mL for each analyte, and the highest CVs obtained at this concentration were 10.2%. The lower limit of quantitation (LLOQ) has not yet been fully investigated. Although cyclosporin, which also has the largest concentration range, achieved CVs of 12.5% at 0.3 ng/mL.

A total of 360 clinical research samples were analyzed by the HRAM method. The results were compared to the current LC-MS/MS method. Analysis of the clinical specimens by both HRAM LC-MS and LC-MS/MS demonstrate good correlation for cyclosporin, tacrolimus, and sirolimus across the required therapeutic range. No clinical research specimens were available for the method comparison of everolimus.

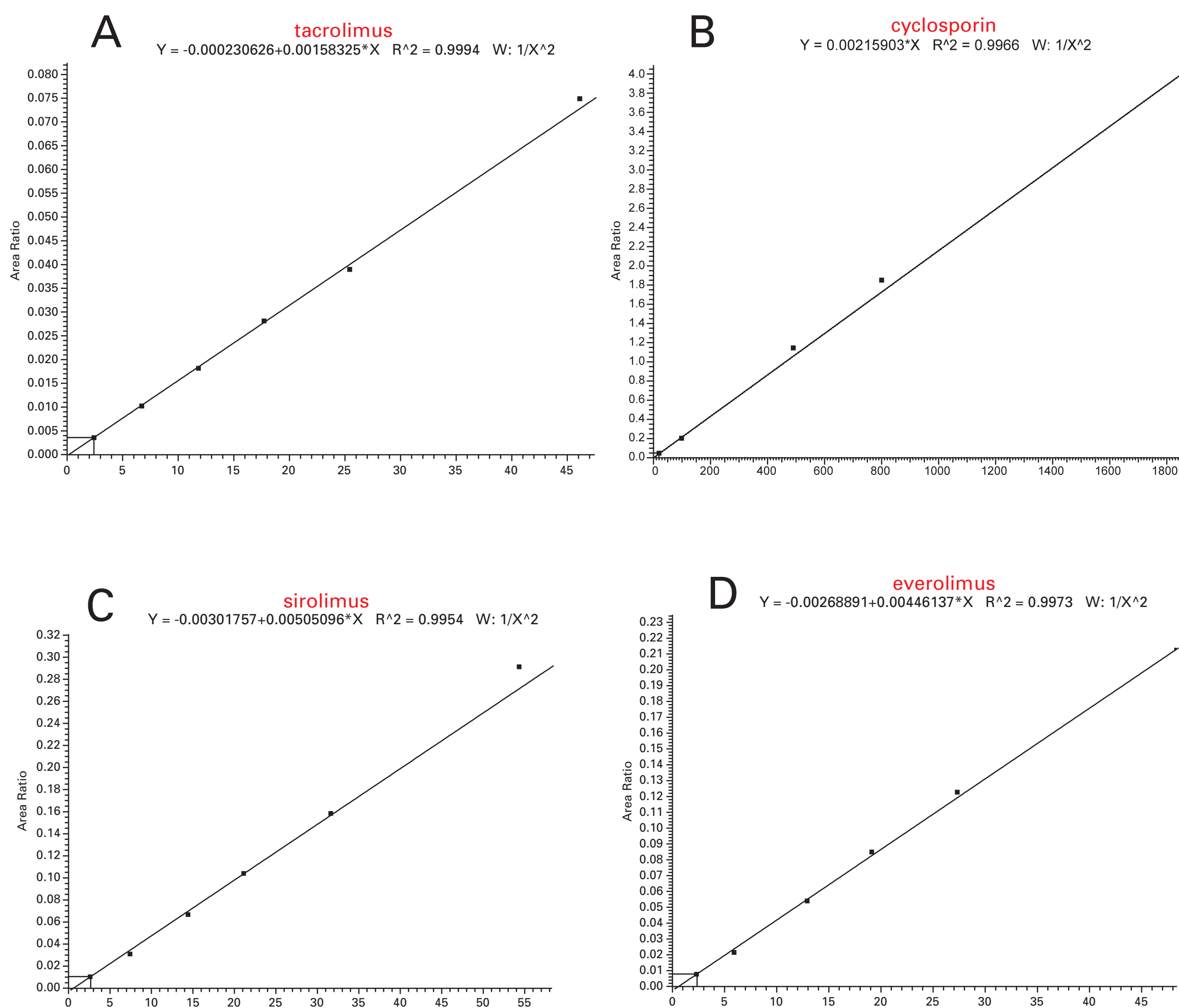

Figure 2. Calibration curves for (A) tacrolimus, (B) cyclosporin, (C) sirolimus, and (D) everolimus

Table 1. Method variability for each analyte

Analyte		Control 1	Control 2	Control 3	Control 4
Tacrolimus	Mean (ng/mL)	1.6	7.4	10.8	20.2
	%CV	12.8	4.2	4.2	2.9
Sirolimus	Mean (ng/mL)	3.3	14.4	25.3	41.6
	%CV	12.1	5.6	6.5	6.6
Everolimus	Mean (ng/mL)	2.9	13.9	24.2	41.8
	%CV	9.4	3.7	4.4	5.5
Cyclosporin	Mean (ng/mL)	83	176	362	787
	%CV	8.1	11.1	7.2	4.7

Conclusion

The HRAM analysis using the Exactive mass spectrometer demonstrates SRM comparable specificity, dynamic range, LOQ and precision in whole blood matrix. There is good correlation between SRM and HRAM results for the immunosuppressant drugs monitored.

The precision of HRAM LC-MS analysis meets current consensus guidelines and has acceptable performance to be used as a candidate clinical research method following further evaluation. All the method development time for this application was associated with the sample preparation and chromatography conditions. The mass analysis method was established in less than 5 minutes since there is no requirement to tune SRM transitions, collision energies or transfer lens voltages.

Acknowledgement

We would like to thank Dr. Mark Harrison for advice during the method set up.

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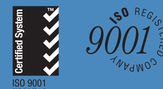

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Improved Quantitative Selectivity of Clenbuterol in Human Urine Using High Resolution on the TSQ Quantum Mass Spectrometer

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Key Words

- TSQ Quantum™
- High Resolution Analysis
- Improved Sensitivity
- Quantitation

The data presented here was acquired on a TSQ Quantum mass spectrometer.

Introduction

Clenbuterol (Figure 1) is a beta-2-adrenergic agonist, an effective bronchodilator drug used for the treatment of human asthma. It relieves bronchial airway smooth muscle contractions caused by Chronic Obstructive Pulmonary Disease (COPD) and allergy-induced respiratory distress.

Clenbuterol has significant anabolic effects and could be used as a drug of abuse in athletes and livestock for its muscle growth stimulant properties. It raises the body temperature and hence facilitates fat tissue catabolism. Due to Clenbuterol having these anabolic properties, it must be routinely monitored in biological samples by veterinary and human doping control laboratories.

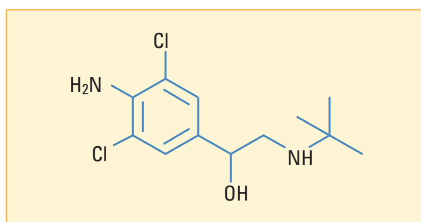

Figure 1: Chemical structure of Clenbuterol

Goal

One of the limitations to quantitation is the unequivocal identification of analytes in biological samples due to endogenous matrix interferents.

This report describes the use of high resolution on the Thermo Scientific TSQ Quantum to exploit the negative mass defect of a compound containing Chlorine, such as Clenbuterol, and hence improve the selectivity of the quantitative assay.

Clenbuterol ($C_{12}H_{18}Cl_2N_2O$, molecular weight 276.08 amu) was infused, 0.1 ng/µL, into the ESI source and the four most abundant product ions for the MS/MS breakdown were determined using the automated compound optimization procedure on the TSQ Quantum (Figure 2).

The transition yielding the most abundant product ion (*m/z* 203.0) was selected for the analysis of Clenbuterol.

Experimental Conditions

Sample Preparation: Human urine extracts were prepared using a C18 Solid Phase Extraction media. The extracted urine was spiked with Clenbuterol in the concentration range 0.1, 0.5, 1, 5, 10, 50 and 100 pg/µL for the calibration standards. No internal standard was used in this study.

Sample Analysis: The spiked urine extracts were chromatographed using a Thermo Scientific Surveyor™ LC on a C18 100 mm × 2.1 mm column at a flow rate of

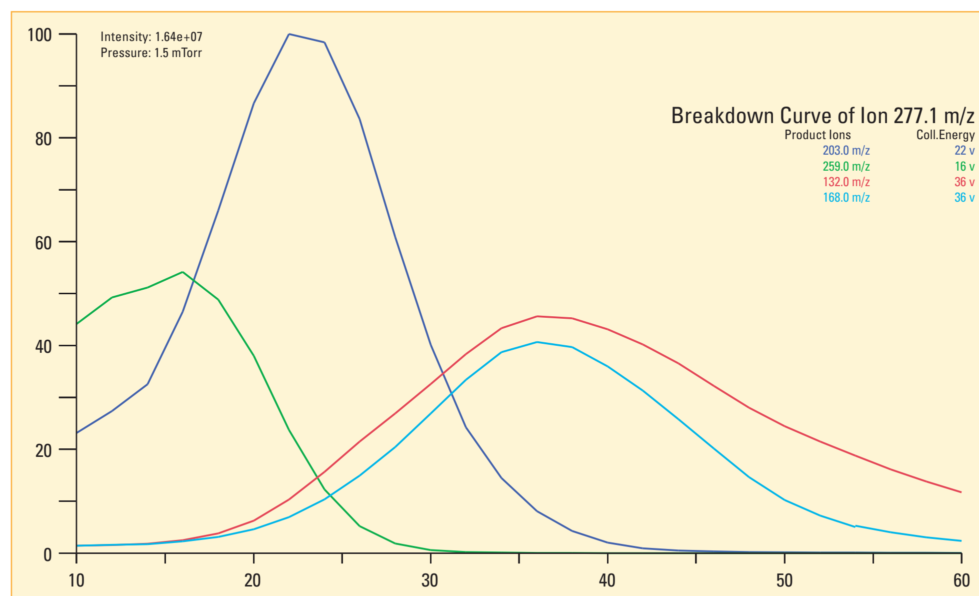

Figure 2: Automated optimization of MS/MS parameters for Clenbuterol

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300 µL/min with a linear gradient of 10% solvent B (Methanol/Ammonium acetate [10 mM] 90/10 v/v) to 100% B over 5 minutes. Solvent A was Ammonium acetate (10 mM). The calibration standards were injected in duplicate at volumes of 10 µL.

MS Conditions

Mass spectrometer: TSQ Quantum

Ionization mode: Electrospray (ESI), positive ion

SRM: Clenbuterol 277.1 → 203.0 ± 0.3 Da, 22 eV
Collision energy Resolution

Experiment 1: 0.7 Da FWHM on Q1 and Q3

Experiment 2: 0.1 Da FWHM on Q1, 0.7 Da FWHM on Q3

Two separate quantitative analyses were performed at peak widths of 0.1 Da and 0.7 Da Full Width Half Maximum (FWHM) on Q1 in SRM mode. A peak width of 0.7 Da FWHM was used on Q3 for all analyses.

Results

The chromatogram of a pure standard of Clenbuterol in aqueous solvent demonstrates the retention time at 5.8 minutes (Figure 3).

Experiment 1: Quantitative Analysis Performed at 0.7 Da FWHM

The data below shows the quantitative analysis of Clenbuterol in Human urine at peak width settings of 0.7 Da FWHM on Q1 and Q3. Chromatograms are shown for blank urine (Figure 4) and urine containing Clenbuterol at 0.1 pg/µL (Figure 5).

A calibration curve of Clenbuterol analyzed at 0.7 Da FWHM was constructed using linear fit of peak area plotted against concentration, weighted 1/x (Figure 6). A correlation coefficient of r^2=0.9990 with an equation of Y=8496.82+266143*X was obtained for the curve.

The peak area, back-calculated values and precision of all calibration standards are shown in Table 1.

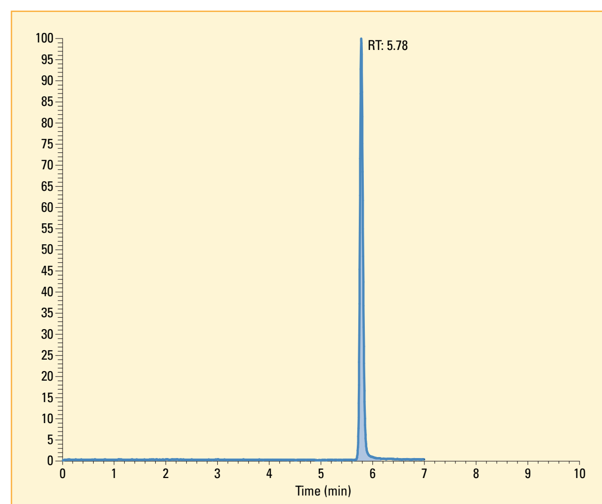

Figure 3: Determination of Clenbuterol retention time

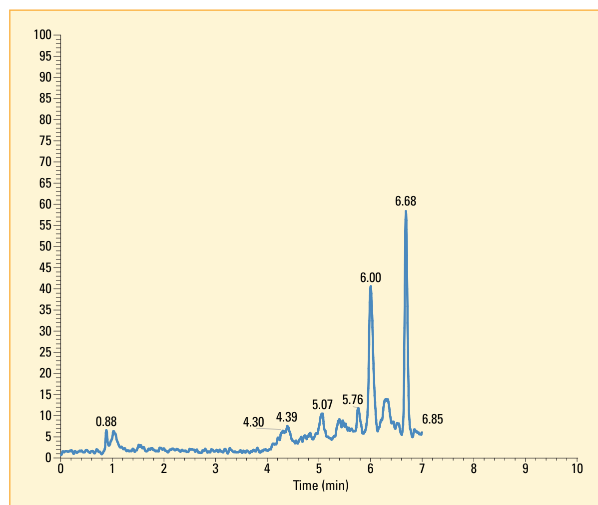

Figure 4: Urine blank, 0.7 Da FWHM

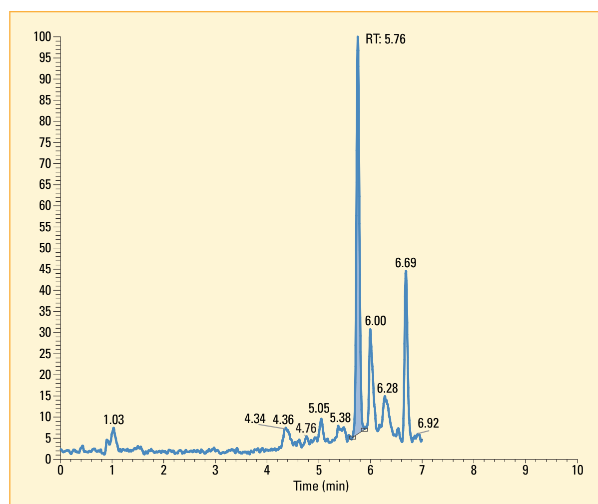

Figure 5: Clenbuterol, 0.1 pg/µL in urine, 0.7 Da FWHM

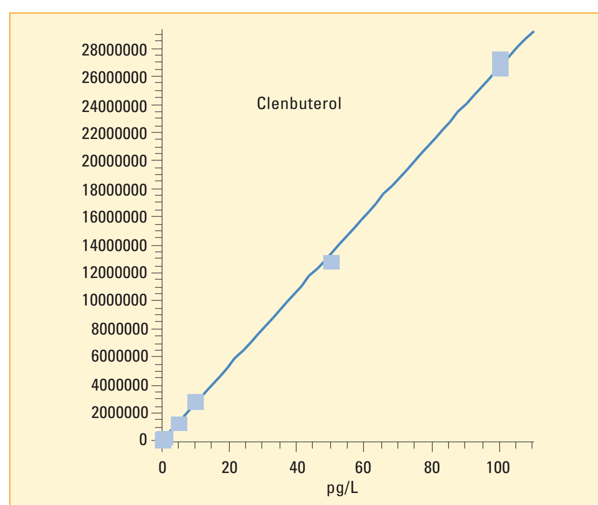

Figure 6: Clenbuterol curve at 0.7 Da FWHM

Experiment 2: Quantitative Analysis Performed at 0.1 Da FWHM

The data below shows the quantitative analysis of Clenbuterol in Human urine at peak width settings of 0.1 Da FWHM on Q1 and 0.7 Da FWHM on Q3. Chromatograms are shown for blank urine (Figure 7) and urine containing Clenbuterol at 0.1 pg/µL (Figure 8).

A calibration curve of Clenbuterol analyzed at 0.1 Da FWHM was constructed using linear fit of peak area plotted against concentration, weighted 1/x (Figure 9). A correlation coefficient of r^2=0.9994 with an equation of Y=2661.76+85951.1*X was obtained for the curve.

The peak area, back-calculated values and precision of all calibration standards are shown in Table 2.

SAMPLE NAME	AREA	CALC AMT	UNITS	%RSD
Urine blank	0.00	0.00	pg/L	
Urine blank	0.00	0.00	pg/L	
Cal 0.1 pg/L	33516.83	0.09	pg/L	4.5%
Cal 0.1 pg/L	31977.14	0.09	pg/L	4.5%
Cal 0.5 pg/L	136967.28	0.48	pg/L	0.6%
Cal 0.5 pg/L	137996.57	0.49	pg/L	0.6%
Cal 1 pg/L	289917.16	1.05	pg/L	1.3%
Cal 1 pg/L	295117.95	1.07	pg/L	1.3%
Cal 5 pg/L	1353210.91	5.05	pg/L	0.8%
Cal 5 pg/L	1338935.79	4.99	pg/L	0.8%
Cal 10 pg/L	2856289.00	10.70	pg/L	0.5%
Cal 10 pg/L	2877525.09	10.78	pg/L	0.5%
Cal 50 pg/L	12837781.41	48.20	pg/L	0.2%
Cal 50 pg/L	12797548.82	48.05	pg/L	0.2%
Cal 100 pg/L	27232776.65	102.29	pg/L	1.7%
Cal 100 pg/L	26578332.48	99.83	pg/L	1.7%

Table 1: Calculated standards at 0.7 Da FWHM

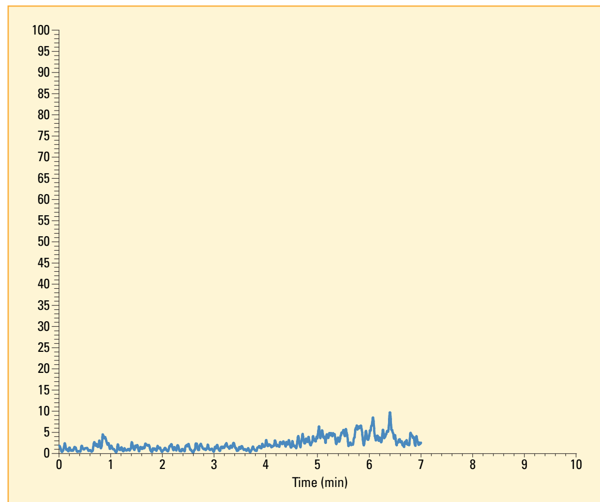

Figure 7: Urine blank, 0.1 Da FWHM

Discussion

Analysis, in SRM mode, of the spiked urine samples at a resolution setting of 0.7 Da FWHM resulted in a Clenbuterol peak eluting from the column upon a broad chemical noise background signal containing interferent peaks from the urine.

The same urine samples analyzed at a peak resolution setting of 0.1 Da FWHM resulted in elimination of the interfering isobaric mass peaks and the broad background chemical noise previously seen in the analysis at a peak width setting of 0.7 Da FWHM. The selected reaction monitoring analysis performed at a higher resolution setting of 0.1 Da FWHM resulted in increased selectivity of the assay and hence an increase in the precision that could be achieved.

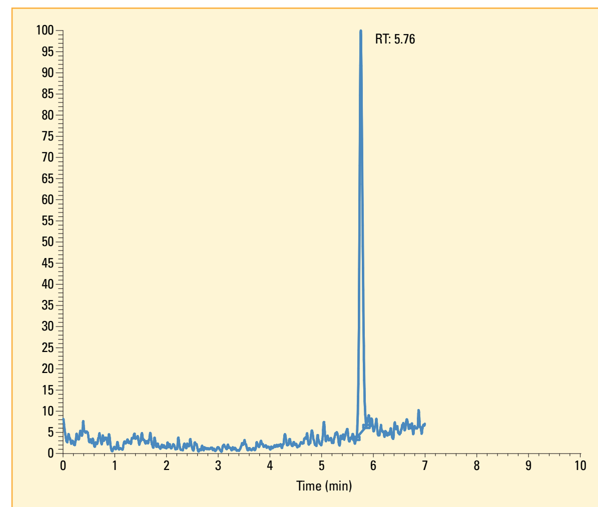

Figure 8: Clenbuterol, 0.1 pg/µL in urine, 0.1 Da FWHM

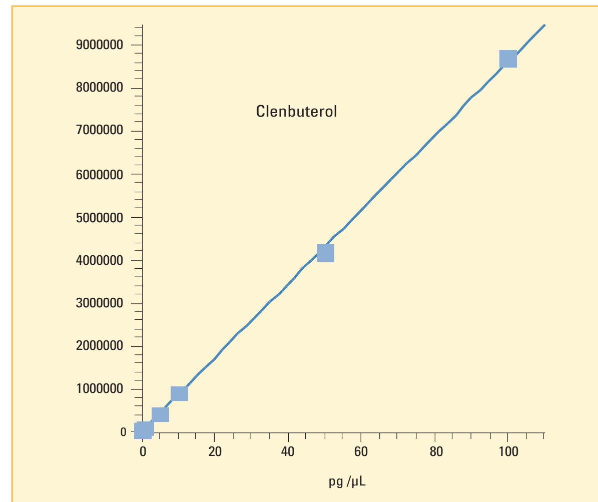

Figure 9: Clenbuterol curve at 0.1 Da FWHM

SAMPLE NAME	AREA	CALC AMT	UNITS	%RSD
Urine blank	0.00	0.00	pg/L	
Urine blank	0.00	0.00	pg/L	
Cal 0.1 pg/L	11245.02	0.10	pg/L	0.2%
Cal 0.1 pg/L	11272.54	0.10	pg/L	0.2%
Cal 0.5 pg/L	41960.02	0.46	pg/L	1.1%
Cal 0.5 pg/L	42592.84	0.46	pg/L	1.1%
Cal 1 pg/L	90353.60	1.02	pg/L	3.4%
Cal 1 pg/L	94633.92	1.07	pg/L	3.4%
Cal 5 pg/L	435920.49	5.04	pg/L	0.4%
Cal 5 pg/L	438538.32	5.07	pg/L	0.4%
Cal 10 pg/L	893656.24	10.36	pg/L	0.9%
Cal 10 pg/L	904758.00	10.49	pg/L	0.9%
Cal 50 pg/L	4120496.02	47.90	pg/L	1.3%
Cal 50 pg/L	4195902.58	48.78	pg/L	.3%
Cal 100 pg/L	8667429.70	100.81	pg/L	0.5%
Cal 100 pg/L	8727427.54	101.50	pg/L	0.5%

Table 2: Calculated standards at 0.1 Da FWHM

The increase in selectivity at a peak width setting of 0.1 Da FWHM is due to the fact that Clenbuterol is a chlorinated compound and thus the negative mass deficiency can be used to eliminate interferents from the urine matrix in SRM mode. This increased selectivity can be achieved without detrimental loss of transmission. Typically only a factor of two to three fold decrease in peak area is observed between analyses performed at 0.7 and 0.1 Da FWHM, however, greater selectivity could then be achieved.

The calibration curves for Clenbuterol concentrations of between 0.1 to 100 pg/µL at resolution settings of 0.1 and 0.7 Da FWHM both demonstrate excellent linearity. The calibration line at 0.7 Da FWHM showed a high intercept due to chemical background in the urine blank. This was significantly reduced by the use of high resolution.

The use of higher resolution to increase selectivity and precision could enable the limit of quantitation of an assay to be lowered and achieves a higher degree of confidence in identification of analytes in biological matrices.

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Versatile Solutions to Current Demands for Automated Sample Cleanup, High Throughput, and High-Resolution Chromatography on a Single LC-MS Platform

Catherine Lafontaine, Keeley Murphy, Joseph DiBussolo
Thermo Fisher Scientific, San Jose, CA

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Overview

Purpose: Use two case studies to demonstrate the versatility of an LC-MS platform for research: high-throughput, online sample cleanup capabilities in a multi-channel UHPLC system coupled to a high-resolution, accurate-mass (HRAM) hybrid quadrupole-Orbitrap™ mass spectrometer (MS).

Methods:

Case Study #1:

An LC/MS accurate-mass screen of antiretrovirals (ARVs) in biological matrix for research was examined using online sample cleanup. In a high-throughput workflow configuration, standards in several biological matrices were injected across two LC channels (each with distinct sample extraction columns) in a cross-sequential manner to one at-source guard column.

Case Study #2:

One LC/MS research method for analysis of four major sulfatide molecular species in an organic standard was optimized for demonstration of two discrete high-throughput data acquisition styles. Four LC channels, each with distinct HPLC columns, were used and injections were made in both a cross-channel and a cross-sequential manner to the HRAM-MS.

Results:

Case Study #1:

A 100 s LC/MS method for ARVs in human biological matrices with online sample cleanup and chromatographic resolution were acquired within a data window of 30 s using a multi-channel LC system. Four standard curve matrices were investigated. All four curves gave linearity between R^2 = 0.96 and 0.99.

Case Study #2:

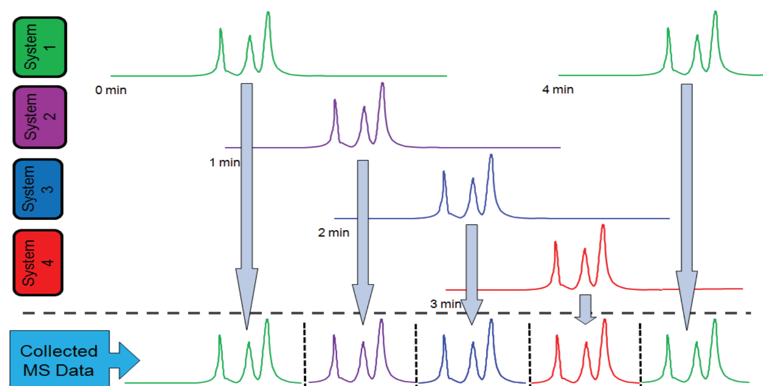
Through the two discrete high-throughput acquisition styles examined, the percent RSDs of each analyte across multiple injections of the organic standard were maintained at less than 5.8%.

Introduction

Investigative demands in both clinical research and pharmaceutical laboratories necessitate the use of simpler and faster LC/MS technologies. Market response to these demands has produced high-throughput technologies that can perform online sample cleanup followed by mass spectral analysis. These chromatographic capabilities, however, can be limiting and their results inconsistent.

Here we present two case studies highlighting the versatility of a single multichannel LC-MS platform as both a research and a pharmaceutical production tool. A multi-channel LC provides the ability to make staggered injections through multiple LC channels while utilizing a single MS, thus increasing sample throughput (Figure 1). This platform also has the capacity to perform online sample cleanup utilizing Thermo Scientific™ TurboFlow™ technology along with either baseline-resolved UHPLC chromatography or resolution based solely on accurate mass, all in a rugged high-throughput workflow.

FIGURE 1. Illustrative staggered chromatograph



Methods

Sample Preparation

Case Study #1: Four standard curve matrices of ARVs (saquinavir, nevirapine, efavirenz, and zidovudine) were prepared by a research [hospital] lab: acetonitrile-crashed plasma, water-diluted plasma, acetonitrile-crashed whole blood, and an acetonitrile-water neat mix. The dynamic range for each ARV was from 5 to 1000 ng/mL. Warfarin was added post-prep as internal standard for use with either the positive- or negative-ionizing ARVs.

Case Study #2: A 100 ng/mL sulfatide (from bovine) standard in neat solution, C8 UHPLC columns, and mobile phase reagents were supplied by a local pharmaceutical lab.

Liquid Chromatography

The multi-channel LC used in these experiments was a Thermo Scientific™ Transcend™ II LX-4 configured with four Thermo Scientific™ Dionex™ UltiMate™ 3000 Binary Rapid Separation HPG Pumps, a dual-valve VIM (valve interface module), and a CTC™ Dual-Arm DLW Autosampler (CTC Analytics AG, Zwingen, Switzerland). In both case studies, LC pump flow to the MS was diverted to waste, except during the data collection window of the method, to allow for faster LC flow rates and, therefore, faster column re-equilibration time.

Case Study #1: In a high-throughput workflow configuration, two LC channels (each with distinct TurboFlow sample extraction columns) were multiplexed to one at-source C18 guard column. The LC method details are outlined in Table 1. Use of the VIM's detector bypass position allowed for high flow rates (up to 5 mL/min) as necessary for TurboFlow technology.[1] Multiple injections of 30 µL were injected in staggered fashion across two channels of the LC system and were driven by Thermo Scientific™ Aria™ MX software version 2.1. Figure 2 illustrates a resultant chromatographic comparison of a neat standard with three in biological matrices.

Case Study #2: The sulfatide LC method details are outlined in Table 2. Multiple injections (10 µL) of the neat standard were injected onto a UHPLC column (C8, 2.1 x 50 mm, 1.7 µm) heated to 60 °C in both a cross-LC channel (four) and a cross-sequential manner and were driven by Aria OS software version 1.6.

TABLE 1. LC method details for Case Study #1

ARVs LC Method Details	
LC Method Length	100 s
Data Window	30 s
TurboFlow Columns	Thermo Scientific™ Cyclone™ column, 0.5 x 50 mm
Analytical Column	C18 guard cartridge, 10 x 4.6 mm
Loading Mobile Phase	10 mM ammonium formate + 0.05% formic acid (aq)
Eluting Mobile Phase	0.1% formic acid in acetonitrile
Extraction Column Wash	45:45:10 acetonitrile/isopropanol/acetone
Injection Volume	30 µL

TABLE 2. LC Method Details for Case Study #2

Sulfatide LC Method Details	
LC Method Length	96 s
Data Window	21 s (or 9 s)
Analytical Columns	C8, 2.1 x 50 mm, 1.7 µm heated to 60 °C
Loading Mobile Phase	5 mM ammonium formate + 0.2% formic acid (aq)
Eluting Mobile Phase	5 mM ammonium formate + 0.2% formic acid in 1:1 acetonitrile/methanol
Injection Volume	10 µL

Mass Spectrometry

All data were collected on the Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap MS (HRAM, high-resolution accurate mass) with heated electrospray ionization source (HESI) and full scan exact mass extraction used to quantitate results.

Case Study #1: The HRAM MS was used in full scan positive (250-700 *m/z*) and negative (250-350 *m/z*) ionization modes at resolution 70,000. A 30 s MS data window, necessary for simultaneous positive/negative switching, was used.

Case Study #2: The Q Exactive HRAM MS was used in full scan negative ionization mode across a mass range of 804-895 *m/z* at resolution 36,000. Two acquisition styles of high-throughput data collection workflows were examined. The first style of acquisition (collection of 1000 individual data files with 21 s data windows) involved a high-throughput collection with complete baseline chromatographic separation of the analytes in order to avoid potential matrix interferences (data not shown). A two-tiered second acquisition style involved, first, 384 injections collected into a single data file using the 21 s data window (Figure 3). The second high-throughput tier demonstrated the use of the Q Exactive MS to theoretically separate compounds based solely on their accurate mass using a 9 s data window (Figure 4).

Data Analysis

Case Study #1: Post-acquisition data analysis was performed using Thermo Scientific™ Xcalibur™ Quan Browser software version 3.0.

Case Study #2: Post-acquisition data processing of single, individually collected data files was performed using Xcalibur Quan Browser software version 3.0. Post-acquisition data processing of the multiple data files collected into a single file was performed using Generic Chromatographic Viewer of Thermo Scientific™ QuickCalc™ software version 8.3.24.

FIGURE 2. Comparison of ARV standard (250 ng/mL) across four matrix preparations

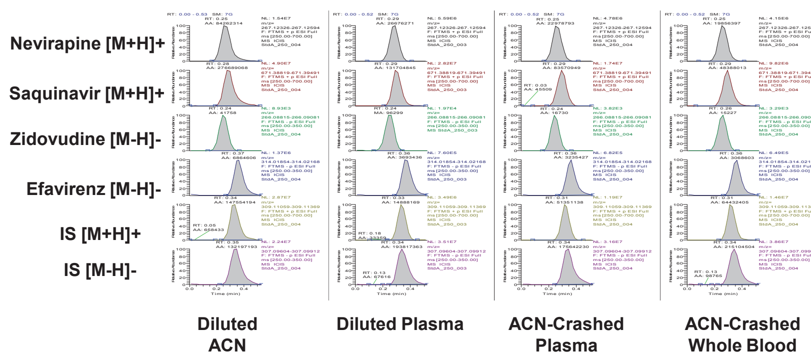


FIGURE 3. Upper trace: View of 384 sulfatide sample injections collected into a single data file, 21 s data window. Lower trace: Scaling to 1.75 min (four injections).

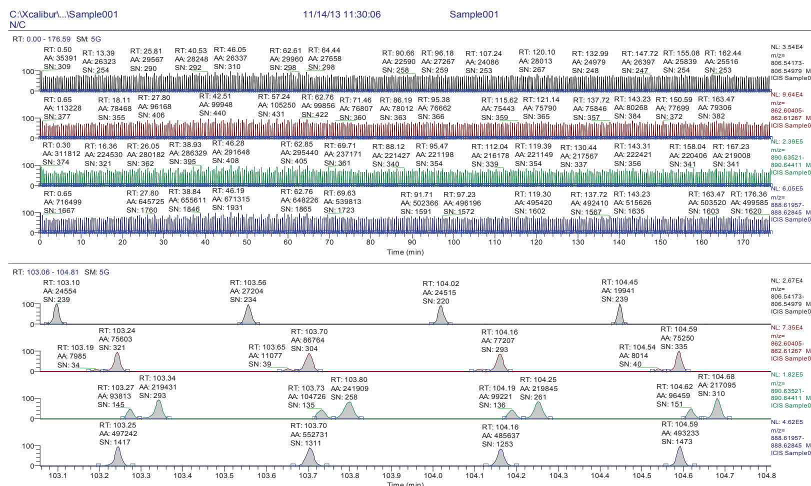
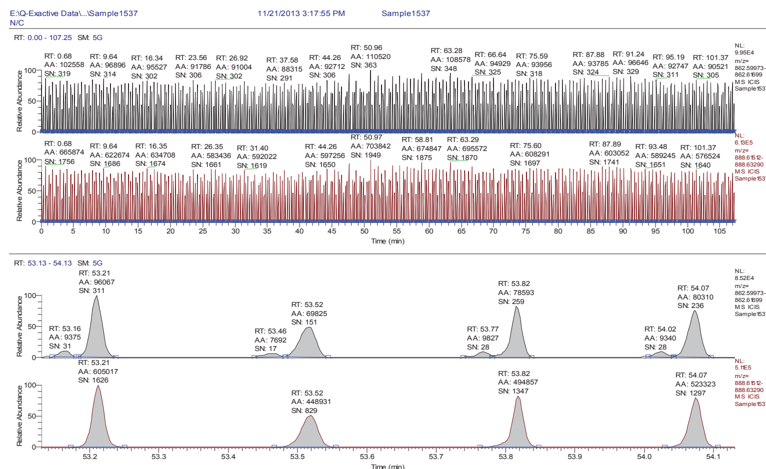


FIGURE 4. Upper trace: 384 sulfatide sample injections collected into a single data file, 9 s data window. Lower trace: Scaling to 1 min (four injections).



Results

Case Study #1:

Using a 100 s LC/MS research method with sample cleanup and chromatographic resolution, antiretrovirals were acquired within a data window of 30 s using a multiplexed (two-channel) LC system. Within the dynamic range investigated, all four standard curve matrices gave linearity between R^2 = 0.96 and 0.99. Internal standard %RSD across two channels was maintained at, or better than, 16% for each curve.

Case Study #2:

The first workflow (1000 separate data files using 21 s data windows) resulted in the acquisition of an average of 1.7 samples per minute with baseline chromatographic resolution of three analytes. The fourth analyte, co-eluting with another sulfatide, was earmarked as the internal standard.

The second workflow (three sets of 384 injections collected into 3 single data files using 21 s data windows) resulted in the acquisition of an average of 2.2 samples per minute with peaks chromatographically resolved.

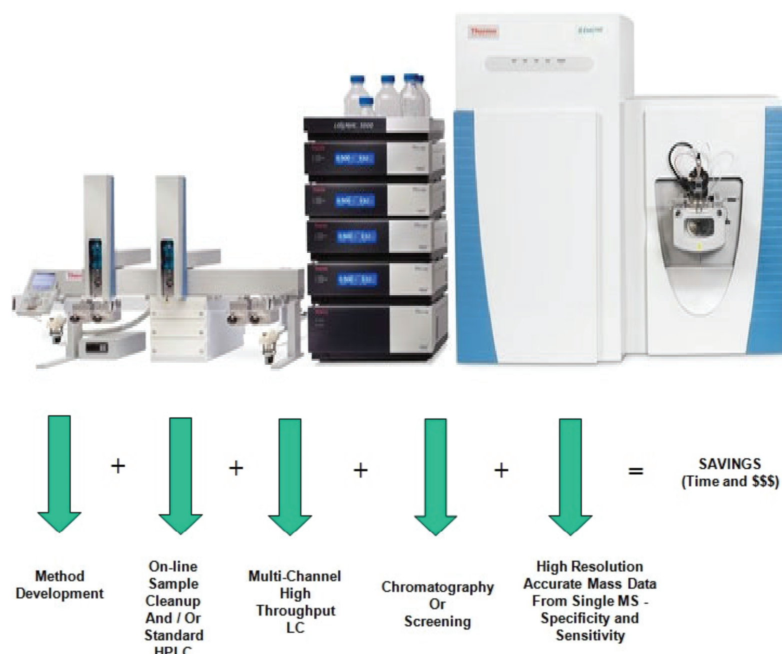
The final workflow (one set of 384 injections into a single file using 9 s data windows) resulted in the acquisition of 4.0 samples per minute with resolution by accurate mass in an ultra-high-throughput workflow. All workflows are summarized in Table 3.

Robustness of the assay was demonstrated with a combined %RSD of multiple injections (1152# multiplexed) for the 21 s data window workflow of ≤5.8% and with %RSDs of multiple injections (384# multiplexed) for the 9 s data window workflow of ≤2.8% .

TABLE 3. Overview of acquisition styles for data collection of 384-sample batches using the CTC Dual-Arm DLW AS with the four-channel Transcend II LX-4 system running cross-channel staggered injections to the Q Exactive HRAM MS.

Acquisition Style	Data Window Length (s)	LC Method Length (min)	Chromatography	Injections per minute
Standard LC-MS w/ 384 data files	N/A	1.6	Baseline separation of 3 analytes	0.63
Transcend II LX-4 system with 384 data files	21	1.6	Baseline separation of 3 analytes	1.7
Transcend II LX-4 system with 1 data file (DT_Submit)	21	1.6	Baseline separation of 3 analytes	2.2
Transcend II LX-4 system with 1 data file (DT_Submit)	9	1.0	Co-elution of 2 analytes	4.0

FIGURE 6. Versatility chart for Transcend II LX-4 system with Q Exactive hybrid quadrupole-Orbitrap MS platform.



Conclusion

- This work has effectively demonstrated the versatility of the Transcend II LX-4 with Q Exactive MS platform (Figure 6), which enables the user to perform fast online sample cleanup as needed or standard HPLC. Additionally, collection of multiple injections into a single data file can accomplish ultra-high sample throughput, whether for research screening applications or for true chromatography. The use of high-resolution, accurate-mass data collection adds in both the sensitivity and selectivity to the assay. The staggered injection capabilities of the multi-channel Transcend II LX-4 LC with the Q Exactive MS platform can be used to continuously acquire data during the window where the compounds are eluting. QuickCalc software offers accurate data parsing of those multiple injections into a single data file.
- In conclusion, it is important to note that any high-throughput workflow determinations must involve considerations to the requirements of the assay, such as chromatographic separation of isobaric compounds, removal of matrix interference, and carryover associated with either the LC column or the autosampler. Each of these considerations may pose restrictions to highest throughput. Overall, the greatest strength of this platform lies in the versatility that it can bring to either clinical research or drug discovery laboratories.

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1. Chassaing, C.; Robinson, S.; *Chromatography Today,* ***2009***, September, 20-24.

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An Improved Immunosuppressant Drug Research Method Based on a Novel SPLC-MS/MS System

Joseph Di Bussolo, Christopher Esposito and Francois Espourteille
Thermo Fisher Scientific, Franklin, MA, USA

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Overview

Purpose: Demonstrate robust and rugged method performance utilizing an automated two-channel sample preparation-liquid chromatography (SPLC) system that minimizes matrix interferences from whole blood when measuring immunosuppressant drugs (ISDs) for research purposes by tandem mass spectrometry (MS/MS) with electrospray ionization (ESI).

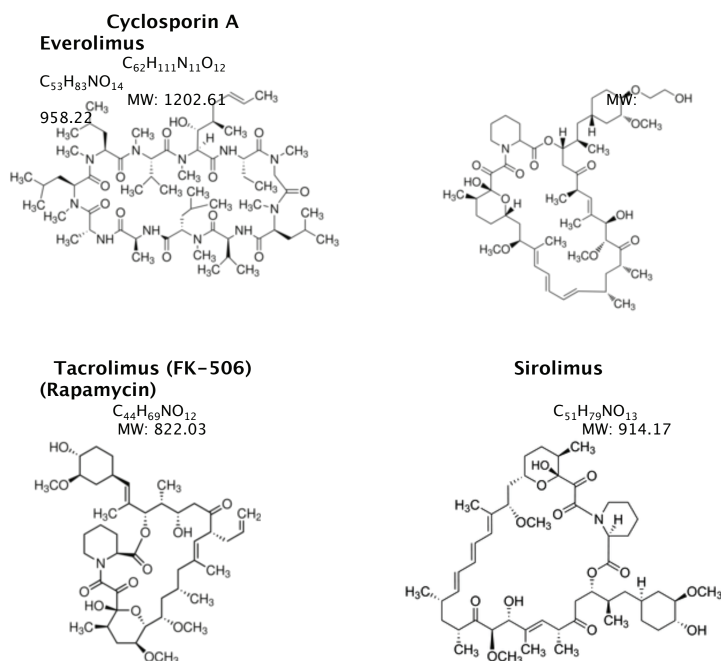
Methods: A 5 minute method involved automated clean up of whole blood preparations (cell rupture and protein precipitation by aqueous zinc sulfate and methanol) using TurboFlow technology followed by high-resolution liquid chromatography using a short Accucore C8, 2.6 µm HPLC column. Reversed-phase extraction, elution and final separations were done in a way that avoided the accumulation and co-elution of phospholipids, which would have suppressed ionization of ISDs in ESI sources. Quantitation of four ISDs was achieved by stable-isotope dilution using two internal standards (IS).

Results: Performance specifications were consistently reproduced within systems and across different laboratories as whole-blood levels were reliably measured: between 2.5 and 50 ng/mL for Everolimus, Sirolimus and Tacrolimus; and between 25 and 1,250 ng/mL for Cyclosporin A. A throughput of 21 samples per hour was achieved when multiplexing across both channels, which generated only 165 mL of solvent waste. No significant carryover between samples was detected.

Introduction

Immunosuppressant drugs (ISDs) are often analyzed in whole-blood using LC-MS with electrospray ionization, which is prone to interference by phospholipids. Although stable isotopes for each ISD are available to compensate, minimizing such interferences would improve data quality. The Thermo Scientific™ Prelude™ SPLC system—a novel dual-channel system that automates sample preparation and liquid chromatography (SPLC), was interfaced to the ESI of a tandem mass spectrometer (MS/MS) for the analysis of ISDs. The Prelude SPLC system incorporated Thermo Scientific™ TurboFlow™ technology and high-efficiency LC utilizing solid-core packing. Stable isotope derivatives D_{12}-Cyclosporin-A and Tacrolimus-$^{13}CD_2$ were used as internal standards in the whole-blood sample preparation procedure. The method was optimized to reliably minimize interferences from phospholipids to improve data quality. The method was also designed to minimize solvent waste.

FIGURE 1. Immunosuppressant Drugs Analysed



Methods

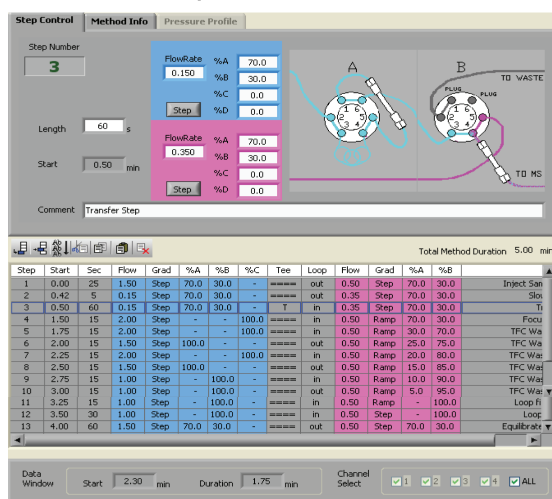
Off-Line Sample Preparation

ChromSystems 6PLUS1® ISD multilevel calibrator set and MassCheck® whole-blood controls as well as in-house test samples were mixed with aqueous zinc sulfate solution and then with methanol containing internal standards: Tacrolimus-$^{13}CD_2$ (Toronto Research Chemicals, Canada) and D_{12}-Cyclosporin A (Alsachim, France). After centrifugation, supernatants were harvested into glass autosampler vials.

On-Line Sample Preparation & Liquid Chromatography (SPLC)

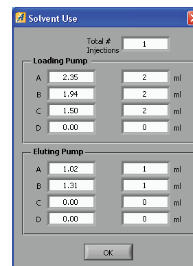
In each channel, 20 µL injections of supernatants were extracted with a Thermo Scientific™ TurboFlow™ Cyclone-P™ TurboFlow column (0.5 x 50mm) using a mobile phase mixture of 7:3 water:methanol containing 10 mM ammonium formate and 0.05% formic acid at 1.5 mL/min. A slow flow of methanol eluted extracted ISDs, which merged with a higher flow of a 7:3 water: methanol mixture, to transfer and focus the ISDs to an Accucore C8, 2.6 um, 3.0 x 30 mm HPLC column, which was maintained at 70 °C by the built-in heater. The ISDs were separated from matrix interferences and eluted to the heated electrospray ionization (HESI) source by a gradient of increasing methanol. Figure 2 shows this focus method.

FIGURE 2. Summary of SPLC Focus Method.



Step	Start	Sec	Flow	Grad	%A	%B	%C	Tee	Loop	Flow	Grad	%A	%B	
1	0.00	25	1.50	Step	70.0	30.0	-	====	out	0.50	Step	70.0	30.0	Inject San
2	0.42	5	0.15	Step	70.0	30.0	-	====	out	0.35	Step	70.0	30.0	Slo
3	0.50	60	0.15	Step	70.0	30.0	-	T	in	0.35	Step	70.0	30.0	Tr
4	1.50	15	2.00	Step	-	-	100.0	====	in	0.50	Ramp	70.0	30.0	Focu
5	1.75	15	2.00	Step	-	-	100.0	====	in	0.50	Ramp	30.0	70.0	TFC Wa
6	2.00	15	1.50	Step	100.0	-	-	====	out	0.50	Ramp	25.0	75.0	TFC Wa
7	2.25	15	2.00	Step	-	-	100.0	====	in	0.50	Ramp	20.0	80.0	TFC Was
8	2.50	15	1.50	Step	100.0	-	-	====	out	0.50	Ramp	15.0	85.0	TFC Was
9	2.75	15	1.00	Step	-	100.0	-	====	in	0.50	Ramp	10.0	90.0	TFC Was
10	3.00	15	1.00	Step	-	100.0	-	====	out	0.50	Ramp	5.0	95.0	TFC Was
11	3.25	15	1.00	Step	-	100.0	-	====	in	0.50	Ramp	-	100.0	Loop fi
12	3.50	30	1.00	Step	-	100.0	-	====	in	0.50	Step	-	100.0	Loop
13	4.00	60	1.50	Step	70.0	30.0	-	====	out	0.50	Step	70.0	30.0	Equilibrate

Solvents:
A: Water + 10mM NH_4OOCH + 0.05% HOOCH
B: Methanol + 10mM NH_4OOCH + 0.05% HOOCH
C: 45% Acetonitrile + 45% Isopropanol + 10% Acetone



Total solvent consumption is 3.37 mL A, 3.25 mL B, 1.5 mL C for each injection.

Mass Spectrometry

The Thermo Scientific™ TSQ Vantage™ triple-stage quadrupole system with heated electro-spray interface (HESI-II) was used to measure the transitions from ammonium-adduct precursor ions to product ions:

Everolimus: 975.7 > 908.4 — Sirolimus: 931.6 > 864.6

Tacrolimus: 821.5 > 824.4 — Tacrolimus IS: 824.4 > 771.0

Cyclosporin A: 1202.8 > 425.3 — Cyclosporin A IS: 1214.9 > 437.2

During method development, the elution of phospholipids and dioctylphthalate were tracked by adding the following transitions:

Dioctylphthalate: 391 >149

Lyso-Phosphotidylcholine;16:0: 496 > 184

Lyso-Phosphotidylcholine;18:0: 524 > 184

Phosphotidylcholine;38:6: 806 > 184

Data Analysis

Thermo Scientific™ TraceFinder™ software with Aria MX was used for instrument control, data acquisition and data processing. The internal standards (IS) shown above were used for quantitation by stable-isotope dilution technique.

Results

Identifying the HPLC Column and Conditions that Minimize Interferences

Because ISDs are as hydrophobic as phospholipids and phthalates, all are extracted and transferred to the HPLC column during the TurboFlow process. Therefore, the HPLC conditions must be optimized to elute the ISDs to the detector in a reasonable timeframe while avoiding co-elution of interferences as well as buildup of interfering compounds in the HPLC column while processing many samples. Figure 3 shows buildup and co-elution from non-optimized conditions, which resulted in poor reproducibility (RSDs > 20%) of peak areas for internal standards in sample batches. Figure 4 shows results from optimized conditions, which resulted in improved IS peak area reproducibility (RSDs < 10%).

FIGURE 3. Non-Optimized HPLC Conditions

Elution from Accucore PFP, 2.6 μm, 3.0 x 50 mm column:

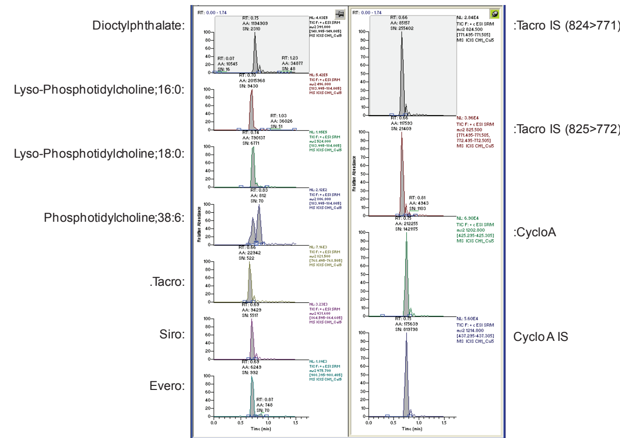
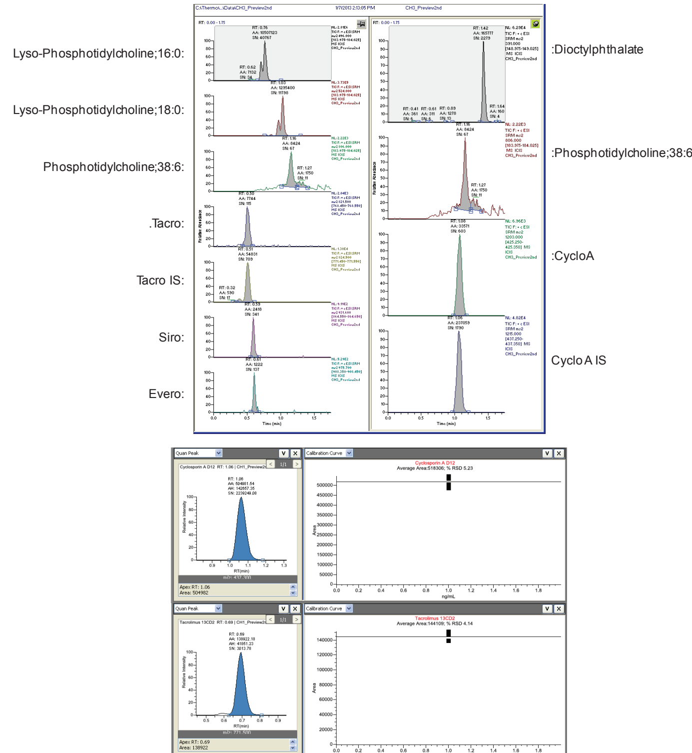


FIGURE 4. Optimized HPLC Conditions

Elution from Accucore C8, 2.6 μm, 3.0 x 30 mm column:



Achieving Required Linear Range with No Significant Carryover

As shown in Figures 5 and 6, the method consistently showed linear responses between 2.5 and 50 ng/mL for Everolimus, Sirolimus and Tacrolimus and between 25 and 1,250 ng/mL for Cyclosporin A. Weighting the data by 1/x minimized differences between expected and calculated concentrations in calibrators.

FIGURE 6. Everolimus Calibrators and QCs

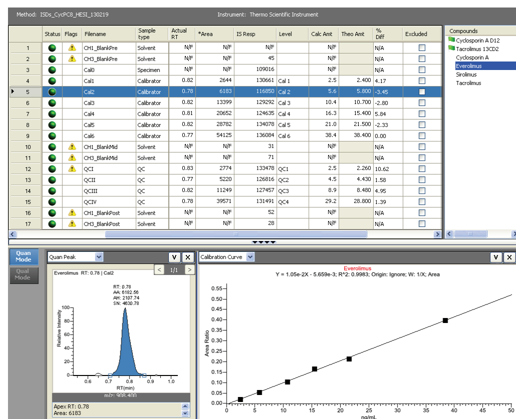
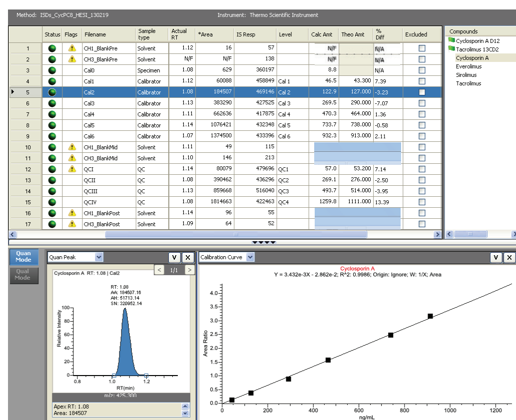


FIGURE 7. Cyclosporin A Calibrators and QCs



Reproducible QC Results were Reported Across 3 Different Test Sites.

As shown in Table 1, very similar results were reported from three different research test sites: Johns Hopkins University, Boston Children's Hospital and The Cleveland Clinic.

TABLE 1. Commercial Quality Control (QC) Reproducibility Results

	CyclosporinA			*Everolimus*		
Level	**Expected**	**Average**	**RSD%**	**Expected**	**Average**	**RSD%**
I	53	53	4.6	2.3	2.3	11.7
II	276	260	3.5	4.4	4.4	11.0
III	514	515	2.1	8.5	8.8	8.4
IV	1111	1172	6.4	28.8	28.6	6.1

	Sirolimus			*Tacrolimus*		
Level	**Expected**	**Average**	**RSD%**	**Expected**	**Average**	**RSD%**
I	2.9	2.9	8.5	2.6	2.8	5.3
II	10.1	10.0	4.6	7.3	7.1	6.1
III	20.4	20.6	5.2	16.7	16.4	4.1
IV	38.5	38.6	6.2	34.2	33.8	4.1

n=15 from 3 systems within 30 days

Matching Results from Legacy Method

As shown in Table 2, the Prelude method produced results that agreed with those produced by a legacy TurboFlow method for ISDs. Furthermore, the Prelude results were reproduced remarkably well from sample preparations that were almost 1 month old.

TABLE 2. Everolimus Calibrators and QCs

		Ran on	1/9/2013	Repeated 1/29/2013				Ran on	1/9/2013	Repeated 1/29/2013
Test Sample	ISD	Legacy Method	Prelude Method	Prelude Method		Test Sample	ISD	Legacy Method	Prelude Method	Prelude Method
8KLE	Cyclosporin A:	86	105	103		120726-001	Everolimus:	3.5	3.0	4.5
8KBG	Cyclosporin A:	186	201	203		120726-002	Everolimus:	2.0	1.7	1.8
8KOU	Cyclosporin A:	84	99	93		120726-003	Everolimus:	2.0	1.8	2.0
8L20	Cyclosporin A:	80	81	75		120904-001	Everolimus:	4.0	4.3	3.9
8LB5	Cyclosporin A:	88	94	94		121227-001	Everolimus:	4.6	3.9	4.4
8JDF	Cyclosporin A:	168	176	176		121227-002	Everolimus:	2.3	2.2	2.5
8I6C	Cyclosporin A:	53	58	58		121227-003	Everolimus:	2.3	2.3	2.1
8KJNK	Sirolimus:	3.6	2.2	1.8		8LO5	Tacrolimus:	7.3	7.6	7.6
8KN6	Sirolimus:	3.0	1.2	2.0		8M3Y	Tacrolimus:	2.6	3.2	2.9
8L5K	Sirolimus:	8.4	9.5	7.3		8M4D	Tacrolimus:	12.5	11.1	12.5
8JB0	Sirolimus:	3.3	3.5	2.8		8M8F	Tacrolimus:	2.3	2.8	2.8
8GOC	Sirolimus:	14.4	12.5	10.9		8MI1	Tacrolimus:	16.2	15.0	17.9
8I27	Sirolimus:	3.2	2.5	1.9		8MDV	Tacrolimus:	8.9	8.8	9.6
86HF	Sirolimus:	5.7	5.5	4.2		8LRH	Tacrolimus:	20.0	17.7	19.0

Conclusion

Improved reliability and economy was achieved for ISD analysis for research purposes by using a novel SPLC-MS/MS system and method.

- Ion suppression of ISDs by co-eluting phospholipids was largely avoided by using the short Accucore C8 HPLC column.
- Using 1/x weighting, correlation coefficients (r2) > 0.995 were typical for:
  - Cyclosporin A, from 25 to 1250 ng/mL,
  - Everolimus, Sirolimus & Tacrolimus, from 2.5 to 50 ng/mL.
- Carryover, measured by peak areas corresponding to the ISDs from blank injections following the highest calibrators, was typically less than 0.1%.
- Reproducible ISD QC results were obtained from three research test sites evaluating this method with the PreludeSPLC-TSQ Vantage system.
- A reduction in solvent waste of about 40% was achieved, comparable to legacy TurboFlow methods for ISDs.

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A Novel On-Line Sample Cleanup and Liquid Chromatography Platform for LC/MS Analysis in the Clinical Research Laboratory

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Overview

Purpose: Describe a reliable and rugged sample preparation liquid chromatography system - Prelude SPLC™ - which utilizes novel pumps and fluidics configuration to multiplex two channels, for high-throughput LC-MS applications.

Methods: TurboFlow™ on-line extraction coupled to high efficiency HPLC utilizing core enhanced technology prior to tandem mass spectrometry were optimized for measuring immunosuppressant drugs, drugs of abuse and steroidal compounds.

Results: Typical throughput was 20 samples per hour while conserving consumables and minimizing user intervention. Quality-control (QC) sample results from three different Prelude SPLC systems operated at three different locations typically varied by less than ten percent coefficient of variation (%CV).

Introduction

Clinical research and forensic toxicology laboratories have a need for rapid and reproducible methods automated by systems that are easy-to-use and maintain. We describe a new system, which encompasses a novel HPLC pump design and fluidics configuration, enabling the user to perform on-line sample cleanup using TurboFlow technology and high-performance liquid chromatography (HPLC) on two channels multiplexed to a mass spectrometer (MS). Reproducibility, linearity, and other performance data are discussed. Several applications (immunosuppressant drugs (ISDs), pain management drugs (PMDs), 25-OH-vitamin D and various steroids in blood) have been satisfactorily tested. They displayed significantly reduced solvent consumption and shortened run times with reproducible results.

Methods

Sample Preparation & Liquid Chromatography

A Prelude SPLC system (Thermo Scientific) processed 20 uL injections of supernatants from protein-precipitated samples using a Cyclone-P™ TurboFlow column, transferred extracted analytes to an Accucore™ PFP HPLC column (2.1 x 50 mm) in which the analytes were separated, and then eluted to the MS system.

Mass Spectrometry

A TSQ Vantage™ tandem mass spectrometer (MS/MS) with heated electrospray ion (HESI-II) source (Thermo Scientific) was used for selective reaction monitoring (SRM) of analytes.

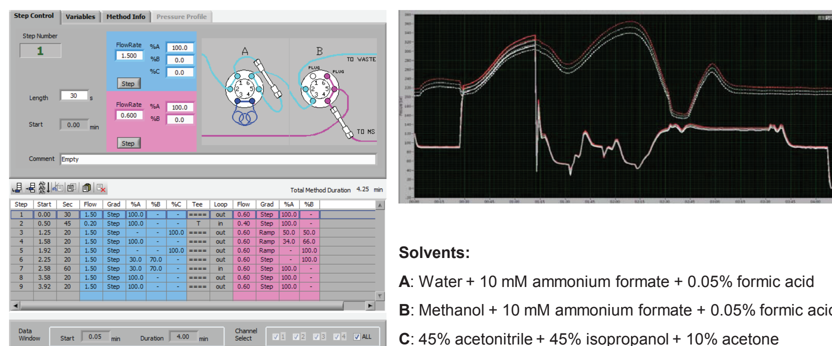
System Control & Data Analysis

TraceFinder™ 2.1 software was used to control the SPLC-MS system and to collect and process the MS/MS data.

System Suitability

A rigorous LC-MS/MS testing protocol was designed to determine inter- and intra-system precision and ruggedness of the system (Figure 1). Using a test mix of four compounds - Atenolol, Warfarin, Lidocaine and Imipramine, in both aqueous and plasma matrices, both channels on multiple Prelude SPLC systems were tested. %RSD values were generated for peak areas as well as retention times across channels and across systems.

FIGURE 1. SPLC System Suitability Method and Representative Pressure Trace.



Solvents:

A: Water + 10 mM ammonium formate + 0.05% formic acid

B: Methanol + 10 mM ammonium formate + 0.05% formic acid

C: 45% acetonitrile + 45% isopropanol + 10% acetone

Results

Whole-System Testing Verified Performance

To simulate a typical bio-analytical application, plasma spiked with our test mix was mixed with a 3-fold volume of acetonitrile and centrifuged. To test the reproducibility and ruggedness of the SPLC-MS/MS system, we ran a batch of 500 injections of the supernatant, which had a duration of 34 hours. The peak retention times and areas for each compound were reproducible as illustrated in Figure 2. Without the benefit of smoothing or internal-standard compensation, peak area RSDs were below 9% (Figure 3).

FIGURE 2. 500 Matrix Injections - ***over 34 hrs of run time!***

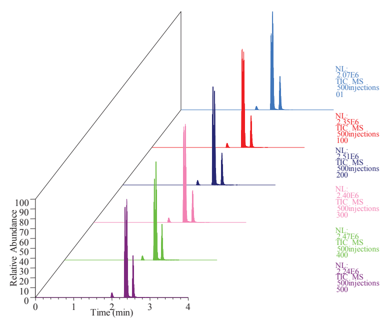
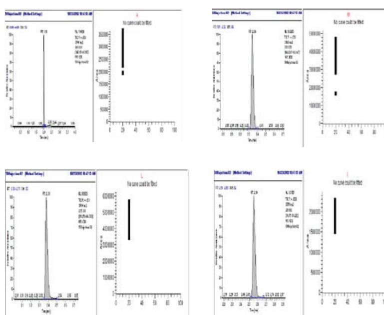


FIGURE 3. Peak Area %RSDs



Pressure and Retention Times were Reproducible

While the reproducibility of raw area counts speaks to the removal of matrix effect and its impact on data, the burden of 500 matrix injections and its impact on the aging of the SPLC system and its columns can be significant. For that reason retention time drift, pressure trace drift and peak shape changes were evaluated for the same data set. As shown by Figures 4, 5 & 6, retention times, pressure traces and peak shapes were remarkably stable throughout the 500-injection 34-hour batch.

FIGURE 4. Retention Time Drift for four compounds over 34 hrs of run time

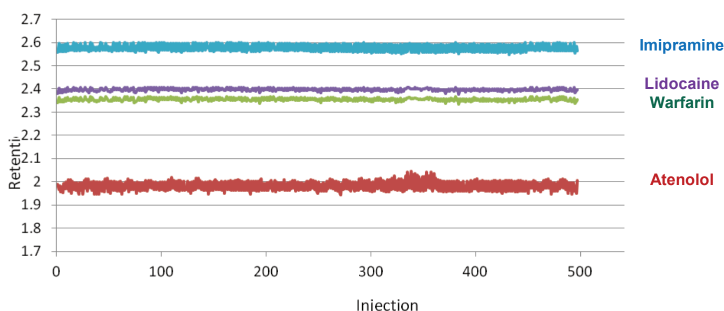
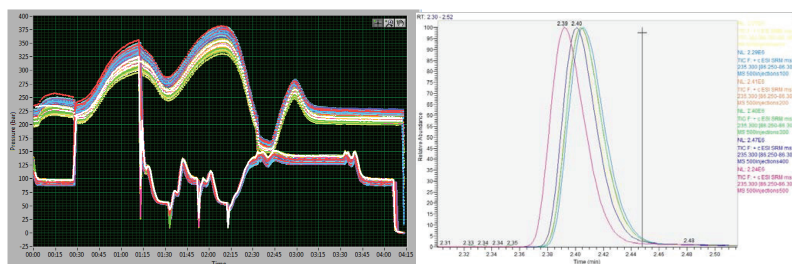


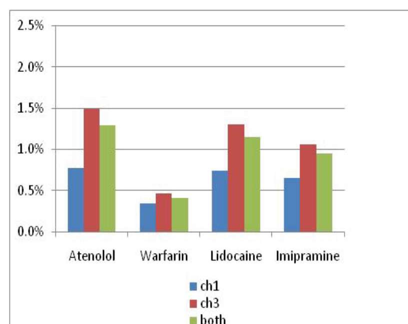
FIGURE 5. Pressure Trace Overlay across 34 hrs & Peak Overlay at injections 1,100, 200, 3 and 500



Inter-System Testing was Acceptable

While performance and ruggedness of any single LC-MS system is essential, inter-system performance is equally important. The typical workflow from Development to Production of a new method relies on inter-system ruggedness and reproducibility. For this reason, data from three prototype SPLC systems were gathered over the course of 5 months of testing and retention time performance across the three systems were analyzed. Reproducibility of retention times for each of the four test compounds generated from both channels of the three systems is summarized in Figure 7. The percent coefficient of variation (%CV) values were calculated from A random selection of 9 data points for each compound.

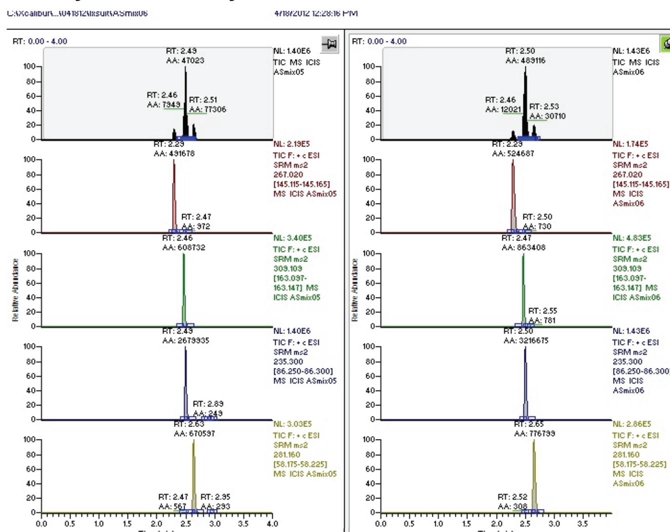
FIGURE 7. Retention Time %CVs from 3 different prototype systems.



A Purpose-Built Method clearly showed System Performance

Knowing that a rigorous LC-MS method with stringent data criteria would be the best test of the Prelude SPLC system, a method that tests for common problems with chromatographic separations was devised. The Suitability test has four compounds the first, Atenolol, the earliest eluter, is used to help elucidate problems that might exist with the refocusing of analytes on the analytical column. Retention time and peak shape differences in Warfarin and Lidocaine peaks will help detect any problems that may exist with gradient formation and/or column deterioration, as their RT shifts with compositional mobile phase differences. Imipramine is highly susceptible to degradation in peak shape if the mobile phases are not fresh or made precisely as prescribed by the method. In concert, the test mix serves as powerful diagnostic tool. The installation protocol for Prelude SPLC systems requires all four compounds to pass 20 injections (10 per channel) with RSD or CV of 10% or less with no internal standard correction for retention times and peak areas. Figure 8 shows typical performance.

FIGURE 8. System Suitability run of both channels of the Prelude SPLC System

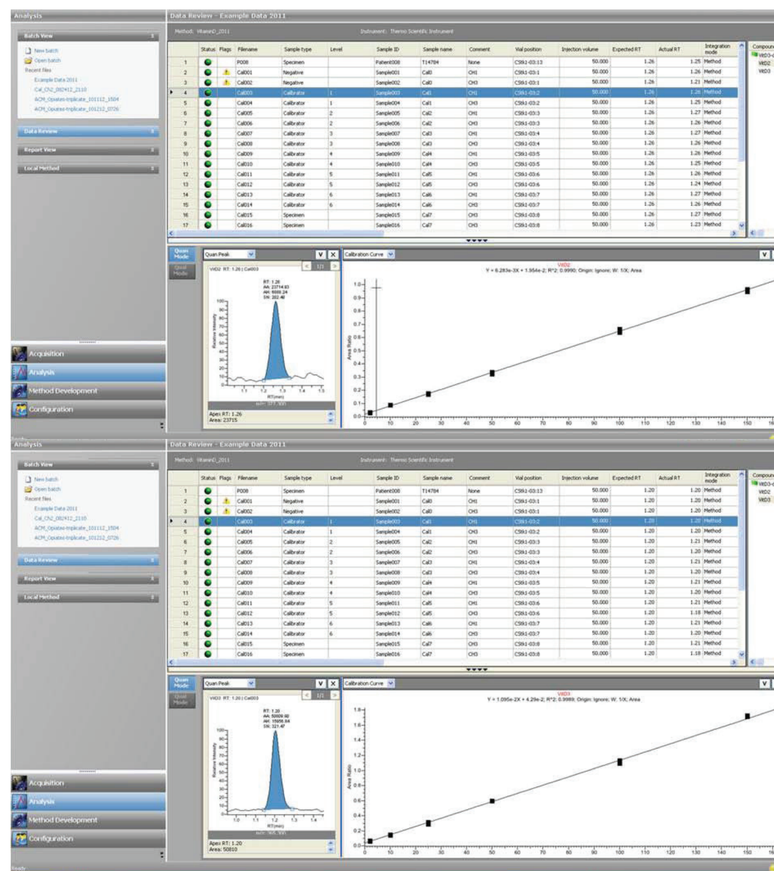


Compound	Area Count RSD%	RT CV%
Atenolol	1.98%	0.31%
Warfarin	3.64%	0.18%
Lidocaine	4.69%	0.15%
Imipramine	3.81%	0.10%

System was Suitable for well-known Clinical Research Methods

In order to asses the scope of applications for the Prelude SPLC system, popular LC-MS methods used in clinical research were considered. Methods for steroids, pain management drugs, immunosuppressant drugs and 25-OH-Vitamin D2 and D3, were developed and evaluated. We monitored linearity within the experimental range, inter- and intra-day reproducibility, long-term system stability, solvent consumption as compared to other platforms, and other relevant parameters. Please see other posters for more details on some of these methods. Figure 9 shows typical quantitative results for the Vitamin D compounds - excellent reproducibility for peak areas and retention times while achieving the desired sensitivity and linearity.

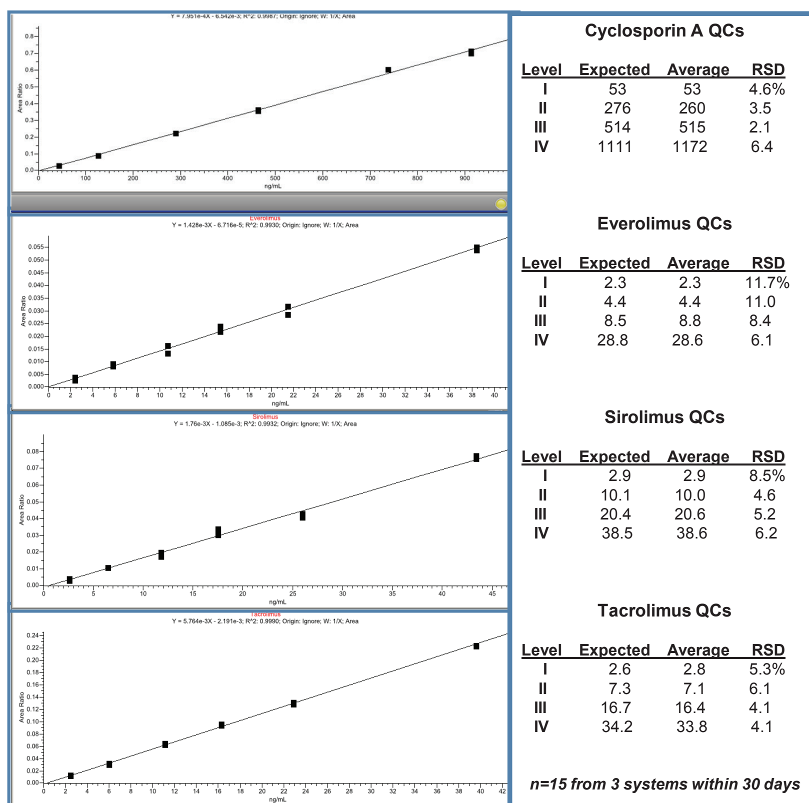
FIGURE 9. Quantitative Results – 25-OH-Vitamin D2 and D3



Even for Immunosuppressant Drug applications

Measuring Everolimus, Sirolimus, Tacrolimus and Cyclosporin A in whole-blood samples presents many challenges, from sample preparation to detection of each analyte and internal standard by the MS/MS system. To evaluate the Prelude SPLC system's ability to handle such an application, ChromSystems® multilevel calibrators and MassCheck® whole blood controls were processed using D_{12}-Cyclosporin A (Alsachim, Illkirch-Graffenstaden, France) as the internal standard (IS) for Cyclosporin A and Tacrolimus-$^{13}CD_2$ (Toronto Research Chemicals, Canada) as the IS for Everolimus, Sirolimus and Tacrolimus. Typical RSDs of peak areas for the two IS compounds were less than 12%.Typical quantitative results, collected over a span of 30 days from three systems in different locations - Cleveland, Baltimore and Boston, are shown in Figure 9.

FIGURE 10. Quantitative Results Immunosuppressant Drugs



Cyclosporin A QCs

Level	Expected	Average	RSD
I	53	53	4.6%
II	276	260	3.5
III	514	515	2.1
IV	1111	1172	6.4

Everolimus QCs

Level	Expected	Average	RSD
I	2.3	2.3	11.7%
II	4.4	4.4	11.0
III	8.5	8.8	8.4
IV	28.8	28.6	6.1

Sirolimus QCs

Level	Expected	Average	RSD
I	2.9	2.9	8.5%
II	10.1	10.0	4.6
III	20.4	20.6	5.2
IV	38.5	38.6	6.2

Tacrolimus QCs

Level	Expected	Average	RSD
I	2.6	2.8	5.3%
II	7.3	7.1	6.1
III	16.7	16.4	4.1
IV	34.2	33.8	4.1

n=15 from 3 systems within 30 days

Conclusion

Far too often LC/MS methods and instruments fall short of the rigorous performance criteria Clinical Research Labs require for everyday testing. The complex nature of the samples being injected on the system and the number of samples which need to be processed tax the instrumentation and columns. The system suitability method we developed proved a valuable whole-system testing procedure and demonstrated consistent performance of the Prelude SPLC systems in three different locations. This purpose-designed testing facilitates the implementation of rigorous evaluation standards for LC-MS systems used for clinical research. Availability of a standard system suitability test allows vendors and scientists to verify LC/MS system performance under controlled conditions which are similar to actual operating circumstances and has proven to be a valuable tool which is utilized from manufacture to installation of Prelude SPLC Systems. System performance was also verified by calibration and QC results for ISDs that matched expected values under typical operating conditions at two different clinical research facilities.

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LC-MS Quantitative Screening Method for 18 Anabolic Steroids in Oral Fluid Using MS2 Spectra Data Collected with Q Exactive Orbitrap Mass Spectrometer

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Overview

Purpose: To develop a sensitive method for quantitative screening of 18 anabolic steroids in oral fluid.

Methods: Samples were processed with LLE, analyzed with a 15 min. LC gradient, and compounds were identified with ion ratio calculated for fragments in MS2 spectrum

Results: The LLOQ was 1ng/mL for all analytes except for 6β-Hydroxyfluoxymesterone (6 ng/mL). The UPLQ was between 60-1500 ng/mL, and it was lower for compounds producing high signal in mass spectrometer detector. Matrix effects were not observed: percent recovery in spiked blank oral fluid and analyzed with calibration standards prepared in solvent was in range 78.5-118%.

Introduction

Androgenic-anabolic steroids (AAS) are drugs which mimic effects of testosterone and dihydrotestosterone in the human body. They increase protein synthesis within cells which results in buildup of cellular tissue, especially in muscles. Use of anabolic steroids by athletes to increase body weight is referred to as doping and is banned by major sporting bodies.

In this work we implemented Thermo Scientific™ Q Exactive™ ultra high resolution mass spectrometer to ensure high method specificity and sensitivity.

Methods

Sample Preparation - LLE

1. To 200 µL of oral fluid (in preservation buffer), add 40 µL of internal standard solution (10 µg/mL Testosterone $^{13}C_3$ in MeOH) and 1 mL MTBE
2. Vortex, let samples rest for 5 min. at room temperature
3. Store samples for 30 min. at -20 °C
4. Transfer solvent upper layer to glass tube
5. Evaporate at 37 °C
6. Reconstitute in 50% MeOH
7. Inject 30 µL of the sample onto LC-MS

Liquid Chromatography

Column: Thermo Acucore C18, 100x3 mm, 2.6 µm

Mobile phase:

A: 0.2% Formic Acid in DIW
B: 0.1% Formic Acid in MeOH
C: ACN/IPA/Acetone=45/45/10 v/v/v

LC gradient:

	Time	A%	B%	C%	D%	µl/min
0	0.00	95.0	5.0	0.0	0.0	1000.0
1	0.49	95.0	5.0	0.0	0.0	1000.0
2	0.50	95.0	5.0	0.0	0.0	500.0
3	2.00	50.0	50.0	0.0	0.0	500.0
4	10.00	0.0	100.0	0.0	0.0	500.0
5	10.01	0.0	100.0	0.0	0.0	1000.0
6	11.00	0.0	100.0	0.0	0.0	1000.0
7	11.01	0.0	0.0	100.0	0.0	1000.0
8	12.00	0.0	0.0	100.0	0.0	1000.0
9	12.01	95.0	5.0	0.0	0.0	700.0
10	13.00	95.0	5.0	0.0	0.0	1000.0
11	15.00	95.0	5.0	0.0	0.0	1000.0

Mass Spectrometer

Ionization source: APCI
Resolution: 35K
Isolations width: 2 mu
AGC target: 2e5
Maximum IT = 250 ms
Acquisition mode: t-MS2

MS2 spectra are collected with optimized collision energies specified in method inclusion list (Figure 1) together with acquisition time windows.

Figure 1. MS method inclusion list

File Edit Help

	Mass [m/z]	Polarity	Start [min]	End [min]	nCE	CS [z]	Comment
1	259.07630	Positive	2.75	3.75	40 %		Clenbuterol
2	259.07630	Positive	7.25	8.25	40 %		19-Norandosterone
3	275.20060	Positive	6.00	7.00	50 %		Nandrolone
4	283.20560	Positive	6.12	7.12	40 %		Methandrosterone
5	285.18490	Positive	3.70	4.70	40 %		6b-Hydroxyboldenone
6	287.20060	Positive	5.70	6.70	35 %		Boldenone
7	287.20060	Positive	6.70	7.70	50 %		DHEA
8	289.21620	Positive	5.90	7.90	40 %		Oxandrolone/Testosterone/Epitestosterone
9	303.19550	Positive	3.70	4.70	45 %		Formestane
10	311.24820	Positive	7.10	8.10	90 %		Stanozolol
11	313.21620	Positive	7.44	8.44	50 %		THG
12	319.22680	Positive	6.70	8.70	50 %		Oxymesterone
13	323.17720	Positive	7.00	8.00	40 %		Clostebol
14	292.22630	Positive	6.40	7.40	40 %		Testosterone_3C13
15	337.21740	Positive	5.90	6.90	50 %		Fluoxymesterone
16	345.25360	Positive	5.80	6.80	80 %		3-Hydroxystanozolol
17	353.21230	Positive	4.20	5.20	45 %		6b-Hydroxyfluoxymesterone
* 18							

Data Processing

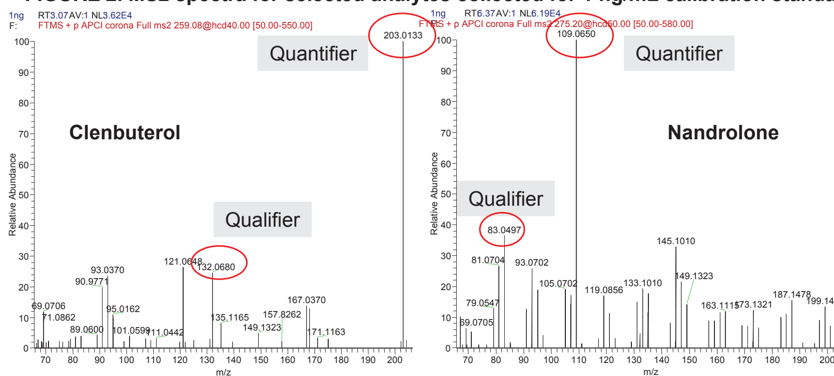
Two most abundant fragments (Table 1) in MS2 spectra (Figure 2) were selected for quantification and confirmation. Ion ratio was calculated and EU guidelines[1] for maximum permitted tolerance were applied.

Table 1. List of analytes, m/z values for parent ion and fragments in MS2 spectrum

Analyte	Formula	m/z	m/z in MS source	Ret Time (min)	Fragment 1	Fragment 2
Clenbuterol	$C_{12}H_{18}Cl_2N_2O$	277.0869	259.0763	3.2	203.0129	132.0679
19-Norandrosterone	$C_{18}H_{28}O_2$	277.2162	259.2056	7.7	241.1942	145.1007
Nandrolone	$C_{18}H_{26}O_2$	275.2006	275.2006	6.5	109.0647	83.0494
Methandrosterone	$C_{20}H_{28}O_2$	301.2161	283.2056	6.6	173.0956	147.0800
6β-Hydroxyboldenone	$C_{29}H_{26}O_3$	303.1955	285.1849	4.3	121.0645	147.0798
Boldenone	$C_{19}H_{26}O_2$	287.2006	287.2006	6.2	121.0648	135.1166
DHEA	$C_{19}H_{28}O_2$	289.2162	287.2006	7.2	97.0653	109.0651
Oxandrolone	$C_{19}H_{30}O_3$	307.2268	289.2162	6.4	135.1165	121.1012
Testosterone	$C_{19}H_{28}O_2$	289.2162	289.2162	6.9	97.0651	109.0650
Epitestosterone	$C_{19}H_{28}O_2$	289.2162	289.2162	7.4	97.0651	109.0650
Formestane	$C_{19}H_{26}O_3$	303.1955	303.1955	4.2	121.0649	171.0802
Stanozolol	$C_{21}H_{32}N_2O$	329.2587	311.2482	7.6	81.0542	107.0857
THG	$C_{21}H_{28}O_2$	313.2162	313.2162	7.9	241.1576	159.0798
Oxymesterone	$C_{20}H_{30}O_3$	319.2268	319.2268	7.2	113.0595	125.0593
Clostebol	$C_{19}H_{27}ClO_2$	323.1772	323.1772	7.5	143.0254	131.0254
Fluoxymesterone	$C_{20}H_{29}FO_3$	337.2173	337.2173	6.4	241.1576	131.0851
3-Hydroxystanozolol	$C_{21}H_{32}N_2O_2$	345.2536	345.2536	6.3	97.0400	107.0855
6β-Hydroxyfluoxymesterone	$C_{20}H_{29}FO_4$	353.2122	353.2123	4.7	95.0857	239.1419
Testosterone-$^{13}C_3$	$C_{16}{}^{13}C_3H_{28}O_2$	292.2263	292.2263	6.9	100.0753	112.0751

Results

FIGURE 2. MS2 spectra for selected analytes collected for 1 ng/mL calibration standard



Linearity Range, LOQ, LOD

Figure 3. Chromatographic peaks reconstructed with m/z accuracy of 5 ppm at LOQ of 1 ng/mL (*3 ng/mL, **6 ng/mL)

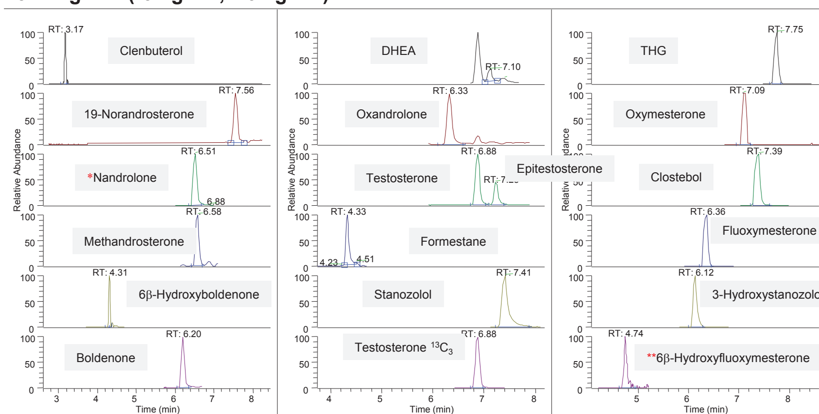
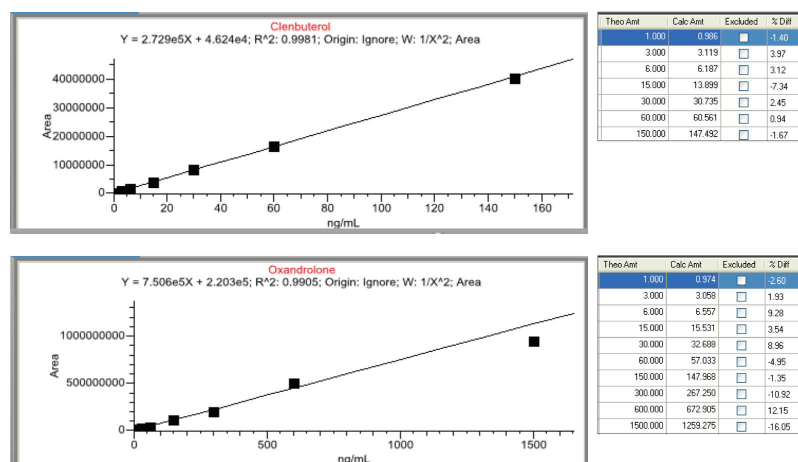


Figure 4. Calibration curves for selected analytes



Theo Amt	Calc Amt	Excluded	% Diff
1.000	0.986		-1.40
3.000	3.119		3.97
6.000	6.187		3.12
15.000	13.899		-7.34
30.000	30.735		2.45
60.000	60.561		0.94
150.000	147.492		-1.67

Theo Amt	Calc Amt	Excluded	% Diff
1.000	0.974		-2.60
3.000	3.058		1.93
6.000	6.557		9.28
15.000	15.531		3.54
30.000	32.688		8.96
60.000	57.033		-4.95
150.000	147.968		-1.35
300.000	267.250		-10.92
600.000	672.905		12.15
1500.000	1259.275		-16.05

Table 2. Linearity ranges, LOQ, LOD

Analyte	Linearity range	R^2	LOQ	LOD
Clenbuterol	1-150 ng/mL	0.9981	1 ng/mL	<1 ng/mL
19-Norandrosterone	1-1500 ng/mL	0.9937	1 ng/mL	< 1 ng/mL
Nandrolone	3-150 ng/mL	0.9926	3 ng/mL	<1 ng/mL
Methandrosterone	1-600 ng/mL	0.9931	1 ng/mL	<1 ng/mL
6β-Hydroxyboldenone	1-600 ng/mL	0.9852	1 ng/mL	<1 ng/mL
Boldenone	1-600 ng/mL	0.9939	1 ng/mL	<1 ng/mL
DHEA	1-600 ng/mL	0.9898	1 ng/mL	<1 ng/mL
Oxandrolone	1-1500 ng/mL	0.9905	1 ng/mL	<1 ng/mL
Testosterone	1-300 ng/mL	0.9896	1 ng/mL	<1 ng/mL
Epitestosterone	1-600 ng/mL	0.9889	1 ng/mL	<1 ng/mL
Formestane	1-600 ng/mL	0.9882	1 ng/mL	<1 ng/mL
Stanozolol	1-300 ng/mL	0.9911	1 ng/mL	<1 ng/mL
THG	1-600 ng/mL	0.9914	1 ng/mL	<1 ng/mL
Oxymesterone	1-300 ng/mL	0.9923	1 ng/mL	<1ng/mL
Clostebol	1-150 ng/mL	0.9961	1 ng/mL	<1 ng/mL
Fluoxymesterone	1-300 ng/mL	0.9916	1 ng/mL	<1 ng/mL
3-Hydroxystanozolol	1-60 ng/mL	0.9952	1 ng/mL	<1 ng/mL
6β-Hydroxyfluoxymesterone	6-150 ng/mL	0.9838	6 ng/mL	3 ng/mL

Method Precision

QC samples with concentrations across calibration range (2 ng/mL, 15 ng/mL, 90 ng/mL, 450 ng/mL) were prepared in blank oral fluid. QC samples were analyzed in 5 replicates in 3 separate batches to obtain intra- and inter- assay precision (Table 3).

Table 3. Intra-assay and inter-assay results

Analyte	Intra assay				Inter assay			
	2 ng/mL	15 ng/mL	90 ng/mL	450 ng/mL	2 ng/mL	15 ng/mL	90 ng/mL	450 ng/mL
Clenbuterol	<10.5	<3.3	<6.2	<15.1	12.6	8.4	5.8	11.0
19-Norandrosterone	<12.4	<11.6	<12.5	17.9	16.3	9.4	12.0	14.1
Nandrolone	NA	<14.2	<12.3	<13.0	NA	12.7	10.0	10.4
Methandrosterone	<13.1	<11.9	<13.9	<18.3	11.5	12.7	13.9	17.3
6β-Hydroxyboldenone	<7.9	<13.3	<11.5	<20.0	14.5	10.8	9.5	13.6
Boldenone	<15.1	<9.4	<11.1	<12.9	12.6	11.3	16.1	18.2
DHEA	<16.6	<13.4	<10.5	<9.7	14.2	10.2	10.2	8.9
Oxandrolone	<11.0	<14.4	<12.9	<19.9	10.6	10.4	10.2	13.7
Testosterone	<14.6	<9.0	<11.9	<19.1	11.5	7.6	9.5	16.7
Epitestosterone	<16.4	<14.4	<10.8	<13.2	14.3	9.8	7.7	8.3
Formestane	<10.4	<10.6	<10.0	<18.1	18.7	13.5	14.3	19.9
Stanozolol	<20.9	<10.9	<10.5	<15.2	19.9	10.9	8.2	13.1
THG	<19.5	<10.1	<11.0	<16.9	16.3	11.1	7.5	13.4
Oxymesterone	<25.0	<12.3	<6.0	<15.0	24.5	9.0	4.9	12.6
Clostebol	<14.8	<12.4	<10.3	<12.8	14.1	11.0	6.6	9.7
Fluoxymesterone	<18.0	<9.6	<11.6	<19.2	24.0	9.0	7.5	14.0
3-Hydroxystanozolol	<15.1	<5.0	<5.3	<12.5	24.8	8.0	5.8	11.0
6β-Hydroxyfluoxymesterone	NA	<12.8	<6.5	<13.7	NA	9.1	9.4	14.2

Matrix Effect

Matrix effects (Table 4) were evaluated by spiking blank oral fluid with all analytes at concentrations of 2 ng/mL, 10 ng/mL, 100 ng/mL and analyzing these samples with calibration standards prepared in solvent.

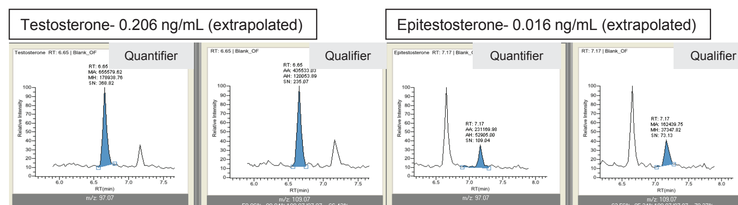
Table 4. Percent recovery in spiked blank oral fluid

Analyte	2 ng/mL	10 ng/mL	100 ng/mL
Clenbuterol	121	131	107
19-Norandrosterone	101	123	101
Nandrolone	ND	97.7	93.5
Methandrosterone	95.0	104	103
6β-Hydroxyboldenone	102	92.4	94.3
Boldenone	101	103	99.6
DHEA	100	127	115
Oxandrolone	93.5	124	109
Testosterone	90.5	105	96.8
Epitestosterone	78.5	99.8	102
Formestane	90.5	92.6	95.3
Stanozolol	80.0	81.5	92.8
THG	94.0	100	95.9
Oxymesterone	89.0	109	113
Clostebol	99.7	110	118
Fluoxymesterone	96.5	101	104
3-Hydroxystanozolol	93.5	92.0	105
6β-Hydroxufluoxymesterone	80.5*	102	104

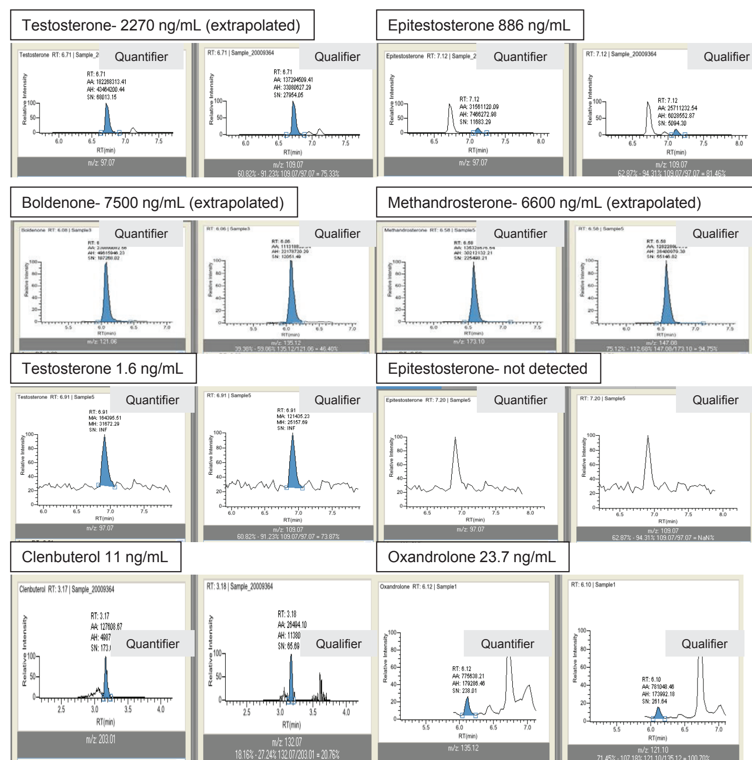
ND: not detected; *concentration (1.61 ng/mL) below LOQ

Donor Samples

Testosterone and Epitestosterone in negative tested oral fluid processed with LLE.



Compounds detected in selected positive tested samples prepared in collaborator lab with protein precipitation method.



Conclusion

We developed sensitive and robust quantitative screening method to analyze anabolic steroids in human oral fluid.

- Implementation of the ultra high resolution Q Exactive mass spectrometer to collect MS2 spectra and ion ratio confirmation results in high confidence in compound identification.
- Method was validated using LLE for sample preparation, but we also detected all analytes in positive tested samples processed with protein precipitation and provided by collaborator laboratory.

Acknowledgement

We would like to thank Erica Guice, Research Director, Western Slope Laboratory, for scientific advice and for providing samples for method testing.

References

1. Draft SANCO 1805/2000 Rev.1 [Revised Commission Decision 93/256 of 14 April 1993] laying down performance criteria for analytical methods to be used for certain substances and residues there of in live animals and animals products according to Council Directive 96/23/EC

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Therapeutic Drug Monitoring of 9 New Anticancer Agents by High-Performance Liquid Chromatography-Tandem Mass Spectrometry

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Introduction

The treatment of some cancers has shifted from conventional chemotherapy drugs to chronic treatment with molecular targeted therapies. Targeted therapies include drugs such as Tyrosine kinase inhibitors (eg: Imatinib, Dasatinib, Nilotinib, Sunitinib, Sorafenib, Vandetanib, Lapatinib, Vatalanib and Erlotinib) that present better efficiency and lower side effects than conventional anti cancer drugs.

Goal

The goal was to develop and validate a fast, specific and sensitive method for the quantitation of Tyrosine kinase inhibitors (eg: Imatinib, Dasatinib, Nilotinib, Sunitinib, Sorafenib, Vandetanib, Lapatinib, Vatalanib and Erlotinib) in plasma samples using liquid chromatography coupled to mass spectrometry.

Method

Equipment

The liquid chromatography consisted of a Thermo Scientific (Courtaboeuf, France) Accela® autosampler and a quaternary pump. Separation was performed on an Hypersil Gold® PFP (2.1x100 mm; pore size 1.9 µm) analytical column placed in a thermostated column heater at 50°C. The chromatographic system was coupled to a triple quadrupole (TSQ) Quantum Ultra mass spectrometer (MS) from Thermo Fisher Scientific, Inc. equipped with an Ion Max electrospray ionization (ESI) interface and operated with XCalibur 2.07 software (Thermo Fisher Scientific, Courtaboeuf France).

LC conditions

The mobile phase used for chromatography was 10 mM ammonium formate buffer containing 0.1% (v/v) formic acid (solution A), and acetonitrile with 0.1 % (v/v) formic acid (solution B). The mobile phase was delivered using the following stepwise gradient elution program: initial conditions of 95:5 (A:B) maintained for 0.5 minutes, run from 95:5 (A:B) at 0.5 minutes to obtain 5:95 (A:B) at 2 minutes, conditions 5:95 (A:B) maintained from 2 to 4 minutes, wash using 100% of phase C from 4 to 7 minutes, run from 5:95 (A:B) at 7.01 minutes to 95:5 (A:B) at 7.5 minutes, conditions 95:5 (A:B) maintained to 10 minutes for equilibration. The flow was 300 µl/min. The thermostated column heater was set at 50°C and the autosampler was maintained at 4°C.

MS conditions

The MS conditions were as follows: ESI in positive mode, capillary temperature: 325 °C: 10V, tube lens voltages range: reported in Table 1; spray voltage: 3500 V; sheath and auxiliary gas (nitrogen) flow-rate: 45 and 25 (arbitrary units), respectively. The Q2 collision gas (argon) pressure was 1.5 mTorr. Data are acquired in selected reaction monitoring (SRM) mode.
The SRM transitions, the collision energy and ions ratio for each analyte are reported in Table 1.

Sample preparation

Calibrators and QCs preparation

For each drug, two primary stock solutions were prepared at 1 mg/ml by dissolving 10-mg base equivalent aliquots of each drug in 10 mL of methanol. Stock solutions were mixed together in order to get 2 methanolic working solutions containing all drugs at 100 µg/mL, 10 µg/mL and 1 µg/mL.
The first set was used for the preparation of the calibration standards ranging from 2 to 250 ng/mL for BORT, DASA and SUNI and from 50 to 3 500 ng/mL for the others drugs. The second set was used for the preparation of the 5 quality controls (QCs): 7, 75, 150, 750 and 1 500 ng/mL for each drug.

Only QCs at 7, 75 and 150 ng/mL were used for BORT, DASA and SUNI while QCs at 75, 150, 750 and 1 500 ng/mL were used for the other TKIs. A 0.5 mg/mL d8-imatinib, internal standard (IS) stock solution was prepared by dissolving 1 mg of the chemical in 2 ml of methanol. Plasma calibration samples and three plasma quality control (QC) samples were prepared by adding the appropriate volume of each working solution to blank plasma.

Analyte	Retention time	Precursor Ion	Product ion	TL/CE	Product ion	CE	Ion Ratio
Bortezomib	3.11	367.1	226.0	192/-18	208.0	-28	60
Dasatinib	3.01	488.2	401.0	184/-29	231.9	-38	40
Erlotinib	3.12	394 .2	277.9	136/-21	336.0	-22	40
Imatinib	2.96	494.3	394.1	170/-25	222.0	-27	20
D8-Imatinib	2.96	502.3	394.1	170/-25			
Lapatinib	3.28	581.1	349.9	185/-36	364.9	-38	75
Nilotinib	3.26	530.1	288.9	199/-29	261.0	-42	45
Sorafenib	3.59	465.1	251.9	176/-31	270.0	-21	75
Sunitinib	3.06	399.2	282.9	134/-28	326.0	-20	60
Vandetanib	2.99	475.1	83.1	142/-32	111.9	-64	15

Table 1: Retention time, precursor molecular ion/product ion for quantification, precursor molecular ion/product ion for confirmation and detection parameters (tube lens voltage (TL)/collision energy(CE)) for each analyte

Plasma sample extraction procedure

Aliquots of 50 μl of the plasma unknowns, blank, calibration standards and QCs were placed in appropriate labeled 1.5 mL microcentrifuge tubes and mixed with 200 μl of acetonitrile containing 20 ng/mL IS. After automatic vortexing for 10 minutes, each sample was centrifuged at 6 000g at 4°C for 15 minutes. Hundred microliters of supernatant were diluted two-fold using the mobile phases A and B in a 50/50 (v/v) ratio. After capping and vortexing, the vials were transferred into the autosampler tray that was maintained at +4°C. Twenty-five microliters aliquots of the extract were injected into the HPLC system.

Results

Chromatograms

The proposed method enables the simultaneous quantification of commonly used TKIs in 50μL-plasma aliquots by liquid chromatography coupled with tandem MS. Typical chromatographic profiles of the highest calibrator sample containing all are shown in Fig. 2.

Internal standard, calibration curve and lower limit of quantification

Imatinib-D8 was used as IS with a satisfactory chromatographic profile and a negligible memory effect. Calibration curves over the entire ranges of concentrations were best described by 1/x weighted linear regression of the peak-area ratio of each TKI to IS *versus* the concentrations of the respective TKI in each standard sample.

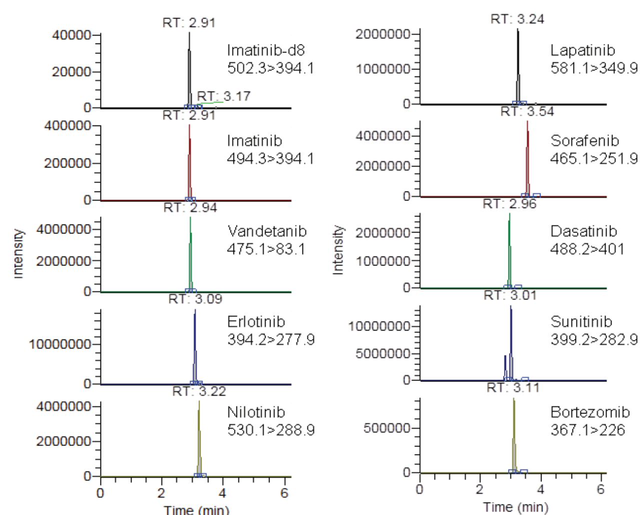

Fig. 1: Chromatogram of the highest calibrator sample containing each TKI.

This model was optimal for the 9 TKIs. Standard curves, prepared from different biological plasmas (EDTA), were performed in plasma on twenty consecutive days. The assay proved to be linear and acceptable, as the regression coefficients were >0.99 for each of the twenty standard curves excepted for sorafenib (mean r^2 0.9894) (Table 2).

A linearity test has been performed to compare theorical values, mean and standard deviations of the back-calculated values to each nominal concentration used in the low and the high standard curves. Then the accuracies were calculated for each analyte. In all cases, slopes of these linear curves were ranging between 0.9987 to 1.019 and statistics showed slopes significantly different from 1 ($p<0.0001$). The LLOQ was established at 2 ng/mL for BORT, DASA and SUNI and 50 ng/mL for the others drugs in human plasma.

Analyte		Slope	Intercept	R^2
	Mean	0.000179	0.0000483	0.9935
BORT	CV	14.8	142.9	0.48
	Mean	0.000989	-0.0004033	0.9967
DASA	CV	9.3	181.7	0.26
	Mean	0.00820	0.2222	0.9913
ERLO	CV	7.1	40.8	0.46
	Mean	0.0198	-0.009083	0.9980
IMAT	CV	5.4	137.8	0.10
	Mean	0.000286	-0.0004005	0.9964
LAPA	CV	11.5	164	0.22
	Mean	0.002519	-0.02377	0.9911
NILO	CV	3.88	91.8	1.44
	Mean	0.000657	-0.020596	0.9894
SORA	CV	10.8	24.0	0.69
	Mean	0.00514	0.00121	0.9919
SUNI	CV	6.9	183.9	0.46
	Mean	0.0000199	-0.002118	0.9943
VAND	CV	12.2	163.0	0.32

Table 2: Data detailing the slopes, intercepts, coefficient correlations (r^2) for 9TKIs (n=20).

Accuracy and precision

Precision and accuracy determined with 3 and 4 controls samples are given in Table 3. The levels of control samples were selected to reflect low, medium and high range of the two sets of calibration curves. They were chosen to encompass the clinically range of concentrations found in patients plasma. The mean intra-assay precision was similar over the entire concentration range and lower than 8.2 %. Overall, the mean inter-day precision was good with CVs within 5.3 and 13.8%. The intra-assay and **inter-assay** bias from the nominal concentrations of QCs for each considered TKI were contained between and 86.8 and 113.5 %. Ratios of ion transitions were reproducible for all TKIs and standard deviation for all of them below 25%.

Concentration	BORT		DASA		SUNI	
	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
2	98.4	19.8	106.9	16.2	119.8	20.0
5	93.2	19.5	101.5	8.2	99.7	6.3
10	93.9	8.9	98.3	11.9	97.6	7.0
20	108.2	13.1	97.8	6.0	93.9	11.0
50	104.0	10.1	97.2	7.7	90.7	5.1
100	98.2	5.8	98.4	5.6	91.3	4.0
250	99.5	3.9	102.2	3.0	105.8	2.1

Concentration	ERLO		IMAT		LAPA	
	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
50	91.8	13.8	93.4	12.8	105.5	7.7
100	94.3	10.5	98.1	9.9	96.8	6.6
200	113.9	7.1	107.6	7.8	109.2	6.1
500	109.0	5.7	98.3	5.9	90.5	7.8
1000	103.5	5.1	100.2	5.1	96.8	6.1
2000	101.4	4.4	99.2	3.8	99.6	4.8
3500	94.9	3.6	100.8	2.3	101.9	2.7

Concentration	NILO		SORA		VAND	
	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision
50	111.5	7.8	113.1	5.4	86.5	16.2
100	99.7	4.6	98.9	3.3	91.7	11.0
200	111.1	4.5	108.2	5.8	111.2	17.4
500	98.5	5.3	91.8	5.7	103.8	10.7
1000	93.0	7.0	91.2	8.4	108.5	5.0
2000	97.5	3.3	98.3	2.7	101.3	2.4
3500	103.2	3.0	105.3	3.3	96.0	3.3

Table 3: Assay performance data of the low calibration samples for BORT, DASA, SUNI and of the high calibration samples for ERLO, IMAT, LAPA, NILO, SORA, VAND in human plasma (n=20)

Selectivity and specificity

No peaks from endogeneous compounds were observed at the drugs retention time in any of the 10 blank plasma extracts evaluated. The endogeneous responses in blank plasma were always below 6.5 % of the signal at the LLOQ of 2 ng/mL for BORT, DASA, SUNI and at 50 ng/mL for the others. The endogeneous responses in plasma provided from polymedicated patients were always less than 7.1% of the signal at each LLOQ. There were no effects of others concomitant treatments (40 mg/l of amikacin, 20 mg/l of gentamycin, 25 mg/l of vancomycin, ceftazidime, imipenem and cisplatin, 0.5 mg/l of morphine, 3 mg/l of docetaxel, 5 mg/l of voriconazole, posaconazole, itraconazole and fluconazole).

Extraction recovery and matrix effect

The assessment of matrix effects and extraction recoveries is reported in Table 5. A value above or below 100% for the matrix effects indicates an ionization enhancement or suppression, respectively. Matrix effects and extraction yields were ranged from 84.6 to 109 % and 84.0 to 101.2% respectively. Overall recoveries were ranged from 77.8 to 93.3 % for lower concentrations, 78.6 to 98.4% for medium concentrations and from 79.8 to 105.6 % for higher concentrations. The extraction recovery of D8-imatinib was 93.7%. There was no effect of hyperbilirubinemia, hyperlipemia and haemolysis on matrix effect as evaluated in medium CQs.

Stability

The stability of TKIs in human plasma samples was studied with low and high QC samples left at room temperature up to 48h. The variations are contained within ± 15% of starting concentrations indicating that TKIs can be considered stable at RT excepted for lapatinib which decreases of -36% at RT after 24h and of -76% after 48h. It has been demonstrated that lapatinib was stable at RT for 6 hours. Sunitinib is sensitive to light and decreases by -15% after 48h even light protection. By contrast, all TKIs in plasma samples left during the same period of time at +4°C were found stable.

QC samples prepared in human plasma undergoing three freeze-thaw cycles showed no significant degradation (variation < 8.2 %) for all analytes.

Long-term stability studies indicated that all analytes were stable in human plasma when stored at -70°C for 150 days (ratios between 96.0 to 100.5%, degradation < 7.9%).

The stability of stock solutions held at -70°C and left in the dark for 10 months showed decrease less than 6% for each analyte.

In neutral extracts, all analytes were stable up to 7h when left in the autosampler without any degradation allowing more than 40 samples to be analyzed simultaneously within a single chromatographic batch.

External quality controls

The external quality controls (low and high concentrations) for imatinib (18 laboratories), nilotinib and dasatinib (9 laboratories) showed a good accuracy (97.2 to 101.4%) in comparison to data obtained from others laboratories.

Application to biological samples

We applied the assay to the analysis of samples obtained from patients receiving imatinib, nilotinib, dasatinib, sunitinib or sorafenib.

DASA, IMAT and NILO were frequently detected in patients with chronic myeloid leukemia (n=75). In 71 patients treated with 400 (84%) or 600 mg imatinib daily, detected though concentrations were around 871 ng/mL (median: 789 ng/mL). Among these 71 patients, 45 % of them presented a major molecular response associated with a trough concentration higher than 1,000 ng/mL such as recommended [50].

We applied the assay to samples provided from an obese patient treated with 50 mg sunitinib for a renal carcinoma. The profile of SUNI concentrations measured in this obese woman showed no difference with AUC (1592 ± 41 ngh/ml) observed in patients without obesity.

Conclusion

In overall, the method that has been developed is precise, accurate and sensitive. It concerns nine inhibitors of tyrosine kinase acquired in a single run Confirmation is performed using confirmation/quantification ion ratios criteria. The method is very simple and therefore used in a routine environment for clinical studies; it is also possible to add new TKIs that could potentially have an interest in clinical practices and performed a partial analytical validation. The dynamic range of the concentrations allow to carry out some pharmacokinetics studies.

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The Utilization of Novel Platform in a LC-MS/MS Workflow for the Analysis of Vitamin D, Testosterone, Immunosuppressants, Chemotherapeutics and Cortisol

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Thermo Fisher Scientific, Franklin, MA, USA

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Overview

Purpose: To demonstrate the validity of the Prelude Sample Preparation Liquid Chromatography (SPLC) system, a new LC/MS/MS platform that reduces solvent consumption, requires less maintenance, and is easier to use then traditional systems.

Methods: Prelude SPLC^{TM}, Turbulent Flow Chromatography, LC/MS/MS, Multiplexing

Results: Methods for 25-hydroxy-vitamin D2 and D3, testosterone, the immunosuppressant drugs Sirolimus, Tacrolimus, Everolimus, and Cyclosporine A, the chemotherapeutic drugs Busulfan, Docetaxel, Methotrexate and Imatinib, and cortisol were validated using a Prelude SPLC^{TM} LC/MS/MS platform.

Introduction

A new LC system was specifically designed to reduce instrument maintenance, down time, and operating costs for high-throughput, LC/MS/MS applications which require sample clean-up prior to HPLC analysis. The Prelude SPLC System utilizes syringe pumps designed to deliver the volume of mobile phase required for each sample analysis with a single push of the piston. This pump design greatly reduces the wear and tear on pump seals and check valves, because the pistons in dual piston reciprocating pumps can move several hundred if not thousands of times per sample run. The majority of maintenance required on traditional HPLC pumps results from the wear of the seals and check valves; therefore, syringe pumps are more robust than traditional HPLC pumps. The Prelude SPLC System's also have extremely low dead volumes making rapid changes in mobile phases possible. The time required for many of the steps in a method to occur are reduced resulting in shorter run times and lower solvent costs for equivalent methods.

In order to prove the utility of the Prelude SPLC platform, several LC/MS methods that are currently used by clinical researchers were validated. The successful validation of such a wide range of analytes using the new platform demonstrates that the Prelude SPLC offers a viable alternative to existing LC/MS systems. Reduced system void volumes resulted in methods that had run times 20-30% shorter then their equivalent methods run on a conventional HPLC and produce a corresponding reduction in mobile phase consumption.

Methods

All samples were vortexed, mixed with internal standard solution and centrifuged. Supernatant was removed and transferred into sampling containers for LC-MS/MS analysis. On-line sample clean-up using a 0.5x50 mm ThermoScientific HTLC-C18 XL TurboFlow column was followed by chromatographic separations of 25-OH-D_2, 25-OH-D_3, immunosuppressants, chemotherapeutics, cortisol and testosterone using a 50x2.1mm, 2.6 μm particle size ThermoScientific Accucore PFP analytical column. The detector was a TSQ Vantage triple quadrupole mass spectrometer with HESI-II ionization probe in positive mode. Mobile phases were (A) 10 mM ammonium formate in water, (B) 10 mM ammonium formate in Methanol, and (C) 45/45/10 acetonitrile/isopropanol/acetone. All run times were 4 minutes or less and when mutiplexed the effective analysis time was reduced to 2 minutes per sample. The Immunosuppresants were run in spiked human whole blood with cell lysis and protein precipitation occurring at the same time as the addition of the internal standard. Testosterone analysis was performed in spiked testosterone depleted human plasma. Chemotherapeutics were run in spiked human plasma. Cortisol was run using synthetic urine but was validated against human urine samples containing known levels of Cortisol

Results

Accuracy and precision experiments were performed for system verification from three separate preparations on calibrators and controls on three different days . The interday and intraday accuracy and precision results were obtained for 25-OH-D_2 and 25-OH-D_3 at a concentration range of range of 2-100 ng/mL. The range for testosterone was 0.02-10 ng/mL. Immunosuppressants and chemotherapeutics were analyzed in ranges from 1-2000 ng/mL. The method range for cortisol was 3.62 - 362 ng/mL (0.1-10 nM). The method precision had RSD values were less than 15.0% for all compounds tested. Additionally, accuracy was ±15% of the theoretical value for all the methods. The correlation coefficient values for all the compounds ranged from 0.991 to 0.999, showing linearity throughout all concentrations and analytes. All the analytes passed carryover, benchtop stability, autosampler stability, and

TABLE 1. Method Range, Linearity and Recovery

Compound Name	Method Range (ng/mL)	Linearity (r^2)	Recovery
Cyclosporin A	10 – 2000	0.992 – 0.998	87.3 – 93.9
Sirolimus	1 – 50	0.998 – 0.999	86.9 – 93.9
Everolimus	1 – 50	0.992 – 0.998	88.5 – 95.2
Tacrolimus	1 – 50	0.998 – 0.999	87.3 – 97.9
Testosterone	0.020 -10.0	0.994 – 0.999	99.9 – 103.5
Cortisol	3.62 - 362	0.997 – 0.999	88.3 – 114.1
Busulfan	20 - 2000	0.995 – 0.998	89.4 – 93.5
Docetaxel	10 - 1000	0.993 – 0.999	96.6 – 102.1
Imibitib	10 - 2000	0.991 – 0.998	92.0 – 110.2
Methotrexate	10 - 750	0.992 – 0.998	102 – 111.8
25-hydroxy Vit D2	2.0 - 100	0.992 – 0.998	92.2 – 94.5
25-hydroxy Vit D3	2.0 - 100	0.992 – 0.996	95.0 - 98.9

TABLE 2. Intraday Accuracy and Precision

Compound Name	Intraday Accuracy Range (% Difference from Theoretical)			Intraday Precession Range (%RSD)		
	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC
Cyclosporin A	2.38 – 12.4	3.61 – 10.9	2.11 – 9.72	1.7 – 4.2	1.1 – 2.9	1.4 – 2.7
Sirolimus	1.78 – 16.5	2.33 – 14.9	0.11 – 13.6	7.5 -10.6	1.8 – 2.8	4.7 – 7.6
Everolimus	1.98 – 18.9	2.66 – 13.4	0.81 – 10.2	5.4 – 8.3	1.7 – 3.5	1.6 – 4.1
Tacrolimus	1.09 – 13.3	0.87 – 5.32	0.34 – 8.38	4.8 – 6.0	1.3 – 2.6	1.4 – 2.3
Testosterone	0.18 – 11.4	0.15 – 5.24	1.63 – 4.84	3.4 – 3.6	1.5 – 2.6	0.8 – 1.2
Cortisol	1.6 - 9.3	0.76 – 12.0	0.03 – 15.1	4.0 – 6.3	2.3 – 3.9	2.6 – 5.1
Busulfan	0.56 - 16.5	0.17 – 8.17	0.22 – 5.83	1.1 – 10.9	1.8 – 3.3	1.6 – 4.2
Docetaxel	0.37 – 11.9	0.14 – 5.61	0.26 – 6.98	1.6 – 9.4	1.1 – 3.7	0.9 – 3.4
Imatinib	1.0 - 9.5	0.3 – 9.8	0.0 – 11.7	1.0 – 1.9	1.1 – 7.4	1.3 – 6.2
Methotrexate	0.13 – 18.5	0.12 – 9.74	0.10 – 10.5	3.3 – 7.5	0.6 – 5.9	2.8 – 7.8
25-hydroxy Vit D2	0.5 – 14.8	0.09 – 12.5	0.3 – 11.2	5.0 – 11.5	2.9 – 6.6	1.9 – 5.1
25-hydroxy Vit D3	1.0 – 17.8	0.3 – 12.9	0.9 – 13.3	6.3 – 6.8	2.3 – 3.9	2.0 – 3.2

TABLE 3. Interday Accuracy and Precision

Compound Name	Interday Accuracy (% Difference from Theoretical)			Interday Precession (%RSD)		
	Low QC	Mid QC	High QC	Low QC	Mid QC	High QC
Cyclosporin A	2.00	0.75	3.06	12.2	9.7	12.2
Sirolimus	2.00	4.00	3.75	7.8	8.1	1.8
Everolimus	2.35	3.11	2.98	9.7	5.4	4.6
Tacrolimus	1.67	0.50	3.75	5.1	3.2	2.9
Testosterone	5.00	0.32	3.12	3.5	1.3	0.15
Cortisol	1.10	1.72	3.50	3.3	3.8	2.7
Busulfan	4.76	0.35	3.85	5.6	5.4	3.9
Docetaxel	2.66	1.51	1.28	4.2	4.4	3.1
Imatinib	11.0	1.33	3.74	4.0	2.0	5.9
Methotrexate	2.33	2.80	0.48	5.5	2.8	7.5
25-hydroxy Vit D2	4.83	2.52	2.87	3.9	4.0	4.8
25-hydroxy Vit D3	5.33	2.53	0.00	3.4	3.1	3.9

FIGURE 1. Standard Curves for Each Compound Tested Using a Prelude SLPC™ LC/MS/MS System

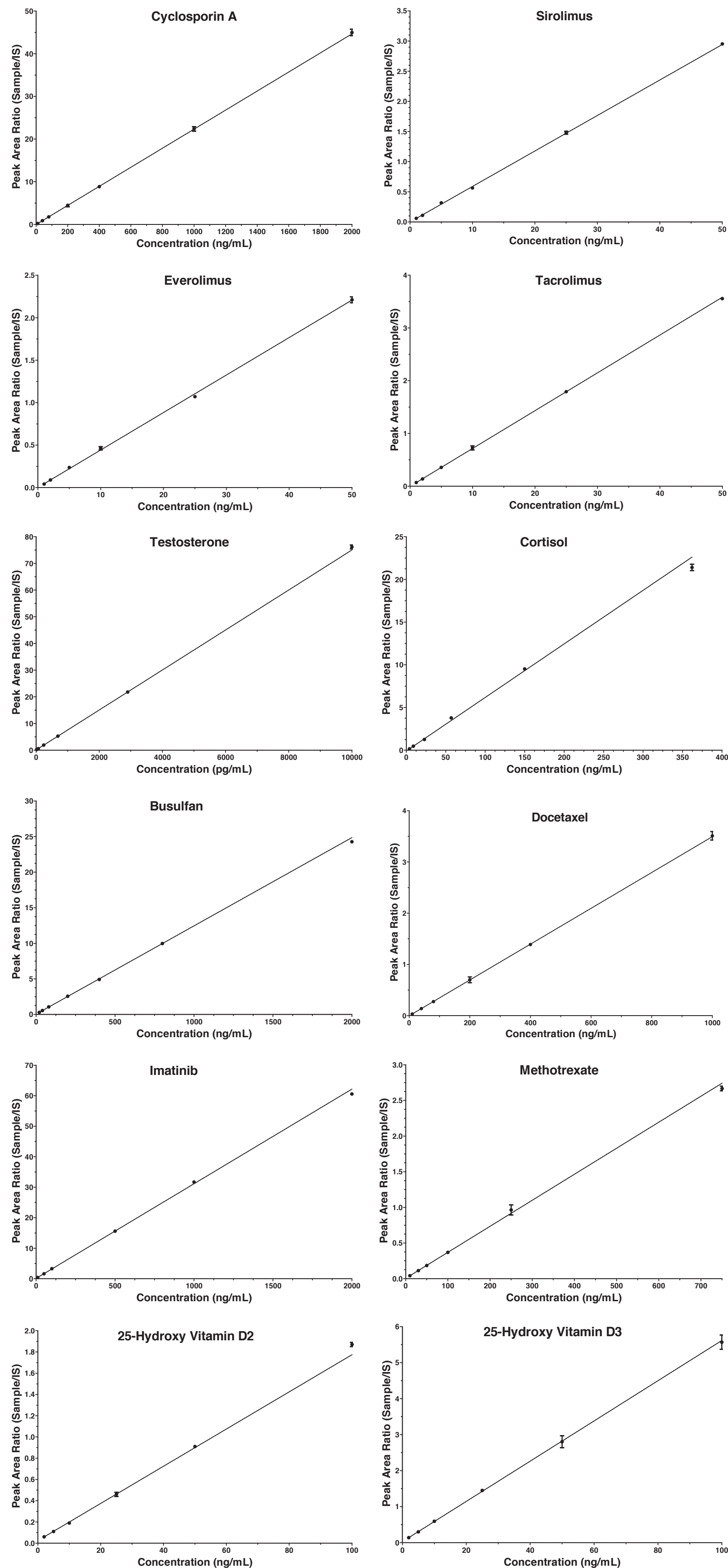


FIGURE 2. Representative Chromatograms at the LOQ for Each Compound Tested Using a Prelude SLPC™ LC/MS/MS System

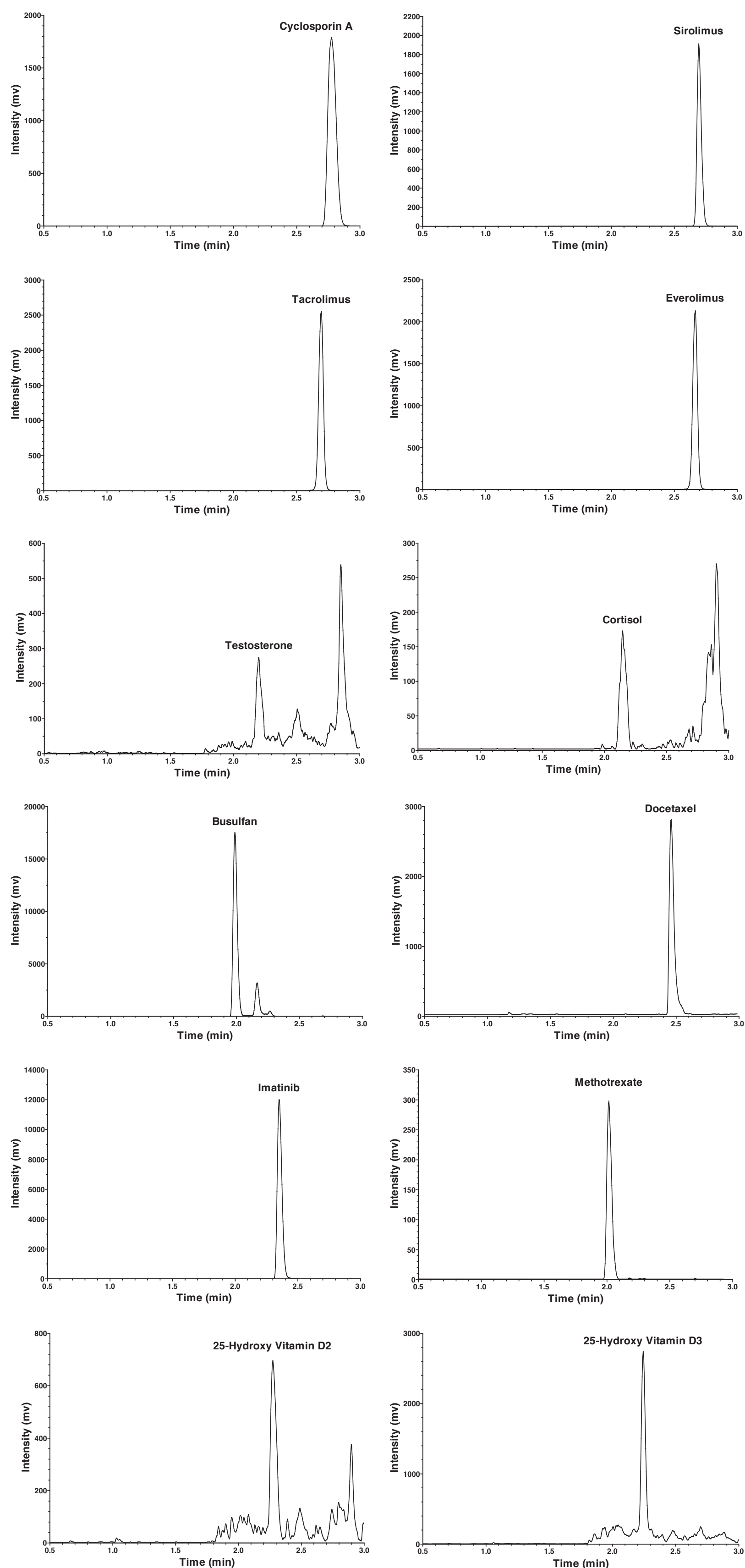
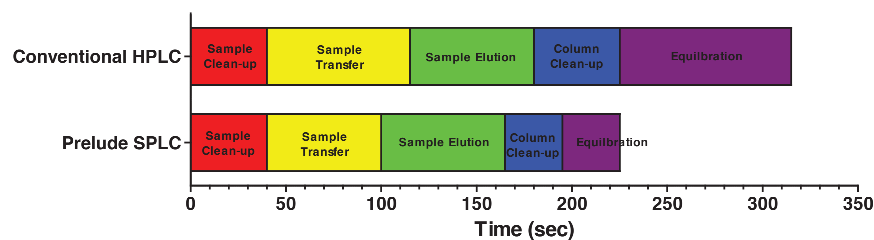


FIGURE 3. Comparison of the Method Run Time for Vitamin D on a Prelude SLPC LC/MS/MS System to that of a Conventional HPLC System



specificity criterion. Recoveries, including matrix effects, were all around 90% or higher. All the data is summarized in Tables 1 to 3. Figure 1 depicts representative standard curves for each compound tested. Representative chromatograms at the lower limit of quantitation (LLOQ) for each compound are shown in Figure 2.

The improvement in run times resulting from the lower void volumes of the Prelude SPLC System verses a conventional HPLC is illustrated in Figure 3 for vitamin D. The same mobile phases and columns were used for the comparison. When using on-line clean-up the duration of certain steps cannot be changed because they are dependent on the chromatographic separation needed. The duration of others steps in the process are related to how long it takes for solvent changes to reach the column. The sample clean-up and sample elution steps are dependent on the chromatography and; therefore, the time for those steps remain the same. However, the transfer, column cleaning and re-equilibration steps can be reduced. On a conventional HPLC the transfer step was 75 sec vs. 60 seconds on the Prelude SPLC. The column clean-up and equilibration steps were reduced from 150 to 60 seconds. The result is a reduction in run time of 29% (5:15 minutes to 3:45 minutes). A shorter run time also reduced solvent consumption by 33%.

Conclusion

- A large number of compounds, with logP values ranging from -1 to 5, have been validated on a new LC/MS/MS platform demonstrating the viability of the Prelude SPLC System for compounds of interest to clinical researchers..
- The Prelude SPLC System's lower void volume results in sample run times that are 20-30% shorter. The reduced run time results in reduced cost due to lower consumption of mobile phases and less waste disposal.
- The Prelude SPLC uses a single syringe fill per sample, which removes the need for pulse dampeners, reduces the mechanical wear and tear on pump parts such as pump seal and active check valves, and does not need proportioning valves. The result is far less required maintenance, reducing operating cost and down time.

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AN601: Quantitative Forensic Analysis of Opiates, Opioids, and Their Metabolites in Human Urine Without Hydrolysis

AN596: Quantitation of Bath Salts/Cathinones in Urine by LC-MS/MS

AN593: Evaluation of an LC-MS/MS Research Method for the Analysis of 33 Benzodiazepines and their Metabolites

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AN588: High Throughput Quantitative LC-MS/MS Analysis of 6 Opiates and 14 Benzodiazepines in Urine

AN576: Simultaneous Quantitation of 43 Drugs in Human Urine with a “Dilute-and-Shoot” LC-MS/MS Method

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AN546: Quantitation of Six Opiates in Urine Using a Triple Stage Quadrupole LC-MS System

AN547: Quantitation of 14 Benzodiazepines and Benzodiazepine Metabolites in Urine Using a Triple Stage Quadrupole LC-MS System

AN536: Targeted Screening of Drugs of Abuse and Toxic Compounds with LC-MS/MS Using Triple Stage Quadrupole Technology

Forensic Toxicology Application Notes (cont.)

AN486b: Simultaneous Analysis of Opiates and Benzodiazepines in Urine in Under 3 Minutes per Sample Using LC-MS/MS

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AN390: A Quantitative Test for Multiple Classes of Illicit Drugs and Their Primary Metabolites in Human Biological Fluids by LC-MS/MS for Forensic Use

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AN461b: Forensic Analysis of Opiates in Whole Blood by LC-MS/MS Using Automated, Online Sample Preparation

AN449: A Complete Toxicology Screening Procedure for Drugs and Toxic Compounds in Urine and Plasma Using LC-MS/MS

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Kristine Van Natta, Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Key Words

Q Exactive Focus, opiates, morphine, codeine, hydromorphone, hydrocodone, oxymorphone, oxycodone, TraceFinder, forensic toxicology, drugs of abuse, PRM, parallel reaction monitoring

Goal

To evaluate the performance of the Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap™ mass spectrometer as a quantitative platform for HPLC-MS analysis of opiates in human urine to low ng/mL levels for forensic toxicology.

Introduction

Forensic toxicologists need an economical instrument capable of both screening a large number of compounds and quantifying smaller panels to industry-established limits. Here we present a method for quantitation of six opiates—morphine, codeine, hydromorphone, hydrocodone, oxymorphone, and oxycodone—in human urine down to low ng/mL levels. This work was performed on a Q Exactive Focus hybrid quadrupole-Orbitrap mass spectrometer.

Methods

Sample Preparation

Samples were processed by enzymatic hydrolysis followed by urine dilution. Briefly, an aliquot of urine was spiked with stable-isotope-labeled internal standards and incubated with ß-glucuronidase enzyme. The resulting mixture was centrifuged and further diluted before an aliquot was analyzed by gradient HPLC and a Q Exactive Focus MS. Calibrators and controls were prepared by spiking compounds into blank synthetic urine in the range of 1 to 5000 ng/mL.

Liquid Chromatography

Gradient elution was performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system with OAS autosampler (Figure 1). Mobile phases consisted of 10 mM ammonium acetate with 0.1% formic acid in water and methanol (Fisher Chemical brand) for solvents A and B, respectively. The column used was a Thermo Scientific™ Accucore™ PFP, 2.6 µm particle size, 50 x 2.1 mm fused core (p/n 17426-052130). The

Figure 1. Q Exactive Focus MS with UltiMate 3000 RSLC HPLC pump and UltiMate 3000 OAS autosampler.

gradient was run from 0 to 70% mobile phase B over 3.3 minutes followed by a column wash at 100% B and re-equilibration to starting conditions. The total run time was 5.3 minutes.

Mass Spectrometry

Compounds were detected on a Q Exactive Focus MS equipped with a Thermo Scientific™ Ion Max™ source and a heated electrospray ionization (HESI-II) sprayer. Data was acquired in parallel reaction monitoring (PRM) mode. In this mode, a single precursor ion is selected in the quadrupole with an isolation width of 3.0 *m/z* and fragmented in the HCD cell. The resulting MS/MS product ions are detected in the Orbitrap detector at a resolution of 35,000.

Method Evaluation

The method precision and accuracy were evaluated by running a calibration curve and quintuplicate replicates of quality controls on three different days. Additionally, internal-standard response was assessed in 58 donor samples obtained from a collaborator laboratory and compared to a sample prepared in water to determine matrix effects.

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Data Analysis

Data was acquired and processed using Thermo Scientific™ TraceFinder™ software. Two product ions were selected as the quantifying and confirming ions for each compound. The resulting chromatograms were extracted and reconstructed with a mass accuracy of 5 ppm for quantification and ion ratio confirmation. Because the entire MS/MS spectrum was collected, multiple confirming ions could be chosen. Figure 2 shows a representative MS/MS spectrum for oxymorphone, highlighting the quantifying and confirming ions with corresponding reconstructed chromatograms.

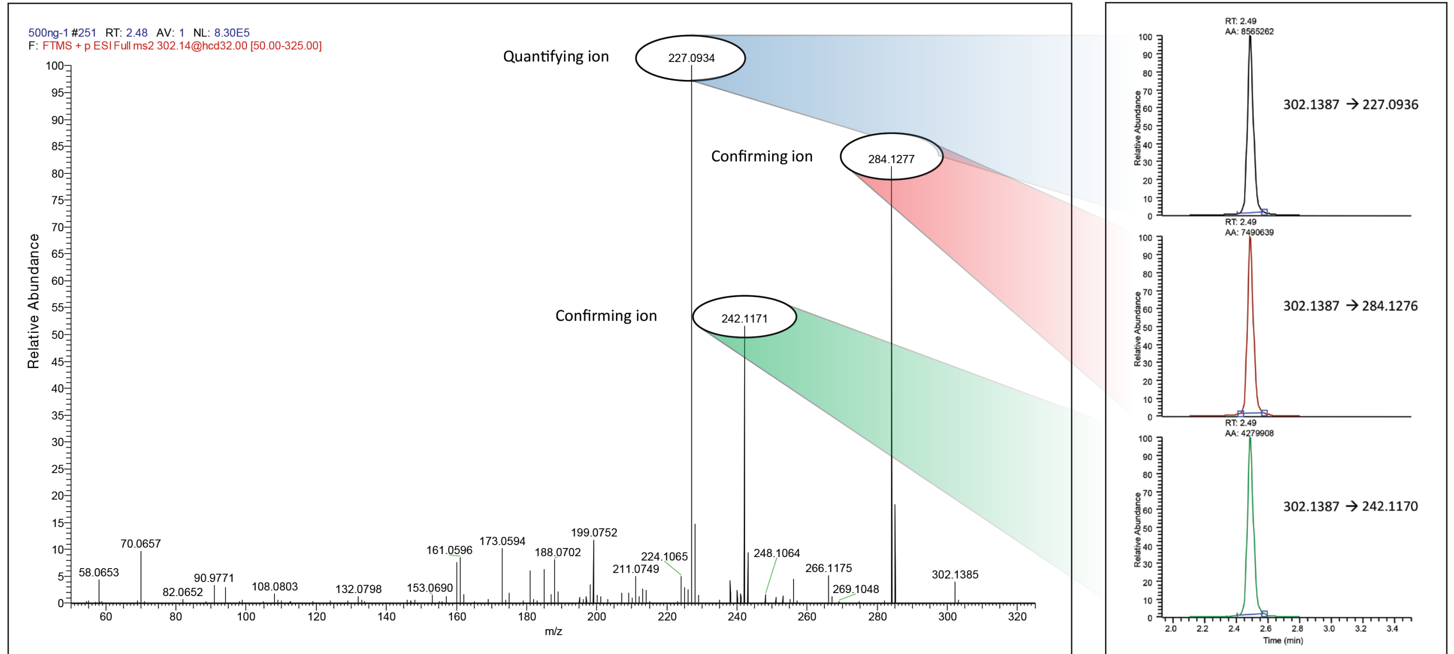


Figure 2. Representative fragmentation spectrum for oxymorphone obtained from a 500 ng/mL calibrator, highlighting the quantifying and confirming ions and showing corresponding chromatograms reconstructed with 5 ppm mass accuracy.

Results

Limits of quantitation (LOQs) were defined as the lowest concentrations that had back-calculated values within 20%, ion ratios within defined tolerance (tolerance dependent upon actual ratio), and quality controls within 20% RSD as well as meeting the above two requirements. Using these criteria, limits of quantitation for codeine, oxycodone, and oxymorphone were determined to be 2.5 ng/mL. For morphine, hydrocodone, and hydromorphone, the limit was 5 ng/mL. Tables 1 and 2 show the inter- and intra-assay statistics, respectively, for quality controls for all compounds in this method. Limited matrix effects were observed. The average recovery across 58 donor urine samples obtained from a collaborator laboratory ranged from 69% to 81% for the six internal standards evaluated. Figure 3 shows a combined chromatogram for analytes at their respective LOQs, and Figure 4 shows chromatograms for each compound with confirming ion ratio at its LOQ. Figure 5 shows representative calibration curves for all compounds. Figure 6 shows representative chromatograms with ion ratio confirmation for donor samples.

Table 1. Inter-assay precision and bias.

	Codeine	Hydrocodone	Hydromorphone	Morphine	Oxycodone	Oxymorphone
5 ng/mL						
% RSD	3.82	3.67	3.89	7.54	2.78	3.43
% Bias	-4.11	7.20	5.28	0.18	-6.19	-2.04
10 ng/mL						
% RSD	4.13	6.06	2.79	4.35	3.35	2.78
% Bias	-7.00	-3.47	-0.20	-6.15	-5.24	-3.60
100 ng/mL						
% RSD	3.00	6.13	2.03	2.28	2.58	1.62
% Bias	7.43	5.51	-0.16	0.98	5.34	0.26
1000 ng/mL						
% RSD	3.63	4.21	1.35	2.52	1.99	2.29
% Bias	6.07	3.64	3.53	4.67	5.44	2.86

Table 2. Intra-assay precision.

Maximum %RSD from Three Runs	5 ng/mL	10 ng/mL	100 ng/mL	1000 ng/mL
Codeine	5.09	4.56	3.61	3.34
Hydrocodone	3.55	5.60	6.38	4.25
Hydromorphone	3.16	1.87	2.41	1.90
Morphine	8.25	4.52	2.77	3.64
Oxycodone	3.27	3.27	2.85	2.31
Oxymorphone	4.28	3.04	1.78	2.73

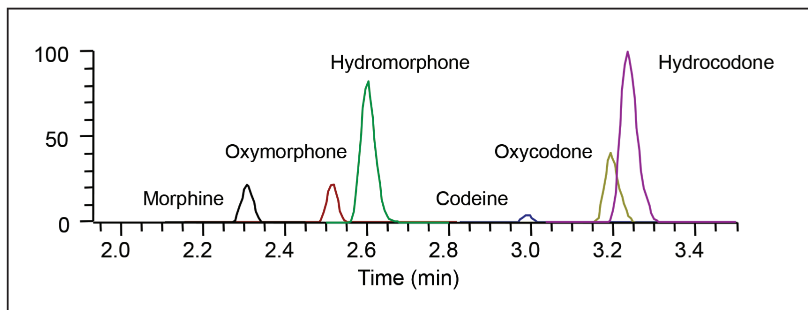
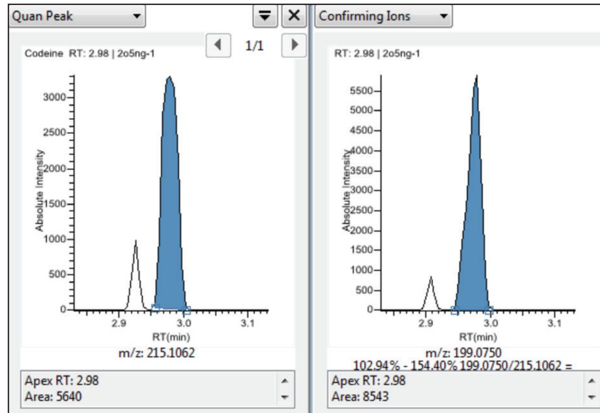


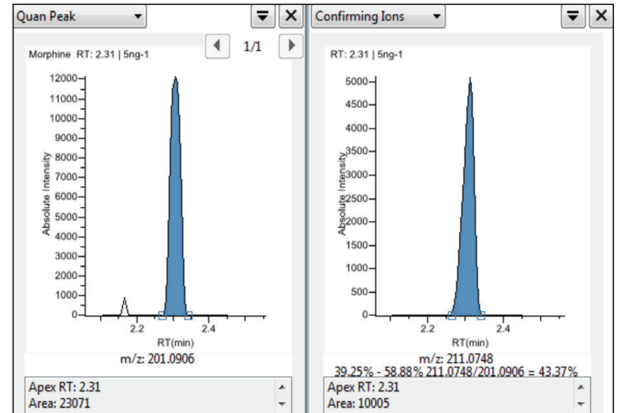
Figure 3. Chromatograms extracted from MS^2 spectra obtained from a confirmation PRM experiment for six opiates at their respective LOQs (2.5 ng/mL for codeine, oxycodone, and oxymorphone , and 5 ng/mL for hydrocodone, hydromorphone, and morphine) in hydrolyzed and diluted urine.

Figure 4. Chromatograms showing quantifying and confirming ions with ion ratio at LOQ for each compound in this method.

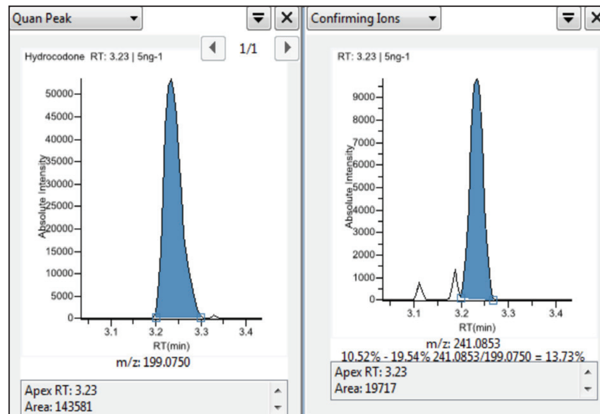
Codeine LOQ = 2.5 ng/mL



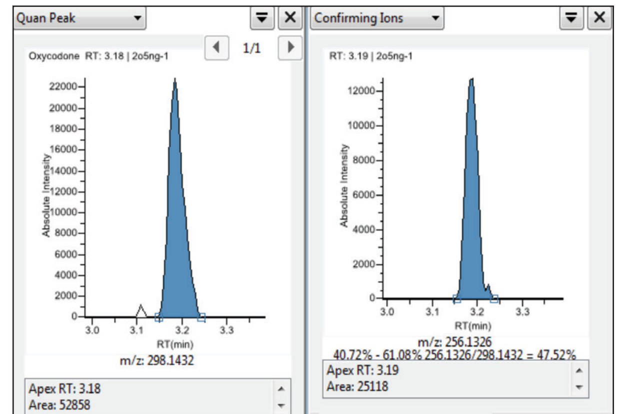
Morphine LOQ = 5 ng/mL



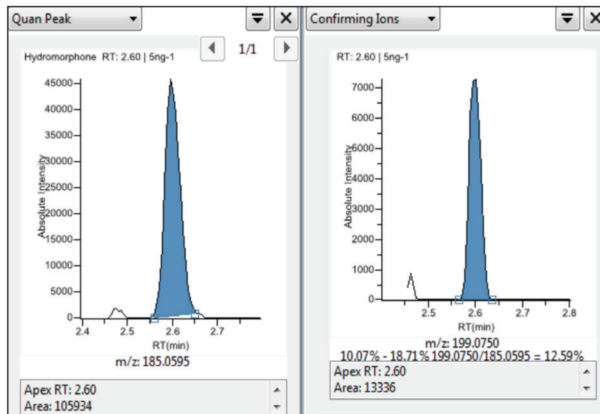
Hydrocodone LOQ = 5 ng/mL



Oxycodone LOQ = 2.5 ng/mL



Hydromorphone LOQ = 5 ng/mL



Oxymorphone LOQ = 2.5 ng/mL

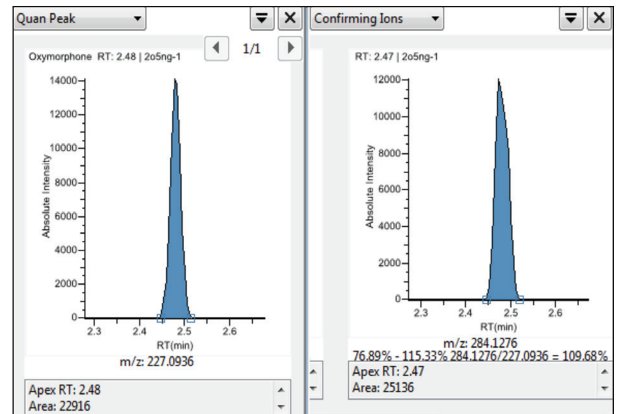
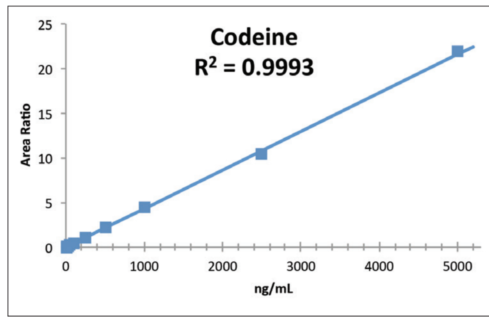
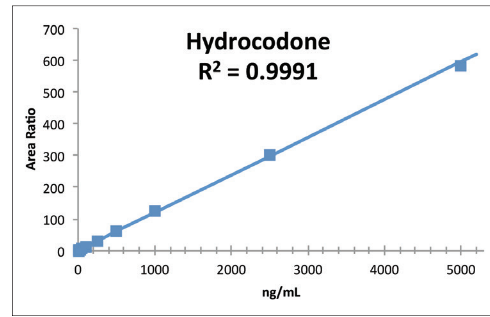


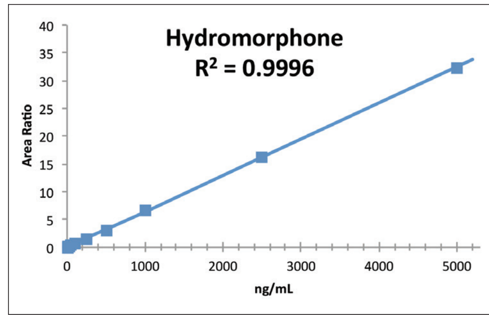
Figure 5. Representative calibration curves from PRM data for six opiates.



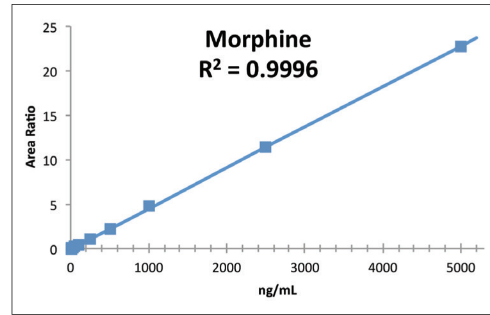
ng/mL	%Diff
5000	1.20
2500	-3.69
1000	3.55
500	-1.29
250	-1.53
100	7.11
50	2.83
25	-5.20
10	-5.64
5	6.42
2.5	-3.76



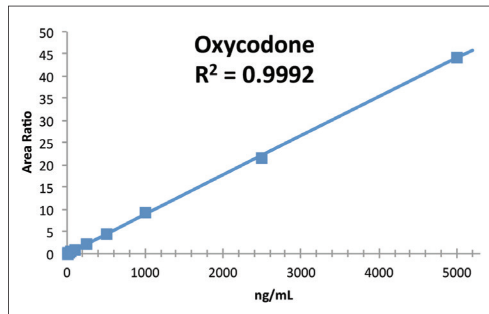
ng/mL	%Diff
5000	-2.24
2500	1.63
1000	5.46
500	1.86
250	-1.18
100	9.49
50	8.09
25	-10.1
10	-6.88
5	-6.10



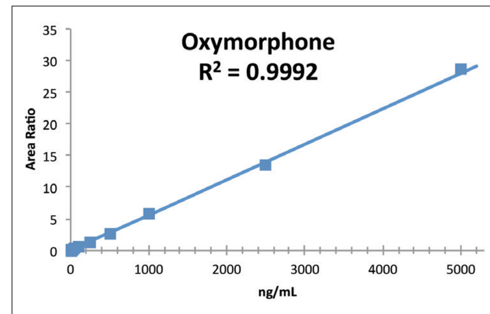
ng/mL	%Diff
5000	-0.390
2500	0.580
1000	3.72
500	-2.77
250	-7.48
100	0.510
50	0.750
25	-4.42
10	0.280
5	9.22



ng/mL	%Diff
5000	-0.140
2500	0.260
1000	4.09
500	-5.75
250	-4.14
100	-1.16
50	3.62
25	-7.44
10	-6.89
5	5.70



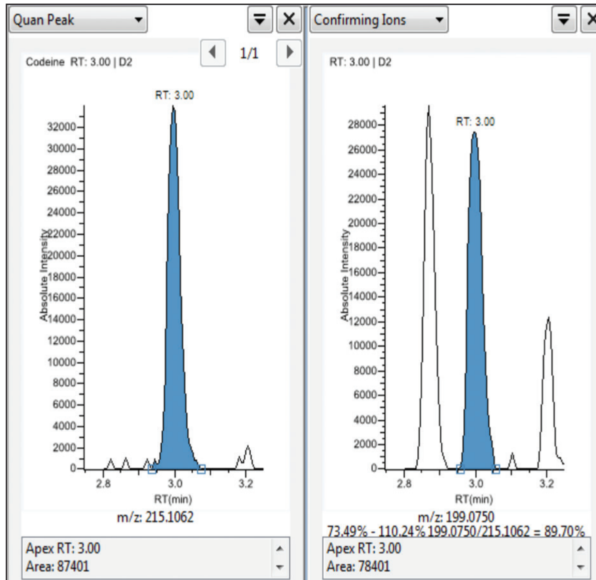
ng/mL	%Diff
5000	-0.0200
2500	-2.61
1000	6.71
500	-0.130
250	-2.87
100	4.51
50	6.80
25	-0.630
10	-4.42
5	-7.32
2.5	-4.00



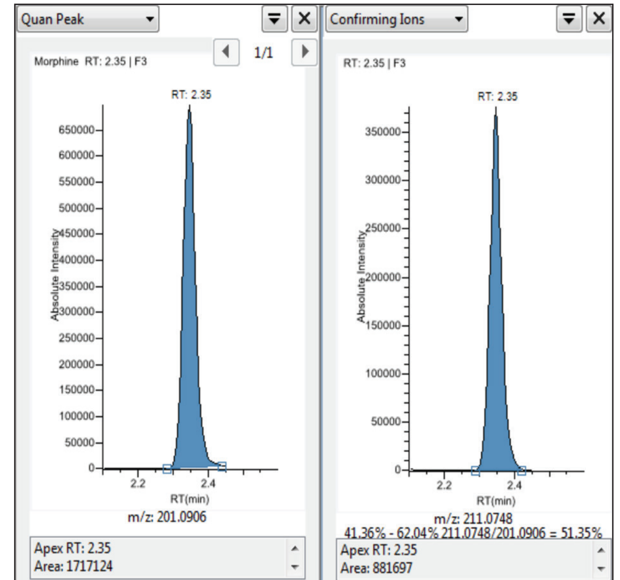
ng/mL	%Diff
5000	1.93
2500	-3.57
1000	2.06
500	-2.66
250	-7.29
100	2.72
50	3.44
25	-4.19
10	-1.57
5	1.08
2.5	8.04

Figure 6. Extracted ion chromatogram from donor sample obtained in confirmation PRM experiment.

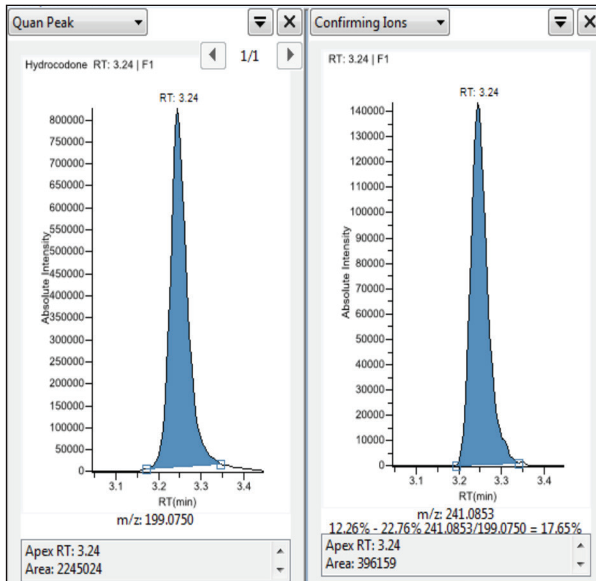
Codeine in Donor D2, 12.1 ng/mL



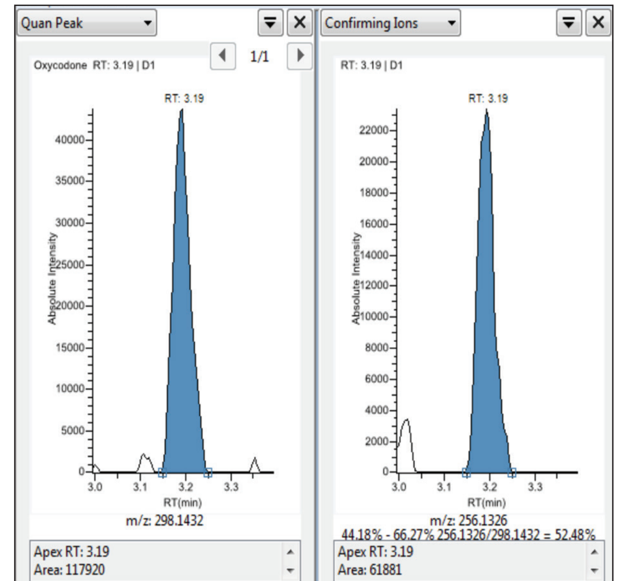
Morphine in donor F3, 217 ng/mL



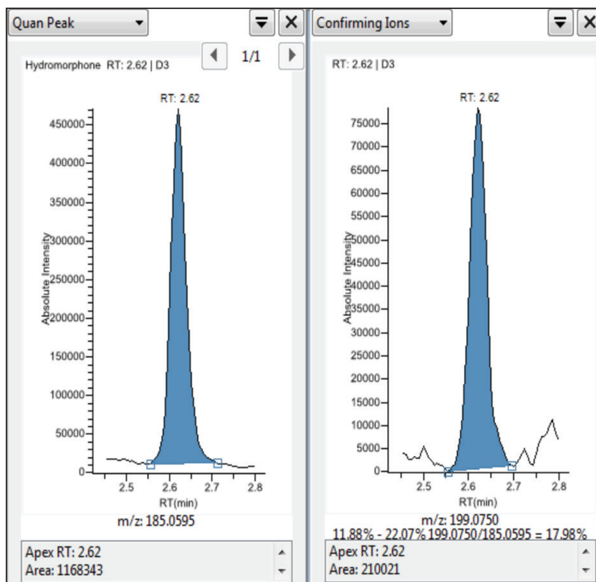
Hydrocodone in Donor F1, 60.6 ng/mL



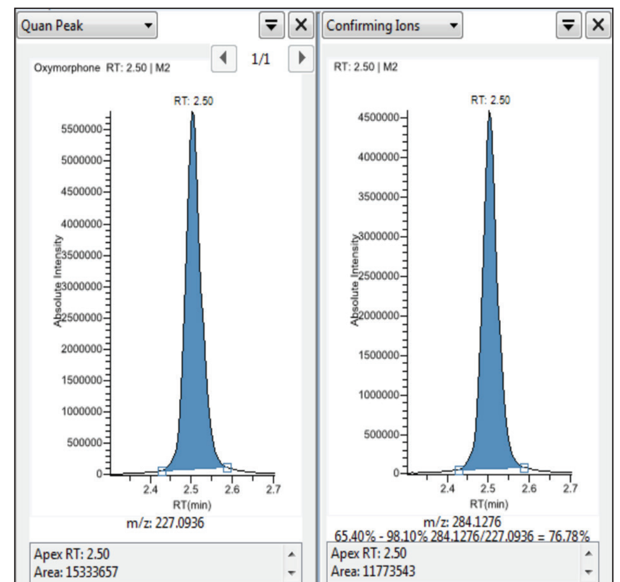
Oxycodone in Donor D1, 4.38 ng/mL



Hydromorphone in Donor D3, 37.1 ng/mL



Oxymorphone in Donor M2, 698 ng/mL



Conclusion

The Q Exactive Focus MS accurately quantitated all six opiates tested to the low ng/mL level in human urine. This new instrument gives forensic laboratories a single versatile platform capable of both screening large panels[1] and quantitative confirmation of specific panels that provides performance with value.

References

1. Kozak, M.; Van Natta, K., Thermo Fisher Scientific Application Note 616: Forensic Screening for Drugs in Urine Using High-Resolution MS/MS Spectra and Simplified High-Performance Screening Software, 2014.

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LC-MS/MS Analysis of EtG and EtS in Dilute Urine on the TSQ Endura Triple Quadrupole MS

Kristine Van Natta, Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Key Words

EtG, EtS, ethyl glucuronide, ethyl sulfate, ion pairing, TSQ Endura, DHAA (dihexylammonium acetate)

Goal

To develop a high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for the forensic toxicological analysis of EtG and EtS in urine with limits of quantitation (LOQs) of 100 and 50 ng/mL, respectively, using only urine dilution as sample preparation.

Introduction

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are long-term biomarkers for ethanol consumption. Although they are minor metabolites of ethanol, their longer half-lives make them useful for detection of past alcohol use in forensic settings. These compounds are highly polar, which makes them retain poorly on most reversed-phase HPLC columns and elute on or near the chromatographic solvent front. This results in poor peak shape and large matrix effects. Here an ion-pairing reagent was used to retain these compounds on an HPLC column long enough to move them off the solvent front. This enabled better peak shape and less matrix interference.

Methods

Sample Preparation

Equal volumes (25 µL) of urine and internal standard (5,000 and 500 ng/mL of EtG-d_5 and EtS-d_5, respectively) were mixed and then diluted with 450 µL of water. For analysis, 30 µL were injected into the HPLC-MS/MS.

Liquid Chromatography

Chromatographic separations were performed under gradient conditions using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system and Thermo Scientific Dionex UltiMate 3000 OAS. The analytical column was a Thermo Scientific™ Hypersil GOLD™ column (50 x 3 mm, 5 µm particle size, catalog #25005-053030). The column was maintained at room temperature. The injection volume was 30 µL. Mobile phases A and B consisted of 5 mM dihexylammoniumacetate (TCI America™) ion-pairing reagent in Fisher Chemical™ water and acetonitrile, respectively. The flow rate was 1 mL/minute, and the total run time was 5 minutes.

Figure 1. UltiMate 3000 RSLC system and TSQ Endura mass spectrometer

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific™ TSQ Endura™ triple-stage quadrupole mass spectrometer equipped with a Thermo Scientific™ Ion Max NG source and heated electrospray ionization (HESI-III) probe (Figure 1). Table 1 shows the mass spectrometer source parameters.

Table 1. TSQ Endura MS source parameters

Parameter	Value
Spray Voltage	3500 V
Sheath Gas	60 Arb
Aux Gas	20 Arb
Sweep Gas	0 Arb
Ion Transfer Tube	380 °C
Vaporizer	475 °C
Divert Valve	1.2–2.5 min

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Two selected-reaction monitoring (SRM) transitions were monitored for EtG, EtS and their deuterated internal standards to provide ion ratio confirmations (IRC). The scans were run in timed selected-reaction monitoring (t-SRM) mode with a cycle time of 0.25 seconds. In this mode, SRM transitions are given a retention time and window in which the mass spectrometer acquires the specified transitions only. This allows the instrument to maximize the amount of time spent acquiring each transition, while maintaining a consistent number of data points across the chromatographic peak.

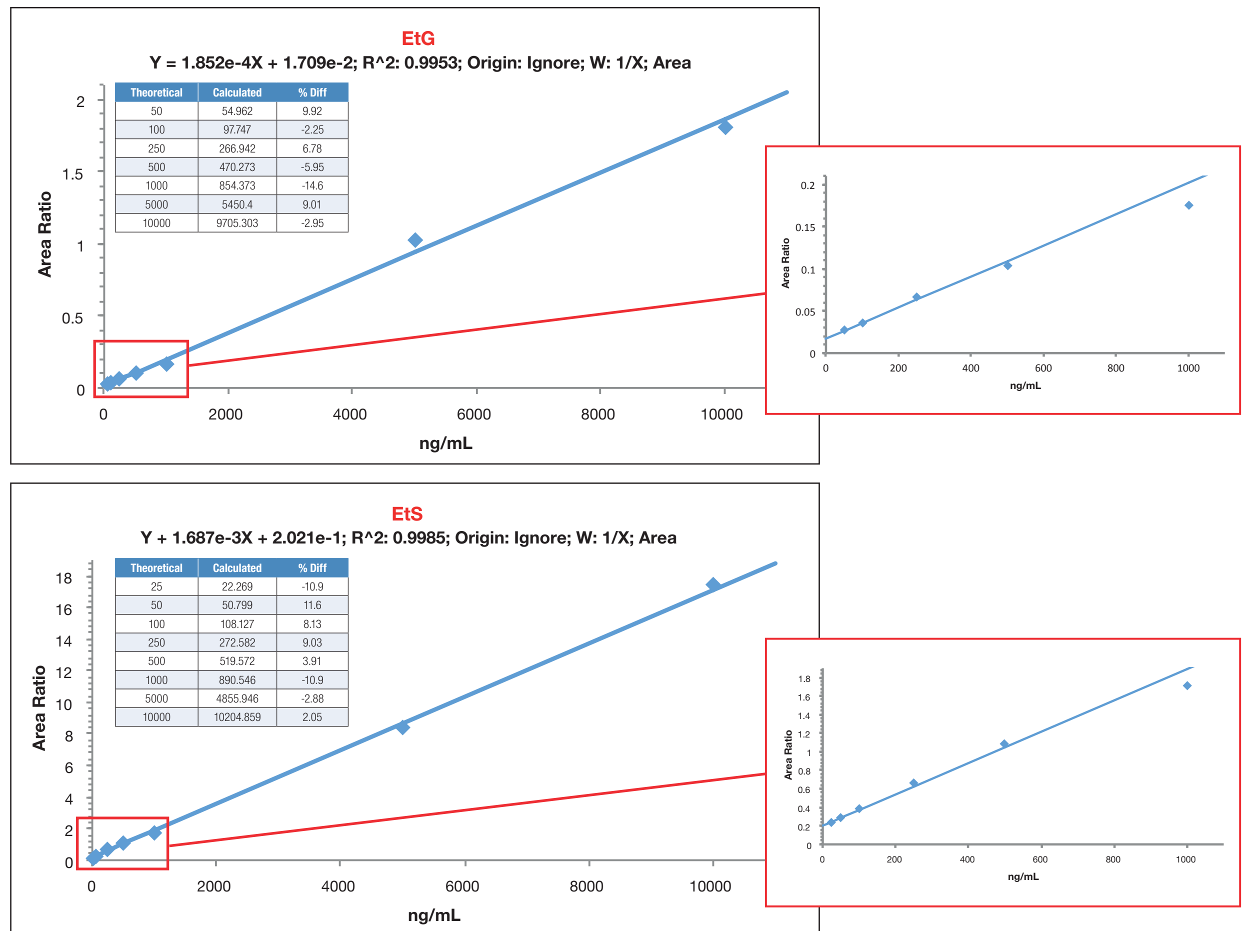
Data was acquired and processed with Thermo Scientific™ TraceFinder™ software.

Validation

Intra-assay precision and accuracy were determined by analyzing a calibration curve along with six replicates of quality control (QC) samples. Inter-assay precision and accuracy were determined by analyzing a calibration curve along with six replicates of QC samples on three different days. Matrix effects were evaluated by observing the internal standard signals in 23 different lots of human urine.

Results

Both compounds were linear over a wide dynamic range. EtG was linear from 50 to 50,000 ng/mL, while EtS had a range of 25 to 50,000 ng/mL. Figure 2 shows representative calibration curves for both compounds. Figure 3 shows representative chromatograms for EtG and EtS at their respective LOQs.



Theoretical	Calculated	% Diff
50	54.962	9.92
100	97.747	-2.25
250	266.942	6.78
500	470.273	-5.95
1000	854.373	-14.6
5000	5450.4	9.01
10000	9705.303	-2.95

Theoretical	Calculated	% Diff
25	22.269	-10.9
50	50.799	11.6
100	108.127	8.13
250	272.582	9.03
500	519.572	3.91
1000	890.546	-10.9
5000	4855.946	-2.88
10000	10204.859	2.05

Figure 2. Representative calibration curves for EtG (top) and EtS (bottom) in urine

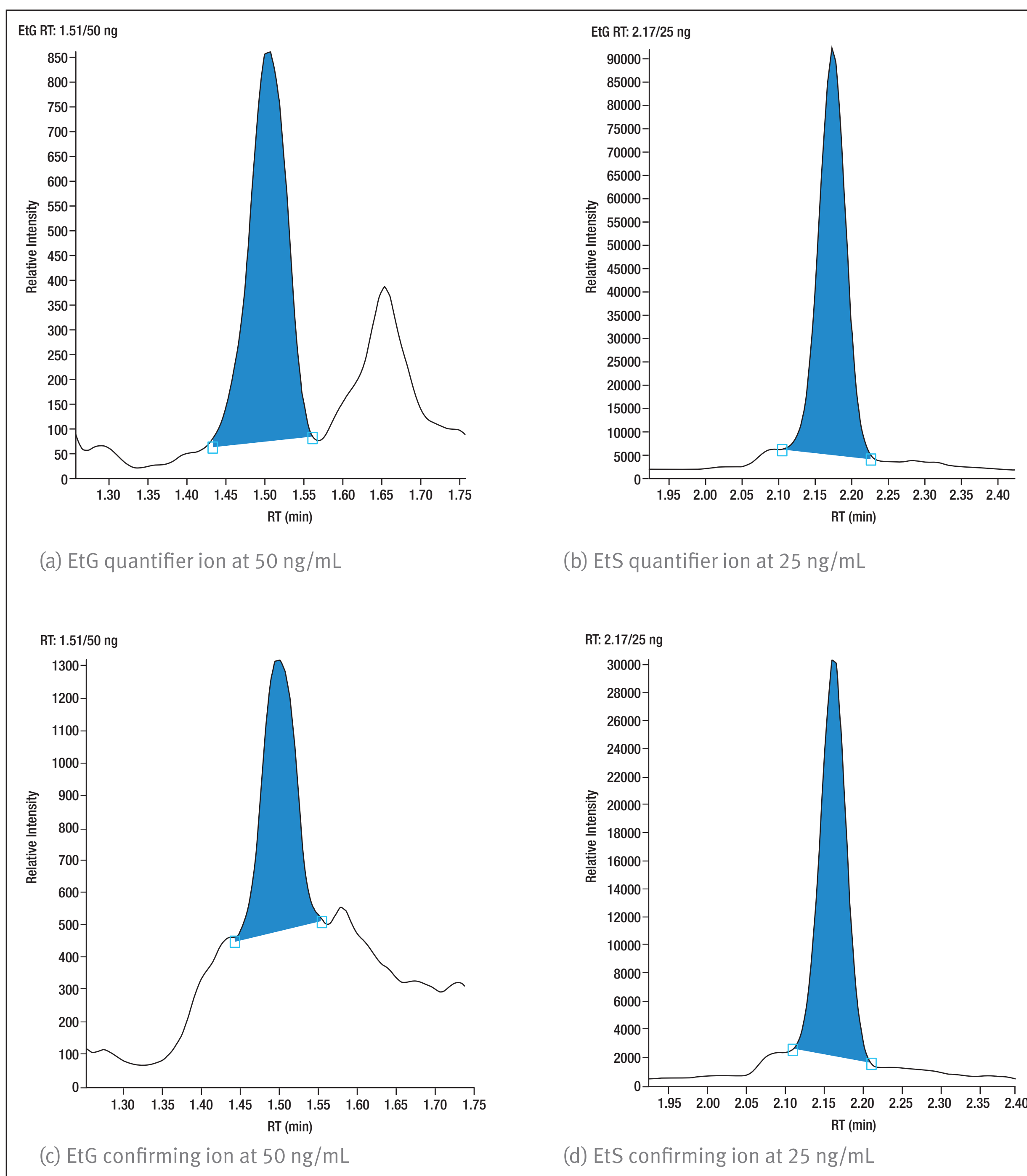


Figure 3. Representative chromatograms showing quantifier (a and b) and confirming (c and d) ions for EtG and EtS at 50 and 25 ng/mL, respectively

Table 2 shows the inter-assay precision and accuracy for EtG and EtS at 50 and 100 ng/mL.

Table 2. Inter-assay precision and accuracy for quality controls of EtG and EtS

	50 ng/mL		100 ng/mL	
	%Bias	%RSD	%Bias	%RSD
EtG	-0.253	12.0	3.31	6.94
EtS	-1.04	8.80	-1.99	5.67

Figure 4 displays the internal standard recovery compared to that of the calibrators.

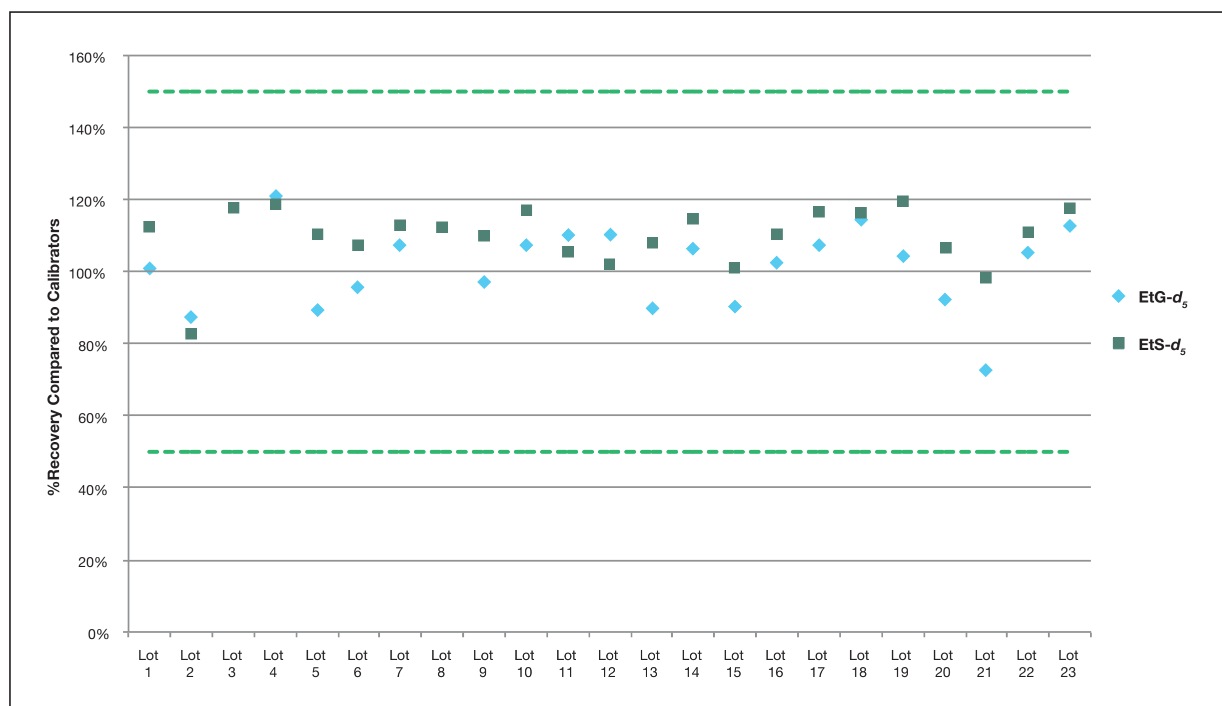


Figure 4. Internal standard recovery in 23 lots of urine compared to calibrators

Conclusion

- This method gives limits of detection in urine of 50 ng/mL for EtG and 25 ng/mL for EtS while still maintaining a wide dynamic range up to 50,000 ng/mL.
- An ion-pairing reagent helps chromatographically separate the compounds from interferences on the solvent front, thereby improving limits of detection.
- The TSQ Endura MS is a robust system that provides accurate results within 5% and good precision all the way down to the LOQ.
- This method is suitable for forensic toxicology use.

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Quantitative Forensic Analysis of Opiates, Opioids, and Their Metabolites in Human Urine Without Hydrolysis

Sarah Fair Wandland, Kerry Hassell, Joseph Herman, Thermo Fisher Scientific, Franklin, MA

Key Words
Opiates, opioids, metabolites, sample preparation liquid chromatography (SPLC), mass spectrometry (MS), forensic toxicology

Goal

To develop a quantitative forensic method for analysis of opiates, opioids, and their metabolites in human urine without the time-consuming step of hydrolysis.

Introduction

Analysis of opiate and opioid metabolites in urine is most often done with a hydrolysis step that make total sample preparation time up to 24 hours. The method described here eliminates the hydrolysis step by analyzing the conjugated metabolites intact using a Thermo Scientific™ Prelude SPLC™ system for sample preparation and a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer for analysis.

Experimental

Sample Preparation

Urine samples, which were free of opiates, were diluted two-fold with water and methanol (95:5) containing internal standards. There were a total of 10 deuterated internal standards in solution at a concentration of 50 ng/mL. After the addition of the internal standards, 50 µL of each sample were injected onto the analytical column at a temperature of 27 °C.

Calibration standards containing all 19 compounds at concentrations ranging from 5 to 500 ng/mL were prepared in urine. Quality control (QC) samples were also prepared in urine at three levels: 12, 225, and 400 ng/mL.

SPLC Method Parameters

Instrumentation	Prelude SPLC system (Figure 1)
Analytical column	Thermo Scientific™ Accucore™ aQ column (100 x 2.1 mm, 2.6 µm particle size), catalog # 17326-102130
Mobile phase A	0.1% formic acid in water (Fisher Chemical brand)
Mobile phase B	0.1% formic acid in methanol (Fisher Chemical brand)
Gradient	Refer to Table 1

Figure 1. Prelude SPLC system with TSQ Endura triple quadrupole mass spectrometer

Table 1. Gradient details

Step	Start (min)	Time (s)	Flow (mL/min)	Grad.	%A	%B
1	0.00	20	0.40	Step	100.0	0.0
2	0.33	5	0.40	Step	92.0	8.0
3	0.42	50	0.40	Step	92.0	8.0
4	1.25	5	0.40	Step	75.0	25.0
5	1.33	130	0.40	Ramp	65.0	35.0
6	3.50	45	0.40	Step	0.0	100.0
7	4.25	100	0.40	Step	100.0	0.0

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MS Method Parameters

Instrumentation	TSQ Endura triple quadrupole MS
Ion source	Heated electrospray (HESI II)
Ionization polarity	Positive
Cycle time	0.200 s
Peak width (Q1)	0.7 Da
Peak width (Q3)	0.7 Da
Chrom peak filter width	3.0
Spray voltage	4500 V
Vaporizer temperature	400 °C
Sheath gas pressure	30 (arbitrary units)
Ion sweep gas pressure	1.0 (arbitrary units)
Aux gas pressure	15 (arbitrary units)
Capillary temperature	325 °C
Collision gas pressure	1.5 mTorr
SRM parameters	Refer to Table 2

Table 2. SRM parameters

Analyte	Precursor Ion (Q1)	Product Ions (Q3)	CE (V)	S-lens (V)
Normorphine	272.0	165.0	59	95
		209.0	40	95
Morphine 3b glucuronide	462.1	286.1	52	148
		185.2	58	139
Oxymorphone 3b glucuronide	478.1	284.1	47	147
		302.1	42	147
Hydromorphone 3b glucuronide	462.1	185.2	58	139
		286.1	52	148
Morphine 6b glucuronide	462.1	286.1	52	148
		185.2	58	139
Codeine 6b glucuronide	476.2	300.2	31	114
		215.2	39	114
6-Acetylmorphine	328.1	165.0	58	112
		211.0	39	112
6-Acetylcodeine	342.1	225.1	27	109
		165.1	47	109
Dihydromorphine	288.1	185.1	48	95
		165.0	59	95
Morphine	286.1	165.1	64	90
		185.0	44	119
Oxymorphone	302.0	227.0	40	116
		199.1	55	116
Hydromorphone	286.1	185.0	44	119
		165.1	64	90
Codeine	300.0	171.0	40	119
		199.1	43	119
Dihydrocodeine	302.0	201.1	42	93
		199.0	52	93
Norcodeine	286.1	165.1	64	90
		181.6	49	90
Oxycodone	316.0	241.1	41	119
		256.0	40	119
Noroxycodone	302.1	227.0	41	116
		187.0	40	116
Norhydrocodone	286.1	199.0	39	119
		241.1	35	119
Hydrocodone	300.0	171.1	40	119
		181.1	51	94
Noroxycodone-D_3	305.1	190.1	25	116
Norhydrocodone-D_3	298.1	152.1	62	116
6acetylmorphine-D_6	334.1	165.1	38	116
Morphine 6b glucuronide-D_3	465.1	298.1	32	140
Morphine-D_3	289.1	152.1	61	116
Dihydrocodeine-D_6	308.1	202.1	34	116
Codeine-D_6	306.1	165.1	43	116
Hydromorphone-D_6	292.1	185.1	32	116
Morphine-3b-glucuronide-D_3	465.1	289.1	31	140
Oxycodone-D_6	322.1	218.1	43	116

Method Validation

Accuracy and precision were tested by using five replicates of three levels of quality controls over four days and quantitating them using calibration curves at the beginning and end of the batch run. The fourth day of accuracy and precision was performed in real urine to cross-verify the use of real matrix. Carryover was calculated by dividing the total analyte signal of the lower limit of quantitation (LLOQ) by the total analyte signal found in the matrix blank after the upper limit of quantitation (ULOQ). This number could not exceed 20% of the total LLOQ signal. Additionally, autosampler stability (24 hours at 4 °C) was determined by running QC samples that were refrigerated overnight in the autosampler to a new calibration curve the following day.

Results and Discussion

The assay precision had %RSD values that were within 20.0% at the LLOQ and low QC, and within 15.0% for all other QC and calibration standard levels. Additionally, accuracy was within 20.0% at the LLOQ and low QC, and within 15% for all other QC and calibration standard levels. All of these results are shown in Table 3.

The short 4.25 minute analytical method provided ample resolution for all isobaric compounds. All the analytes passed acceptance criteria for carryover, matrix effects, and autosampler stability. Example chromatograms for each of the compounds are shown in Figure 2.

Table 3. Accuracy and precision results

Analyte	Accuracy	Precision (%RSD)	
		Intra-Assay	Inter-Assay
Normorphine	94.6	<14.3	<5.7
Dihydromorphine	102	<14.1	<8.2
Morphine	99.2	<8.8	<4.8
Oxymorphone	103	<10.3	<3.5
Hydromorphone	102	<14.1	<5.8
Norcodeine	98.6	<9.6	<4.1
Dihydrocodeine	99.5	<11.1	<5.3
Codeine	99.2	<13.6	<5.7
Norhydrocodone	98.2	<13.5	<9.2
Oxycodone	99.4	<14.1	<5.8
Noroxycodone	100	<11.6	<10.4
Hydrocodone	95.2	<7.4	<5.0
6-Acetylmorphine	103	<9.7	<4.4
Codeine 6B glucuronide	102	<8.5	<4.1
Oxymorphone 3B glucuronide	100	<14.4	<4.4
Hydromorphone 3B glucuronide	108	<7.9	<5.7
Morphine 3B glucuronide	98.5	<14.9	<4.1
Morphine 6B glucuronide	99.0	<10.8	<3.7
6-Acetylcodeine	102	<6.1	<6.9

Figure 2. Representative chromatograms for all 19 compounds

Conclusion

A forensic method for analysis of opiates, opioids, and their metabolites without hydrolysis has been developed using the Prelude SPLC system and TSQ Endura MS. By eliminating the hydrolysis step, the sample preparation time and analysis cost was drastically reduced. The LC method on the Prelude SPLC system/TSQ Endura MS provided ample resolution for all isobaric compounds and an outstanding increase in overall speed of analysis. The high sensitivity that the TSQ Endura MS provided allowed for low limits of quantitation of even the least responsive analytes, like the gluronidated metabolites. The fast SRM acquisition rate yielded a successful, simultaneous analysis of 19 compounds with 10 internal standards.

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Quantitation of Bath Salts/Cathinones in Urine by LC-MS/MS

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Key Words

Cathinone, bath salts, designer drugs, MDPV, methylone, mephedrone, ethylone, butylone, naphyrone, methedrone, TSQ Quantum Ultra, liquid/liquid extraction, forensic toxicology

Goal

To develop an LC-MS/MS method for the analysis of the three Schedule I cathinones (MDPV, methylone and mephedrone), as well as other substituted cathinones (methedrone, ethylone, butylone and naphyrone) in urine with LOQs of 1 ng/mL for forensic toxicology.

Introduction

Substituted cathinones, sometimes know as "bath salts," have become the latest abused designer drugs. Based on cathinone, a substance found in the African *Catha edulis* (khat) plant, substituted cathinones are stimulants with amphetamine- and cocaine-like effects. As with many designer drug classes, variations on base structure abound (Figure 1). On October 21, 2011 the United States Drug Enforcement Agency (US DEA) listed three of the most common substituted cathinones: methylenedioxy-pyrovalerone (MDPV), methylone, and mephedrone, as Schedule I drugs, thereby making them illegal. As these drugs are not detected by current ELISA drug screening tests, new methods are needed to detect and quantify them.

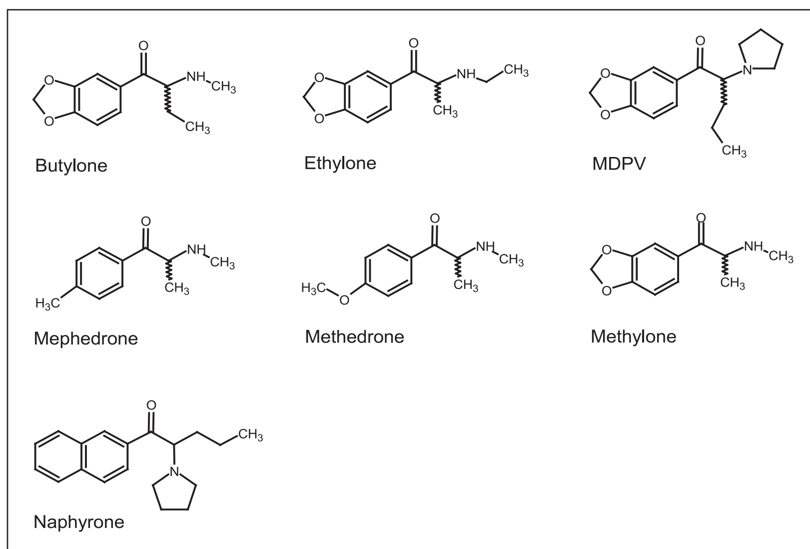

Figure 1. Structures of substituted cathinones (bath salts)

Experimental

Sample Preparation

Deuterated internal standards were available for all compounds except methedrone and naphyrone. Butylone-d_3 was used as internal standard for methedrone and MDPV-d_8 was used for naphyrone.

Sample preparation was a liquid-liquid extraction (LLE). First, 200 µL of urine and 10 µL of internal standard mix solution (2 µg/mL of each deuterated IS) were basified with 100 µL of 1 N NaOH. Extraction was performed by adding 1 mL of ethylacetate/hexane (1:1), mixing, and centrifuging. Then, an 800 µL aliquot of the resulting supernatant was transferred to a clean test tube containing 20 µL of DMSO to prevent complete evaporation of solvent. Analytes have low molecular weight, are slightly volatile, and will evaporate if left too long in the evaporator. The supernatant was evaporated at 37 °C under nitrogen for 15 minutes. Samples were diluted with 200 µL of 5% methanol and transferred to an HPLC vial equipped with a limited-volume insert. Finally, 20 µL was injected into the LC-MS system.

Liquid Chromatography

Chromatographic separations were performed under gradient conditions using a Thermo Scientific™ Accela™ 1250 pump and Accela Open autosampler. The analytical column was a Thermo Scientific™ Hypersil GOLD™ column (50 x 2.1 mm,1.9 µm particle size). The column was maintained at room temperature. The injection volume was 20 µL. Mobile phases A and B consisted of 10 mM ammonium formate with 0.1% formic acid in water and methanol, respectively. Mobile phase C was acetonitrile/1-propanol/acetone (45:45:10). All mobile phases were Fisher Chemical™ brand solvents. A shallow gradient at a flow rate of 500 µL/min was used to separate isomeric ethylone and butylone. The total run time was 5 minutes.

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Mass Spectrometry

MS analysis was carried out using a Thermo Scientific™ TSQ Quantum Ultra™ triple-stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. Two selected-reaction monitoring (SRM) transitions were monitored for each analyte and each deuterated internal standard to provide ion ratio confirmations (IRC). Data acquisition and processing were performed using Thermo Scientific™ TraceFinder™ software.

Validation

Standard calibration curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at low (LQC), middle (MQC), and high (HQC) concentrations. Intrarun variability and robustness were determined by processing six replicates of each QC level along with a calibration curve, as outlined in the Sample Preparation section, on three different days. Matrix effects were investigated by comparing peak areas of analyte at 10 ng/mL and internal standard prepared in twelve different lots of urine to those of a sample prepared in water.

Results and Discussion

MDPV, methylone, mephedrone, methedrone, ethylone, and butylone were all linear from 1–1000 ng/mL. Figure 2 shows representative calibration curves for all compounds tested. Figure 3 shows representative chromatograms at 1 ng/mL for all compounds. Interassay quality control statistics shown in Table 1 demonstrate the method to be reproducible across the calibration range for the above compounds. Limited matrix effects were seen for the above compounds. These effects were largely mediated by deuterated internal standards. The absolute recoveries of all cathinones tested in various lots of urine, compared to a sample prepared in water, ranged from 85% to 132%. Relative recoveries ranged from 107% to 124%. Precision across all lots also improved when deuterated internal standards were used. Table 2 shows average statistics for all lots showing improvement in both precision and accuracy when internal standards were used.

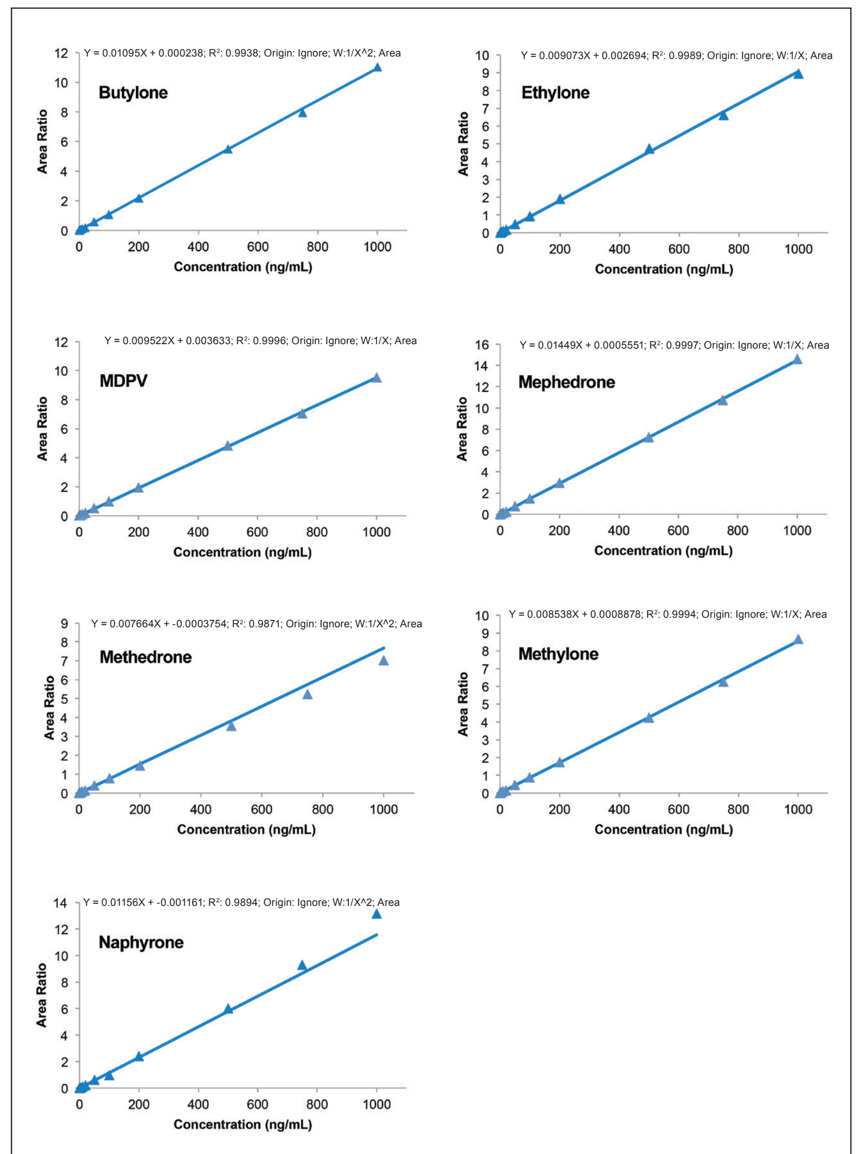

Figure 2. Representative calibrations curves for cathinones in urine

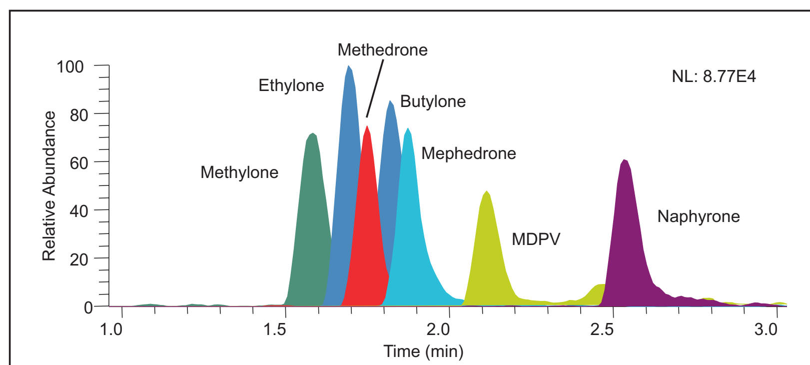

Figure 3. Representative chromatogram of cathinones at 1 ng/mL

Table 1. Interassay QC results

	LQC		MQC		HQC	
n = 18	%Bias	%CV	%Bias	%CV	%Bias	%CV
butylone	-5.80%	5.13%	1.40%	4.81%	-2.86%	3.76%
ethylone	-7.36%	5.93%	6.21%	1.72%	0.777%	1.71%
MDPV	-8.32%	5.48%	5.48%	2.89%	-0.907%	2.92%
mephedrone	-3.23%	2.79%	7.89%	2.64%	0.978%	2.00%
methedrone	-0.0565%	6.21%	8.09%	2.85%	-2.44%	1.79%
methylone	-3.95%	4.86%	6.06%	2.55%	0.394%	2.07%
naphyrone	-40.2%	59.7%	-18.2%	10.9%	-10.3%	8.68%

Table 2. Average imprecision and bias across all lots of urine

	Absolute Recovery		Relative Recovery	
	Imprecision	Bias	Imprecision	Bias
butylone	4.2%	12%	2.5%	13%
butylone-d_3	4.0%	-1.2%	na	na
ethylone	4.8%	16%	1.9%	18%
ethylone-d_5	4.9%	-1.5%	na	na
MDPV	3.0%	25%	1.6%	17%
MDPV-d_8	2.9%	7.1%	na	na
mephedrone	4.7%	21%	2.2%	16%
mephedrone-d_3	5.4%	4.3%	na	na
methedrone[1]	5.4%	18%	2.7%	19%
methylone	6.3%	15%	1.4%	17%
methylone-d_3	6.0%	-1.9%	na	na
naphyrone[2]	17%	49%	16%	39%

[1] Butylone-d_3 used as IS
[2] MDPV-d_8 used as IS

Although naphyrone was detected at 1 ng/mL, it showed more variability than the other compounds and a greater matrix effect from lot to lot. Absolute recoveries for naphyrone ranged from 113% to 207% while relative recoveries using MDPV-d_8 as internal standard ranged from 111% to 191%. All available internal standards were tried, and MDPV-d_8 showed the best results. A lack of a deuterated analog for naphyrone does not allow for matrix effect corrections and negatively affects method precision. In this assay, naphyrone should be considered qualitative.

Conclusion

We achieved our goal of a 1 ng/mL LOQ for the three DEA-regulated cathinones, MDPV, mephedrone, and methylone, as well as methylone, ethylone, and butylone in urine for forensic toxicology. Naphyrone, which shows greater variability, can be detected down to 1 ng/mL in a qualitative manner. Deuterated internal standards are essential for rigorous quantitation of these compounds.

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Evaluation of an LC-MS/MS Research Method for the Analysis of 33 Benzodiazepines and their Metabolites

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Key Words
TSQ Vantage, Transcend TLX-1, benzodiazepines

Goal
The goal of this work was to evaluate the RECIPE® ClinMass® LC-MS/MS Complete Kit for research purposes using an online method analysis of 33 benzodiazepines and benzodiazepine metabolites in serum.

Introduction
Benzodiazepines are prescribed for the management of anxiety, sleeping disorders, muscle spasms, and seizures. Benzodiazepines are widely viewed as safe drugs that have relatively few side effects. However, high dosages over prolonged periods can lead to tolerance, which leads to a loss of efficacy and/or physical and psychological dependence, resulting in severe withdrawal symptoms. Benzodiazepines can also be abused in cases of crime, suicide, and drug-facilitated sexual assault. While benzodiazepine intoxication alone is rarely fatal, concurrent use with alcohol or other drugs can be life-threatening. For all of these reasons, additional research into dosage and effects of benzodiazepines is necessary.

Benzodiazepines are active at very low concentrations and have short half lives; therefore, it is of great interest to clinical researchers and forensic toxicologists to simultaneously analyze benzodiazepines and their metabolites in biological samples. However, the analysis is not always easy because of very low blood concentrations and the complexity of detecting multiple drugs at the same time, especially in a biological matrix. Typically, benzodiazepines are quantified in serum.

In this work, the RECIPE kit for benzodiazepines analysis in serum was evaluated for research purposes using a Thermo Scientific™ Transcend™ TLX-1 system for performing both the online solid-phase extraction (SPE) and the chromatography. The Transcend system was coupled to a Thermo Scientific™ TSQ Vantage™ triple-stage quadrupole mass spectrometer for quantitative analysis. The RECIPE kit allows the analysis of 33 benzodiazepines and their metabolites in 12–15 minutes for research applications, such as those discussed in this application note. It includes the SPE column, analytical column, mobile phases, optimization mixtures, calibrators, quality controls, and an internal standards solution that integrates 20 deuterated internal standards. One kit is sufficient for up to 200 samples.

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Experimental

The research method was applied as described in the RECIPE ClinMass LC-MS/MS Complete Kit instructions, with the exception of the loading flow rate, which is described in the HPLC method.

Sample Preparation

As described in the kit instructions, 50 µL of each calibrator and quality control was vortexed for 10 s in a sample preparation tube with 50 µL of internal standards solution. The sample was then centrifuged for 10 min at 10,000 rpm, and 20 µL of the supernatant was injected into the LC-MS/MS system.

HPLC Method

The kit includes an online solid phase extraction (SPE) column and an HPLC separation column that are integrated in a valve system to operate by column switching. A Transcend TLX-1 system was used to perform this column switching. The plumbing diagram is shown in Figure 1.

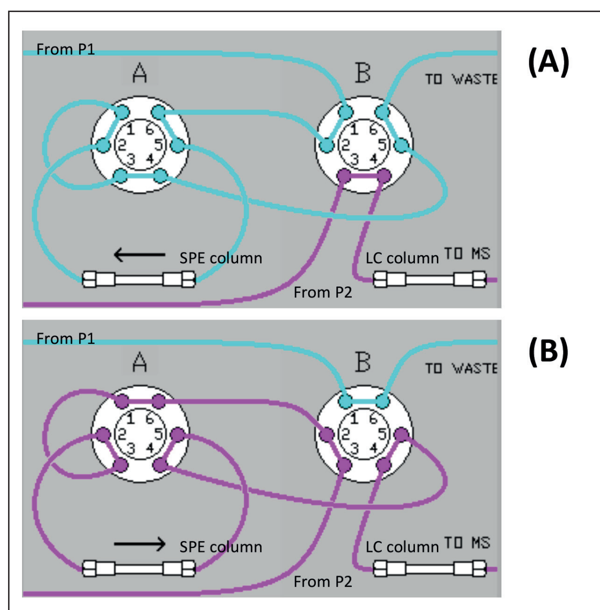

Figure 1. Plumbing of the Transcend system to perform column switching, with load (A) and inject (B) position, P1 is the loading pump and P2 the elution pump

The RECIPE kit was used as described in the instructions for this research purpose; however, the loading flow rate was 2 mL/min instead of the 5 mL/min described in the kit manual. This increased the time of analysis from 11 min to 12–15 min, but it did not impact the quality of the obtained data. In the first step, the valves are in load position (Figure 1A) where the sample is loaded onto the SPE column for the extraction of the analytes from the biological matrix. This step takes 1.9 min. In step two, the valves are switched to the inject position (Figure 1B) where the analytes extracted on the SPE column are eluted to the HPLC column by backflushing with mobile phase for 7.5 min. The analytes are then chromatographically separated with a gradient. For the last step, the valves are switched back to a loading position (Figure 1A) and both columns are re-equilibrated for the next injection. This step lasts 2.75 min.

MS Method

Mass spectrometric analysis was performed using a TSQ Vantage triple-stage quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source. Source parameters are summarized in Table 1. MS analysis was performed in positive-ion selected-reaction monitoring (SRM) mode. The optimized SRM parameters for all the analytes and internal standards are presented in Table 2. The cycle time was set to 600 ms with a data acquisition window of 4 min for each analyte.

Table 1. Optimized source parameters

Ion Source	APCI, Positive
Resolution Q1 and Q3	0.7 amu
Discharge Current	5.0 µA
Vaporizer Temperature	450 °C
Sheath Gas Pressure	30 au
Aux Gas Pressure	10 au
Capillary Temp	250 °C
Collision Pressure	1.5 mTorr

Results and Discussion

Calibration curves were plotted for each analyte with the three calibrators provided in the kit. The regression model for all the analytes was linear with different weighting according to the analyte. The limits of quantification (LOQ) were obtained by diluting the first calibrator with blank serum either two times or five times (the blank serum is the 0 calibrator solution). The LOQ were then determined as the lowest concentration for which the %RSD for 5 injections was less than 20% and the bias was less than 20%. The weighting, internal standards, correlation factor, and LOQ of the analytes are presented in Table 3. Examples of chromatograms obtained at the LOQ for some of the analyzed compounds are presented in Figure 2. As can be seen in Table 3, good linearity was obtained for all of the analytes in the concentration ranges of the kit calibrators. A blank sample injected after the upper limit of quantification (ULOQ) was used to evaluate carryover. The carryover was less than 10% of the signal obtained for the LOQ for all the analytes tested with this kit.

Table 2. SRM parameters used for the analysis

Compound	Retention Time (minutes)	Precursor Ion	Product Ion	S-Lens	Collision Energy
7-Aminoclonazepam	4.56	286.1	222.2	114	24
7-Aminoflunitrazepam	4.70	284.1	135.1	101	26
7-Aminonitrazepam	4.56	252.1	121.1	96	26
α-OH-Alprazolam	6.20	325.1	297.1	102	24
α-OH-Midazolam	7.24	342.1	324.1	102	19
α-OH-Triazolam	5.95	359.1	331.0	101	23
Alprazolam	6.66	309.1	205.1	112	38
Bromazepam	5.73	316.0	182.1	100	31
Chlordiazepoxide	7.64	300.1	227.1	95	24
Clobazam	6.35	301.1	259.1	96	19
Clonazepam	6.10	316.1	270.1	114	23
Demoxepam	5.67	287.1	178.0	91	21
Desalkylflurazepam	7.02	289.1	140.1	123	28
Desmethylflunitrazepam	5.80	300.1	254.1	95	23
Diazepam	8.47	285.1	193.1	91	28
Estazolam	6.31	295.1	267.1	119	22
Flunitrazepam	6.20	314.1	268.1	89	25
Flurazepam	9.16	388.2	315.1	117	20
Lorazepam	6.67	323.0	277.1	100	21
Lormetazepam	7.32	335.1	289.1	88	21
Medazepam	10.57	271.1	207.1	86	26
Midazolam	8.41	326.1	291.1	129	25
Nitrazepam	6.20	282.1	236.1	95	23
Norclobazam	5.96	287.1	245.1	81	19
Nordiazepam	7.98	271.1	140.1	99	27
Oxazepam	6.76	287.1	241.1	106	21
Prazepam	10.03	325.1	271.1	100	21
Temazepam	7.12	301.1	255.1	96	22
Tetrazepam	9.96	289.1	225.2	117	28
Trazodone	9.34	372.2	148.1	115	32
Triazolam	6.49	343.1	308.1	146	25
Zaleplone	5.43	306.1	236.1	100	26
Zolpidem	7.10	308.2	235.2	116	33

Table 3. Calibration parameters and LOQ

Compound	Internal Standard	Weighting	R^2	LOQ (µg/L)
7-Aminoclonazepam	7-Aminoclonazepam-D4	1/X	0.999	2.6
7-Aminoflunitrazepam	7-Aminoflunitrazepam-D7	$1/X^2$	0.995	2.6
7-Aminonitrazepam	7-Aminoclonazepam-D4	1/X	0.995	22.1
α-OH-Alprazolam	α-OH-Alprazolam-D5	1/X	0.993	2.7
α-OH-Midazolam	α-OH-Midazolam-D4	1/X	0.995	10.8
α-OH-Triazolam	α-OH-Triazolam-D4	1/X	0.997	4.6
Alprazolam	Alprazolam-D5	1/X	0.996	1.1
Bromazepam	7-Aminoflunitrazepam-D7	1/X	0.997	15.8
Chlordiazepoxide	Chlordiazepoxide-D5	$1/X^2$	0.993	111.5
Clobazam	Triazolam-D4	$1/X^2$	0.992	9.5
Clonazepam	Clonazepam-D4	$1/X^2$	0.994	2.5
Demoxepam	Clonazepam-D4	1/X	0.997	216.0
Desalkylflurazepam	Temazepam-D5	$1/X^2$	0.993	9.7
Desmethylflunitrazepam	Clonazepam-D4	$1/X^2$	0.990	4.5
Diazepam	Diazepam-D5	$1/X^2$	0.993	20.4
Estazolam	Estazolam-D5	1/X	0.996	21.4
Flunitrazepam	Flunitrazepam-D7	1/X	0.990	5.2
Flurazepam	Prazepam-D5	Equal	0.996	4.3
Lorazepam	Lorazepam-D4	$1/X^2$	0.996	20.6
Lormetazepam	Nordiazepam-D5	$1/X^2$	0.989	1.8
Medazepam	Nordiazepam-D5	Equal	0.997	8.6
Midazolam	Midazolam-D4	Equal	0.995	15.5
Nitrazepam	Nitrazepam-D5	$1/X^2$	0.997	10.5
Norclobazam	α-OH-Triazolam-D4	1/X	0.997	133.0
Nordiazepam	Nordiazepam-D5	1/X	0.997	16.7
Oxazepam	Oxazepam-D5	1/X	0.995	62.5
Prazepam	Prazepam-D5	1/X	0.999	41.7
Temazepam	Temazepam-D5	$1/X^2$	0.996	21.7
Tetrazepam	Prazepam-D5	Equal	0.999	8.2
Trazodone	Prazepam-D5	1/X	0.997	82.5
Triazolam	Triazolam-D4	1/X	0.988	4.0
Zaleplone	7-Aminoflunitrazepam-D7	1/X	0.999	4.2
Zolpidem	Zolpidem-D6	Equal	0.998	21.2

The intraday and interday analytical accuracies and variability are presented in Tables 4 and 5, respectively. For each analyte, intraday variability and accuracy were determined by analyzing five samples at each QC level. For interday variability and accuracy, the process performed for intraday data was repeated on three different days. For the intraday study, the accuracy obtained was between 94% and 112% with a %RSD less than 12%. In the case of interday assays, the accuracy was between 93% and 111% with a %RSD less than 13%. The obtained results show a low variability for the two QC levels as well as good accuracy.

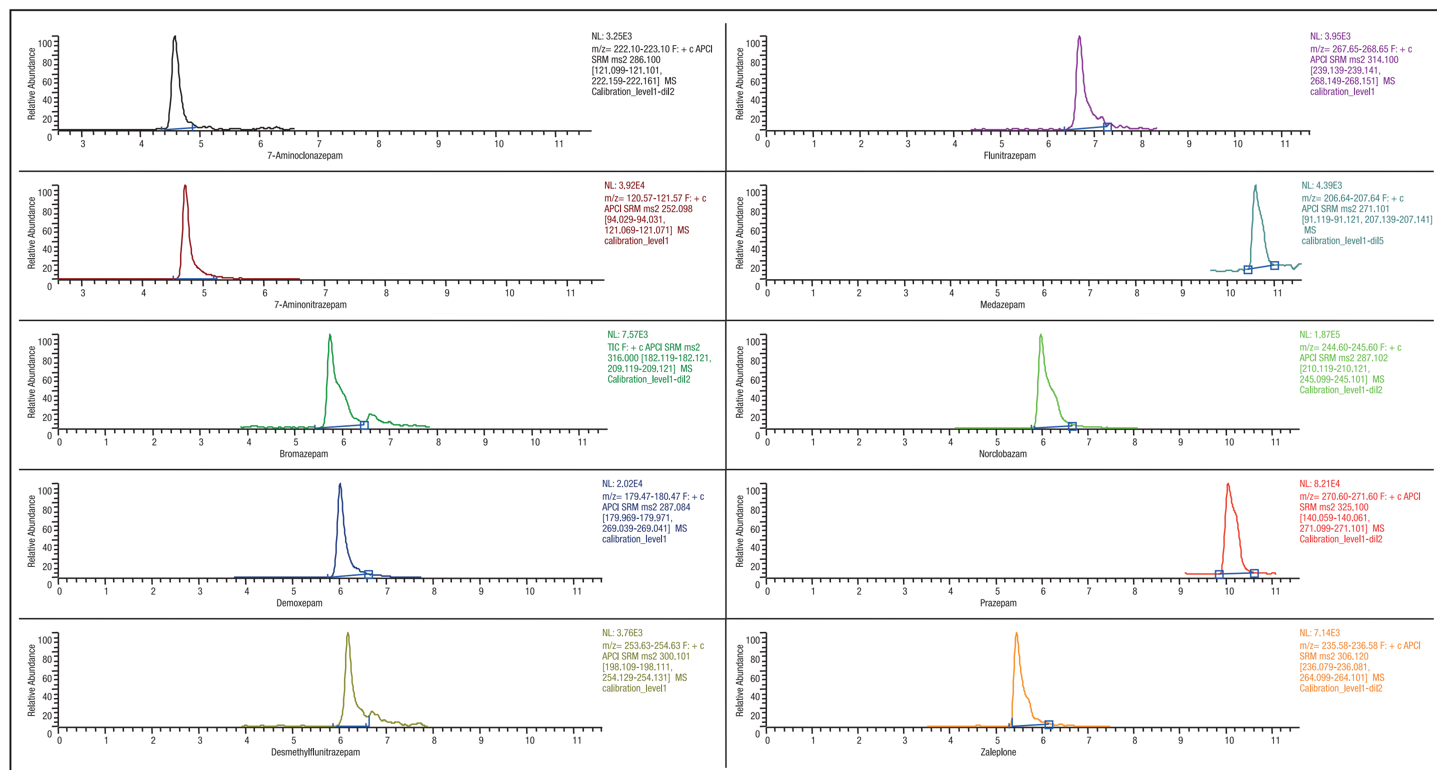

Figure 2. Chromatograms of the SRM transitions of some commonly found benzodiazepines at the LOQ

Table 4. Intraday repeatability and accuracy for QC1 and QC2

Compound	QC 1				QC 2			
	Specified Concentration	Measured Concentration	%RSD	%Accuracy	Specified Concentration	Measured Concentration	%RSD	%Accuracy
7-Aminoclonazepam	16.0	15.5	3.8	97	52.4	54.6	5.2	104
7-Aminoflunitrazepam	16.6	16.8	8.6	101	54.5	55.9	3.0	103
7-Aminonitrazepam	66.4	68.5	3.9	103	221	237	5.1	107
α-OH-Alprazolam	16.7	17.4	7.1	104	57	59.4	3.9	104
α-OH-Midazolam	64.6	65.4	3.8	101	203	217	4.1	107
α-OH-Triazolam	13.6	13.7	8.8	101	43.3	45.7	2.9	105
Alprazolam	15.4	15.7	9.2	102	55.5	55.2	5.8	99
Bromazepam	94.3	97.0	6.9	103	296	320	2.3	108
Chlordiazepoxide	621	602	3.1	97	2040	2150	3.3	105
Clobazam	143	144	7.7	101	491	489	4.4	100
Clonazepam	14.6	14.6	5.2	100	49.7	54.7	7.5	110
Demoxepam	682	673	5.5	99	2270	2350	4.4	103
Desalkylflurazepam	31.4	29.3	9.6	94	102	107	5.0	94
Desmethylflunitrazepam	15.2	14.4	4.6	95	51.1	54.2	3.8	106
Diazepam	292	297	3.4	102	949	941	5.2	99
Estazolam	132	127	2.4	96	441	447	6.6	101
Flunitrazepam	15.9	16.8	12.1	106	53.5	54.7	8.2	102
Flurazepam	25.9	26.9	5.4	104	86.2	93.0	4.7	108
Lorazepam	62.1	67.3	5.6	108	205	210	4.2	103
Lormetazepam	5.46	5.70	9.0	104	18.2	18.9	9.3	104
Medazepam	127	126	5.1	99	426	437	6.1	102
Midazolam	93.5	91.1	4.7	97	308	316	4.1	103
Nitrazepam	62.6	66.5	4.6	106	206	215	3.0	104
Norclobazam	835	811	3.2	97	2670	2770	3.8	104
Nordiazepam	239	245	4.2	103	821	804	4.3	98
Oxazepam	377	383	7.6	101	1240	1240	6.9	100
Prazepam	262	256	1.7	98	843	862	1.6	102
Temazepam	128	135	3.7	105	409	459	1.8	112
Tetrazepam	123	124	1.1	101	409	422	1.6	103
Trazodone	516	503	4.3	98	1630	1810	9.0	111
Triazolam	12.1	11.6	8.4	96	40.2	40.0	11.1	100
Zaleplone	26.8	27.3	8.5	102	88.6	94.1	5.6	106
Zolpidem	139	131	2.3	94	468	463	3.4	99

Table 5. Interday repeatability and accuracy for QC1 and QC2

	QC 1				QC 2			
Compound	**Specified Concentration**	**Measured Concentration**	**%RSD**	**%Accuracy**	**Specified Concentration**	**Measured Concentration**	**%RSD**	**%Accuracy**
7-Aminoclonazepam	16.0	16.7	6.3	104	52.4	53.9	5.3	103
7-Aminoflunitrazepam	16.6	17.4	6.6	105	54.5	53.2	6.5	98
7-Aminonitrazepam	66.4	70.7	5.8	107	221	224	6.1	101
α-OH-Alprazolam	16.7	17.8	7.4	107	57.0	56.8	5.7	100
α-OH-Midazolam	64.6	68.0	9.2	105	203	212	8.0	104
α-OH-Triazolam	13.6	13.4	6.3	98	43.3	43.5	5.3	100
Alprazolam	15.4	16.0	10.3	104	55.5	52.5	6.9	95
Bromazepam	94.3	91.9	12.9	97	296	310	7.9	105
Chlordiazepoxide	621	607	8.8	98	2040	2090	8.2	103
Clobazam	143	146	6.8	102	491	473	5.9	96
Clonazepam	14.6	15.0	5.6	103	49.7	52.7	5.9	106
Demoxepam	682	711	7.5	104	2270	2280	4.3	100
Desalkylflurazepam	31.4	29.5	10.1	94	102	102	7.3	100
Desmethylflunitrazepam	15.2	15.3	7.3	101	51.1	51.8	6.3	101
Diazepam	292	309	8.8	106	949	945	9.5	100
Estazolam	132	129	3.7	98	441	437	7.1	99
Flunitrazepam	15.9	17.4	9.5	109	53.5	52.6	8.7	98
Flurazepam	25.9	27.3	13.4	106	86.2	89.5	8.1	104
Lorazepam	62.1	66.0	6.1	106	205	200	5.7	97
Lormetazepam	5.46	6.00	11.0	111	18.2	18.8	8.8	103
Medazepam	127	131	6.9	103	426	437	12.5	102
Midazolam	93.5	89.0	4.4	95	308	307	4.3	100
Nitrazepam	62.6	69.3	6.9	111	206	203	6.7	99
Norclobazam	835	835	6.3	100	2670	2640	5.6	99
Nordiazepam	239	262	11.6	110	821	802	8.1	98
Oxazepam	377	387	9.3	103	1240	1250	6.5	101
Prazepam	262	265	3.7	101	843	845	4.1	100
Temazepam	128	134	5.8	105	409	437	6.2	107
Tetrazepam	123	127	4.4	103	409	416	7.5	102
Trazodone	516	501	6.6	97	1630	1750	11.6	107
Triazolam	12.1	11.7	6.1	97	40.2	39.7	6.8	99
Zaleplone	26.8	27.1	9.8	101	88.6	85.2	11.2	96
Zolpidem	139	130	7.8	93	468	450	8.0	96

Conclusion

The analysis of benzodiazepines for research purposes presents a challenge due to their low concentrations, their short half life, and their diversity. In this work, the RECIPE ClinMass® complete kit for the quantification of 33 benzodiazepines and metabolites in serum was evaluated for research use with a TSQ Vantage triple quadrupole. The online SPE configuration was achieved by using a Transcend TLX-1 system. HPLC coupled to tandem mass spectrometry may be a suitable tool for the quantification of benzodiazepines in research applications since it can deal with the differences in polarities of the benzodiazepines and their metabolites. The use of SRM detection increases the analytical specificity needed to attain the low quantification ranges for these types of molecules. Finally, the use of LC-MS/MS simplifies sample preparation since the matrix clean-up is performed by online SPE.

This research method showed good results in terms of variability, precision, and dynamic range requirements for a clinical research method.

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Quantitation of EtG and EtS in Urine by Ion-Pairing LC-MS/MS

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Key Words

EtG, EtS, ethyl glucuronide, ethyl sulfate, ion pairing, SPE, DHAA, dihexylammonium acetate, TSQ Ultra, HyperSep, Hypercarb, forensic toxicology

Goal

To develop an HPLC-MS/MS method for forensic toxicological analysis of EtG and EtS in urine with limits of quantitation (LOQs) of 100 and 50 ng/mL, respectively.

Introduction

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are long-term biomarkers for ethanol consumption. Although they are minor metabolites of ethanol, their longer half-lives make them useful for forensic detection of past alcohol use. These compounds are highly polar; they retain poorly on most reversed-phase HPLC columns and elute on or near the chromatographic solvent front. The result is poor peak shape and large matrix effects. This application note demonstrates the use of solid-phase extraction (SPE) sample preconcentration to remove interferences and an ion-pairing reagent to retain these compounds on the HPLC column long enough to move them off the solvent front. This enables better peak shape, less matrix interference, and baseline resolution of both analytes for less risk of ion suppression.

Experimental

Sample Preparation

A 1 mL volume of urine, 25 µL of internal standard solution (50 and 5 ng/mL of EtG–d_5 and EtS–d_5, respectively), and 50 µL of formic acid were mixed. The 200 mg Thermo Scientific™ HyperSep™ Hypercarb™ SPE column (P/N 60106-301) was conditioned with 2 mL of 1% formic acid in water. The sample was loaded at a rate of 1–2 mL/min. Next, the column was washed with 2 mL of water and dried under nitrogen at 10–15 mm Hg for 10 min. The sample was eluted with 2 mL of 1% formic acid in methanol. The eluent was evaporated to dryness under nitrogen at 37 °C and reconstituted in 0.2 mL of water.[1] Finally, 20 µL was injected onto the HPLC-MS/MS.

Liquid Chromatography

Chromatographic separations were performed under gradient conditions using a Thermo Scientific™ Accela™ 1250 pump and Accela Open autosampler. The analytical column was a Thermo Scientific Hypersil GOLD™ column (50 x 3 mm, 5 µm particle size). The column was maintained at room temperature. The injection volume was 20 µL. The flow rate was 1 mL/min, and the total run time was 5 min. Other size columns can be used for this application with the appropriate adjustment in injection volumes and flow rates (as in AN488b[2]). Mobile phases A and B consisted of 5 mM dihexylammoniumacetate (TCI America) ion pairing reagent in water and acetonitrile (Fisher Chemical), respectively. Mobile phase C was acetonitrile/1-propanol/acetone (45:45:10).

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra™ triple-stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. Two selected-reaction monitoring (SRM) transitions each were monitored for EtG, EtS, and their deuterated internal standards to provide ion ratio confirmations (IRC). Two scan segments, one for EtG and its internal standard and one for EtS and its internal standard, were used to maximize the time the mass spectrometer spent scanning each compound.

Results and Discussion

EtG demonstrated linear response from 100 to 100,000 ng/mL with an LOQ of 100 ng/mL. EtS demonstrated linear response from 25 to 50,000 ng/mL with an LOQ of 25 ng/mL. Figure 1 shows representative calibration curves for both compounds. Figure 2 shows representative chromatograms for EtG and EtS and their respective LOQs.

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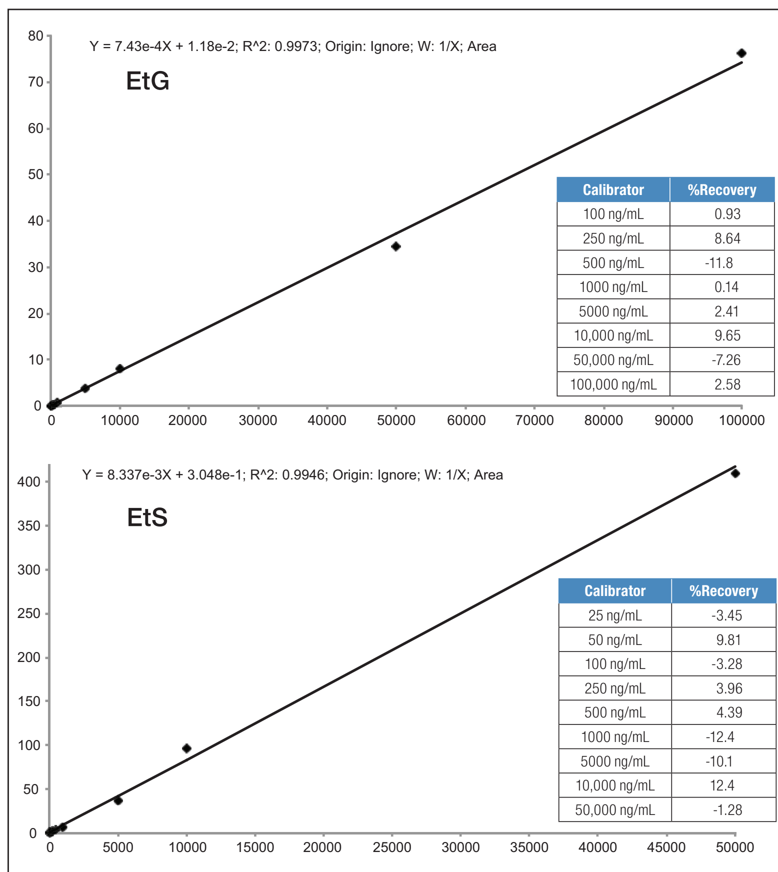

Calibrator	%Recovery
100 ng/mL	0.93
250 ng/mL	8.64
500 ng/mL	-11.8
1000 ng/mL	0.14
5000 ng/mL	2.41
10,000 ng/mL	9.65
50,000 ng/mL	-7.26
100,000 ng/mL	2.58

Calibrator	%Recovery
25 ng/mL	-3.45
50 ng/mL	9.81
100 ng/mL	-3.28
250 ng/mL	3.96
500 ng/mL	4.39
1000 ng/mL	-12.4
5000 ng/mL	-10.1
10,000 ng/mL	12.4
50,000 ng/mL	-1.28

Figure 1. Representative calibration curves for EtG and EtS in urine

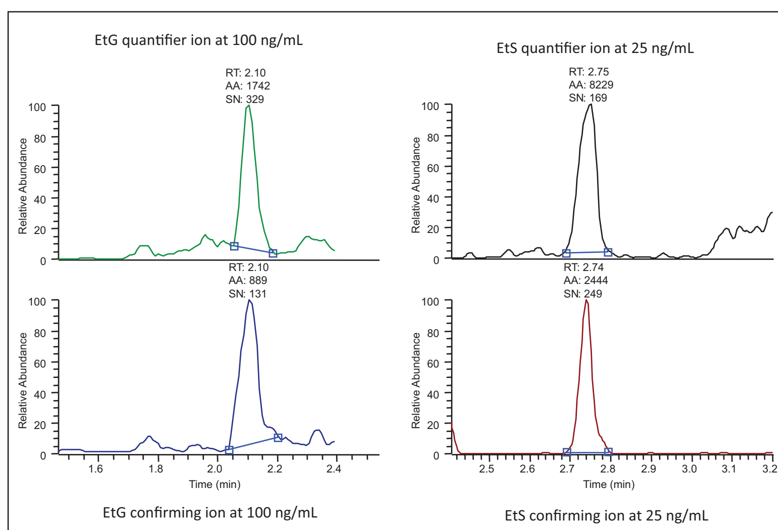

Figure 2. Representative chromatograms showing quantifier and confirming ions for EtG and EtS at 100 and 25 ng/mL, respectively

Conclusion

- This method provides limits of detection in urine of 100 ng/mL for EtG and 25 ng/mL for EtS.
- SPE extraction helps remove interferences in urine.
- Addition of an ion-pairing reagent helps chromatographically separate the compounds from interferences on the solvent front, thereby improving limits of detection.
- This method is suitable for forensic toxicology.

References

1. *LC/MS Method For Extracting Ethyl Glucuronides From Urine Using: 200 mg Clean Screen® Extraction Column;* Part #: CSETG203; UTC, Inc. Bristol, PA; 2008

2. Thermo Scientific Application Note 488b, Quantitation of Urinary Ethyl Glucuronide and Ethyl Sulfate Using Ultrahigh Resolution LC-MS, http://planetorbitrap.com/data/uploads/ZFS1327868383151_AN488b.pdf

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Application Note 588

High-Throughput Quantitative LC-MS/MS Analysis of 6 Opiates and 14 Benzodiazepines in Urine

Bill Yu, Kristine Van Natta, Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Key Words

Opiates, benzodiazepines, Prelude SPLC

Goal

Develop a high-throughput, low solvent consumption, easy-to-run method for quantitative forensic analysis of six opiates and fourteen benzodiazepines in urine.

Introduction

Analyses of opiate and benzodiazepine panels are some of the highest-volume applications in forensic toxicology labs. In order to meet the need for high throughput, a fast, simple, and cost-effective method was developed, consisting of hydrolysis, simple urine dilution, separation by liquid chromatography (LC), and analysis by mass spectrometry (MS). The method incorporated the Thermo Scientific™ Prelude SPLC™ system (Figure 1), which features two independent channels of sample preparation and liquid chromatography (SPLC). With the Prelude SPLC system, LC methods can be executed in parallel with a different method on each channel (Figure 2) or the same method on both channels (Figure 3) and multiplexed into a mass spectrometer for serial detection. Serial MS detection of multiplexed methods improves mass spectrometer utilization time, increases throughput of forensic toxicology laboratories, and reduces analysis cost. The syringe pumps and high-pressure, low-volume gradient mixing used in Prelude SPLC system provide enhanced HPLC performance: improved peak shape and resolution as well as stable retention times.

Figure 1. Prelude SPLC system

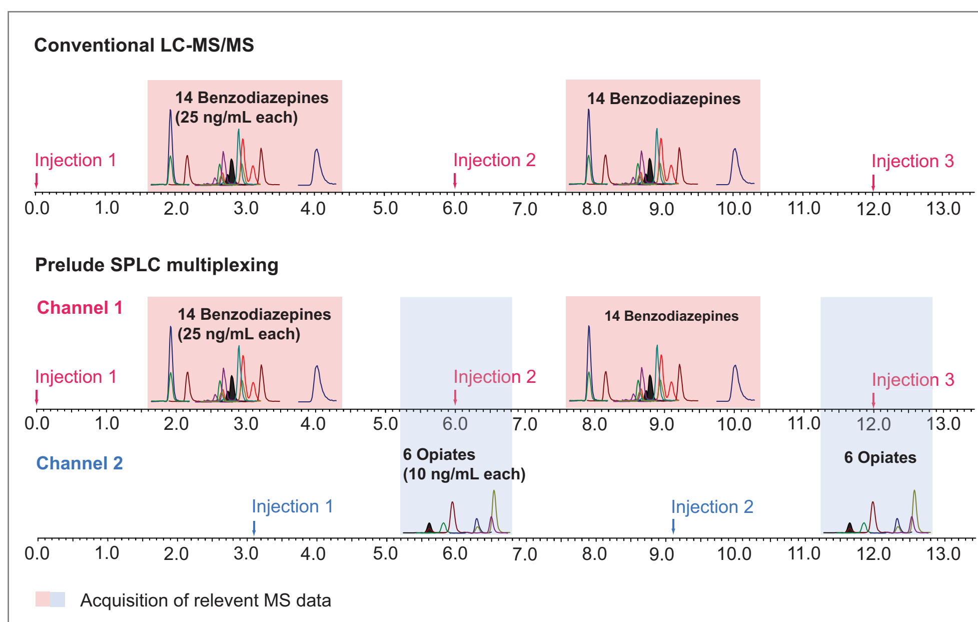

Figure 2. Parallel analysis of 6 opiates (10 ng/mL) and 14 benzodiazepines (25 ng/mL) in multiplexed mode

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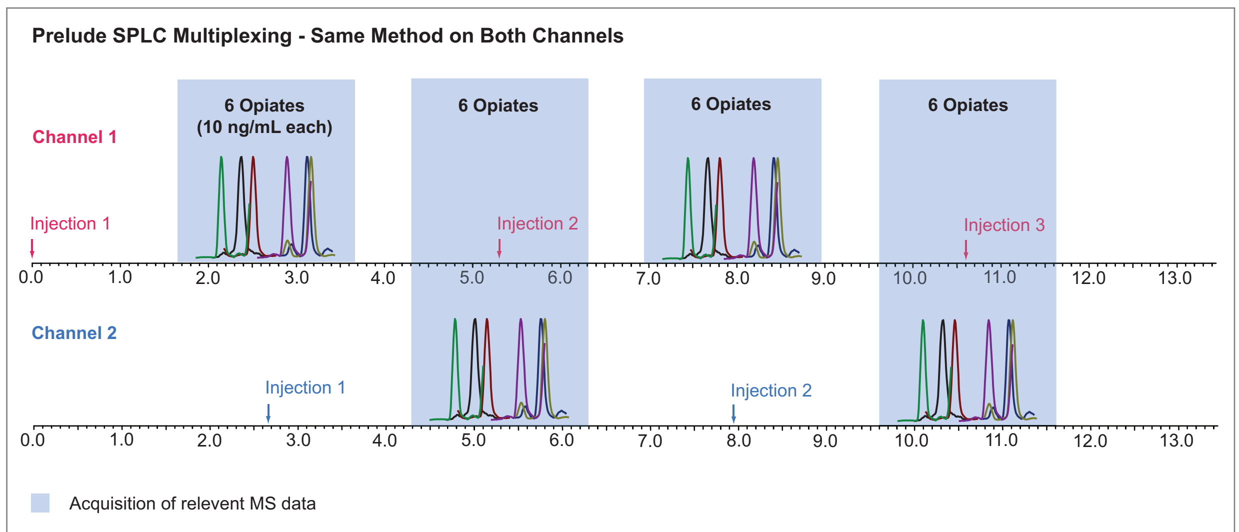


Figure 3. Analysis of six opiates (10 ng/mL) using both channels in multiplexed mode

Experimental

Sample Preparation

Tables 1 and 2 contain the lists of opiates and benzodiazepines analyzed. Sample preparation consisted of glucuronide hydrolysis followed by dilution. For each sample, a 200 µL aliquot of urine was spiked with 10 µL of internal standards solution and 100 µL of β-glucuronidaze enzyme in an ammonium acetate buffer (pH = 5.0). The samples were incubated at 60 °C for 2 hours. A 200 µL aliquot of methanol was added to each sample to stop enzymatic reaction. Samples were cooled, centrifuged, and diluted 20 times with deionized water. Then, 20 µL of sample was injected into the liquid chromatograph-mass spectrometer (LC-MS) system.

Table 1. SRM transitions for opiates method

Compound	Precursor Ion *m/z*	Quantifier Ion *m/z*	Qualifier Ion *m/z*
Morphine	286.1	152.1	185.1
Morphine-d_3	289.1	152.1	185.1
Oxymorphone	302.1	227.1	198.1
Oxymorphone-d_3	305.1	230.1	201.1
Hydromorphone	286.1	185.0	157.1
Hydromorphone-d_6	292.1	185.1	157.1
Codeine	300.2	152.1	165.1
Codeine-d_3	303.1	152.1	215.1
Oxycodone	316.2	241.1	256.1
Oxycodone-d_3	319.2	244.1	259.1
Hydrocodone	300.1	199.1	171.1
Hydrocodone-d_3	303.1	199.1	171.1

Table 2. SRM transitions for benzodiazepines method

Compound	Precursor Ion *m/z*	Quantifier Ion *m/z*	Qualifier Ion *m/z*
7-Aminoclonazepam	286.1	222.1	250.1
7-Aminoclonazepam-d_4	290.1	226.1	254.1
7-Aminonitrazepam	252.1	121.1	224.1
7-Aminoflunitrazepam	284.1	135.1	227.1
7-Aminoflunitrazepam-d_7	291.2	138.2	230.1
α-Hydroxytriazolam	359.0	331.0	239.0
Lorazepam	321.0	275.0	229.0
α-Hydroxyalprazolam	325.0	297.1	229.0
α-Hydroxyalprazolam-d_5	330.1	302.1	216.1
Oxazepam	287.1	241.1	221.1
Oxazepam-d_5	292.1	246.1	104.1
2-Hydroxyethylflurazepam	333.1	109.1	109.1
2-Hydroxyethylflurazepam-d_4	337.1	215.1	113.1
Desalkylflurazepam	289.0	140.0	226.1
Desalkylflurazepam-d_4	293.1	140.0	230.1
Temazepam	301.1	255.1	177.0
Temazepam-d_5	306.1	260.1	177.0
Nordiazepam	271.1	140.1	208.1
Nordiazepam-d_5	276.1	213.1	140.0
Alprazolam	309.1	281.1	205.1
Alprazolam-d_5	314.1	286.1	210.1
Diazepam	285.1	193.1	154.0
Diazepam-d_5	290.1	198.1	154.1
Midazolam	326.1	291.1	249.1
Midazolam-d_4	330.1	295.1	253.1

Liquid Chromatography

Chromatographic separations were performed with a Prelude SPLC system by direct injections onto Thermo Scientific™ Accucore™ PFP 50 x 2.1 mm, 2.6 µm analytical columns. The columns were maintained at room temperature. Mobile phases A and B consisted of 10 mM ammonium formate with 0.1% formic acid in water and methanol, respectively. Mobile phase usage was about 3.8 mL per sample. The total gradient run time was 5.3 min for opiates analysis (Figure 4) and 6 min for benzodiazepines analysis (Figure 5). The data acquisition windows were 2 min and 2.8 min for opiates and benzodiazepines, respectively.

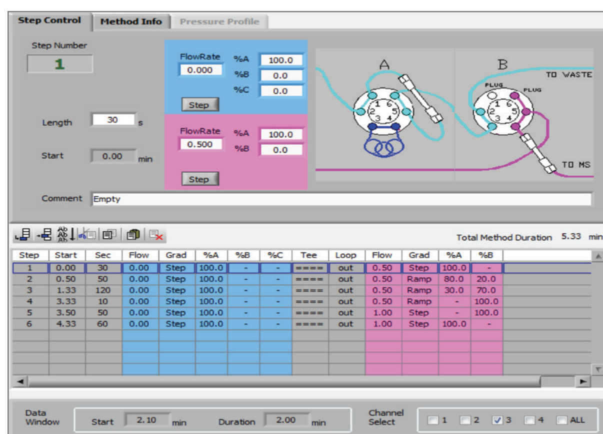


Figure 4. LC gradient for opiates analysis

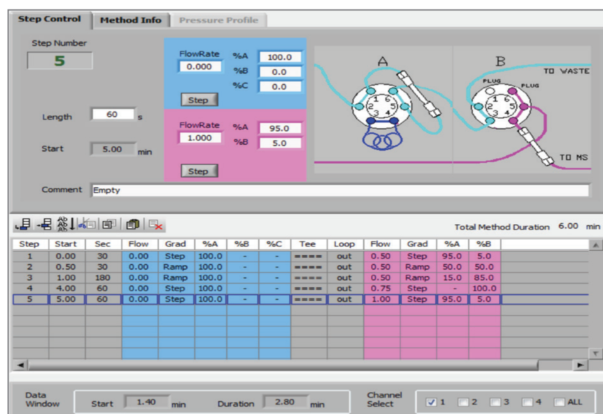


Figure 5. LC gradient for benzodiazepines analysis

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific™ TSQ Quantum Ultra™ triple quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. The mass spectrometer was operated in selected-reaction monitoring (SRM) mode. Two SRM transitions were collected for each analyte and each internal standard (Tables 1 and 2) to calculate the ion ratio.

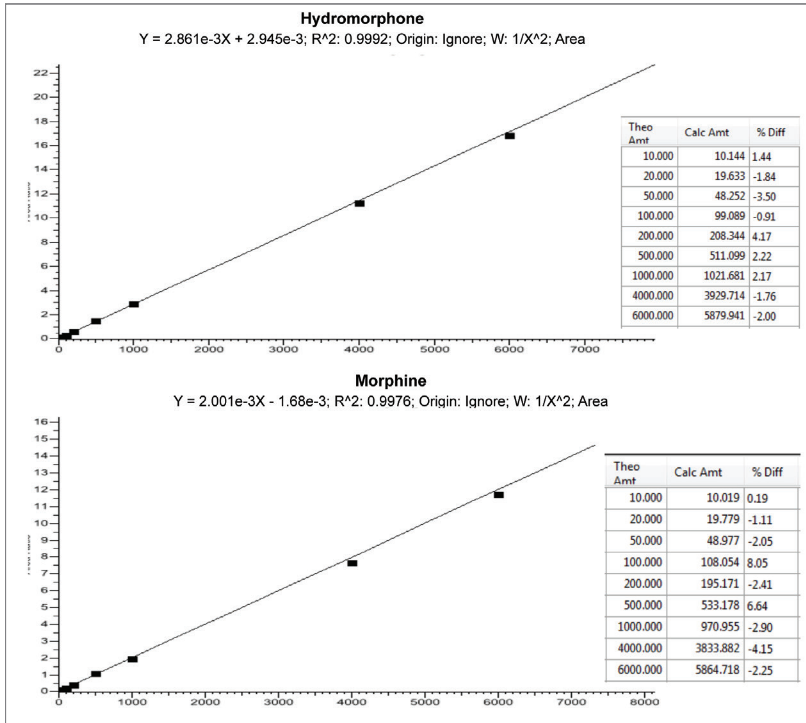
Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at concentrations corresponding to the low (LQC), middle (MQC), and high (HQC) ranges of the calibration curve. Intra-run precisions were determined by processing six replicates of each QC level along with a calibration curve on three different days. Matrix effects were investigated by analyzing seven donated urine samples spiked at concentrations of 27.5 ng/mL for opiates and 50 ng/mL for benzodiazepines. The method performance was compared with method validated in a forensic toxicology lab by analyzing the same donor samples. Method validation experiments were run by executing opiates and benzodiazepines methods in parallel on two channels in multiplexed mode.

Results and Discussion

Opiates Analysis

The limits of quantitation were 10 ng/mL and calibration ranges were 10–6000 ng/mL for all opiates. Figure 6 shows representative calibration curves for selected opiates. Figure 7 shows representative chromatograms at 10 ng/mL for all opiates tested. Intra- and inter-assay quality control statistics shown in Table 3 demonstrate the method to be reproducible across the calibration range for the opiates. Limited matrix effects were seen, and those were largely mediated by deuterated internal standards (Table 4). The data collected with this method correlated well with data collected using an LC/MS method previously validated in a collaborating laboratory (Figure 8).



Hydromorphone

Theo Amt	Calc Amt	% Diff
10.000	10.144	1.44
20.000	19.633	-1.84
50.000	48.252	-3.50
100.000	99.089	-0.91
200.000	208.344	4.17
500.000	511.099	2.22
1000.000	1021.681	2.17
4000.000	3929.714	-1.76
6000.000	5879.941	-2.00

Morphine

Theo Amt	Calc Amt	% Diff
10.000	10.019	0.19
20.000	19.779	-1.11
50.000	48.977	-2.05
100.000	108.054	8.05
200.000	195.171	-2.41
500.000	533.178	6.64
1000.000	970.955	-2.90
4000.000	3833.882	-4.15
6000.000	5864.718	-2.25

Figure 6. Calibration curves for selected opiates

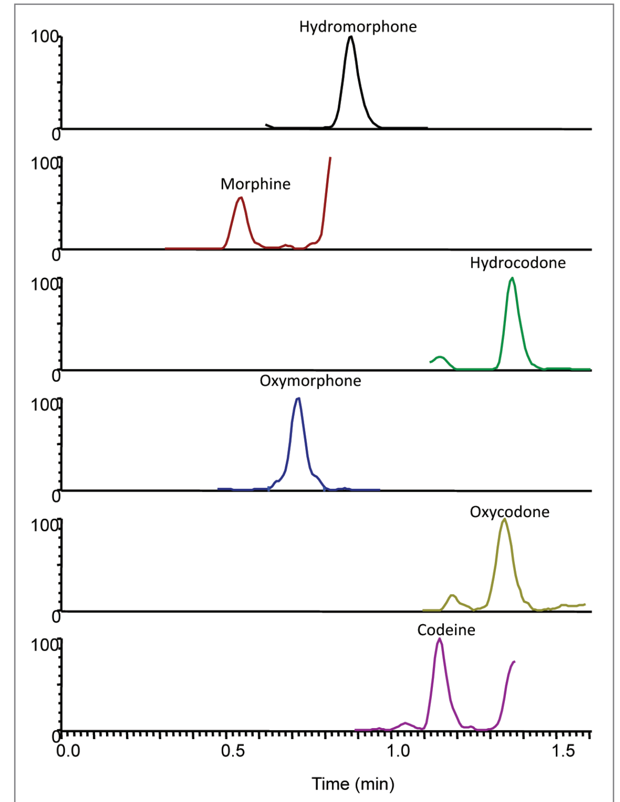


Figure 7. Chromatograms of the lowest opiates calibration standard (10 ng/mL)

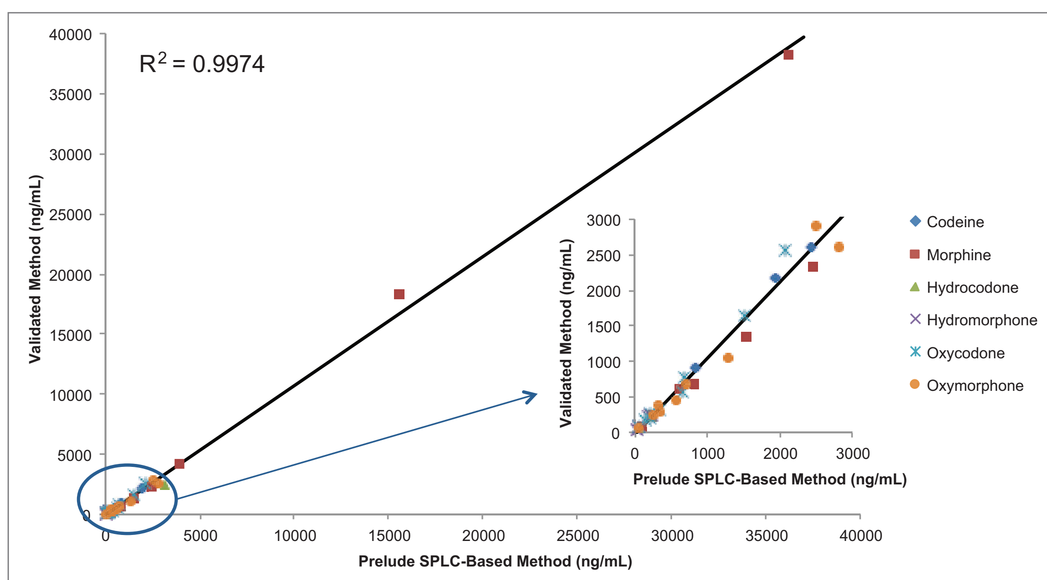


Figure 8. Data correlation between Prelude SPLC-based opiates method and a previously validated LC/MS method

Table 3. Intra- and inter-assay precision for opiates analyses

Compound	Precision % RSD					
	Intra-assay			Inter-assay		
	LQC	MQC	HQC	LQC	MQC	HQC
Morphine	<7.0	<2.8	<2.3	8.3	2.5	3.3
Hydromorphone	<3.7	<1.8	<1.7	4.7	2.2	2.7
Oxymorphone	<5.9	<4.8	<4.9	9.8	4.9	9.8
Codeine	<8.2	<11	<2.2	8.2	4.8	3.0
Hydrocodone	<4.7	<3.8	<2.8	4.7	3.9	4.2
Oxycodone	<7.4	<3.9	<2.8	7.1	3.8	3.6

Table 4. Results of matrix effect experiment showing percent recovery of opiates in spiked urine

Urine Lot#	% Recovery					
	Morphine	Hydromorphone	Oxymorphone	Codeine	Hydrocodone	Oxycodone
1	96.1	93.7	104	102	99.7	93.9
2	99.8	93.8	101	100	99.7	99.6
3	91.0	98.5	101	102	98.1	93.8
4	90.7	96.5	105	103	95.8	101
5	93.9	103	94.9	99.7	97.0	96.9
6	92.3	100	107	109	106	103
7	92.0	97.8	108	109	100	103

Benzodiazepines Analysis

The limits of quantitation were 25 ng/mL and calibration ranges were 25–2000 ng/mL for all benzodiazepines. Figure 9 shows representative calibration curves for selected benzodiazepines. Figure 10 shows representative chromatograms at 25 ng/mL for all benzodiazepines tested. Intra- and inter-assay quality control statistics shown in Table 5 demonstrate the method to be reproducible across the calibration range for these benzodiazepines. Use of deuterated internal standard eliminated the small matrix effects we experienced with the method (Table 6). The data collected with this method correlated well with data collected using an LC/MS method previously validated in a collaborating laboratory (Figure 11).

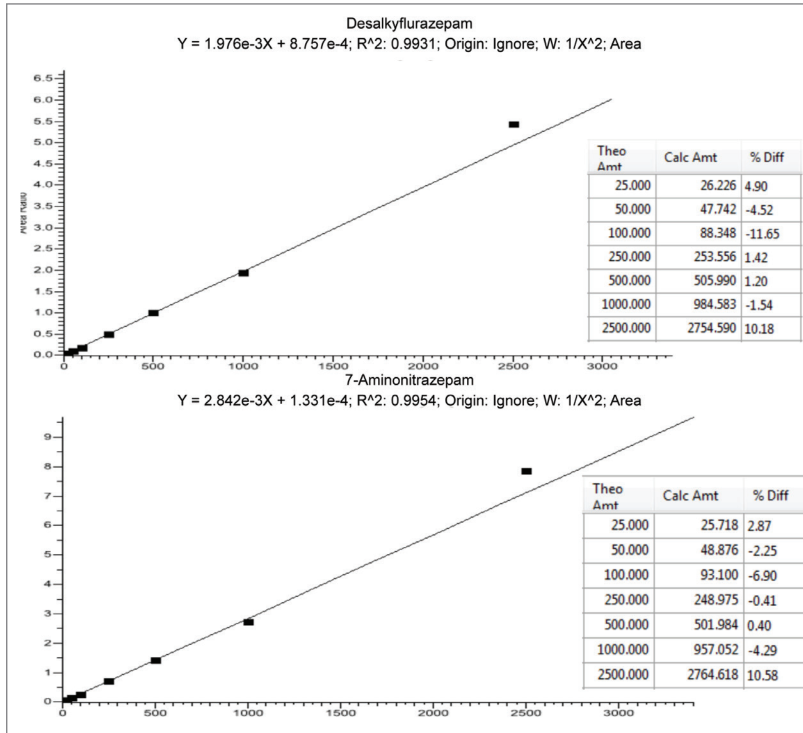


Figure 9. Calibration curves for selected benzodiazepines

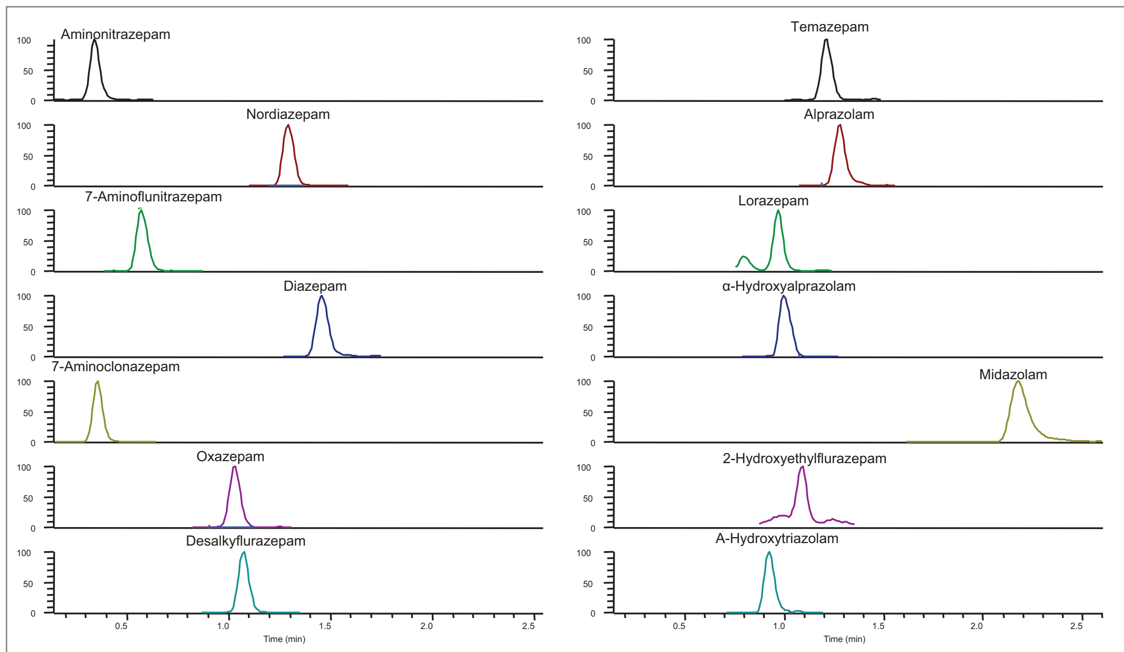


Figure 10. Chromatogram of the lowest benzodiazepines calibration standard (10 ng/mL)

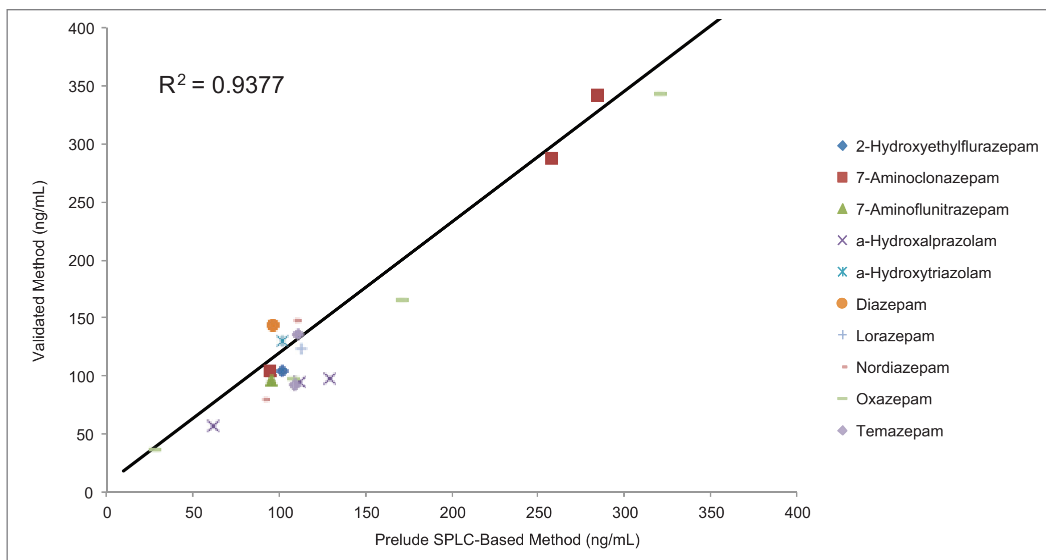


Figure 11. Data correlation between Prelude SPLC-based benzo-diazepines method and a previously validated LC/MS method

Table 5. Intra and inter-assay precision for benzodiazepines analyses

Compound	Precision % RSD			
	Intra-assay		Inter-assay	
	LQC	HQC	LQC	HQC
2-Hydroxyethylflurazepam	<10	<3.3	8.9	4.2
7-Aminoclonazepam	<2.5	<2.7	2.5	2.0
7-Aminoflunitrazepam	<3.6	<3.2	3.1	2.9
7-Aminonitrazepam	<2.7	<3.6	2.4	2.7
α-Hydroxyalprazolam	<5.8	<4.1	5.8	4.5
α-Hydroxytriazolam	<5.9	<4.1	7.2	3.8
Alprazolam	<5.2	<2.1	3.5	2.3
Desalkyflurazepam	<5.3	<5.9	3.6	2.3
Diazepam	<2.8	<3.0	3.1	2.2
Lorazepam	<5.3	<4.5	6.7	3.3
Midazolam	<1.2	<5.4	2.8	1.6
Nordiazepam	<4.0	<4.5	5.1	2.5
Oxazepam	<3.3	<3.2	3.3	3.9
Temezepam	<5.3	<3.1	4.3	3.6

Table 6. Results of matrix effect experiment showing percent recovery of benzodiazepines in spiked urine

Urine Lot#	% Recovery						
	2-Hydroxy-ethylflurazepam	7-Aminoclona-zepam	7-Aminoflunitra-zepam	7-Aminonitra-zepam	α-Hydroxy-alprazolam	α-Hydroxy-triazolam	Alprazolam
1	112	104	103	99.2	111	114	116
2	102	99.8	103	104	112	116	113
3	106	103	101	102	113	116	108
4	112	104	106	100	108	111	114
5	100	102	102	95.8	110	111	108
6	118	105	109	104	113	118	111
7	106	101	99.7	104	111	124	110
8	107	97.8	98.5	101	112	93.0	107

Urine Lot#	% Recovery						
	Desalkyflura-zepam	Diazepam	Lorazepam	Midazolam	Nordiazepam	Oxazepam	Temezepam
1	114	118	110	117	110	105	111
2	109	111	104	116	112	99.8	105
3	108	112	103	117	113	103	102
4	107	114	108	114	118	105	106
5	105	115	108	117	112	99.0	106
6	114	113	109	115	111	104	105
7	108	113	96.2	117	112	98.4	103
8	107	107	101	112	111	95.7	103

Conclusion

Using the Prelude SPLC system, high-throughput, cost-efficient solutions were developed for forensic analysis of opiates and benzodiazepines in urine. The methods met industry requirements for precision, accuracy, and robustness. Implementation of the method on a Prelude SPLC simplified the work flow and resulted in a 40–60% reduction of solvent usage due to the ability of the system to utilize high efficiency, small diameter columns. The mobile phase volumes in developed methods were approximately 3.8 mL per sample, which reduced cost of reagents and waste disposal. Multiplexing into a single mass spectrometer increased MS utilization and reduced overall system hardware costs relative to two independent LC-MS systems. The Prelude SPLC system makes multiplexing of two different methods, with or without on-line sample prep, possible and enabled a throughput of 480 samples in 24 hours. The implementation of methods was facilitated by the many ease-of-use features incorporated into the system.

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Simultaneous Quantitation of 43 Drugs in Human Urine with a "Dilute-and-Shoot" LC-MS/MS Method

Xiang He and Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Key Words

TSQ Quantum Access MAX, forensic toxicology, drugs of abuse, pain management drugs, urine, quantitation

Goal

The goal of this work was to develop a simple "dilute-and-shoot" liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantitation of 43 drugs of abuse, including pain management drugs, in human urine for forensic toxicology purposes. The drugs to be analyzed included opioids, amphetamines, benzodiazepines, cocaine, buprenorphine, methadone, and some of their metabolites. An additional objective was to use ultra-high-pressure liquid chromatography (UHPLC) to improve throughput and sensitivity of the method.

Introduction

LC-MS/MS has become more accepted as the tool for quantitative analysis of drugs in forensic toxicology laboratories. This technique enables simultaneous detection of multiple analytes of interests and is compatible with a simple "dilute-and-shoot" sample preparation method for urine samples.

Methods

Sample Preparation

Nine individual human urine and pure water samples were spiked with 20 and 200 ng/mL of the 43 drugs of abuse, pain management drugs, and with internal standards (IS). The samples were then mixed with β-glucuronidase and incubated at 60 °C for hydrolysis. Methanol was added to the mixture and the supernatant was diluted with water. The final dilution factor was 20. The mixture was centrifuged at 17,000 *g* for 5 minutes. Fifty microliter injections of the supernatant were analyzed by LC-MS/MS.

Blank human urine was used as the matrix for calibration samples. The concentrations of the calibrators were 1, 2, 5, 10, 20, 50, 100, 200, 500, and 1000 ng/mL. Concentration of the internal standards in all samples was 250 ng/mL.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific™ Accela™ 1250 pump and Accela Open autosampler coupled to a Thermo Scientific TSQ Quantum Access MAX™ triple stage quadrupole mass spectrometer. The analytical column was a Thermo Scientific Accucore™ PFP column (50 × 2.1 mm, 2.6 µm particle size) maintained at room temperature. Details of the LC gradient and mobile phases (MP) are as follows:

Time (min)	Flow rate (mL/min)	Gradient	MPA (%)	MPB (%)	MPC (%)
0.00	0.75	Step	95	5	0
0.50	0.75	Ramp	60	40	0
2.60	0.75	Ramp	5	95	0
4.50	1.00	Step	0	100	0
5.50	1.00	Step	0	0	100
5.75	1.00	Step	95	5	0

MPA: 10 mM NH_4Ac and 0.1% formic acid in water
MPB: 10 mM NH_4Ac and 0.1% formic acid in methanol
MPC: acetonitrile/isopropanol/acetone 9:9:2 (v/v/v)

The mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode. The MS conditions were as follows:

Parameter	Value
Spray voltage (V)	4000
Vaporizer temperature (°C)	300
Sheath gas pressure (arbitrary units)	50
Auxiliary gas pressure (arbitrary units)	15
Capillary temperature (°C)	300

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Data were acquired in selected-reaction monitoring (SRM) mode. SRM transitions for the 43 drugs and their internal standards are shown in Table 1. For each analyte and internal standard, two SRM transitions were monitored. One of transition was used as the quantifier and the other as the qualifier. The signal ratio between the qualifier and the quantifier was used to evaluate the validity of the results.

Validation

The validation procedure included tests for the following: 1) matrix effects; 2) lower limit of quantitation (LLOQ), linear range, accuracy, and precision; and 3) carryover.

Table 1. Drug analytes, their corresponding internal standards, and the SRM transitions for both analytes and internal standards

Analyte	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Qualifier Ion (*m/z*)	Ion Ratio (%)	Corresponding Internal Standard	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Qualifier Ion (*m/z*)
6-MAM	328.10	165.10	211.00	86.0	6-MAM-d3	331.20	165.10	211.10
7-Amino-clonazepam	286.00	222.10	250.10	95.0	7-Amino-clonazepam-d4	290.11	226.10	254.10
7-Amino-flunitrazepam	284.10	135.10	226.10	52.0	7-Amino-flunitrazepam-d7	291.11	138.20	230.10
7-Aminonitrazepam	252.10	121.10	224.10	16.0	7-Amino-clonazepam-d4	290.11	226.10	254.10
α-Hydroxy-alprazolam	325.10	216.10	297.10	52.0	α-Hydroxy-alprazolam-d5	330.10	221.10	302.10
Alprazolam	309.40	205.00	281.00	76.0	Temazepam-d5	306.10	260.10	288.10
Amphetamine	136.10	65.30	91.20	10.0	Amphetamine-d5	141.10	92.20	93.20
Benzoylecgonine	290.10	105.10	168.10	30.0	Benzoylecgonine-d3	293.10	105.10	171.10
Benzylpiperazine	177.10	65.30	91.20	16.0	Benzylpiperazine-d7	184.10	70.20	98.20
Buprenorphine	468.30	396.30	414.30	120.0	Diazepam-d5	290.12	198.10	227.10
Carisprodol	261.20	62.10	97.10	58.5	α-Hydroxy-alprazolam-d5	330.10	221.10	302.10
Clonazepam	315.90	214.00	270.00	26.6	Temazepam-d5	306.10	260.10	288.10
Cocaine	304.10	82.20	182.10	17.0	Amphetamine-d5	141.10	92.20	93.20
Codeine	300.10	165.00	215.00	91.0	Codeine-d3	303.20	165.10	215.10
Diazepam	285.10	193.10	222.10	72.0	Diazepam-d5	290.12	198.10	227.10
EDDP	279.20	235.20	250.20	54.5	Temazepam-d5	306.10	260.10	288.10
Fentanyl	337.20	105.20	188.20	67.0	Temazepam-d5	306.10	260.10	288.10
Flunitrazepam	314.40	239.10	268.10	32.5	Temazepam-d5	306.10	260.10	288.10
Flurazepam	388.10	288.10	315.10	11.5	Temazepam-d5	306.10	260.10	288.10
Hydrocodone	300.20	171.00	199.00	34.5	MDA-d5	185.10	110.20	137.10
Hydromorphone	286.11	157.10	185.10	64.0	Benzylpiperazine-d7	184.10	70.20	98.20
Lorazepam	321.00	275.00	303.00	64.0	α-Hydroxy-alprazolam-d5	330.10	221.10	302.10
MDA	180.10	105.20	135.10	79.0	MDA-d5	185.10	110.20	137.10
MDEA	208.10	135.10	163.00	24.0	Nordiazepam-d5	276.10	165.00	213.10
MDMA	194.10	135.10	163.10	40.0	MDMA-d5	199.10	135.10	165.10
Meperidine	248.20	174.20	220.10	28.0	Diazepam-d5	290.12	198.10	227.10
Methadone	310.20	105.10	265.10	29.0	Diazepam-d5	290.12	198.10	227.10
Methamphetamine	150.10	65.30	91.20	9.5	Methamphetamine-d5	155.10	91.20	92.20
Midazolam	326.10	249.20	291.20	28.0	Diazepam-d5	290.12	198.10	227.10
Morphine	286.10	152.10	165.00	78.0	Morphine-d3	289.10	152.10	165.10
Naloxone	328.21	212.00	310.10	23.0	7-Amino-clonazepam-d4	290.11	226.10	254.10
Naltrexone	342.20	270.10	324.20	16.0	MDA-d5	185.10	110.20	137.10
Norbuprenorphine	414.30	187.10	340.30	99.0	Temazepam-d5	306.10	260.10	288.10
Nordiazepam	271.00	140.10	208.10	100.5	Nordiazepam-d5	276.10	165.00	213.10
Norfentanyl	233.20	55.30	84.30	16.0	MDMA-d5	199.10	135.10	165.10
Normeperidine	234.20	111.10	160.10	0.3	Temazepam-d5	306.10	260.10	288.10
Oxazepam	287.00	241.00	269.00	82.0	Oxazepam-d5	292.10	246.10	274.10
Oxycodone	316.20	241.20	298.20	22.5	Benzoylecgonine-d3	293.10	105.10	171.10
Oxymorphone	302.10	227.10	284.20	35.0	7-Amino-clonazepam-d4	290.11	226.10	254.10
PCP	244.20	86.20	159.10	84.5	Diazepam-d5	290.12	198.10	227.10
Propoxyphene	340.20	58.20	91.10	15.0	Diazepam-d5	290.12	198.10	227.10
Temazepam	301.00	255.00	283.00	36.0	Temazepam-d5	306.10	260.10	288.10
Tramadol	264.20	58.30	246.10	3.0	Temazepam-d5	306.10	260.10	288.10

Results and Discussion

Matrix Effects

Matrix effects were assessed with the nine individual human urine samples. Absolute recovery was determined by comparing the signals of unlabeled drugs in urine and water samples. Relative recovery was determined by comparing the analyte/IS ratio in urine and water samples. The recovery/matrix effects results are summarized in Table 2. All 43 drugs had almost full absolute recovery (between 80% and 120%), except morphine for which the matrix effect was compensated by the use of its internal standard, morphine-d3. The observed precision from the nine individual human urine samples was below 15% for most of the 43 drugs.

Table 2. Summary of matrix effects

Drug	Average Absolute Recovery (%, n=9)		CV (%, n=9)		Average Relative Recovery (%, n=9)		CV (%, n=9)	
	20 ng/mL	200 ng/mL	20 ng/mL	200 ng/mL	20 ng/mL	200 ng/mL	20 ng/mL	200 ng/mL
6-MAM	86.7	92.3	16.2	12.2	95.1	100.2	5.6	5.7
7-Amino-clonazepam	96.4	108.4	11.0	11.6	90.3	103.7	6.1	5.8
7-Amino-flunitrazepam	86.8	90.5	11.4	8.4	97.1	102.1	6.3	5.1
7-Aminonitrazepam	86.0	85.5	12.3	9.6	80.6	81.9	9.9	8.2
α-Hydroxy-alprazolam	87.4	87.4	12.9	6.7	99.4	96.2	10.4	4.1
Alprazolam	94.0	89.1	26.0	16.6	95.1	84.8	22.9	13.9
Amphetamine	109.5	112.3	11.8	8.0	112.4	110.8	16.7	3.8
Benzoylecgonine	82.7	85.7	13.0	12.7	98.7	100.9	4.6	3.6
Benzylpiperazine	87.3	85.4	10.0	7.2	100.4	100.6	8.7	7.5
Buprenorphine	108.4	96.9	15.0	6.2	118.1	97.2	14.6	5.4
Carisprodol	88.0	96.3	13.1	11.0	100.5	105.8	13.6	8.4
Clonazepam	100.7	98.4	9.5	6.9	103.4	94.4	13.6	9.5
Cocaine	93.6	93.5	7.4	8.2	95.5	92.2	5.3	5.0
Codeine	93.9	98.9	8.6	8.2	99.3	98.0	3.3	7.2
Diazepam	98.0	96.6	14.0	9.1	106.5	96.7	11.7	6.5
EDDP	103.8	99.2	6.8	2.9	106.8	95.0	13.7	6.2
Fentanyl	98.6	100.9	4.1	2.8	101.4	96.7	10.7	5.8
Flunitrazepam	85.7	86.9	18.8	14.7	87.1	82.9	14.6	12.1
Flurazepam	97.5	103.1	4.2	3.9	100.2	98.8	11.7	5.8
Hydrocodone	91.5	96.4	15.1	13.5	95.2	97.9	7.8	9.6
Hydromorphone	91.2	94.5	11.0	10.4	104.6	110.8	7.2	5.4
Lorazepam	105.7	90.5	16.5	6.0	120.7	99.7	17.2	5.7
MDA	96.6	105.8	16.1	9.6	100.6	107.9	8.7	6.4
MDEA	95.6	94.0	11.8	10.1	99.0	82.8	9.7	10.6
MDMA	92.3	94.3	9.3	7.8	106.1	102.4	2.4	4.1
Meperidine	88.4	88.4	9.8	9.9	96.2	88.5	7.4	7.8
Methadone	101.6	103.2	3.2	3.4	111.1	103.6	8.9	5.4
Methamphetamine	94.6	86.2	12.3	11.1	105.5	94.1	8.4	5.6
Midazolam	98.4	97.4	9.5	5.5	107.1	97.6	6.0	3.5
Morphine	48.1	53.8	6.0	8.2	90.5	98.4	6.9	5.4
Naloxone	124.2	129.4	17.9	16.1	116.1	123.5	9.9	7.7
Naltrexone	96.1	100.2	12.6	10.9	100.3	101.9	5.2	6.1
Norbuprenorphine	76.9	104.6	19.4	14.2	78.9	99.9	20.4	11.6
Nordiazepam	102.8	107.1	21.3	7.3	106.3	94.2	19.8	7.5
Norfentanyl	89.5	92.2	11.4	8.2	103.2	100.1	12.4	4.5
Normeperidine	81.7	92.0	11.9	11.6	83.1	87.7	7.8	8.4
Oxazepam	93.8	91.3	10.8	5.1	113.4	102.1	6.8	4.7
Oxycodone	80.4	84.7	8.8	10.9	97.0	100.1	9.7	6.1
Oxymorphone	107.0	101.4	15.2	12.9	100.1	97.0	8.2	9.0
PCP	100.8	100.5	4.2	4.3	110.3	100.9	8.8	5.1
Propoxyphene	101.3	103.8	6.8	5.8	111.2	104.1	15.3	4.4
Temazepam	95.3	102.2	14.2	7.1	97.3	97.7	12.3	4.6
Tramadol	77.8	84.8	14.1	12.9	78.9	80.8	10.1	10.4

Lower Limit of Quantitation, Linear Range, Accuracy, and Precision

The LLOQ of these 43 drugs and other aspects of analytical performances of this method are summarized in Table 3. Linear fit with 1/X weighting was used for calibration curves of all the 43 drugs. The LLOQ for these 43 drugs was determined to be between 2 and 20 ng/mL except for tramadol, which was 50 ng/mL. At the LLOQ, the accuracy ranged between 89.9% and 118.4%, and precision ranged between 3.6% and 19.5%. The method was linear to 1000 ng/mL for all the drugs. Figure 1 shows the calibration curves of six typical pain management drugs in human urine.

Table 3. Lower limit of quantitation, linear range, accuracy, and precision

Drug	Retention Time (min)	LLOQ (ng/mL)	Accuracy at LLOQ (%, n=4)	CV at LLOQ (%, n=4)	Linear Range (ng/mL)	R^2	Precision 20 ng/mL (%, n=6)	Precision 200 ng/mL (%, n=6)
6-MAM	2.97	2	95.0	14.6	2–1000	0.9955	5.8	2.8
7-Amino-clonazepam	2.76	5	95.1	10.1	5–1000	0.9988	3.4	4.0
7-Amino-flunitrazepam	3.31	5	101.0	13.7	5–1000	0.9980	5.3	4.0
7-Aminonitrazepam	2.51	2	102.0	9.6	2–1000	0.9972	3.7	3.2
α-Hydroxy-alprazolam	3.87	20	94.0	10.0	20–1000	0.9972	6.9	5.9
Alprazolam	4.11	5	94.1	13.1	5–1000	0.9950	2.7	1.2
Amphetamine	2.97	20	94.9	7.7	20–1000	0.9944	5.4	5.5
Benzoylecgonine	2.99	5	92.3	3.6	5–1000	0.9990	2.7	2.0
Benzylpiperazine	2.70	10	96.0	10.4	10–1000	0.9979	9.5	5.0
Buprenorphine	4.50	20	94.7	17.3	20–1000	0.9976	6.0	6.1
Carisprodol	3.80	10	104.5	11.3	10–1000	0.9903	9.5	6.3
Clonazepam	4.00	20	92.7	7.7	20–1000	0.9954	9.1	6.1
Cocaine	4.23	5	101.2	7.4	5–1000	0.9969	4.0	3.7
Codeine	2.82	10	110.4	18.3	10–1000	0.9978	6.6	3.8
Diazepam	4.24	5	93.0	11.9	5–1000	0.9979	7.0	3.4
EDDP	4.90	10	106.5	3.9	10–1000	0.9944	4.5	2.2
Fentanyl	4.62	2	108.9	3.7	2–1000	0.9975	4.8	1.8
Flunitrazepam	4.12	20	93.7	17.2	20–1000	0.9904	6.4	4.4
Flurazepam	4.57	2	118.4	3.6	2–1000	0.9961	4.9	2.4
Hydrocodone	3.16	2	106.6	9.6	2–1000	0.9988	7.8	2.8
Hydromorphone	2.25	2	89.9	13.2	2–1000	0.9979	8.2	3.1
Lorazepam	3.86	20	92.5	17.3	20–1000	0.9943	2.2	9.6
MDA	3.16	10	93.1	6.8	10–1000	0.9974	1.1	3.1
MDEA	3.97	2	104.3	4.5	2–1000	0.9937	7.5	4.4
MDMA	3.61	5	97.3	4.3	5–1000	0.9975	7.6	2.2
Meperidine	4.20	5	101.2	9.6	5–1000	0.9986	5.5	4.6
Methadone	4.95	5	100.3	3.8	5–1000	0.9982	4.2	3.0
Methamphetamine	3.51	5	106.0	5.1	5–1000	0.9979	5.0	4.0
Midazolam	4.48	2	117.1	12.7	2–1000	0.9983	7.0	4.3
Morphine	1.71	5	93.0	13.6	5–1000	0.9990	5.0	3.3
Naloxone	2.86	10	102.3	10.9	10–1000	0.9944	3.3	2.9
Naltrexone	3.11	5	101.9	7.0	5–1000	0.9985	5.1	1.6
Norbuprenorphine	4.13	20	101.4	14.4	20–1000	0.9955	3.9	8.4
Nordiazepam	4.06	10	97.1	19.5	10–1000	0.9948	8.4	3.8
Norfentanyl	3.68	10	102.5	6.3	10–1000	0.9985	7.1	2.3
Normeperidine	4.00	2	116.2	11.2	2–1000	0.9982	7.3	4.2
Oxazepam	3.88	20	108.0	15.0	20–1000	0.9970	10.9	6.4
Oxycodone	3.03	5	91.9	11.7	5–1000	0.9982	2.6	2.3
Oxymorphone	2.01	2	93.3	9.5	2–1000	0.9946	10.0	2.6
PCP	4.83	2	100.9	4.0	2–1000	0.9981	7.8	3.0
Propoxyphene	4.70	10	113.6	4.1	10–1000	0.9978	7.3	5.2
Temazepam	4.05	5	104.6	16.9	5–1000	0.9981	5.6	2.2
Tramadol	4.04	50	98.8	2.5	50–1000	0.9970	NA	2.5

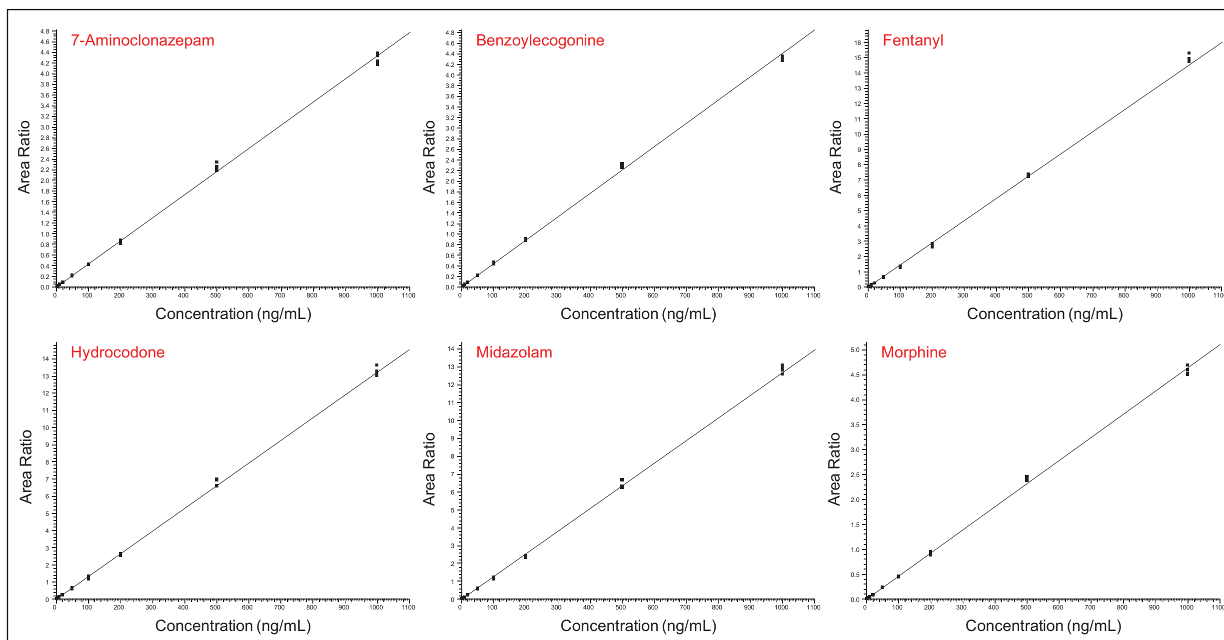

Figure 1. Calibration curves of six selected drugs in spiked human urine

Method precision was also assessed with spiked human urine samples at low and high quality control (QC) concentrations of 20 and 200 ng/mL, respectively (Table 2). Precision values at low (20 ng/mL) and high (200 ng/mL) quality control concentrations ranged between 1.1% and 10.9% (Table 2). Figure 2 shows both the quantifier and qualifier SRM chromatograms of 20 selected pain management drugs spiked at 20 ng/mL in human urine.

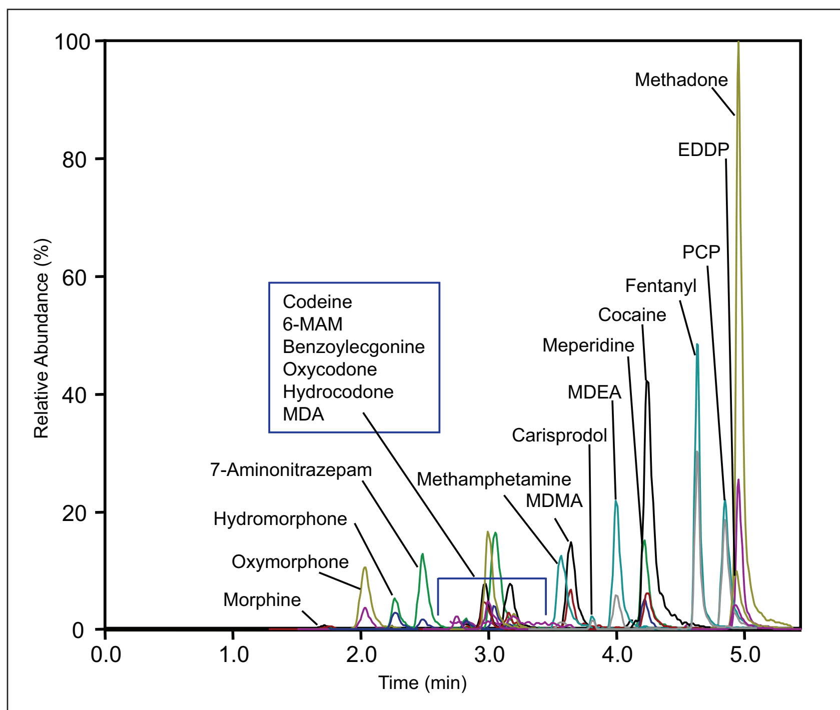

Figure 2. SRM chromatograms of 20 selected drugs at 20 ng/mL in spiked human urine

Carryover

The lowest calibrator was analyzed after the highest calibrator. No carryover causing elevated measurements of the drugs in the lowest calibrator was observed.

Conclusion

The developed method provides a simple, fast, and sensitive way for forensic toxicology labs to simultaneously quantify 43 drugs of abuse, including pain management drugs, in human urine by LC-MS/MS. The method provided LLOQ values of 2–20 ng/mL for 42 of the 43 drugs, and was linear to 1000 ng/mL. Minimal ion suppression and no carryover were observed in matrix samples. At the LLOQ, the accuracy ranged between 89.9% and 118.4%. Method precision ranged between 1.1% and 10.9% at low and high QC samples.

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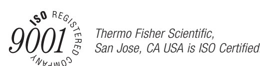

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Application Note 571

Simultaneous Quantitation of 19 Drugs in Human Plasma and Serum by LC-MS/MS

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Key Words

TSQ Vantage, drug monitoring research, clinical research, CSS, plasma, serum

Goal

To develop a simple, fast, and sensitive LC-MS/MS method for the simultaneous quantitation of 19 drugs in human plasma and serum.

Introduction

Liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) has become an accepted tool for quantitative analysis of drugs in clinical research laboratories. LC-MS/MS enables simultaneous, sensitive detection and quantitation of multiple analytes of interest. In this study, 19 drugs of various types, including antipsychotics, antiepileptics/anticonvulsants, antianginals, and antidepressants, were monitored and simultaneously quantitated using LC-MS/MS.

Experimental

Sample Preparation

Nineteen drugs (Table 1) and 15 isotopically labeled internal standards of the drugs were used in this research.

Table 1. Drug analytes

Analytes		
Amitriptyline	Dothiepin	Nortriptyline
Bromazepam	Doxepin	Oxazepam
Clobazam	Flunitrazepam	Perhexilline
Clomipramine	Imipramine	Temazepam
Clonazepam	Lamotrigine	Trimipramine
Clozapine	Levetiracetam	
Diazepam	Nitrazepam	

To assess signal recovery and determine the best dilution factor, 9 randomly chosen individual human-donor plasma samples were spiked with the 19 drugs at 40 ng/mL and 15 isotopically labeled internal standards at 100 ng/mL. These samples were mixed (1:3, v/v) with a 1:1 methanol/acetonitrile mixture. The samples were vigorously vortexed and stored at -30 °C for 30 min. The samples were then centrifuged at 17,000 *g* for 5 min. Supernatant (20 µL) was drawn off and diluted 10-fold, 20-fold, and 50-fold with 10% methanol in water to final dilution factors of 40x, 80x, and 200x.

Calibration and linearity standards were prepared by spiking a matrix of charcoal-stripped human serum (CSS) with the 15 internal standards at 100 ng/mL and the 19 drug analytes at 4, 10, 20, 40, 100, 200, and 400 ng/mL. The samples were processed as above and diluted to a final dilution factor of 200x.

For accuracy and precision testing, CSS samples were spiked with the 15 isotopically labeled internal standards at 100 ng/mL and the 19 drugs at both 40 ng/mL and 200 ng/mL. The samples were processed as above and diluted to a final dilution factor of 200x.

Also for accuracy and precision testing, 9 individual human-donor plasma samples were spiked with the 15 isotopically labeled internal standards at 100 ng/mL and the 19 drugs at 40 ng/mL. The samples were processed as above and diluted to a final dilution factor of 200x.

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Liquid Chromatography

Chromatographic separations were performed with a Thermo Scientific Accela 1250 pump and Accela Open autosampler. The analytical column was a Thermo Scientific Accucore PFP column (50 × 2.1 mm, 2.6 µm particle size). The column was maintained at room temperature. Details of the LC gradient and information on the mobile phases (MP) are shown in Table 2. The injection volume was 40 µL.

Table 2. LC gradient

Time (min)	Flow rate (mL/min)	Gradient	MPA (%)	MPB (%)	MPC (%)
0.00	0.4	Step	95	5	0
0.50	0.4	Step	90	10	0
1.50	0.4	Ramp	50	50	0
2.00	0.4	Ramp	5	95	0
6.50	0.4	Step	0	100	0
7.75	0.6	Step	0	0	100
8.00	0.6	Step	95	5	0

MPA: 10 mM ammonium acetate and 0.1% formic acid in water
MPB: 10 mM ammonium acetate and 0.1% formic acid in methanol
MPC: acetonitrile:isopropanol:acetone 9:9:2 (v/v/v)

Mass Spectrometry

MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer. The mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode. The MS conditions were as follows:

Spray voltage (V):	4000
Vaporizer temperature (°C):	300
Sheath gas pressure (arbitrary units)	50
Auxiliary gas pressure (arbitrary units)	15
Capillary temperature (°C)	300

Data were acquired in selected-reaction monitoring (SRM) mode. Detailed SRM settings for the 19 drugs and their internal standards are shown in Table 3. For each analyte and internal standard, two SRM transitions were monitored. One was used as the quantifier and the other as the qualifier. The signal ratio between the qualifier and the quantifier was used to evaluate the validity of the results. Results that varied by more than 20% of the nominal ratio were considered invalid data points.

The validation procedure included tests for: 1) signal recovery, 2) lower limit of quantitation (LLOQ) and linear range, 3) accuracy and precision, and 4) carryover.

Table 3. SRM settings for the analytes and internal standards

Analyte	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Collision Energy (V)	Qualifier Ion (*m/z*)	Collision Energy (V)	S-Lens (V)
Amitriptyline	278.10	202.10	56	233.10	16	74
Bromazepam	316.11	182.10	31	209.10	26	95
Clobazam	301.10	259.10	20	224.10	32	90
Clomipramine	315.10	86.00	17	58.00	35	74
Clonazepam	316.00	270.10	25	214.00	37	101
Clozapine	327.10	270.10	23	192.00	42	94
Diazepam	285.10	193.10	32	154.00	27	88
Dothiepin	296.10	202.10	53	221.10	45	71
Doxepin	280.10	165.10	51	107.00	23	80
Flunitrazepam	314.10	268.10	26	239.10	34	92
Imipramine	281.20	86.00	16	58.00	35	69
Lamotrigine	256.00	211.00	26	109.00	49	89
Levetiracetam	171.10	126.10	14	69.00	28	36
Nitrazepam	282.10	236.10	24	207.10	34	97
Nortriptyline	264.20	233.20	13	91.10	32	66
Oxazepam	287.10	269.10	14	104.10	33	81
Perhexilline	278.20	95.10	28	67.00	34	87
Temazepam	301.11	255.10	22	283.10	13	72
Trimipramine	295.20	100.10	16	58.10	35	71
Internal Standards						
Amitriptyline-D3	281.21	91.10	32	233.20	16	85
Clomipramine-D3	318.20	89.10	18	61.10	36	75
Clonazepam-D4	320.10	274.10	26	218.10	35	102
Clozapine-D4	331.20	272.20	25	192.10	45	102
Diazepam-D5	290.10	198.10	31	154.00	26	89
Doxepin-D3	283.20	107.00	23	77.00	46	78
Flunitrazepam-D7	321.10	275.20	26	246.20	35	96
Imipramine-D3	284.20	89.10	16	61.10	35	69
Lamotrigine-13C, 15N4	261.00	214.00	26	109.10	50	104
Levetiracetam-D6	177.10	132.20	14	69.10	30	38
Nitrazepam-D5	287.11	185.10	37	212.10	34	100
Nortriptyline-D3	267.20	91.00	33	233.20	14	66
Oxazepam-D5	292.10	246.10	22	274.10	15	84
Temazepam-D5	306.10	260.10	23	288.10	13	83
Trimipramine-D3	298.20	103.10	16	61.10	35	72

Results and Discussion

Signal Recovery

Plasma and serum are complex matrices. The matrix content in them can significantly affect the detection of drugs by ESI MS. Therefore, three different dilution factors after protein precipitation (40-fold, 80-fold, and 200-fold) were compared. The LC-MS/MS signals of the analytes in the plasma samples were compared to LC-MS/MS signals from solvent blanks with the same spikes. The 200-fold sample dilution produced the best signal recovery and minimum ion suppression (Table 4 and Figures 1 and 2). For all of the subsequent analyses, all samples were prepared with a 200-fold final dilution factor.

Table 4. Absolute mean signal recovery of 19 drugs at 40 ng/mL in 9 human plasma samples diluted 40-fold, 80-fold, and 200-fold, as compared to a similarly spiked solvent blank

Analyte (40 ng/mL)	Absolute mean signal recovery (%)		
	n=9 200x dilution	n=9 80x dilution	n=9 40x dilution
Amitriptyline	107.9	53.9	79.4
Bromazepam	125.7	49.7	56.6
Clobazam	78.6	43.4	54.4
Clomipramine	103.6	57.5	84.1
Clonazepam	65.9	36.0	32.3
Clozapine	81.5	60.4	56.7
Diazepam	78.4	45.6	57.9
Dothiepin	124.6	53.4	83.9
Doxepin	110.8	57.4	84.0
Flunitrazepam	77.8	44.1	51.9
Imipramine	107.2	50.6	82.8
Lamotrigine	71.5	45.1	52.8
Levetiracetam	86.7	48.2	58.3
Nitrazepam	77.8	38.4	41.7
Nortriptyline	83.7	44.5	62.2
Oxazepam	74.5	41.9	52.7
Perhexilline	94.9	152.8	190.0
Temazepam	74.7	44.6	55.1
Trimipramine	98.4	49.1	76.4

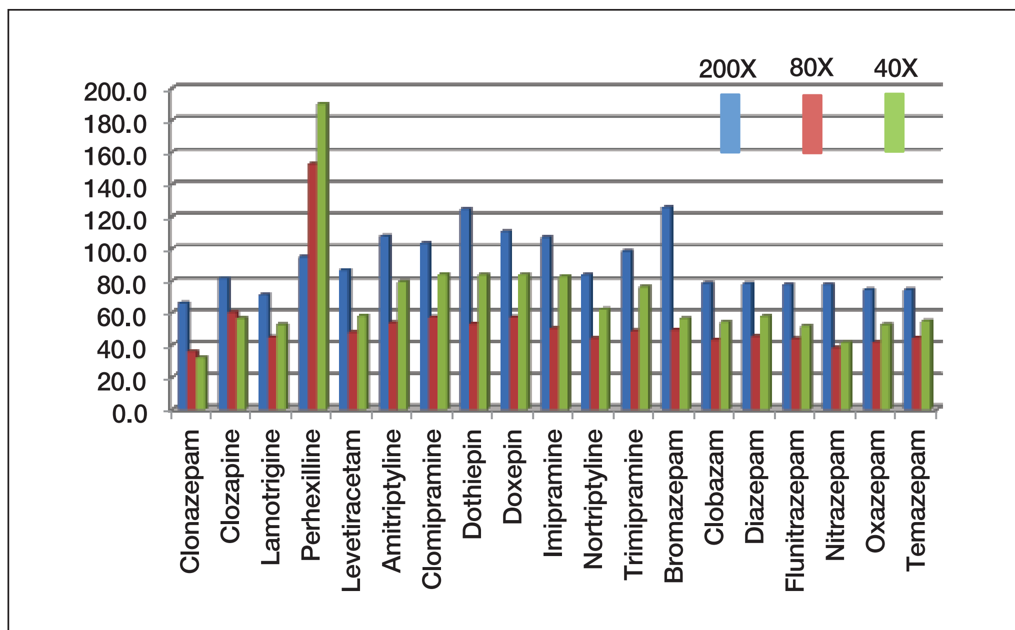

Figure 1. Mean signal recovery of 19 drugs at 40 ng/mL in 9 human plasma samples diluted 40-fold, 80-fold, and 200-fold, as compared to a similarly spiked solvent blank

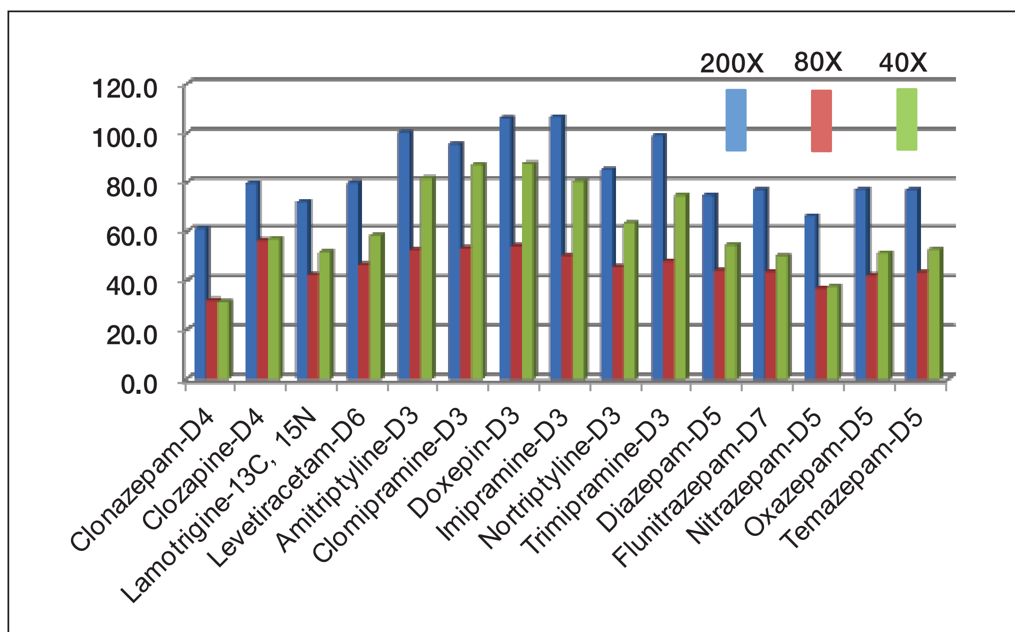

Figure 2. Mean signal recovery of 15 internal standards at 100 ng/mL in 9 human plasma samples diluted 40-fold, 80-fold, and 200-fold, as compared to a similarly spiked solvent blank

Lower Limit of Quantitation and Linear Range

The lower limit of quantitation (LLOQ), linearity, and ion ratio test parameters for the 19 drugs are summarized in Table 5. For calibration curves, a linear fit with 1/X weighting was used. The LLOQ for these 19 drugs were determined to be between 4 and 20 ng/mL. The method was linear to 400 ng/mL for all the drugs. Figure 3 shows the calibration curve of clozapine in CSS. Figure 4 shows the overlaid SRM chromatograms (quantifier and qualifier) of all the 19 drugs at 20 ng/mL in CSS.

Table 5. LLOQ and linearity summary for 19 drugs

Analyte	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Qualifier Ion (*m/z*)	Ion Ratio (%)	Ion Ration Window (±%)	LLOQ (ng/mL)	Linear Range (ng/mL)	R^2
Amitriptyline	278.10	202.10	233.10	105	21	4	4–400	0.9941
Bromazepam	316.11	182.10	209.10	90	18	10	10–400	0.9955
Clobazam	301.10	259.10	224.10	37	7	10	10–400	0.9967
Clomipramine	315.10	86.00	58.00	35	7	4	4–400	0.9933
Clonazepam	316.00	270.10	214.00	35	7	10	10–400	0.9960
Clozapine	327.10	270.10	192.00	70	14	4	10–400	0.9974
Diazepam	285.10	193.10	154.00	67	13	4	4–400	0.9951
Dothiepin	296.10	202.10	221.10	84	17	10	10–400	0.9937
Doxepin	280.10	165.10	107.00	180	36	4	4–400	0.9955
Flunitrazepam	314.10	268.10	239.10	39	8	4	4–400	0.9973
Imipramine	281.20	86.00	58.00	35	7	4	4–400	0.9972
Lamotrigine	256.00	211.00	109.00	50	10	10	10–400	0.9881
Levetiracetam	171.10	126.10	98.10	4.6	2	10	10–400	0.9945
Nitrazepam	282.10	236.10	207.10	35	7	4	4–400	0.9980
Nortriptyline	264.20	233.20	91.10	73	15	4	4–400	0.9948
Oxazepam	287.10	269.10	104.10	13	4	10	10–400	0.9943
Perhexilline	278.20	95.10	67.00	66	13	20	20–400	0.9755
Temazepam	301.11	255.10	283.10	25	5	4	4–400	0.9948
Trimipramine	295.20	100.10	58.10	44	9	4	4–400	0.9968

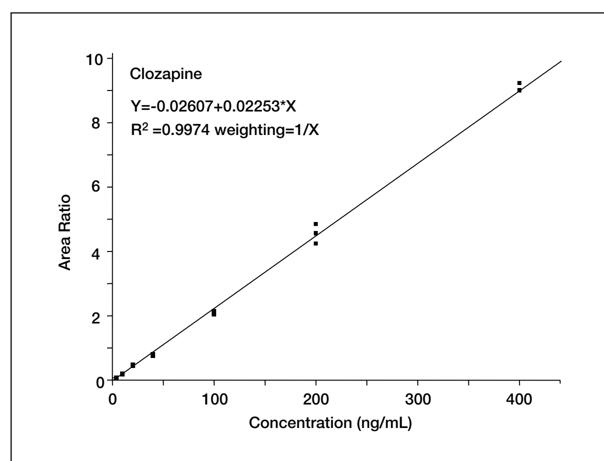

Figure 3. Calibration curve of clozapine in CSS

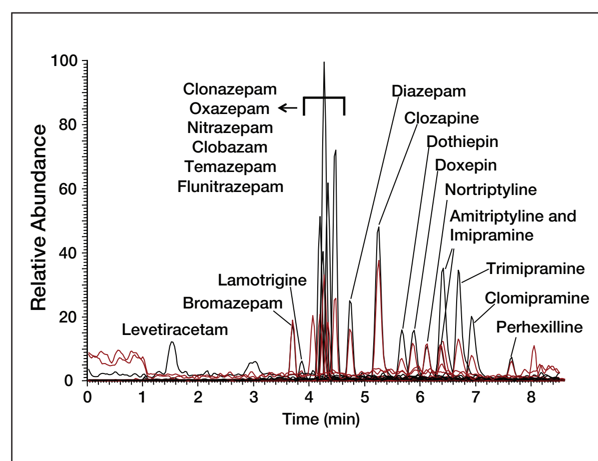

Figure 4. SRM chromatograms of all 19 drugs at 20 ng/mL in CSS after 200-fold dilution

Accuracy and Precision

Accuracy and precision were first assessed with CSS spiked at concentrations of 40 and 200 ng/mL (Table 6). Overall accuracy ranged between 82.4% and 111.3%. Inter- and intra-batch precision (coefficient of variation) values at low (40 ng/mL) and high (200 ng/mL) concentrations varied between 1.4% and 13.5%. Accuracy and intra-batch precision were also assessed in the 9 individual human-donor plasma samples spiked with 40 ng/mL drugs. The results were satisfactory (Table 7).

Table 6. Accuracy and precision summary for analysis of 19 drugs in CSS

Analyte	40 ng/mL					200 ng/mL				
	Precision				Accuracy	Precision				Accuracy
	Intra1 (%) n=5	Intra2 (%) n=5	Intra3 (%) n=5	Inter (%) n=15	Inter (%) n=15	Intra1 (%) n=5	Intra2 (%) n=5	Intra3 (%) n=5	Inter (%) n=15	Inter (%) n=15
Amitriptyline	8.5	10.4	11.5	9.7	87.8	4.6	3.8	9.7	6.3	100.7
Bromazepam	10.1	2.9	3.8	6.9	89.9	3.1	4.0	2.1	3.3	104.2
Clobazam	2.5	3.4	8.6	5.1	90.6	5.5	4.0	4.3	4.6	101.7
Clomipramine	8.3	8.1	6.4	8.0	106.3	3.2	3.1	3.1	4.8	109.4
Clonazepam	3.6	6.4	6.1	5.2	101.4	5.6	2.1	3.3	4.4	107.4
Clozapine	5.7	3.4	5.1	5.4	96.5	4.4	4.3	2.4	3.6	111.3
Diazepam	4.9	6.9	5.9	5.9	88.8	2.7	4.7	3.6	3.6	101.7
Dothiepin	3.7	8.9	5.4	6.1	99.5	4.2	2.5	4.0	4.9	108.2
Doxepin	5.8	10.8	11.9	10.0	96.4	4.5	4.5	2.9	4.5	108.8
Flunitrazepam	1.4	7.0	4.2	5.1	82.4	4.7	4.2	4.3	4.5	100.8
Imipramine	3.1	2.9	2.0	2.8	87.0	1.6	3.1	3.2	2.9	102.2
Lamotrigine	7.0	5.2	8.9	7.2	96.9	3.9	2.5	3.4	3.8	105.8
Levetiracetam	10.9	3.9	9.5	8.3	99.1	5.4	3.0	8.9	5.9	107.8
Nitrazepam	3.8	4.1	6.0	5.2	85.1	5.7	3.8	5.4	4.7	97.3
Nortriptyline	6.9	4.9	4.6	5.2	97.7	2.3	3.9	4.3	3.9	110.5
Oxazepam	8.3	5.5	9.2	7.6	96.5	5.0	7.1	1.7	5.2	106.3
Perhexilline	8.0	12.7	12.7	13.5	86.5	2.2	1.9	6.9	4.4	107.7
Temazepam	7.7	5.5	3.7	6.1	95.3	2.7	2.5	4.8	3.4	104.7
Trimipramine	3.6	3.0	6.1	4.1	89.0	2.9	3.7	3.9	3.7	103.4

Table 7. Accuracy and precision summary for analysis of 19 drugs in 9 individual human-donor plasma samples

Analyte (40 ng/mL)	Mean Measured (ng/mL), n=9	Accuracy (%) n=9	Precision (%) n=9
Amitriptyline	47.2	118.1	7.2
Bromazepam	33.7	84.3	18.0
Clobazam	42.8	107.0	15.0
Clomipramine	41.7	104.2	12.9
Clonazepam	41.4	103.4	13.4
Clozapine	38.6	96.4	9.0
Diazepam	37.2	93.1	8.8
Dothiepin	38.3	95.8	8.1
Doxepin	41.2	102.9	18.5
Flunitrazepam	34.8	87.0	7.7
Imipramine	37.4	93.4	6.5
Levetiracetam	40.0	100.1	7.7
Lamotrigine	37.2	93.0	18.2
Nitrazepam	38.9	97.3	7.0
Nortriptyline	36.2	90.5	6.2
Oxazepam	35.3	88.2	7.2
Perhexilline	42.8	106.9	9.6
Temazepam	36.1	90.3	9.1
Trimipramine	35.9	89.8	7.9

Carryover

The lowest calibrator was analyzed after the highest calibrator, and we did not observe any carryover causing elevated measurements of the drugs in the lowest calibrator.

Conclusion

We have developed a simple, fast, and sensitive LC-MS/MS clinical research method for simultaneously quantitation of 19 drugs in human plasma. The method had LLOQ values of 4–20 ng/mL for all 19 drugs and was linear to 400 ng/mL. Ion suppression was not observed in matrix samples. Accuracy and precision of the method were successfully accessed in both CSS and human plasma samples.

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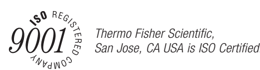

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Quantitation of Six Opioids in Urine with Super-Dilution and Microflow LC-MS/MS

Xiang He and Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Key Words

TSQ Vantage, Microflow, LC-MS/MS, Forensic Toxicology

Goal

To quantitate six opioids in urine with 500-fold urine dilution and microflow LC-MS/MS for forensic toxicology use, using the Thermo Scientific Dionex UltiMate 3000 RSLCnano LC system and the Thermo Scientific TSQ Vantage mass spectrometer.

Introduction

Morphine, codeine, hydromorphone, hydrocodone, oxymorphone and oxycodone are some of the most abused opioids in the United States. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been widely used for their quantitation in forensic toxicology. The analytical methods typically use normal LC flow rates (~0.5 mL/min) and sample preparation usually involves solid phase extraction (SPE) for sensitive detection. Microflow LC uses significantly lower flow rates (15 to 50 µL/min). With the same sample amount and identical LC peak width, the reduction in LC flow rate results in a much-improved detection limit for concentration-dependent detection techniques such as electrospray ionization (ESI) mass spectrometry. Because of this sensitivity increase, we can achieve a similar analytical performance for sensitive measurements of urine opioids for forensic toxicology purposes with a simple "dilute-and-shoot" approach.

Our goal was to use a super-dilution approach to improve the dilute-and-shoot detection of opioids in urine by minimizing matrix effects, and to compensate the sensitivity decrease from super-dilution by using microflow LC. We anticipated savings in solvent consumption and the cost of waste disposal, better environmental conservation, and improved longevity of the LC-MS/MS system.

Methods

Sample Preparation

Urine samples were spiked with internal standards (IS) and then mixed with β-glucuronidase and incubated at 60 °C for hydrolysis. Methanol was added to the mixture and the supernatant was diluted. The tested dilution factors were 100, 250 and 500. The mixture was centrifuged at 17,000 *g* for 5 minutes, and 20 µL of supernatant was injected for microflow LC-MS/MS analysis.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a TSQ Vantage™ triple stage quadrupole mass spectrometer coupled to an UltiMate™ 3000 RSLCnano LC system equipped with a microflow flow rate selector. The microflow LC plumbing was set up in "pre-concentration on a trapping column" mode (Figure 1). The temperature of the columns was maintained at 35 °C. The trapping column was a Thermo Scientific Hypersil GOLD PFP drop-in guard cartridge (10 × 1 mm, 5 µm particle size) in the guard holder, and the analytical column was a Hypersil GOLD™ PFP column (100 × 0.32 mm, 5 µm particle size). LC connections were made with Thermo Scientific Dionex nanoViper fingertight fittings. The LC gradients for sample loading and analytical elution are shown in Figure 2. The mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode. Data was acquired in selected-reaction monitoring (SRM) mode. Detailed source parameters and SRM settings are shown in Figure 3. For each analyte, two SRM transitions were monitored. One of them was used as the quantifier and the other as qualifier. The signal ratio between the qualifier and the quantifier was used to evaluate the validity of the results, and any ratio outside 20% (relative to the ratio) was considered an invalid data point.

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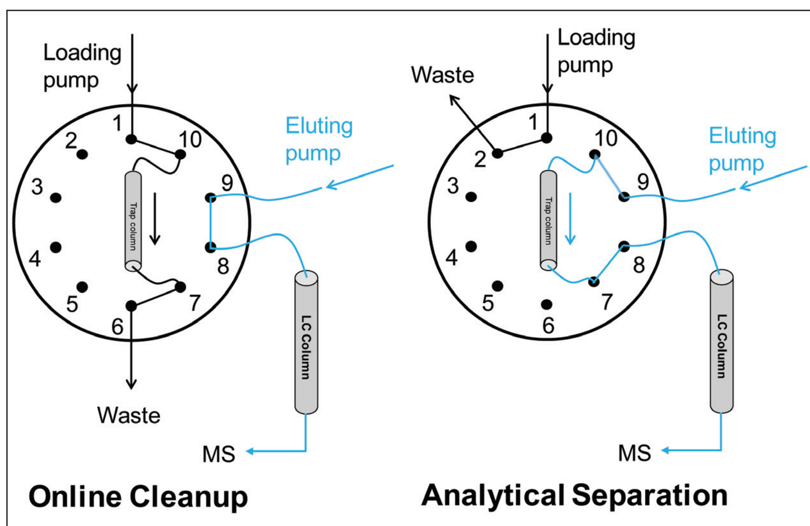

Figure 1. Microflow LC setup with pre-concentration trapping column

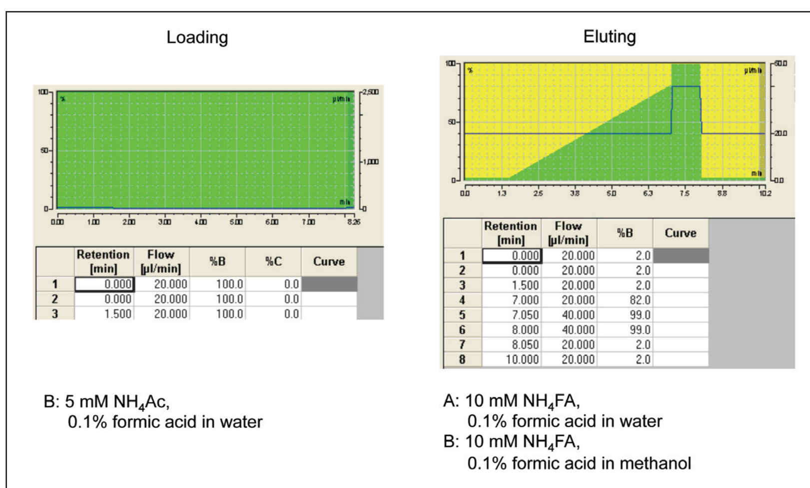

Figure 2. LC gradients of microflow LC with online clean-up

Polarity: positive
Spray Voltage (V): 4000
Vaporizer Temperature (°C): 150
Capillary Temperature (°C): 270
Sheath Gas (AU): 15
Aux Gas (AU): 2

Scan Mode: SRM
Scan Width (*m/z*): 0.02
Scan Time (Sec): 0.1
Q1 (FWHM, *m/z*): 0.7
Q3 (FWHM, *m/z*): 0.7
Collision Gas (Torr): 1.5

Analytes	Precursor (*m/z*)	Quantifier (*m/z*)	Qualifier (*m/z*)	Ion Ratio (%)	IR Window (%)
Morphine	286.1	152.2	201.1	90.0	18.0
Codeine	300.1	165.1	215.1	68.0	13.6
Hydromorphone	286.11	185.1	157.1	79.0	15.8
Hydrocodone	300.11	199.1	171.1	39.0	7.8
Oxymorphone	302.1	227	198.1	69.0	13.8
Oxycodone	316.1	298.1	256.1	22.5	4.5

Figure 3. MS source parameters and SRM transitions

Results and Discussion

Validation

The validation procedure includes tests for 1) recovery; 2) lower limit of quantitation (LLOQ), dynamic range, accuracy; 3) precision; and 4) carryover.

Recovery

First, we determined the optimal dilution factor for urine sample preparation. Twelve lots of blank human urine samples, six lots of donor urine samples, and two water samples were spiked with the IS, hydrolyzed, and diluted 100-, 250- and 500-fold with water. The SRM signals of the internal standards from the urine samples and the water samples were compared for absolute recovery. Table 1 shows the average recoveries (n=18) for the six opioids using different dilution factors. Clearly, the 500-fold dilution led to the highest recoveries for all six opioids.

We used the 500-fold dilution to determine the recoveries for unlabeled opioids spiked into 12 lots of blank urine samples. Two concentrations of opioids at 100 and 500 ng/mL were tested. The absolute recovery was determined by comparing the signals of unlabeled opioids in urine and water samples. The relative recovery was determined by comparing the analyte/IS ratio in urine and water samples. The recovery results are summarized in Table 2. There was minimum ion suppression for morphine, codeine, hydromorphone and hydrocodone. Although there was moderate ion suppression for oxymorphone and oxycodone even after 500-fold dilution, the relative recoveries against their IS were nearly 100% in both concentration levels after compensation from the IS.

Table 1. Dilution factor test results

Recovery (%, n=18)	500x	250x	100x
Morphine-d3	101.2	86.6	85.4
Codeine-d3	99.5	88.0	79.7
Hydromorphone-d6	85.9	73.1	63.7
Hydrocodone-d3	78.0	68.2	67.2
Oxymorphone-d3	59.9	45.1	43.2
Oxycodone-d3	68.2	52.3	42.3

Table 2. Summary of recoveries with 500-fold dilution

		100 ng/mL[a]		500 ng/mL[a]	
Analyte	Recovery (%)	Average (%, n=12[b])	Standard Deviation (%, n=12)	Average (%, n=12)	Standard Deviation (%, n=12)
Morphine	Absolute	76.4	6.8	78.6	5.4
	Relative	92.1	10.9	96.1	9.6
Codeine	Absolute	86.5	6.0	89.7	6.2
	Relative	88.7	10.6	95.6	8.2
Hydromorphone	Absolute	74.4	7.1	73.2	6.6
	Relative	92.8	8.1	89.9	7.0
Hydrocodone	Absolute	82.6	9.0	71.8	6.7
	Relative	101.9	17.1	83.6	13.4
Oxymorphone	Absolute	57.5	7.6	57.9	7.0
	Relative	103.7	17.8	103.0	15.1
Oxycodone	Absolute	63.4	9.9	68.7	8.1
	Relative	90.6	8.5	103.8	8.5

[a] Two levels of spiked opioids concentrations were tested.

[b] Twelve different individual urine lots were tested and compared to water samples (n=2).

Lower Limit of Quantitation (LLOQ), Dynamic Range, and Accuracy

Blank human urine samples were spiked with the six opioids and their IS. Concentrations of the opioids ranged from 20 to 5000 ng/mL. At each concentration level, three individually processed replicates were tested. The concentration of IS was 100 ng/mL for all samples. Linearity samples were analyzed in triplicate along with one set of calibrators, which were also prepared in blank human urine. The calibration curves for morphine and codeine (Figures 4 and 5) were constructed by plotting the analyte/IS peak area ratio vs. analyte concentration.

The linearity was determined to be 20 to 5000 pg/mL for all six opioids. The LLOQ for the six opioids were determined to be 20 ng/mL. At LLOQ, the accuracy (n=3) ranged from 99.2% to 115.5% for the six opioids and the precision (n=3) ranged from 3.9% to 8.8% (Table 3). Within the linear range, the accuracies (at higher than LLOQ levels) were within 11.2% for the six opioids (data not shown). Figures 4 and 5 show the calibration curves for morphine and codeine. Figure 6 shows the SRM chromatograms of the six opioids at their LLOQ in spiked human urine. The signal-to-noise ratios for all six opioids at their LLOQs were excellent.

Table 3. LLOQ, linear range and accuracy for the six opioids in urine

Analyte	LLOQ (ng/mL)	Linear range (ng/mL)	Accuracy at LLOQ (%, n=3)	Precision at LLOQ (%, n=3)
Morphine	20	20-5000	100.8	6.1
Codeine	20	20-5000	102.1	6.9
Hydromorphone	20	20-5000	115.5	8.8
Hydrocodone	20	20-5000	99.2	3.9
Oxymorphone	20	20-5000	102.3	6.2
Oxycodone	20	20-5000	107.4	4.4

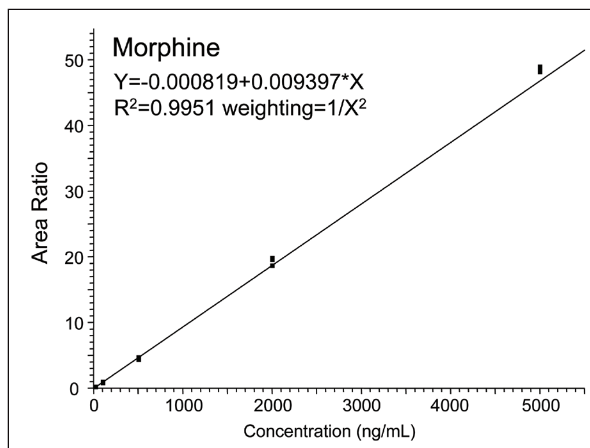

Figure 4. Calibration curve of morphine in human urine

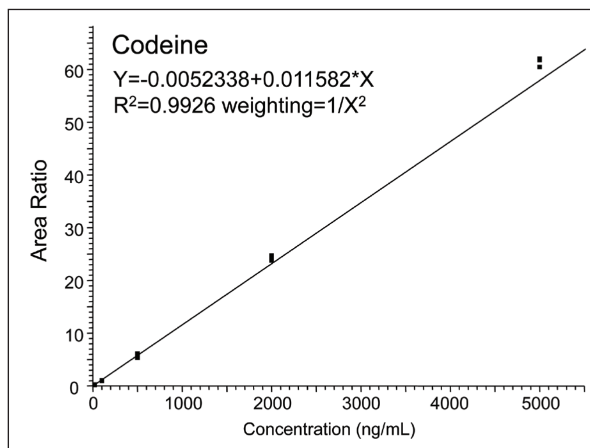

Figure 5. Calibration curve of codeine in human urine

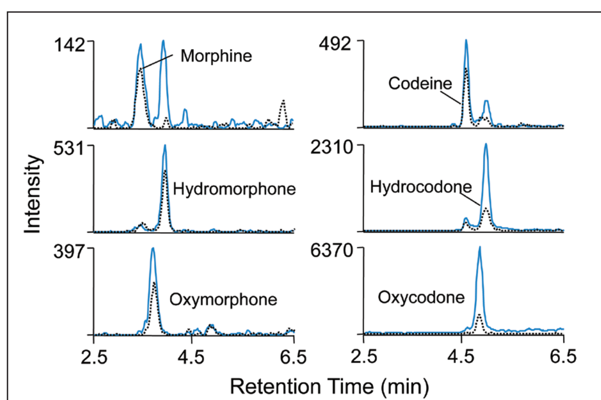

Figure 6. SRM chromatograms (quantifier: solid line; and qualifier: dotted line) of the six opioids at LLOQ in spiked human urine

Precision

Precision was assessed with spiked human urine at concentrations of 40 and 200 ng/mL. Inter- and intra-assay CV values at low and high quality-control concentrations varied between 5.0% and 12.9% (Table 4).

Table 4. Precision data

Precision (%)	Intra (n=5)	Inter (n=15)	Intra (n=5)	Inter (n=15)
Concentration (ng/mL)	40	40	200	200
Morphine	12.0	10.8	9.7	7.4
Codeine	6.8	6.4	9.3	8.0
Hydromorphone	7.0	7.7	5.9	5.0
Hydrocodone	8.3	8.2	12.9	10.0
Oxymorphone	14.1	11.4	7.9	6.4
Oxycodone	5.1	6.3	6.7	5.8

Carryover

No carryover was observed.

Solvent Usage

The method used only 5%–10% of the solvent amount used at a normal flow rate setting (0.5 mL/min). This dramatically lower solvent use will significantly lower both initial solvent cost and the cost of disposing of solvent waste.

Conclusion

We have used a novel approach for sensitive quantitation of six opioids in urine for forensic toxicology purposes. This approach used super-dilution to minimize frequently observed ion suppression in urine samples and used a microflow LC setup (Ultimate 3000 RSLCnano LC system and TSQ Vantage mass spectrometer) to compensate for sensitivity losses from super-dilution. This robust method was linear between 20 and 5000 ng/mL for the six opioids and highly accurate and precise. The method used only 5%–10% of the solvent amount used at a normal LC flow rates, significantly lowering both solvent purchase and waste disposal costs.

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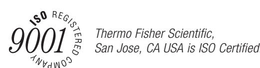

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Application Note: 561

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Quantitation of Amphetamines in Urine for SAMHSA Mandated Workplace Drug Testing Using a Triple Stage Quadrupole LC-MS System

Kristine Van Natta, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TSQ Quantum Ultra
- Hypersil Gold
- NIDA
- Methamphetamine
- Phentermine

Introduction

Federal employees and public transportation workers are required to pass a pre-employment drug screen known as the NIDA5, which refers to the five drugs of abuse that are required to be tested for by the National Institute of Drug Abuse (NIDA), or the Substance Abuse and Mental Health Services Administration (SAMHSA) panel. The assays are divided into 5 groups: opiates, amphetamines, cocaine (benzoylecgonine), cannabis (THCA) and PCP. In the past, these five groups have been screened by immunoassay and confirmed by gas chromatography-mass spectrometry (GC/MS). In October 2010, SAMHSA approved the use of liquid chromatography-mass spectrometry (LC/MS) for confirmation of workplace drug testing samples. Here we will focus on the amphetamine group which consists of amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA or Ecstasy) and methylenedioxyethylamphetamine (MDEA).

Goal

To develop a specific and robust dilute-and-shoot quantitative method for the confirmation of amphetamine, methamphetamine, MDA, MDMA, MDEA in urine that meets SAMHSA cutoffs. Additionally, the method should be able to discriminate between the structural isomers methamphetamine and phentermine.

Methods

Sample Preparation

Urine was spiked with internal standards and hydrolyzed with β-glucuronidase. While amphetamines do not require hydrolysis, other compounds in the SAMHSA panel such as the opiates and THC do require hydrolysis. Adding this step enables all SAMHSA panel compounds to be processed with one method. Methanol was added to the hydrolysis mixture and the resulting mixture was centrifuged. The supernatant was further diluted and subjected to LC-MS analysis.

HPLC Conditions

Chromatographic analysis was performed using Thermo Scientific Accela 600 HPLC pumps and a Thermo Scientific Hypersil GOLD aQ column (50 x 4.6 mm, 1.9 µm particle size). The mobile phase consisted of 5 mM ammonium formate with 0.1% formic acid in both water and methanol. The flow rate was 1.5 mL/min and the column was maintained at 30 °C. The total run time was 4.5 minutes.

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. Two selected reaction monitoring (SRM) transitions were monitored for each compound to provide ion ratio confirmations (IRC).

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at concentrations corresponding to the low (LQC), middle (MQC) and high (HQC) end of the calibration range. Intra-run variability and robustness were determined by analyzing six replicates of each QC level with a calibration curve on three different days. Matrix effects were investigated by comparing peak area of analytes prepared in multiple lots of urine to those of a sample prepared in water.

Results and Discussion

The limits of quantitation (LOQs) for all compounds meet the SAMHSA confirmation requirements. (Table 1). The method is linear up to 5,000 ng/mL with R2 values > 0.99 for all compounds. Figure 1 shows representative calibration curves for all compounds. Quality control results for the validation are shown in Table 2. Figure 2 shows an SRM chromatogram at LOQ. Peak areas of analytes in samples prepared from seven different lots of blank human urine compared to that of a sample prepared in water were all within 15% for amphetamine, methamphetamine, MDMA and MDEA. The peak areas were within 30% for MDA.

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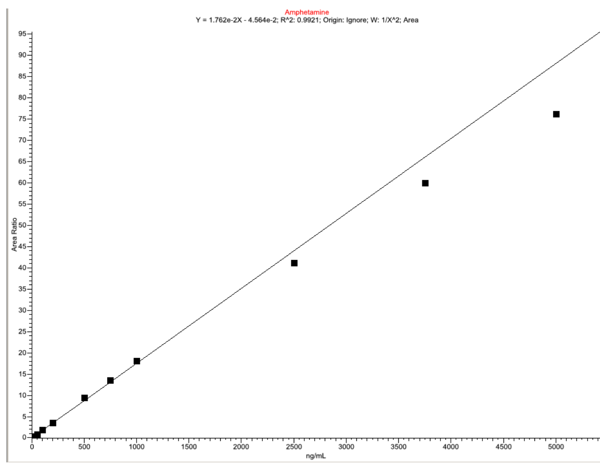
Table 1. Method summary for quantitation of amphetamines in urine

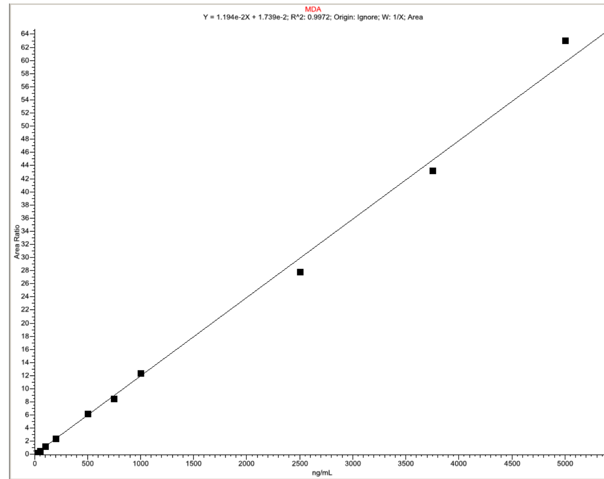
Compound	LOQ	ULOQ	SAMHSA Cutoff
Amphetamine	10 ng/mL	5000 ng/mL	250 ng/mL
Methamphetamine	5 ng/mL	5000 ng/mL	250 ng/mL
MDA	20 ng/mL	5000 ng/mL	250 ng/mL
MDMA	5 ng/mL	5000 ng/mL	250 ng/mL
MDEA	5 ng/mL	5000 ng/mL	250 ng/mL
Phentermine	Not quantitated, but chromatographically well-separated from isomeric methamphetamine.		
Total run time: 4.5 minutes			

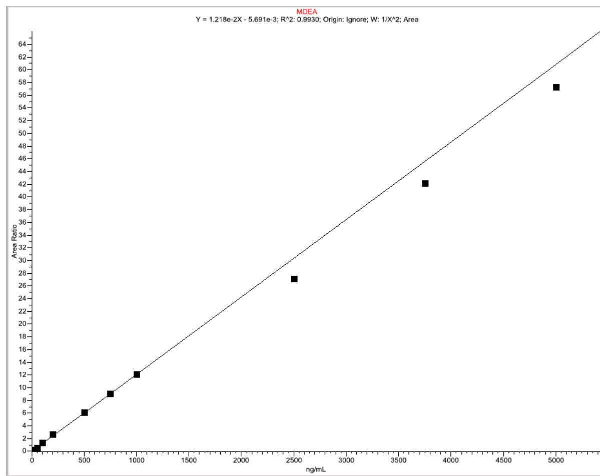
Table 2. %CV/%Bias for QCs analyzed during validation of amphetamines in urine

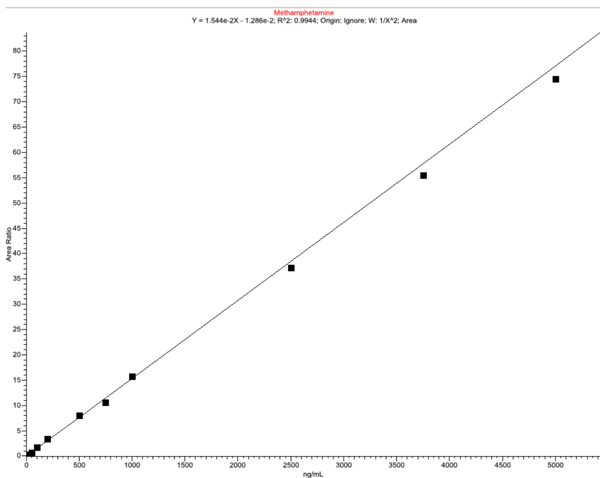
Compound	LQC (10 ng/mL)	MQC (100 ng/mL)	HQC (500 ng/mL)
Amphetamine	10.9/-2.24	4.45/6.39	2.56/0.431
Methamphetamine	7.03/0.420	3.02/7.78	4.26/1.67
MDA	NA	5.97/3.46	4.17/-0.196
MDMA	5.88/0.737	3.31/7.88	4.95/3.45
MDEA	4.51/3.35	2.96/8.20	4.34/2.54

NA: LQC concentration is below LOQ for MDA; data not reported.









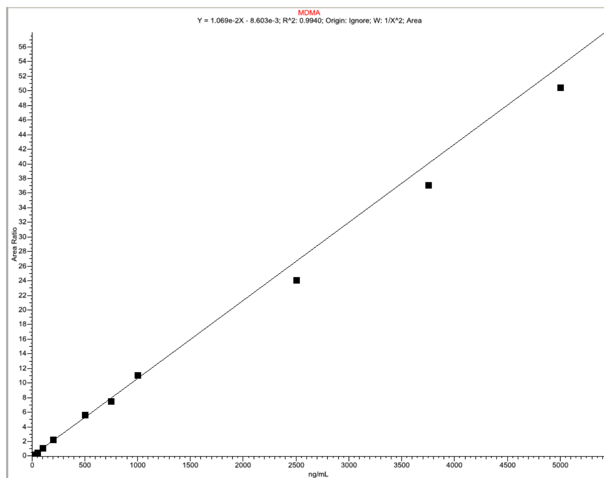


Figure 1. Representative calibration curves for amphetamine, methamphetamine, MDA, MDMA, MDEA in urine

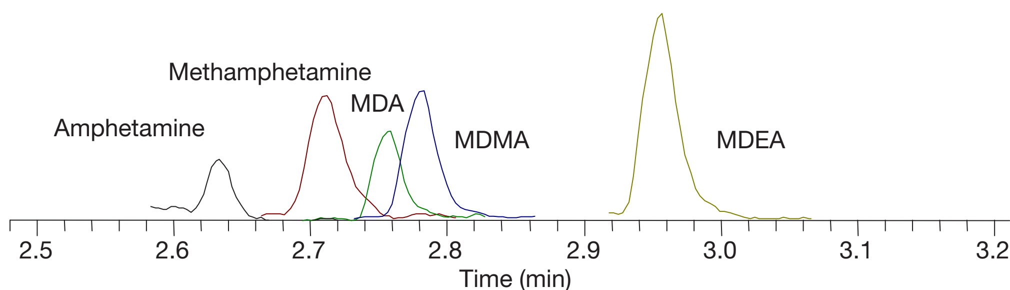


Figure 2. SRM chromatogram of amphetamine, methamphetamine, MDA, MDMA and MDEA in urine at their respective LOQs

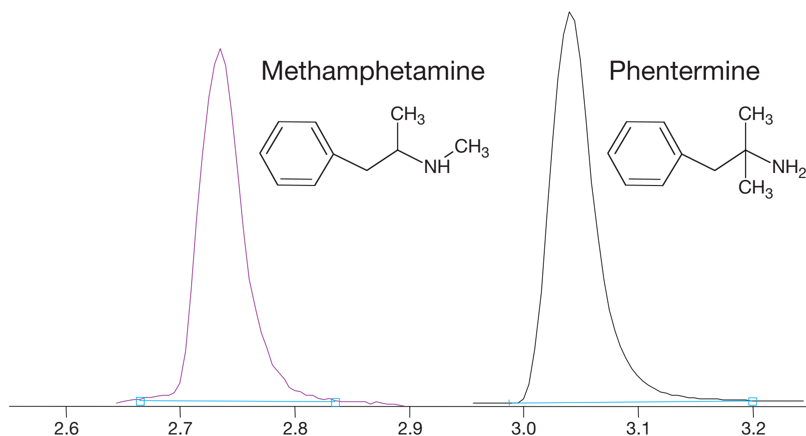


Figure 3. SRM chromatogram showing excellent resolution between structural isomers methamphetamine and phentermine

Methamphetamine and phentermine (an anti-obesity drug) are structural isomers with identical molecular masses and similar fragments. To avoid false positives, they must be separated chromatographically. As seen in Figure 3, these two compounds are well-resolved and will not interfere with each other.

Conclusion

A method with simple dilute-and-shoot sample preparation for the confirmation of amphetamines in urine was developed. This method is suitable for SAMHSA-mandated workplace drug testing, meeting cutoff and specificity requirements within a 4.5-minute run. The sample processing method also enables all SAMHSA panels to be processed at once.

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Quantitation of Synthetic Cannabinoids in Urine Using a Triple Stage Quadrupole LC-MS System in Forensic Toxicology

Kristine Van Natta, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TSQ Quantum Ultra
- JWH-018
- JWH-073
- Spice
- K2
- Forensic Toxicology

Introduction

Synthetic cannabinoids are compounds made to mimic the effects of natural cannabinoids found in the cannabis plant (marijuana). They were first synthesized by pharmaceutical companies seeking to mimic the beneficial analgesic and anti-nausea effects of cannabis while trying to eliminate the psychoactive euphoric effects for which the plant is so abused. In the mid 1980's, these compounds began appearing in herbal incense, marketed as "legal highs" under the names "Spice" and "K2." Effects are similar to those of cannabis, but with reports of increased anxiety and paranoia. In early 2011, the U.S. Drug Enforcement Administration (DEA) regulated five of these compounds as Schedule I drugs.

Simple, robust and precise analytical methods are needed to quantitate these now illegal compounds in biological matrices for forensic purposes. Here we will focus on JWH-018 and JWH-073. Research has shown that parent compound is not excreted in urine. The reported metabolites seen in urine are the alkyl-hydroxy and alkyl-carboxy metabolites of each compound.

Goal

To develop a specific and robust dilute and shoot quantitative method for the analysis of the alkyl-hydroxy and alkyl-carboxy metabolites of JWH-018 and 073: JWH-018-OH, JWH-018-COOH, JWH-073-OH and JWH-073-COOH in urine.

Figure 1. TSQ Quantum Ultra triple stage quadrupole mass spectrometer with Accela HPLC system

Methods

Sample Preparation

Urine was spiked with internal standards and hydrolyzed with β-glucuronidase. Fisher Chemical acetonitrile was added to the hydrolysis mixture and the resulting mixture was centrifuged. Supernatant was further diluted and subjected to liquid chromatography-mass spectrometry (LC-MS) analysis.

HPLC Conditions

Chromatographic analysis was performed using Thermo Scientific Accela 600 HPLC pumps and a Thermo Scientific Hypersil GOLD column (100 x 2.1 mm, 3 µm particle size). Mobile phase consisted of 5 mM ammonium formate in both water and methanol. The total run time was 15.5 minutes.

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe (Figure 1). Two selected reaction monitoring (SRM) transitions were monitored for each compound to provide ion ratio confirmations (IRC).

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at concentrations corresponding to the low, middle and high end of the calibration range. Inter- and intra-run variability and robustness were determined by analyzing replicates of each QC level with a calibration curve on three different days.

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Results and Discussion

The method is linear from 2 to 1,000 ng/mL with R^2 values greater than 0.99 for all compounds (Figure 2). Table 1 shows QC precision and bias data for the validation runs.

A 15-minute run was required to chromatographically separate the analytes of interest from endogenous interferences. Figures 3 and 4 show this chromatographic resolution in a 2-ng/mL and 100-ng/mL standard, respectively. Figure 5 shows a SRM chromatogram from a self-confessed consumption sample.

Table 1. Inter-Assay %CV and % Bias for Quality Control Samples

	LQC	MQC	HQC
JWH-018-OH	10.4/-0.790	3.50/-2.21	7.81/2.51
JWH-018-COOH	8.07/11.6	3.82/6.38	6.37/6.29
JWH-073-OH	9.02/3.72	3.42/-0.359	5.99/0.847
JWH-073-COOH	11.8/14.0	3.75/9.46	4.78/7.34

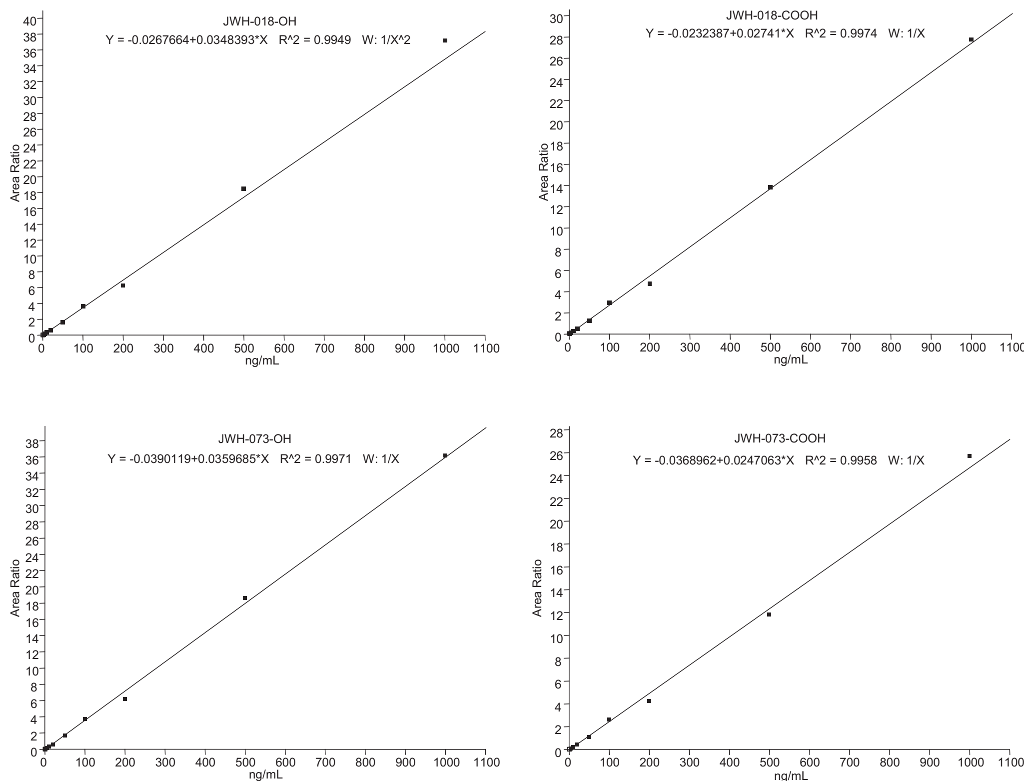

Figure 2. Representative calibration curves for JWH-018 and JWH-073 metabolites showing linearity from 2-1,000 ng/mL in urine

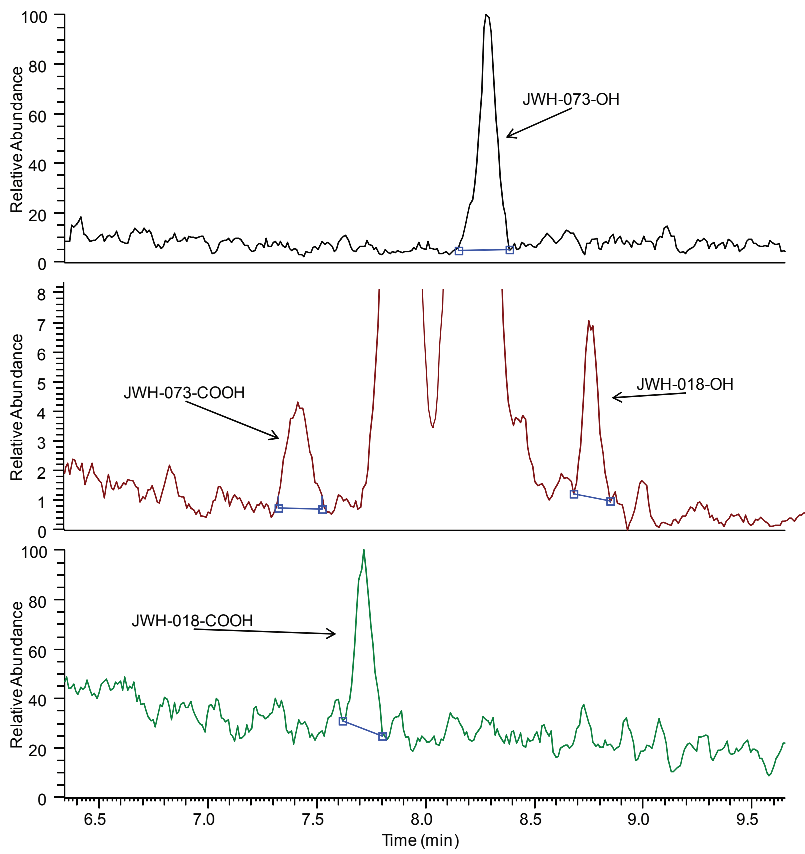

Figure 3. SRM chromatogram of a 2 ng/mL standard showing resolution of analytes from unknown endogenous interferences.

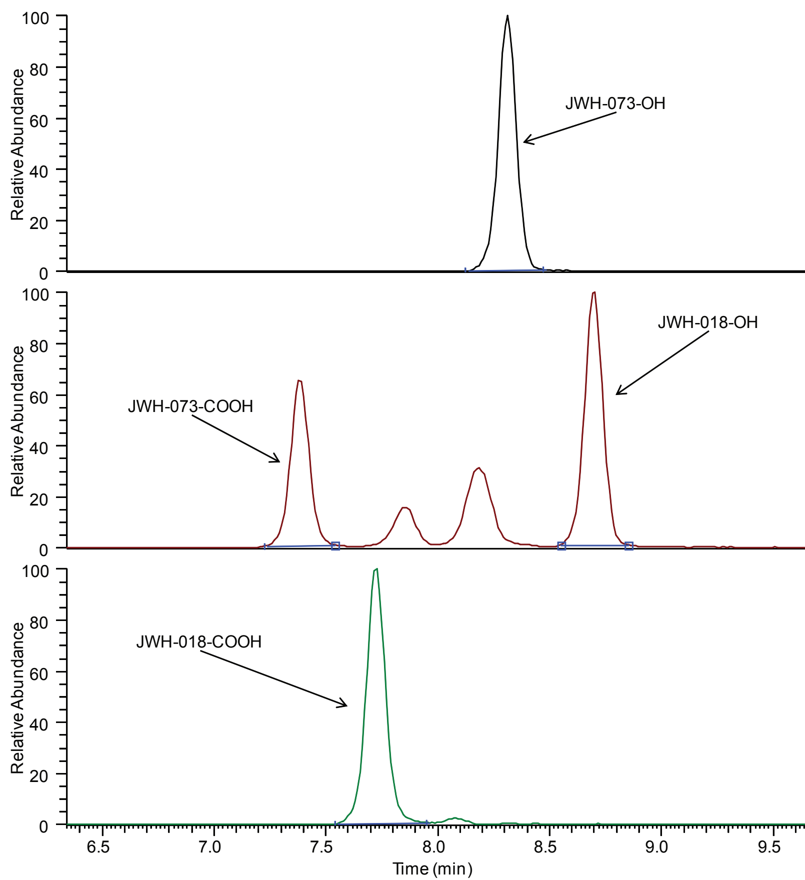

Figure 4. SRM chromatogram of a 100 ng/mL standard.

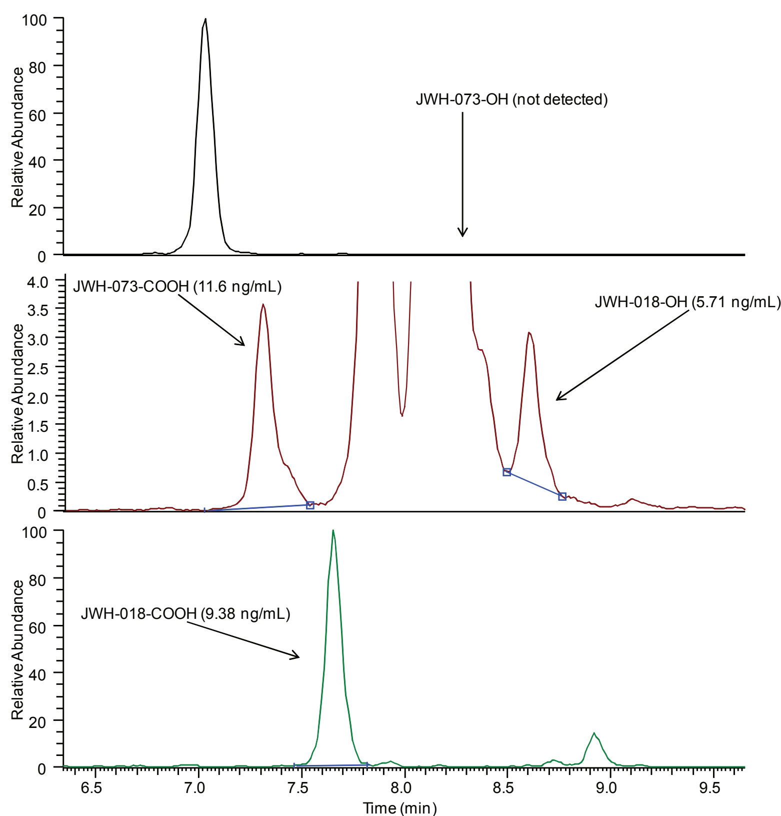

Figure 5. SRM chromatogram of self-confessed human in vivo sample. JWH-073-N-(4-hydroxybutyl), a compound validated in this assay, is not seen in this sample. The unidentified peak in the JWH-073-OH channel is JWH-073-N-(3-hydroxybutyl), a major metabolite not known at the time of this validation.

Conclusion

A simple dilute and shoot method for the analysis of synthetic cannabinoid metabolites in urine was developed for forensic toxicology use. Since analysis of these compounds is relatively new to forensic applications, cut-off values have not been established. The current method has an LOQ of 2 ng/mL for all compounds. Based on published research, using an SPE or liquid/liquid extraction processing method will lower the current LOQ to 0.2 ng/mL, if required.

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Application Note: 548

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THC-COOH Quantification in Urine Using Dilute and Shoot LC-MS/MS Method for Forensic Toxicology

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Maura Brambilla, Ludovico D'Amato, Paolo Brambilla, University Department of Laboratory Medicine, Hospital of Desio, Desio, Italy

Key Words

- TSQ Quantum Access MAX
- Accela Pump
- Cannabinoids
- Forensic Toxicology

Introduction

Cannabis sativa is a widely used drug of abuse. Tetrahydrocannabinol (THC) is the major psychoactive chemical compound in the cannabis plant. After smoke inhalation, THC is absorbed and distributed in blood. Subsequently, it is rapidly metabolized to THC-COOH, conjugated with glucuronic acid, and excreted through urine. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is considered a useful tool to establish the consumption of cannabis by the assessment of THC-COOH in urine for forensic toxicology purposes.

Goal

To develop a reliable and fast analytical method for the quantitative determination of THC-COOH in urine using a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer.

Experimental

Sample Preparation

A urine sample was hydrolyzed with 10M NaOH and heated at 60 °C for 15 minutes. The pH was restored with Fisher Chemical acetic acid. Hydrolyzed samples as well as calibrators were diluted 1:10 in Fisher Chemical water/acetonitrile (1:1). Then, 10 µL were directly injected. Quantitative analysis was performed on the basis of calibration curves prepared in urine, ranging from 7.8 to 1000 ng/mL. Calibrators were injected in duplicate.

UHPLC conditions

Liquid chromatography separation was performed using a Thermo Scientific Accela autosampler and pump. The sample was injected directly on a Thermo Scientific Hypersil GOLD column (50 × 2.1 mm, 1.9 µm). A gradient LC method used mobile phases A (0.1% aqueous formic acid) and B (Fisher Chemical Optima LC/MS acetonitrile) at a flow rate of 300 µL/min. The run time was 6 minutes.

Mass Spectrometry

MS analysis was carried out on a TSQ Quantum Access MAX™ triple stage quadrupole mass spectrometer equipped with a Thermo Scientific Ion Max source with a heated electrospray ionization (HESI) probe. The MS conditions were as follows:

Scan type:	SRM
Divert valve:	2 - 4 min to source
Selected ions for quantification:	*m/z* 343 → 299 + 245 for THC-COOH in negative mode

Results and Discussion

Figures 1 and 2 show the ion chromatograms of the lowest and highest calibration points. Excellent linearity ($r^2 = 0.99$) fits for the calibration curve were observed over the range of 7.8-1000 ng/mL urine, with a Coefficient of Variation (%CV) at the lower end of 6.5%. The limit of quantitation (LOQ) was established as 7.8 ng/mL in urine.

Figure 4 reports an ion chromatogram of a real urine sample positive for cannabinoids (225 ng/mL urine), analyzed as described.

To examine the difference between hydrolyzed and non-hydrolyzed urine, we analyzed the same urine sample without the hydrolysis step. When urines were not hydrolyzed, the portion excreted as free THC-COOH was detected at 3.06 minutes, while THC-COOH-glucuronide was detected at 2.58 minutes (Figure 5). The precursor ion *m/z* 343 was generated as result of an in-source fragmentation and a consequent loss of glucuronic acid.

Because THC-COOH is mainly excreted as glucuronic acid conjugate, it is always necessary to perform urine hydrolysis before the LC-MS analysis to obtain an accurate quantification of THC-COOH.

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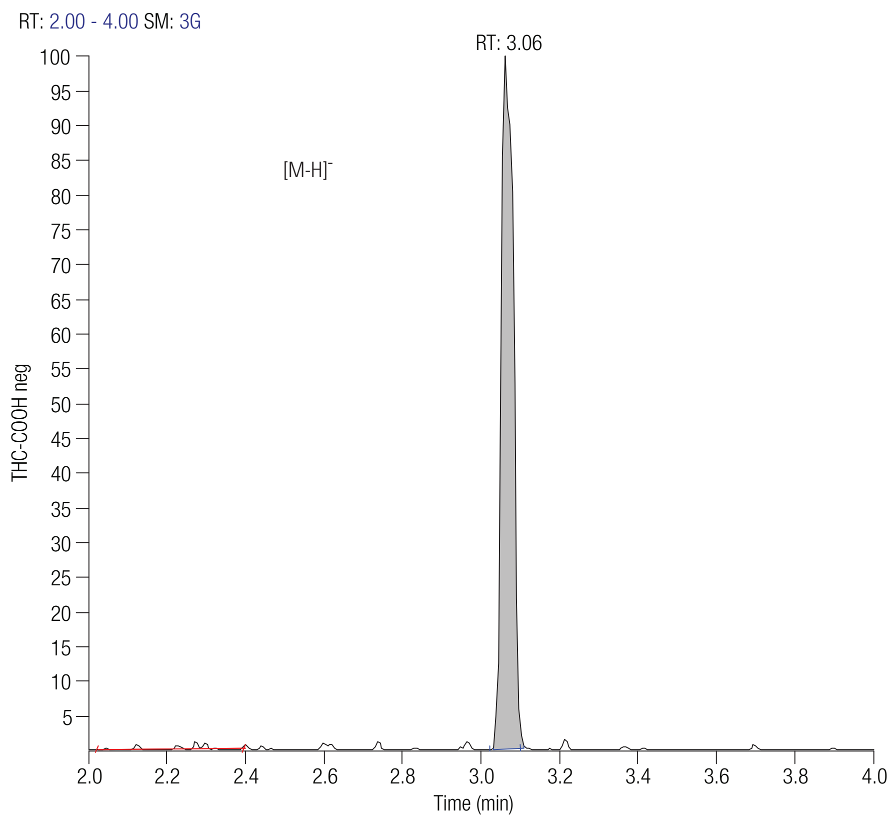

Figure 1. Ion chromatogram of 7.8 ng/mL urine calibration standard

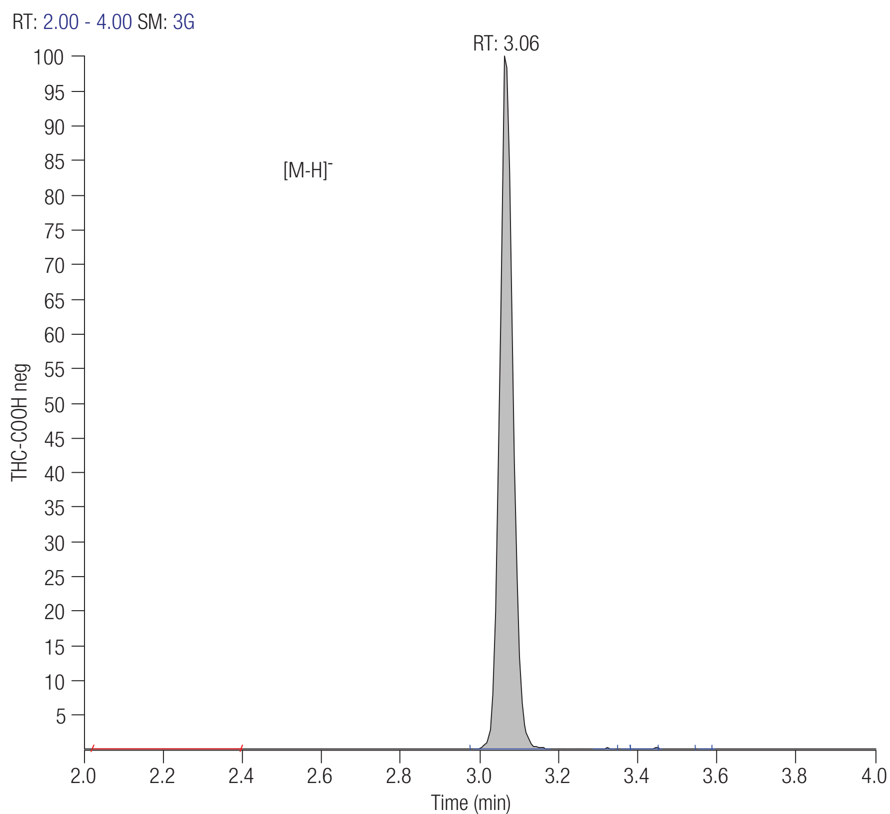

Figure 2. Ion chromatogram of 1000 ng/mL urine calibration standard

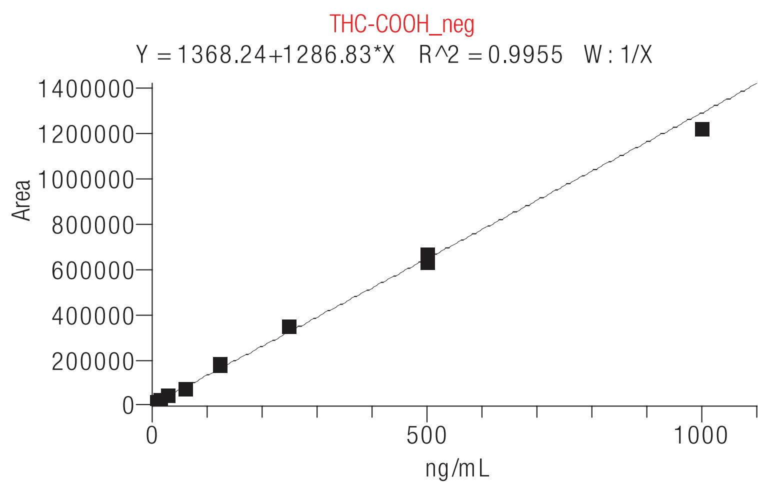

Figure 3. Calibration curve of THC-COOH in negative ionization mode

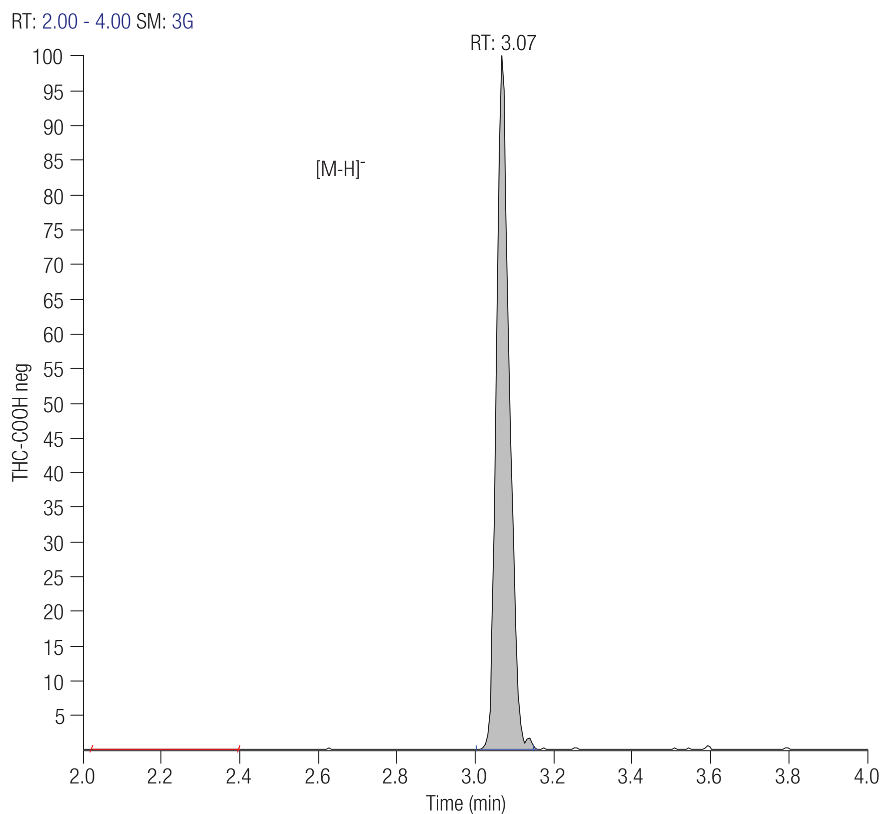

Figure 4. Ion chromatogram of urine sample containing 225 ng/mL. Sample was hydrolyzed and diluted 1:10 before the analysis.

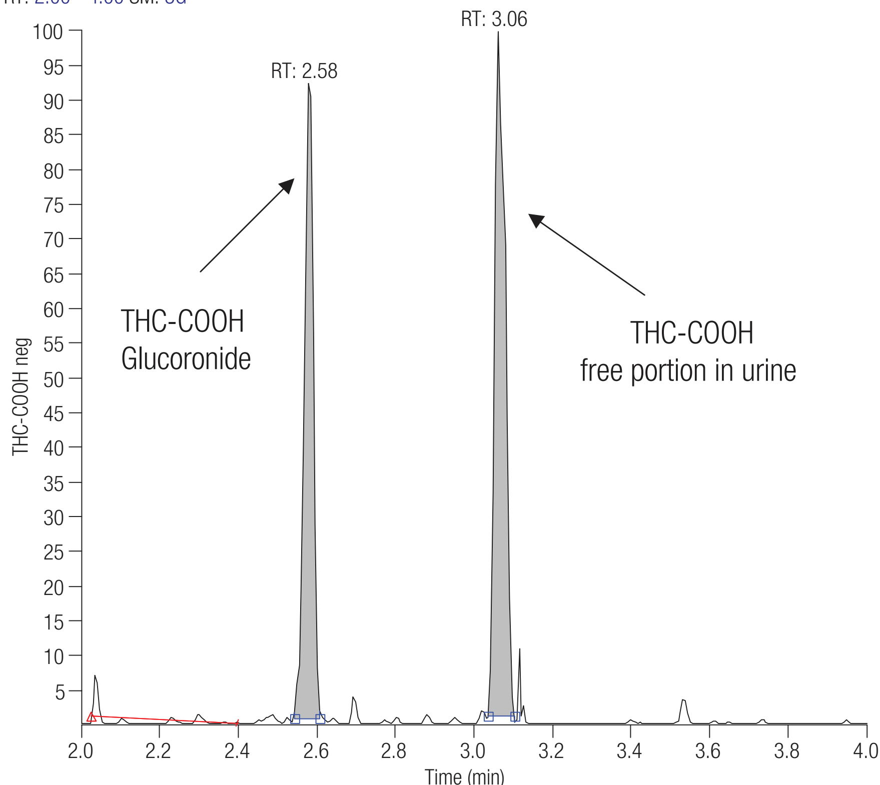

Figure 5. Ion chromatograms of urine sample containing 225 ng/mL. Sample was diluted 1:10 before the analysis; no hydrolysis was performed.

Conclusion

A robust 6-minute method for the quantification of THC-COOH with a dynamic range of 7.8-1000 ng/mL urine has been developed using the TSQ Quantum Access MAX mass spectrometer for forensic toxicology purposes.

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Demonstrating High-Performance Quantitative Analysis of Benzodiazepines using Multiplexed SIM with High-Resolution, Accurate Mass Detection on the Q Exactive LC/MS

Kevin J. McHale; Thermo Fisher Scientific, Somerset, NJ

Key Words

- Q Exactive
- Accela UHPLC
- Selected Ion Monitoring
- Drug Quantitation

Introduction

In today's modern forensic toxicology laboratories, there is a growing demand to have a mass spectrometer with the power and flexibility to perform experiments both for the identification of unknown compounds and for trace-level quantification of target analytes. Additionally, this platform must execute these analyses with minimal sample preparation, provide consistent results and be easily assimilated into the laboratory workflows. With the introduction of the Thermo Scientific Q Exactive high-performance benchtop quadrupole-Orbitrap mass spectrometer, the most stringent qualitative and quantitative objectives can be met. By using high-resolution, accurate mass (HRAM) detection with quadrupole selected ion monitoring (SIM), targeted quantification of benzodiazepines in urine can be accomplished with sensitivity that rivals triple stage quadrupole instruments in selected reaction monitoring (SRM) mode.

Goal

To demonstrate the feasibility of high sensitivity liquid chromatography-mass spectrometry (LC/MS) quantification of benzodiazepines in urine by combining multiplexed SIM with high-resolution, accurate mass detection on the Q Exactive™ high-performance benchtop quadrupole-Orbitrap mass spectrometer.

Experimental

Sample Preparation

Eight benzodiazepines were spiked into blank human urine containing acetonitrile at 10% (v/v) from 0.0125 to 250 ng/mL prior to LC/MS.

UHPLC

Ultra high performance LC (UHPLC) analyses were performed using a Thermo Scientific Accela 1250 liquid chromatography system with an Open Accela™ autosampler. Gradient elution with a Thermo Scientific Hypersil GOLD PFP column (50 x 2.1 mm; 1.9 µm particle size) was used at a flow rate of 500 µL/min. The injection volume was 5 µL.

Mass Spectrometry

MS measurements were accomplished on a Q Exactive mass spectrometer with a heated electrospray ionization (HESI) source in positive ion mode. Quadrupole isolation was set to 1.5 *m/z* with subsequent detection at a mass resolution of 140,000 FWHM via external mass calibration.

Results and Discussion

SIM is a well-established technique for targeted LC/MS quantitation using single quadrupole mass spectrometers. However, its utility is limited owing to the low specificity of unit mass resolution on single quads. The Q Exactive mass spectrometer, which employs Orbitrap-based high-resolution, accurate mass detection, overcomes this limitation. Additionally, the duty cycle on the Q Exactive MS is enhanced by measuring multiple SIM ions simultaneously in the Orbitrap mass analyzer. The process of multiplexed SIM is illustrated in Figure 1. Four different ions are selected by the quadrupole and stored in the C-trap while the Orbitrap analyzer measures the ions from the previous cycle. This process is repeated by passing the four SIM ions from the C-trap to the Orbitrap analyzer for the next mass measurement. The Q Exactive mass spectrometer has the capability to multiplex between two and ten SIM ions.

Table 1 lists the eight benzodiazepines quantified by HRAM LC/MS with their multiplexed SIM time windows, the measured mass errors using external mass calibration, and the lower limits of quantitation (LLOQs) in urine on the Q Exactive mass spectrometer. Two key points to highlight in Table 1 are that (1) mass errors on the Q Exactive system are significantly less than 5 ppm without the need of an internal calibration mass, and (2) the LLOQs of the eight benzodiazepines analyzed in urine are in the pg/mL range.

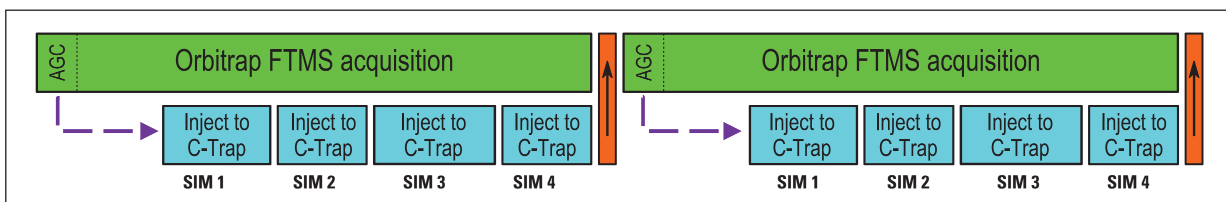

Figure 1. Schematic of multiplexed SIM on the Q Exactive mass spectrometer

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Table 1. List of benzodiazepines quantified by HRAM LC/MS on the Q Exactive mass spectrometer

Compound	SIM Time Window (min)	Exact *m/z*	Measured *m/z*	Error (ppm)	LLOQ (ng /mL)
Oxazepam	0.00-3.45	287.05818	287.05829	+0.4	0.0625
Lorazepam	0.00-3.65	321.01921	321.01926	+0.2	0.1250
Nitrazepam	0.00-3.65	282.08732	282.08746	+0.5	0.0625
Clonazepam	0.00-3.85	316.04835	316.04828	-0.2	0.0625
Temazepam	3.45-6.00	301.07383	301.07410	+0.9	0.0250
Flunitrazepam	3.65-6.00	314.09355	314.09296	-1.9	0.0625
Alprazolam	3.65-6.00	309.09015	309.09024	+0.3	0.0125
Diazepam	3.85-6.00	285.07892	285.07901	+0.3	0.0125

Figure 2 presents an example LC/MS analysis of benzodiazepines at 0.125 ng/mL in urine using multiplexed SIM on the Q Exactive mass spectrometer. By acquiring these data at a mass resolution of 140,000 FWHM, little or no chemical noise is observed for the ± 5 ppm extracted ion chromatograms of the benzodiazepines in urine. The selectivity afforded by the Q Exactive mass spectrometer at a resolution of 140,000 FWHM is illustrated in the SIM spectrum for oxazepam (Figure 3). In addition to the oxazepam ion at *m/z* 287.05829, there are at least 12 other ions observed within a 0.25 *m/z* range. Yet, the oxazepam ion is easily separated from the other chemical interference ions with the high resolving power of the Q Exactive mass spectrometer.

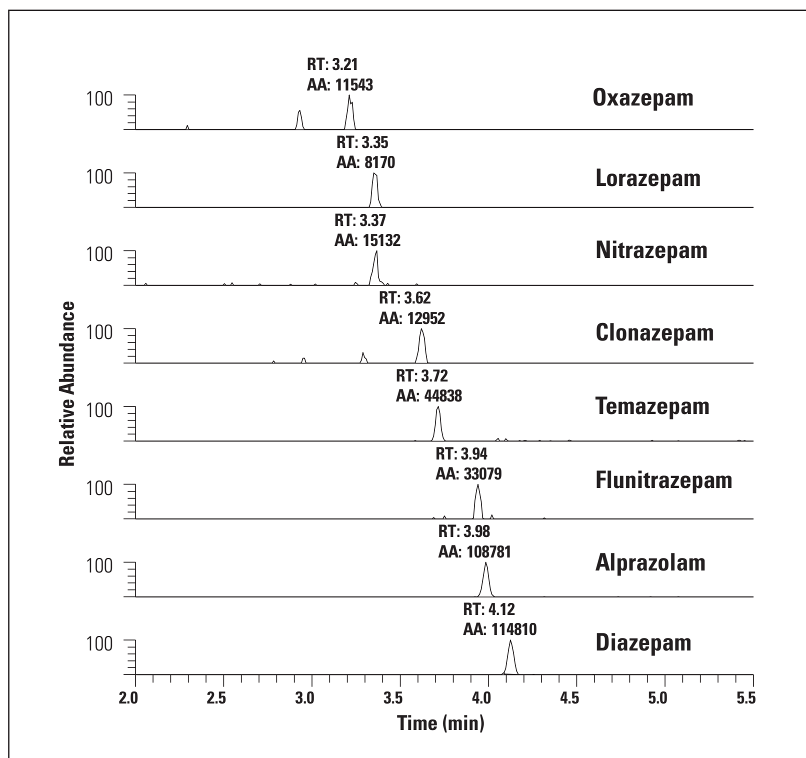

Figure 2. Extracted ion chromatograms (5 ppm) for 0.125 ng/mL benzodiazepines in urine

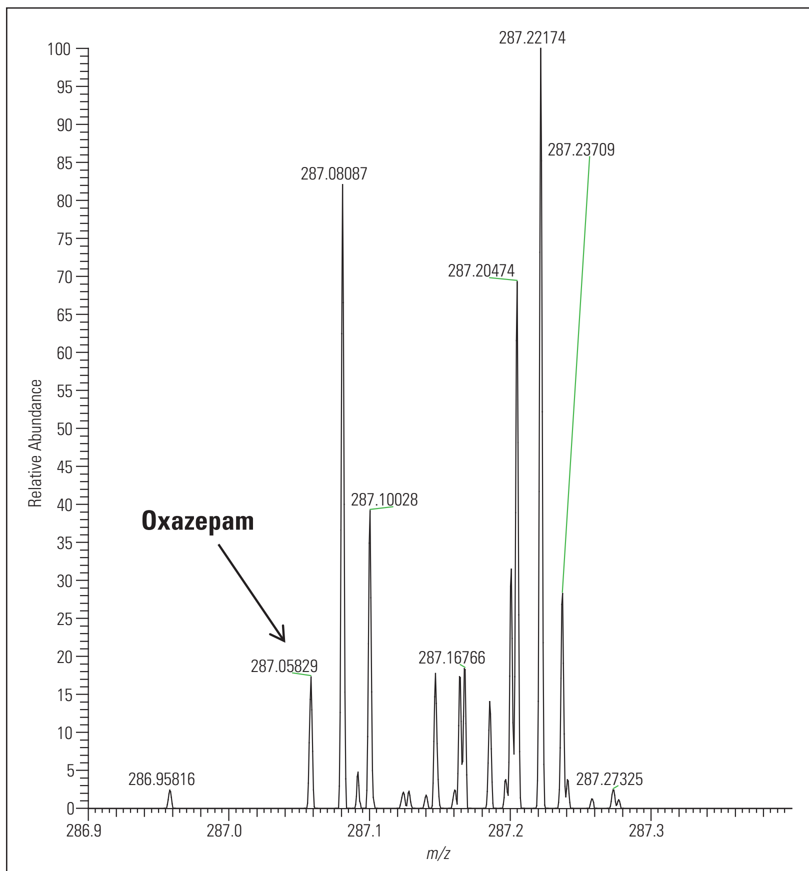

Figure 3. SIM spectrum of oxazepam in urine at mass resolution of 140,000 FWHM

Figure 4 and Table 2 demonstrate the overall quantitative performance of the Q Exactive mass spectrometer for diazepam in urine. The calibration curve for diazepam in Figure 4 shows a linear dynamic range of over four decades (0.0125 – 250 ng/mL), including the inset from 0.0125 to 0.25 ng/mL, with

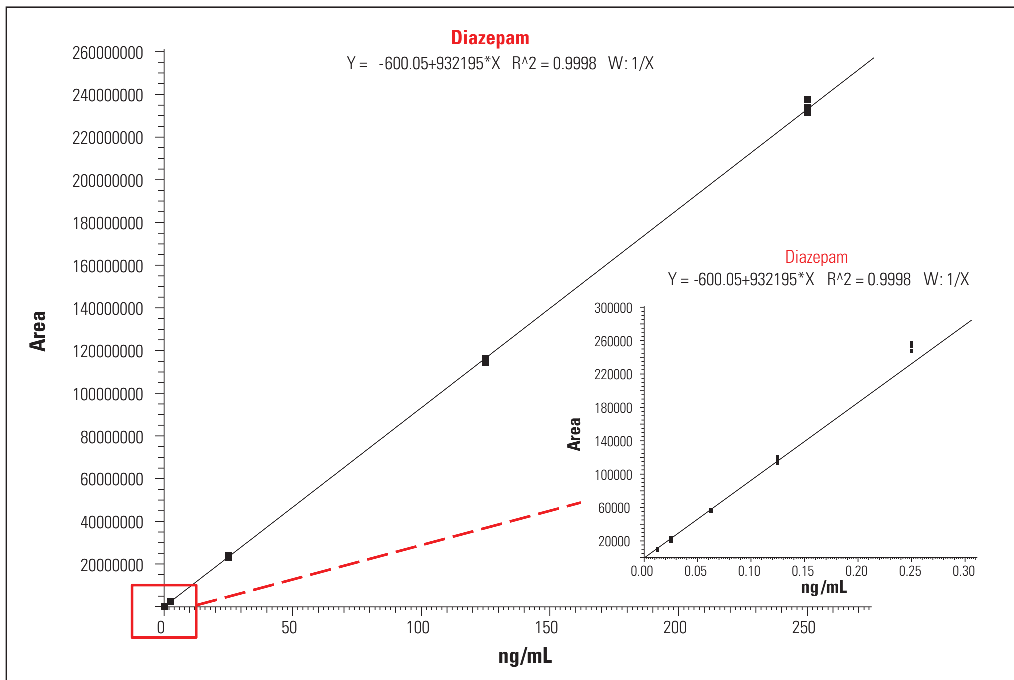

Figure 4. Calibration curve for diazepam in urine from 0.0125 – 250 ng/mL

an R^2 regression value of 0.9998 using 1/x weighting. Table 2 presents the statistical results for the HRAM quantification of diazepam. The quantitative accuracy and precision values obtained by the Q Exactive mass spectrometer using multiplexed SIM are comparable to those observed on triple stage quadrupole mass spectrometers in SRM mode.

Table 2. Statistical results for HRAM LC/MS quantitation of diazepam in urine

Specified Amount (ng /mL)	Mean Calculated Amt. (ng/mL)	%Accuracy	%CV
0.0125	0.0113	90.1	6.0
0.0250	0.0236	94.3	7.9
0.0625	0.0610	97.6	1.4
0.1250	0.127	101.9	2.9
0.250	0.273	109.1	1.7
2.50	2.65	105.9	1.0
25.0	25.5	102.0	1.7
125.0	123.5	98.8	0.8
250.0	250.8	100.3	1.2

Conclusion

The Q Exactive HRAM LC/MS system is a powerful and flexible instrument that can provide both sample identification and quantitative information for forensic toxicology with a single sample analysis. By using the method of multiplexed SIM, eight benzodiazepines in urine were quantified with LLOQs at the pg/mL level and with linear dynamic ranges of 3 to 4 orders of magnitude.

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Antidepressants and Neuroleptics Quantitation Using Tandem Mass Spectrometry and Automated Online Sample Preparation

Hans-Rudolf Kuhn; Unilabs, Switzerland
Bénédicte Duretz; Thermo Fisher Scientific, Les Ulis, France

Key Words

- TurboFlow Technology
- TSQ Quantum Access MAX
- Triple Quadrupole
- Clinical Research

Introduction

Liquid chromatography-mass spectrometry (LC/MS) is a powerful technique applied in clinical research for the analysis of a broad number of analytes. Offline sample preparation techniques (solid phase extraction and liquid-liquid extraction) are widely used but are often time consuming and labor intensive. The Thermo Scientific Transcend system powered by TurboFlow™ technology provides an alternative approach simplifying sample preparation.

Goal

To develop a fast and efficient LC-MS/MS method using Thermo Scientific TurboFlow technology for the analysis of 18 antidepressants and neuroleptics.

Experimental

Sample Preparation

A 100 µL aliquot of serum or plasma sample was mixed with 300 µL of methanol containing internal standards (Venlafaxine-d6 and Sertraline-d3) at 100 ng/mL. The resulting mixture was thoroughly vortexed, allowed to stand for 10 minutes at room temperature and then centrifuged at 4 °C for 10 minutes.

Chromatography and Mass Spectrometry

High pressure LC (HPLC) was performed using the Transcend™ TLX system. Serum and plasma samples were extracted using a TurboFlow Cyclone P (0.5 x 50 mm) extraction column. Chromatographic separation was performed using a Thermo Scientific Hypersil GOLD column (50 x 3 mm, 3 µm particle size). Gradient elution was used. Total analysis time was 8 minutes.

The TurboFlow method conditions were as follows:

Eluent A:	0.1% Formic acid in water
Eluent B:	0.1% Formic acid in methanol
Eluent C:	Acetonitrile, isopropanol and acetone (45/45/10, v/v/v)
Eluent D:	Acetonitrile, water (90/10, v/v)

The analytical LC conditions were as follows:

Eluent A:	0.1% Formic acid in water
Eluent B:	0.1% Formic acid in methanol

The entire LC effluent from the sample injections was directed to the Thermo Scientific Ion Max source, utilizing heated electrospray ionization (HESI), on a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer in positive ion selected reaction monitoring (SRM) mode.

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Results and Discussion

For each analyte, linearity and quantitative results were obtained using SRM transitions. Quantitation of the 18 drugs was performed with a calibration range of 5 to 500 ng/mL for 5 compounds, 10 to 1000 ng/mL for 9 compounds, 2 to 200 ng/mL for 3 compounds, and 1 to 100 ng/mL for 1 compound. The R^2 value for each of the calibration curves was above 0.998, which indicates an excellent linear fit over the dynamic range. Figure 1 shows the chromatogram of the lowest calibration standard. Calibration curves for risperidone and clozapine are reported in Figure 2. Table 1 displays the calibration ranges and method precision for all analyzed drugs.

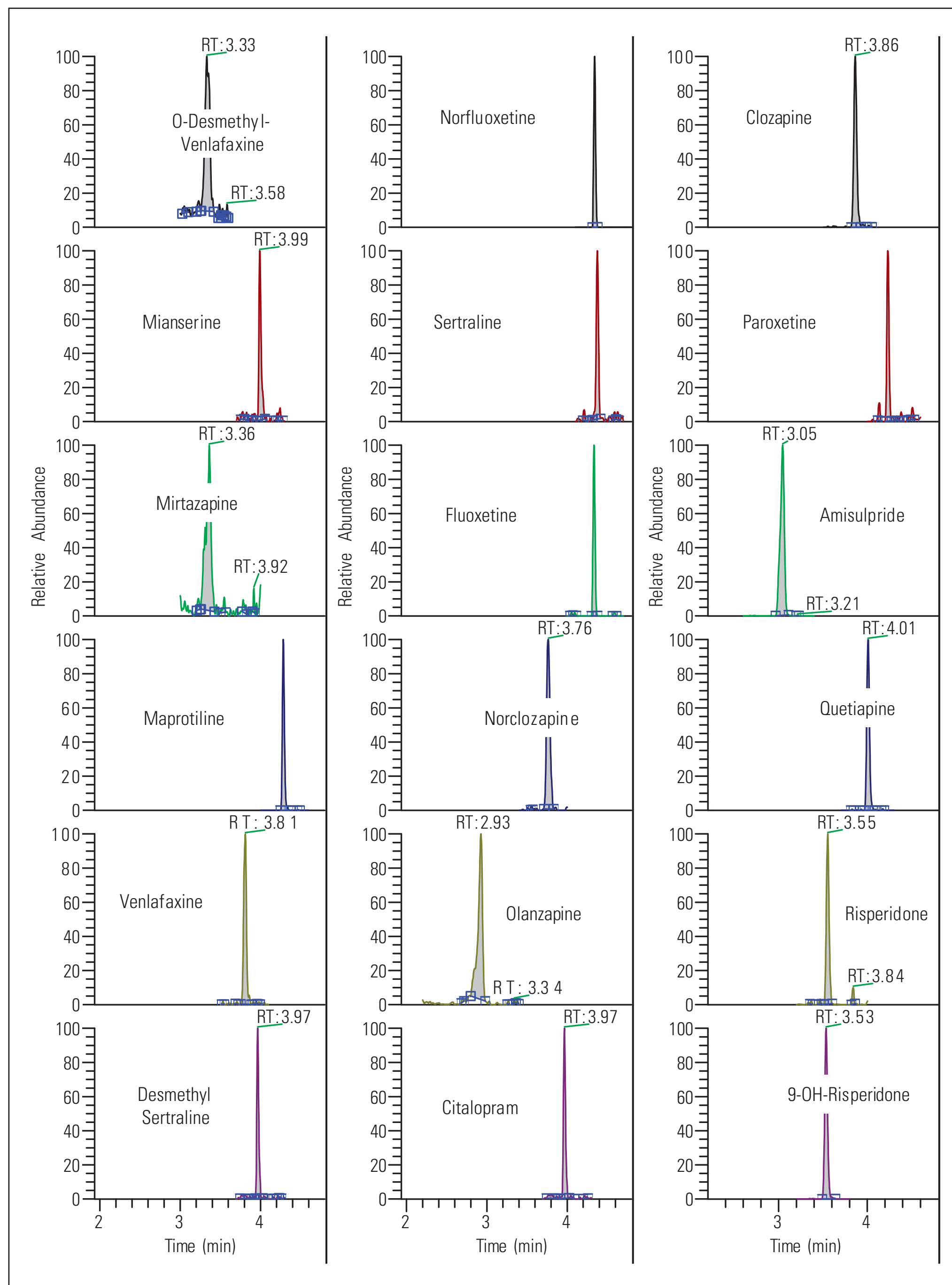

Figure 1. Representative chromatograms for the methods at the low end of the calibration curve

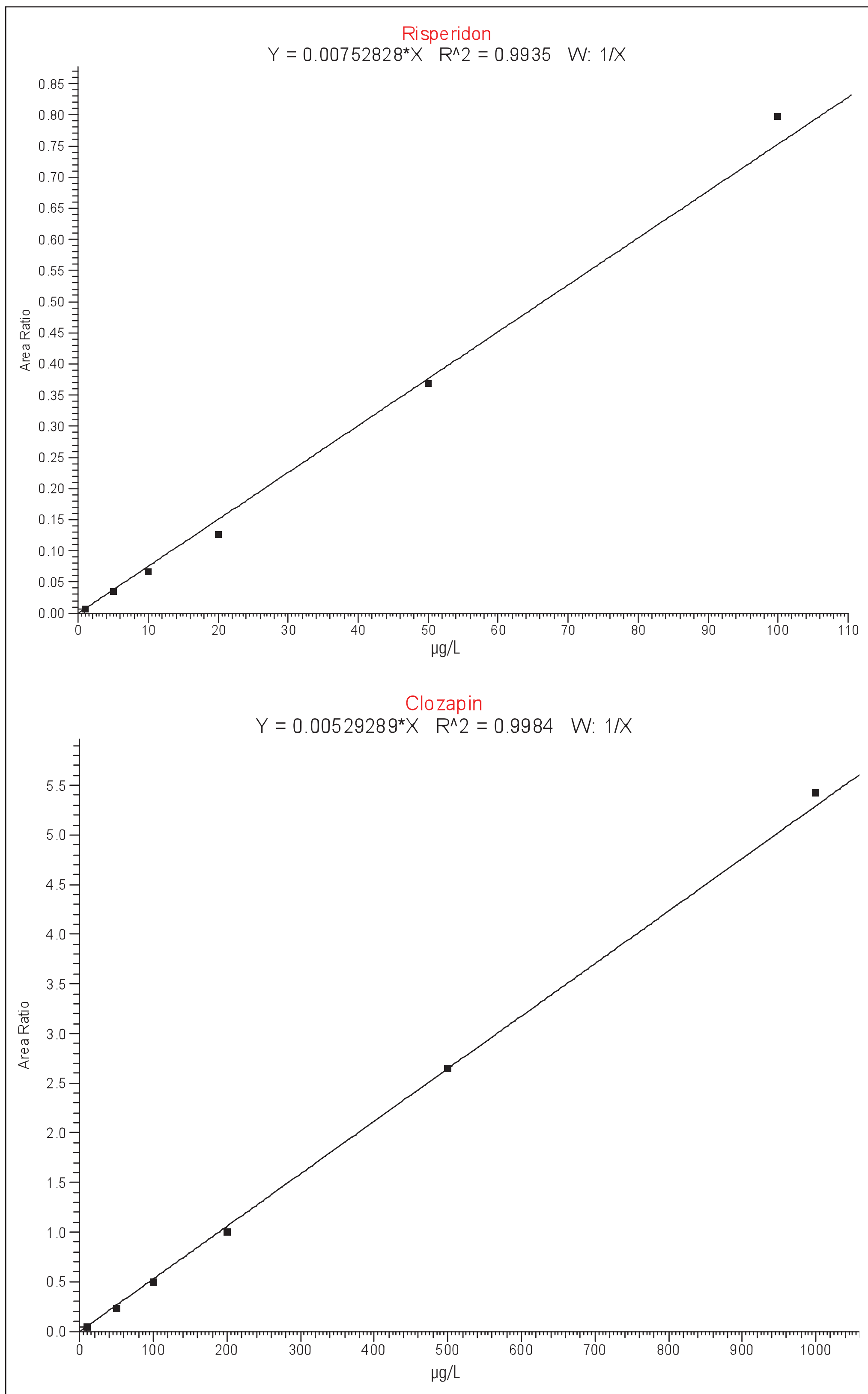

Figure 2. Calibration curves for risperidone and clozapine

Table 1. Calibration ranges and method precision for all the analytes

Analyte	Calibration range (ng/mL)	Within-day (%RSD)*	Between-days (%RSD)**
9-OH-Risperidone	5-500	7.1	5.5
Amisulpride	10-1000	3.9	3.4
Citalopram	5-500	4.9	5.1
Clozapine	10-1000	5.8	4.3
Desmethyl Sertraline	2-200	6.3	6.0
Fluoxetine	10-1000	3.4	3.4
Maprotiline	10-1000	4.2	4.1
Mianserine	5-500	6.2	5.1
Mirtazapine	2-200	5.9	4.4
Norclozapine	10-1000	5.9	3.6
Norfluoxetine	10-1000	6.5	5.0
O-Desmethyl-Venlafaxine	10-1000	4.3	4.8
Olanzapine	5-500	6.2	3.3
Paroxetine	5-500	6.2	5.3
Quetiapine	10-1000	5.2	3.5
Risperidone	1-100	5.8	5.4
Sertraline	2-200	4.5	3.4
Venlafaxine	10-1000	4.5	3.4

* Replicates analyzed each day = 10

** Days averaged = 10

Conclusion

A fast and analytically sensitive method for the detection of 18 antidepressants and neuroleptics is described. The Transcend TLX automated online sample preparation system allows minimal sample preparation and time saving in the absence of SPE sample preparation for clinical research laboratories.

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Application Note: 541

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Software Driven Quantitative LC-MS Analysis of Opioids in Urine for Forensic Laboratories

Kristine Van Natta, Xiang He, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TraceFinder Software
- TSQ Quantum Ultra
- Drugs of Abuse

Introduction

Thermo Scientific TraceFinder software provides an integrated workflow approach for routine forensic screening and quantitation from method development and data acquisition to data processing and on through reporting. The TraceFinder™ software supports all Thermo Scientific quantitative liquid chromatography-mass spectrometry (LC-MS) systems with fully integrated support for Thermo Scientific Transcend multiplexing systems. The software also provides integrated levels of security from a lab manager to a routine user.

Goal

To demonstrate the software driven quantitative analysis of six opioids in urine using the Thermo Scientific TSQ Quantum Ultra mass spectrometer and TraceFinder software.

Experimental

Sample Preparation

Urine was spiked with internal standards and hydrolyzed with β-glucuronidase. Fisher Chemical Optima® LC/MS Methanol was added to the hydrolysis mixture and the resulting mixture was centrifuged. The supernatant was further diluted and subjected to LC-MS analysis.

LC-MS/MS conditions

LC-MS analysis was performed on a TSQ Quantum Ultra™ mass spectrometer equipped with a heated electrospray ionization (HESI) probe coupled with a Transcend™ TLX system operating in LX mode. Two selected reaction monitoring (SRM) transitions were monitored for each compound. High pressure liquid chromatography (HPLC) was carried out on a Thermo Scientific Hypersil GOLD aQ column (50 × 4.6 mm, 1.9 µm particle size) at 30 °C. The MS source conditions were as follows:

Spray Voltage	3500 V
Vaporizer Temp	350 °C
Sheath Gas	80 (arbitrary units)
Ion Sweep Gas	0 (arbitrary units)
Aux Gas	5 (arbitrary units)
Capillary Temp	250 °C

Software

TraceFinder software was used for method development and routine analysis during validation.

Main Tabs in TraceFinder

Figure 1 shows the four main tabs in TraceFinder software: Acquisition, Data Review, Method Development and Configuration.

Compound Data Store

Figure 2 shows the Compound Data Store (CDS) for this opioid application. Entries of the analytes in this CDS contain the quantifier ion, qualifier ion and retention times for easy addition to a Master Method.

Master Method

The Master Method contains all of the information needed for an assay including that for instrument acquisition, data processing and reporting. The five main categories of information are: General (including assay type, injection volume, and instrument method), Compound (including acquisition list selected from the CDS, detection parameters, calibration and control levels), Flags, Groups and Reports. Selected tabs in the General and Compounds sections are shown in Figure 3. Many flagging parameters are available to customize data review and reports. Some of these parameters are shown in Figure 4.

Instrument Method

Instrument methods including autosampler, HPLC, and mass spectrometer parameters can be directly edited within TraceFinder 1.1 through a Thermo Scientific Xcalibur software interface.

Batch

Creating a batch involves assigning a project, linking to the master method, building a run-sequence and finally submitting the batch. Multiplexing channels are also controlled in the batch creation as seen in Figure 5.

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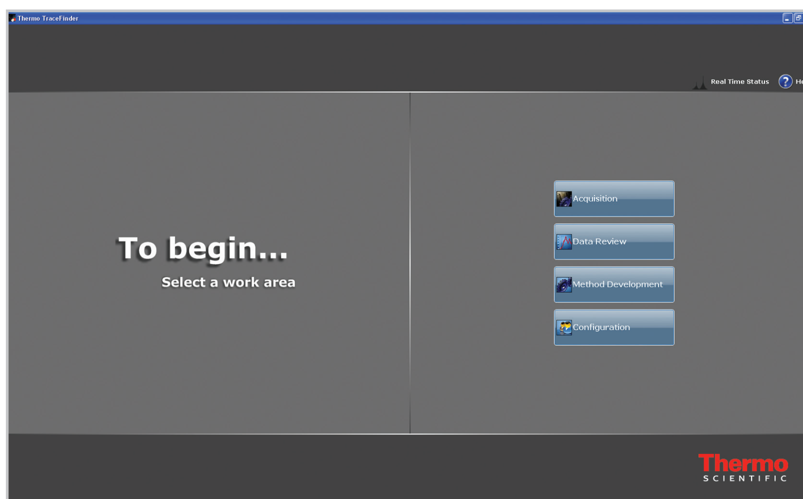


Figure 1. TraceFinder 1.1 welcome screen

	Compound Name	ExperimentType	Category	Ionization	Chemical Formula
1	EDDP	SRM		None	

Compound Name	Precursor Mass	Product Mass	Collision Energy	RT (min)	Window (s
EDDP	278.192	234.080	0.00	2.340	30.00

Compound Name	Precursor Mass	Product Mass	Collision Energy
EDDP	278.192	249.10001	0.00

	Compound Name	ExperimentType	Category	Ionization	Chemical Formula
2	EDDP-d3	SRM		None	
3	Meperidine	SRM		None	
4	Meperidine-d4	SRM		None	
5	Methadone	SRM		None	
6	Methadone-d3	SRM		None	
7	Normeperidine	SRM		None	
8	Normeperidine-d4	SRM		None	
9	Norpropoxyphene	SRM		None	
10	Norpropoxyphene-d5	SRM		None	
11	Propoxyphene	SRM		None	
12	Propoxyphene-266	SRM		None	
13	Propoxyphene-d5	SRM		None	

Figure 2. CDS showing quantifier and qualifier ions

Data Acquisition and Real Time Status

During acquisition, the data may be viewed in real time per the parameters set up in the Master Method. The status of the acquisition, pressure profile, event log, devices, multiplexing status (Figure 6) and sample queue are also monitored in the Real Time Status view.

Data Review

As soon as data are acquired, they are automatically processed per parameters set in the Master Method. Any sample parameters out of range are automatically flagged in the data review. Figure 7 shows the review pane for one compound.

Reporting

TraceFinder 1.1 software comes with over 50 report templates with additional custom reports available. Figures 8 and 9 show examples of standard reports.

Figure 3. Master Method creation process showing general parameters, peak detection settings including retention times, mass filters, ion ratio settings and calibration curve settings

	RT	Compound	LOD (Detection limit)	LOQ (Quantitation	LOR (Reporting limit)	ULOL (Linearity limit)	Carryover limit
1	1.56	Meperidine	10.000	20.000	20.000	1e4	0e0
2	1.70	Normeperidine	2.000	5.000	20.000	1e3	0e0
3	2.34	EDDP	10.000	20.000	20.000	1e4	0e0
4	2.64	Norpropoxyphene	10.000	20.000	20.000	1e4	0e0
5	2.86	Propoxyphene	10.000	20.000	20.000	1e4	0e0
6	2.86	Propoxyphene-266	10.000	20.000	20.000	1e4	0e0
7	2.94	Methadone	10.000	20.000	20.000	1e4	0e0

	RT	Compound	R^2 threshold	Max RSD (%)	Min RF	Max Amt Diff (%)
1	1.56	Meperidine	0.9800	20.00	0.000	20.00
2	1.70	Normeperidine	0.9800	20.00	0.000	20.00
3	2.34	EDDP	0.9800	20.00	0.000	20.00
4	2.64	Norpropoxyphene	0.9800	20.00	0.000	20.00
5	2.86	Propoxyphene	0.9800	20.00	0.000	20.00
6	2.86	Propoxyphene-266	0.9800	20.00	0.000	20.00
7	2.94	Methadone	0.9800	20.00	0.000	20.00

	RT	Compound	Method	Percentage	Max Conc
1	1.56	Meperidine	None		
2	1.70	Normeperidine	Concentration		6.670
3	2.34	EDDP	% of LOD	50.00	5.000
4	2.64	Norpropoxyphene	% of LOQ	20.00	4.000
5	2.86	Propoxyphene	% of LOR	10.00	2.000
6	2.86	Propoxyphene-266	None		
7	2.94	Methadone	None		

Figure 4. Many flagging parameters can be set for samples, standards, controls and blanks. The user can later select which flags to use for reporting (selected tabs).

Define a sample list for the batch

	Status	Filename	Sample type	Sample level	Sample ID	Sample name	Comment	Vial position	Injection volume	Conv Factor	Channel
1		Unknown1	Unknown						10.0	1.000	Auto
2		Unknown2	Unknown						10.0	1.000	Auto
3		Unknown3	Unknown						10.0	1.000	Auto
4		Unknown4	Unknown						10.0	1.000	Auto
5		Unknown5	Unknown						10.0	1.000	Auto
6		Unknown6	Unknown						10.0	1.000	Auto
7		Unknown7	Unknown						10.0	1.000	Auto
8		Unknown8	Unknown						10.0	1.000	Auto
9		Unknown9	Unknown						10.0	1.000	Auto
10		Unknown10	Unknown						10.0	1.000	Auto
11		Unknown11	Unknown						10.0	1.000	Auto
12		Unknown12	Unknown						10.0	1.000	Auto
13		Unknown13	Unknown						10.0	1.000	Auto

Figure 5. A sample batch ready for acquisition including assignment of multiplexing channels

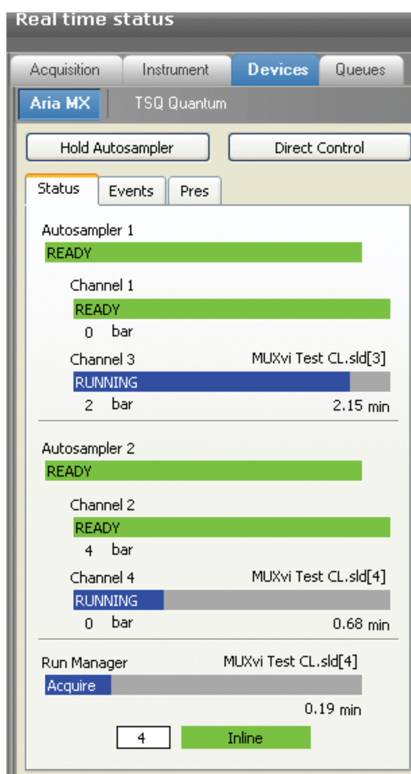


Figure 6. Real Time Status view displaying multiplexing status

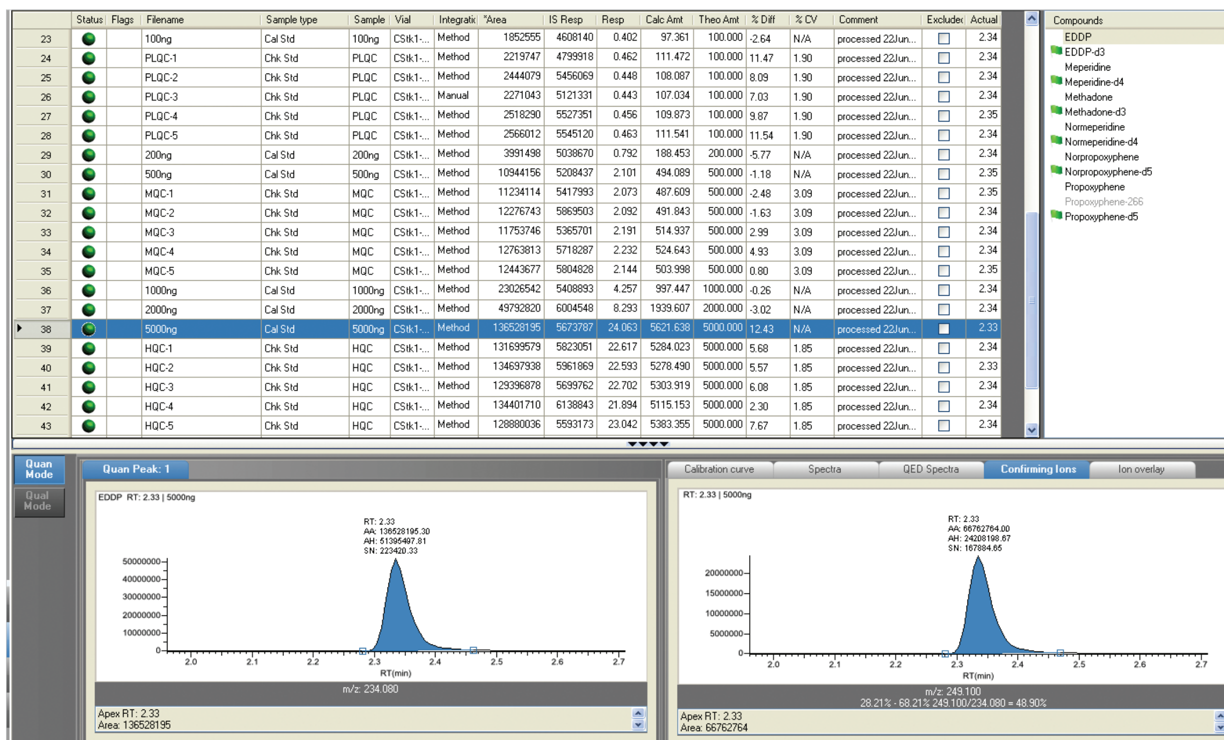
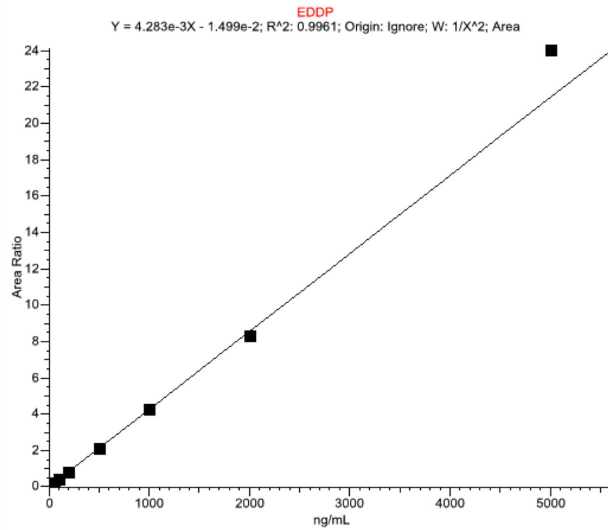


Figure 7. Data Review Confirming Ion window for EDDP, one of the six synthetic opioids, showing injection results, quantifier ion chromatogram and qualifier ion chromatogram

Compound Calibration Report

Lab Name:	Clinical Marketing		Page 1 of 2
Instrument:	TSQ Quantum Ultra		Method: 20110707_Opiods3
User:	Thermo Scientific		
Batch:	20110707		Cali File: 20110707.calx



Linear Pass

Level	Std Amount	Std Area	IS Amount	IS Area	Response ratio	Calc Amt	Units	%CV	%RSD
20ng	20.000	367529	200	5101141	0.072	20.321	ng/mL	N/A	N/A
50ng	50.000	1045386	200	5315652	0.197	49.415	ng/mL	N/A	N/A
100ng	100.000	1852555	200	4608140	0.402	97.361	ng/mL	N/A	N/A
200ng	200.000	3991498	200	5038670	0.792	188.453	ng/mL	N/A	N/A
500ng	500.000	10944156	200	5208437	2.101	494.089	ng/mL	N/A	N/A
1000ng	1000.000	23026542	200	5408893	4.257	997.447	ng/mL	N/A	N/A
2000ng	2000.000	49792820	200	6004548	8.293	1939.607	ng/mL	N/A	N/A
5000ng	5000.000	136528195	200	5673787	24.063	5621.638	ng/mL	N/A	N/A

Figure 8. Compound Calibration Report for EDDP

Status	Compound Name	Compound Type	Quan Peak m/z	Total Response	Quan Peak Response	Quan Peak RT	Theoretical amount	Concentration	Confirming 1 Mass	Confirming 1 Response	Confirming 1 Ion Ratio Flag	Confirming 1 Ion Ratio	Confirming 1 Range
	Meperidine-d4	Internal Standard	224.200	3213110	3213110	1.56	200.000	200.000	178.200	2201547	False	68.52 %	56.04 % - 84.06 %
✓	Meperidine	Target Compound	220.110	6823552	6823552	1.57	500.000	471.029	174.130	4565657	False	66.91 %	53.69 % - 80.54 %
✓	Normeperidine	Target Compound	160.120	490038	490038	1.72	50.000	50.311	188.200	66808	False	13.63 %	5.99 % - 17.98 %
	Normeperidine-d4	Internal Standard	164.200	2346250	2346250	1.71	200.000	200.000	192.200	247015	False	10.53 %	5.00 % - 14.99 %
✓	EDDP	Target Compound	234.080	11234114	11234114	2.35	500.000	490.069	249.100	5551494	False	49.42 %	39.95 % - 59.93 %
	EDDP-d3	Internal Standard	234.100	5417993	5417993	2.34	200.000	200.000	249.120	2628125	False	48.51 %	33.46 % - 55.76 %
	Norpropoxyphene-d5	Internal Standard	100.100	479874	479874	2.64	200.000	200.000	147.100	63747	False	13.28 %	9.79 % - 18.18 %
✓	Norpropoxyphene	Target Compound	100.100	1083361	1083361	2.64	500.000	501.453	143.100	259174	False	23.92 %	18.53 % - 30.89 %
	Propoxyphene-d5	Internal Standard	271.300	1193078	1193078	2.85	200.000	200.000	58.220	824192	False	69.08 %	56.55 % - 84.83 %
✓	Propoxyphene	Target Compound	58.190	2152782	2152782	2.87	500.000	491.610	266.170	1402361	False	65.14 %	50.41 % - 75.62 %
	Methadone-d3	Internal Standard	268.140	5655433	5655433	2.95	200.000	200.000	105.020	1407969	False	24.90 %	18.30 % - 30.50 %
✓	Methadone	Target Compound	265.130	11460288	11460288	2.95	500.000	530.605	105.050	3388653	False	29.57 %	22.35 % - 37.25 %

Figure 9. Sample Report showing ion ratio confirmation

High Density Sample Report 1 Long

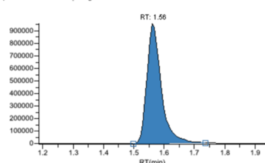
Lab Name: Clinical Marketing
Instrument: TSQ Quantum Ultra
User: Thermo Scientific
Batch: 20110707

Page 1 of 1

Method: 20110707_Opiods3

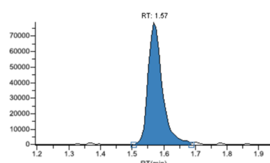
Cali File: 20110707.calx

Vial Pos	Sample ID	File Name	Level	Sample Name	File Date	Comment
CStk1-03:2		20ng	20ng		7/8/2011 4:23:59 PM	processed 22Jun2011

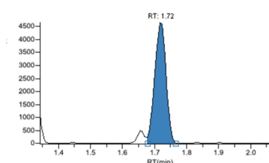


Meperidine-d4
Quan *m/z*: 224.20
Total Area: 3159582
Peak Area: 3159582
RT: 1.56min (1.55)

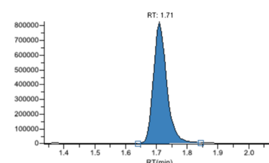
Amount: 1.000



Meperidine
Quan *m/z*: 220.11
Total Area: 246644
Peak Area: 246644
RT: 1.57 min (1.56)
TAmount: 20.000 ng/mL
Amount: 22.452 ng/mL

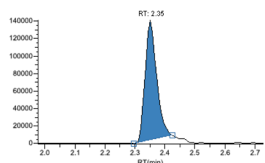


Normeperidine
Quan *m/z*: 160.12
Total Area: 11446
Peak Area: 11446
RT: 1.72min (1.70)
TAmount: 2.000 ng/mL
Amount: 1.844 ng/mL

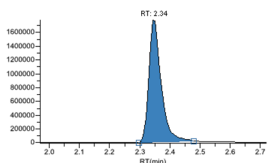


Normeperidine-d4
Quan *m/z*: 164.20
Total Area: 2478539
Peak Area: 2478539
RT: 1.71min (1.70)

Amount: 1.000

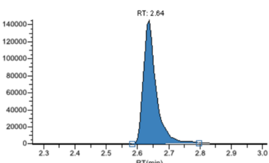


EDDP
Quan *m/z*: 234.08
Total Area: 367529
Peak Area: 367529
RT: 2.35 min (2.34)
TAmount: 20.000 ng/mL
Amount: 20.321 ng/mL



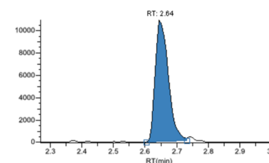
EDDP-d3
Quan *m/z*: 234.10
Total Area: 5101141
Peak Area: 5101141
RT: 2.34 min (2.34)

Amount: 1.000

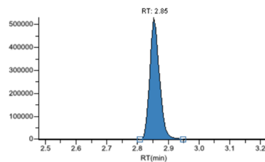


Norpropoxyphene-d5
Quan *m/z*: 100.10
Total Area: 412479
Peak Area: 412479
RT: 2.64min (2.63)

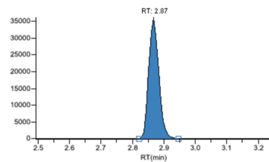
Amount: 1.000



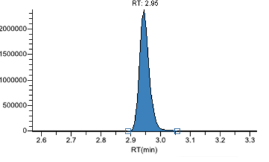
Norpropoxyphene
Quan *m/z*: 100.10
Total Area: 31463
Peak Area: 31463
RT: 2.64 min (2.64)
TAmount: 20.000 ng/mL
Amount: 22.669 ng/mL



Propoxyphene-d5
Quan *m/z*: 271.30
TotalArea: 1188454
Peak Area: 1188454
RT: 2.85min (2.85)
Amount: 1.000

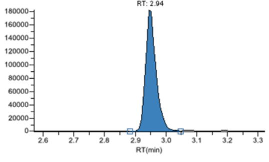


Propoxyphene
Quan *m/z*: 58.19
Total Area: 80927
Peak Area: 80927
RT: 2.87 min (2.86)
TAmount: 20.000 ng/mL
Amount: 22.697 ng/mL



Methadone-d3
Quan *m/z*: 268.14
Total Area: 5496688
Peak Area: 5496688
RT: 2.95min (2.94)

Amount: 1.000



Methadone
Quan *m/z*: 265.13
Total Area: 436828
Peak Area: 436828
RT: 2.94min (2.94)
TAmount: 20.000 ng/mL
Amount: 20.943 ng/mL

Figure 10. SRM chromatograms of six synthetic opiates in urine at 20 ng/mL

Results and Discussion

The method was linear from 20 to 5000 ng/mL for five of the six compounds. Normeperidine was linear from 2 to 1000 ng/mL. Standard accuracy ranged between 87.3% and 115%. Matrix effects were investigated by analyzing QCs prepared from six different lots of blank human urine. All samples showed recoveries within 20% at 50 ng/mL. The assay performance is summarized in Table 1. Figure 10 shows the SRM chromatograms of all six synthetic opiates at the limit of quantitation (LOQ).

Table 1. Assay performance for six synthetic opiates in urine

	% Recovery										
	Lot 1	Lot 3	Lot 4	Lot 5	Lot 6	Lot 7	Lot 8	Lot 9	R^2	Linear Range	LOQ
Methadone	105.0%	92.5%	97.3%	103.0%	98.5%	98.5%	99.3%	98.4%	0.9945	20-5000 ng/mL	20
EDDP	94.9%	97.1%	95.1%	96.0%	105.0%	92.4%	94.9%	98.2%	0.9951	20-5000 ng/mL	20
Meperidine	104.0%	97.6%	105.0%	98.4%	110.0%	104.0%	98.7%	102.0%	0.9935	20-5000 ng/mL	20
Normeperidine	117.0%	115.0%	111.0%	94.7%	105.0%	111.0%	108.0%	118.0%	0.9998	2-1000 ng/mL	2
Propoxyphene	98.7%	97.3%	96.4%	99.8%	98.7%	89.1%	95.5%	102.0%	0.9994	20-5000 ng/mL	20
Norpropoxyphene-dehydrate	97.8%	92.2%	87.3%	101.0%	94.5%	96.4%	97.1%	104.0%	0.9989	20-5000 ng/mL	20

Conclusion

TraceFinder 1.1 software was effectively used to perform routine analysis of the synthetic opiates in urine. The software enabled easy method setup, batch creation and submission, and real time monitoring. The data review functionality was useful for quick review and verification of the data. The generated reports had all the necessary information for record keeping for forensic laboratories.

In addition to these offices, Thermo Fisher Scientific maintains a network of representative organizations throughout the world.

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China +86 10 8419 3588
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland/Norway/Sweden +46 8 556 468 00
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9434
Italy +39 02 950 591
Japan +81 45 453 9100
Latin America +1 561 688 8700
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
New Zealand +64 9 980 6700
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Application Note: 545

DOWNLOAD

Quantitation of Six Synthetic Opioids in Urine Using a Triple Stage Quadrupole LC-MS System

Kristine Van Natta, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TSQ Quantum Ultra
- Transcend LX-2 system
- Forensic Toxicology

Introduction

Synthetic opioids have analgesic, antitussive and anti-addictive effects. However, they are also abused for their psychoactive effects and are often diverted from lawful prescriptions to unlawful recreational use. Simple, robust and precise analytical methods are needed to quantify these compounds in biological matrices for forensic purposes.

Goal

To develop a specific and robust dilute and shoot quantitative method for the analysis of six synthetic opioids and their primary metabolites in urine. These compounds include: methadone, EDDP, merperidine, normeperidine, propoxyphene and norpropoxyphene.

Methods

Sample Preparation

Urine was mixed with methanol containing deuterated analog internal standards. The supernatant was diluted with water prior to liquid chromatography-mass spectrometry (LC-MS) analysis.

HPLC Conditions

Chromatographic analysis was performed using Thermo Scientific Accela 600 HPLC pumps and a Thermo Scientific Hypersil GOLD aQ column (50 x 4.6 mm, 1.9 µm particle size). The total run time was 5 minutes.

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. Two selected reaction monitoring (SRM) transitions were monitored for each compound to provide ion ratio confirmations (IRC).

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at concentrations corresponding to the low (LQC), a middle (MQC) and high (HQC) end of the calibration range. Intra- and Inter- run variability and robustness were determined by analyzing five replicates of each QC level with a calibration curve on three different days. Matrix effects were investigated by spiking seven different lots of human urine with analytes at 50 ng/mL and calculating peak area recovery.

Results and Discussion

The method is linear from 20 to 5,000 ng/mL with R^2 values > 0.99 for all six compounds. Figure 1 shows the representative calibration curves. All IRCs passed within 20% of the standards average. All calibrators back calculate to within 15% of nominal, 20% for the limit of quantitation (LOQ). All quality controls quantitated to within 15% of nominal for the middle and high controls and within 20% for the low control. Inter-assay %CV was less than 10% for all QC levels. Table 1 shows quality control statistics for the validation runs.

No matrix effects were observed during validation. All samples showed recoveries within 20% of nominal. Internal standard variation was less than 5% between the different lots. Table 2 shows matrix effects testing results.

Figure 2 shows a reconstructed SRM chromatogram at LOQ.

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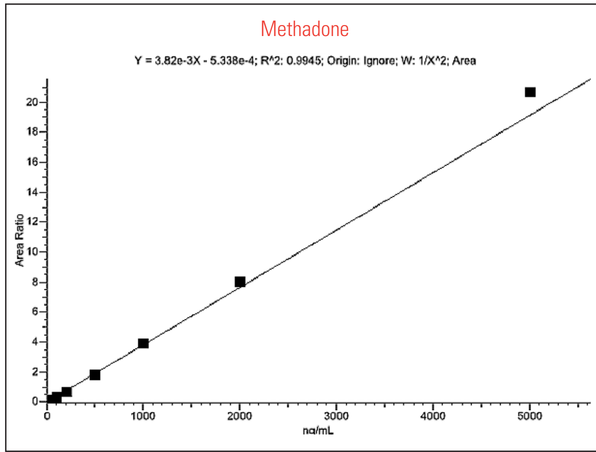

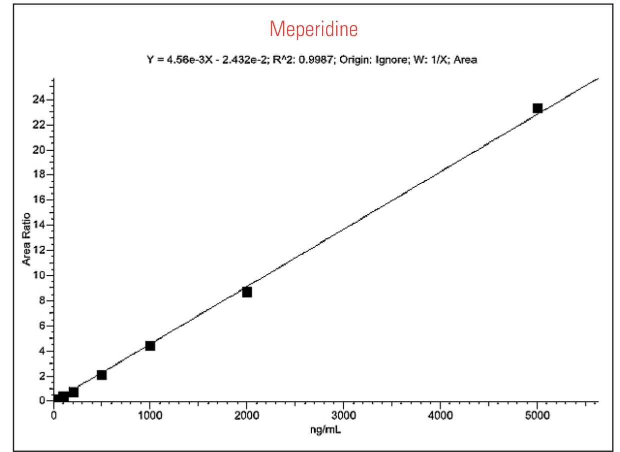

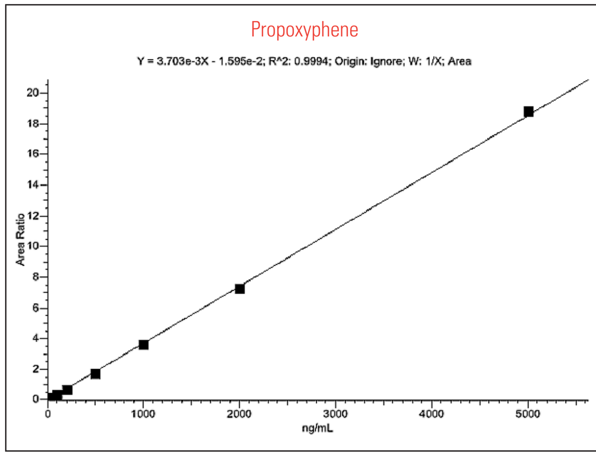

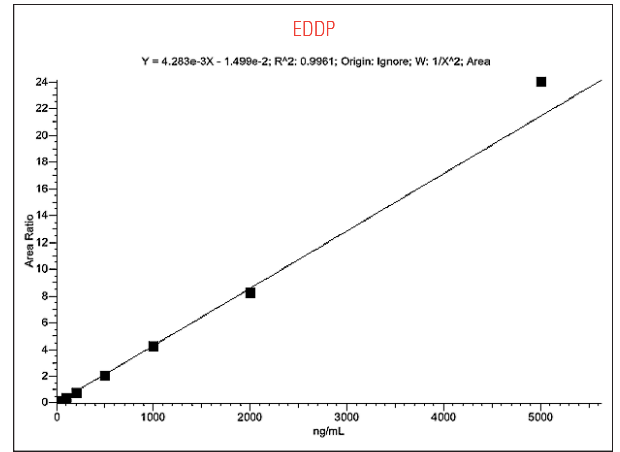

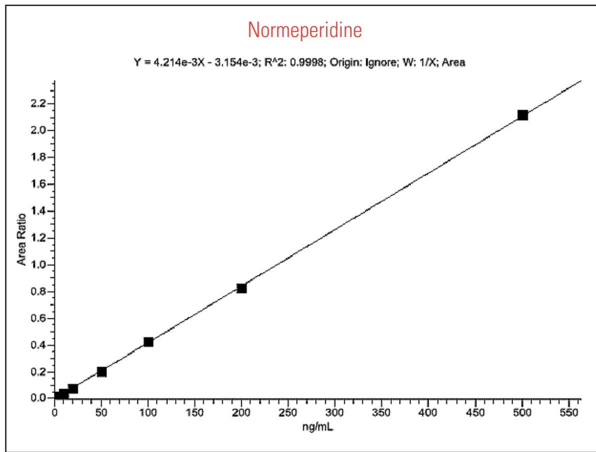

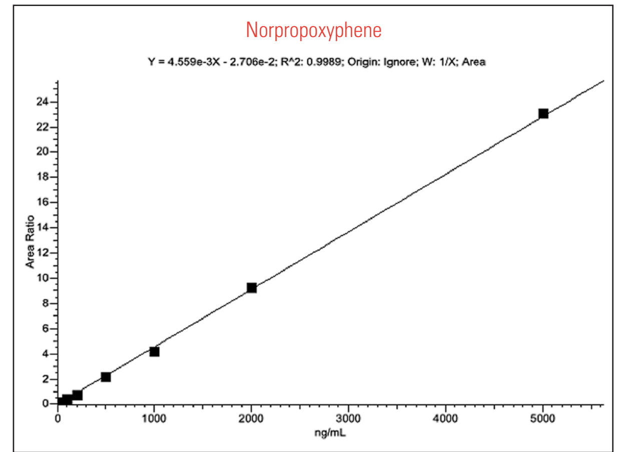

Figure 1. Representative calibration curves for methadone, EDDP, merperidine, normeperidine, propoxyphene and norpropoxyphene

Table 1. Inter-assay quality control statistics for validation runs

	%Bias/%CV					
	Methadone	**EDDP**	**Meperidine**	**Normeperidine**	**Propoxyphene**	**Norpropoxyphene**
LQC	4.16/4.71	2.67/6.04	0.493/5.93	7.04/9.17	5.45/3.42	3.28/8.64
MQC	7.32/2.47	-5.72/4.48	3.55/5.31	-0.747/7.61	4.36/5.28	0.933/3.88
HQC	10.9/2.69	-0.587/2.28	5.67/4.13	3.93/5.92	1.81/5.96	-3.77/8.18

Table 2. Percent recovery of six synthetic opioids in eight lots of human urine

	% Recovery							
Compound	**Lot A**	**Lot B**	**Lot C**	**Lot D**	**Lot E**	**Lot F**	**Lot G**	**Lot H**
Methadone	105	92.5	97.3	103	98.5	98.5	99.3	98.4
EDDP	94.9	97.1	95.1	96.0	105	92.4	94.9	98.2
Meperidine	104	97.6	105	98.4	110	104	98.7	102
Normeperidine	117	115	111	94.7	105	111	108	118
Propoxyphene	98.7	97.3	96.4	99.8	98.7	89.1	95.5	102
Norpropoxyphene	97.8	92.2	87.3	101	94.5	96.4	97.1	104

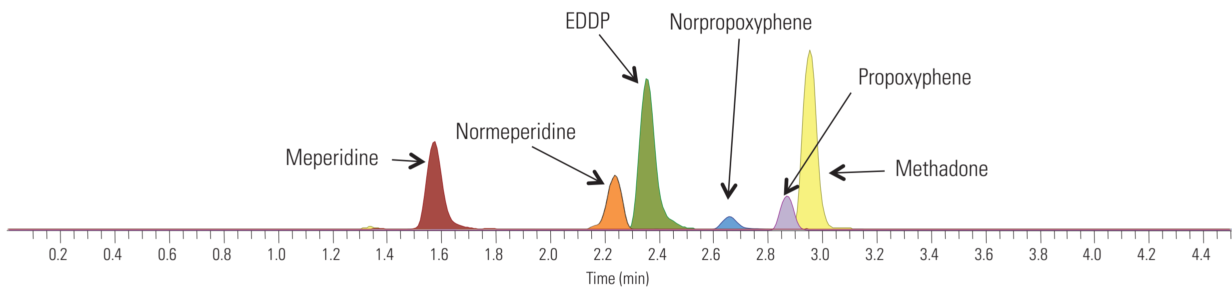

Figure 2. SRM chromatogram of six synthetic opioids and metabolites in urine at 20 ng/mL

Conclusion

A robust method with simple and easy sample preparation was developed and validated for forensic toxicology laboratories. The data window and total run time make this method amenable to multiplexing with the Thermo Scientific Transcend LX-2 LC system. Multiplexing with the Transcend™ LX-2 LC system would result in a run time of 2.5 minutes per sample.

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Application Note: 546

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Quantitation of Six Opiates in Urine Using a Triple Stage Quadrupole LC-MS System

Kristine Van Natta, James Byrd, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- TSQ Quantum Ultra
- Transcend LX-2 system
- Forensic Toxicology

Introduction

The natural opiates morphine and codeine are widely prescribed drugs for their analgesic, antitussive and antidiarrheal effects. However, they are also widely abused for their psychoactive effects and are often diverted from lawful prescriptions to unlawful recreational use. Simple, robust and precise analytical methods are needed to quantify these compounds in biological matrices for forensic purposes.

Goal

To develop a specific and robust dilute and shoot quantitative method for the analysis of primary natural opiates and their metabolites in urine. These compounds include: morphine, codeine, oxymorphone, oxycodone, hydromorphone and hydrocodone.

Methods

Sample Preparation

Urine was spiked with deuterated analog internal standards and hydrolyzed with ß-glucuronidase. Methanol was added to the hydrolysis mixture and the resulting mixture was centrifuged. Supernatant was further diluted and subject to LC-MS analysis.

HPLC Conditions

Chromatographic analysis was performed using Thermo Scientific Accela 600 HPLC pumps and a Thermo Scientific Hypersil GOLD aQ column (50 x 4.6 mm, 1.9 µm particle size). The total run time was 5 minutes.

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. Two selected reaction monitoring (SRM) transitions were monitored for each compound to provide ion ratio confirmations (IRC).

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at concentrations corresponding to the low (LQC), a middle (MQC) and high (HQC) end of the calibration range. Intra-run variability and robustness were determined by analyzing six replicates of each QC level with a calibration curve. Matrix effects were investigated by spiking seven different lots of human urine with analytes at 50 ng/mL and calculating peak area recovery.

Results and Discussion

The method is linear from 10 to 6,000 ng/mL with R^2 values > 0.99 for all six compounds. Figure 1 shows calibration curves for the six compounds. All calibrators back calculate to within 15% of nominal (20% for LOQ). All quality controls quantitated to within 15% of nominal for the middle and high controls and within 20% for the low control. %CV was less than 10% for all QC levels, except for codeine LQC which was 17.2%. Table 1 shows quality control statistics for the validation runs.

No matrix effects were observed during validation. All samples showed recoveries within 20% of nominal. Table 2 shows matrix effects testing results.

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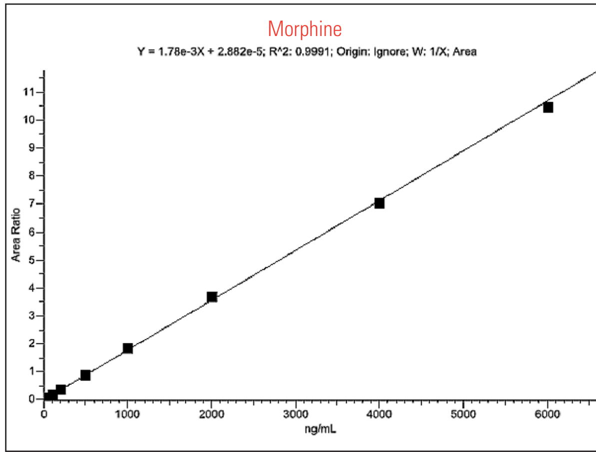

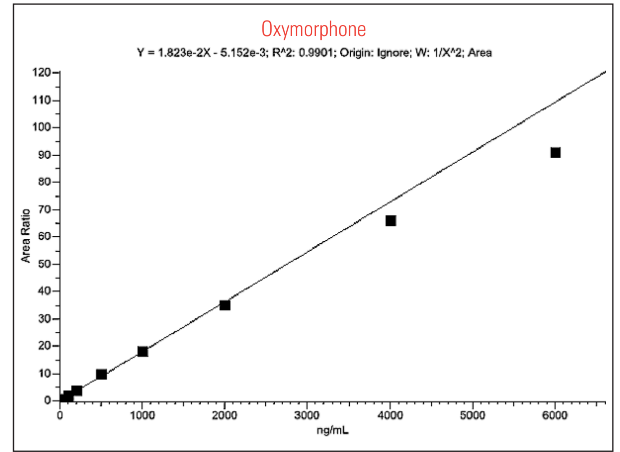

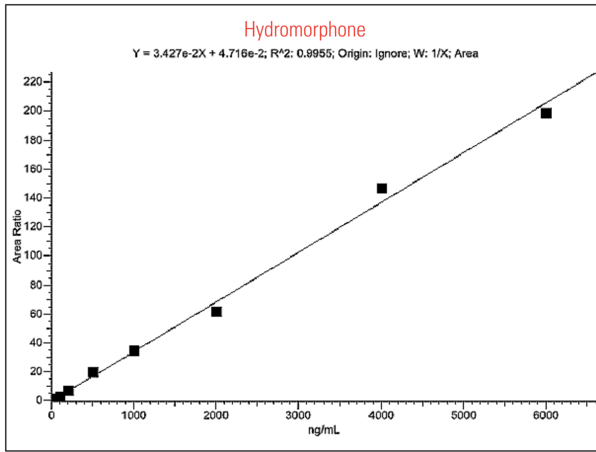

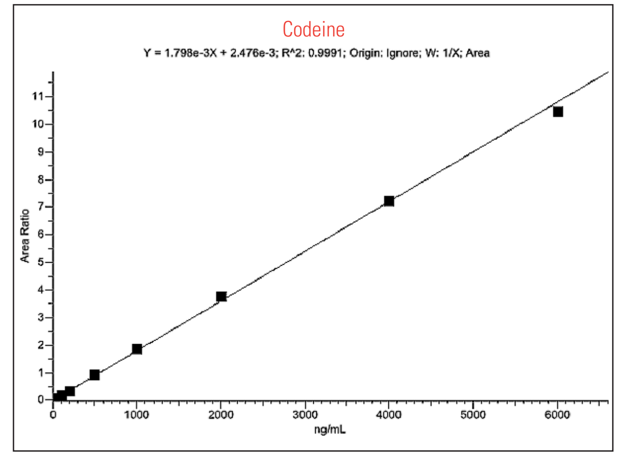

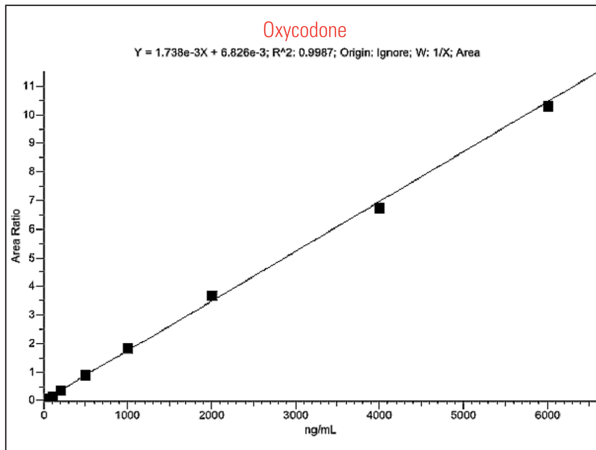

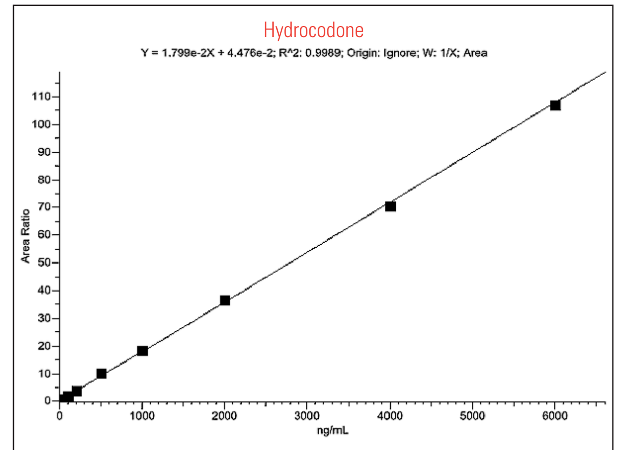

Figure 1. Representative calibration curves for opiates in urine

Table 1. Intra-assay quality control %Bias and %CV

	LQC		MQC		HQC	
	%Bias	%CV	%Bias	%CV	%Bias	%CV
Morphine	-9.42	8.72	2.92	3.29	4.50	2.24
Oxymorphone	12.2	3.45	7.50	2.35	0.00	4.16
Hydromorphone	-1.92	9.79	0.0833	6.63	-4.17	4.93
Codeine	-7.92	17.2	1.50	3.21	3.25	3.46
Oxycodone	-8.08	8.99	8.17	2.24	5.17	2.44
Hydrocodone	-2.42	8.84	7.25	3.60	5.58	4.07

Table 2. Percent recovery of six synthetic opioids in seven lots of human urine

	% Recovery						
Compound	**Lot 1**	**Lot 2**	**Lot 3**	**Lot 4**	**Lot 5**	**Lot 6**	**Lot 7**
Morphine	92.0	98.9	98.9	91.6	96.4	103	94.2
Oxymorphone	105	109	110	116	115	107	113
Hydromorphone	117	93.5	81.5	89.5	101	98.9	92.7
Codeine	113	113	104	98.9	112	108	103
Oxycodone	85.8	97.8	100	103	101	84.4	89.8
Hydrocodone	103	95.6	99.6	99.3	86.5	119	118

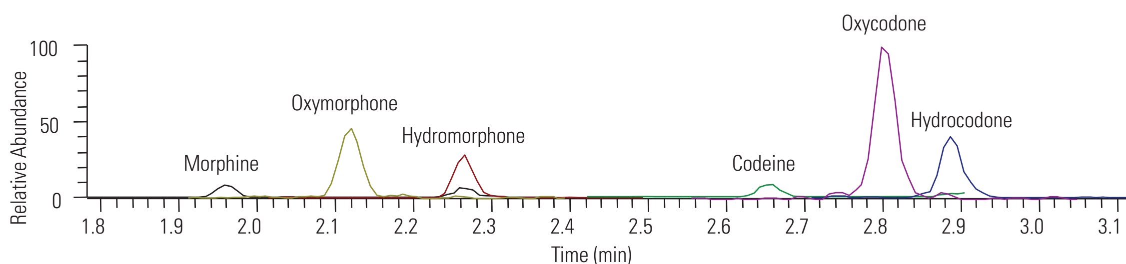

Figure 2. Representative chromatogram of six opiates in urine at LOQ of 10 ng/mL

Conclusion

A robust method with simple and easy sample preparation was developed for forensic toxicology laboratories. The data window and total run time make this method amenable to multiplexing with the Thermo Scientific Transcend system. Multiplexing with the Transcend™ LX-2 LC system would result in a run time of 2.5 minutes per sample. With an LX-4 LC system, the run time could be further reduced to 1.53 minutes per sample.

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Quantitation of 14 Benzodiazepines and Benzodiazepine Metabolites in Urine Using a Triple Stage Quadrupole LC-MS System

Kristine Van Natta, Marta Kozak; Thermo Fisher Scientific, San Jose, CA

Key Words

- **TSQ Quantum Ultra**
- **Forensic Toxicology**

Introduction

Benzodiazepines have a broad range of therapeutic use and are widely prescribed as safe drugs for the treatment of insomnia, anxiety and seizures and for their amnesic effects prior to medical procedures. They are also abused for their psychoactive effects, in suicide and in drug-facilitated sexual assault. Simple, robust and precise analytical methods are needed to quantitate these compounds in biological matrices for forensic purposes.

Goal

To develop a specific and robust dilute and shoot quantitative method for the analysis of 14 benzodiazepines and metabolites in urine. These compounds include: 2-hydroxyethylflurazepam, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, α-hydroxyalprazolam, α-hydroxytriazolam, alprazolam, desalkylflurazepam, diazepam, lorazepam, midazolam, nordiazepam, oxazepam and temazepam.

Methods

Sample Preparation

Urine was spiked with internal standards and hydrolyzed with β-glucuronidase. Deuterated analog internal standards were used for all compounds except α-hydroxytriazolam and lorazepam. Isotopic contribution from the di-chlorinated parent interfered with the d4 internal standards. Deuterated α-hydroxyalprazolam and oxazepam, respectively, were used instead. After hydrolysis, methanol was added to the hydrolysis mixture and the resulting mixture was centrifuged. Supernatant was further diluted and subject to LC-MS analysis.

HPLC Conditions

Chromatographic analysis was performed using Thermo Scientific Accela 600 HPLC pumps and a Thermo Scientific Hypersil GOLD aQ column (50 x 4.6 mm, 1.9 µm particle size). The total run time was 6.5 minutes.

MS Conditions

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. Two selected reaction monitoring (SRM) transitions were monitored for each compound to provide ion ratio confirmations (IRC).

The timed selected reaction monitoring (T-SRM) was used. T-SRM allows the instrument to scan only for those compounds that are expected to be eluting at a certain time. The data for a particular target compound is acquired only in a short window around the known retention time, not throughout the entire run. Using T-SRM significantly reduces the number of SRM transitions that are monitored in parallel at a certain retention time. At a constant acquisition rate (cycle time) a significantly longer scan time (dwell time) is available for each transition resulting in higher sensitivity and lower quantitation limits, improved RSDs and more data points per chromatographic peak.

Validation

Standard curves were prepared by fortifying pooled blank human urine with analytes. Quality control (QC) samples were prepared in a similar manner at concentrations corresponding to the low, middle and high end of the calibration range. Intra-run variability and robustness were determined by analyzing six replicates of each QC level with a calibration curve. Matrix effects were investigated by preparing samples in 8 different lots of human urine at twice the limit of quantitation (LOQ) of the method and monitoring peak area recovery compared to samples prepared in water.

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Results and Discussion

The method is linear from 25 to 10,000 ng/mL with R^2 values > 0.99 for all 14 compounds (Figure 1). All calibrators back calculate to within 15% of nominal (20% for LOQ). All quality controls quantitated to within 15% of nominal for the middle and high controls and within 20% for the low control. The %CV was less than 10% for all QC levels.

No matrix effects were observed during validation. All samples showed recoveries within 20% of nominal. Table 1 shows the matrix effect results.

Figure 2 shows an SRM chromatogram at LOQ.

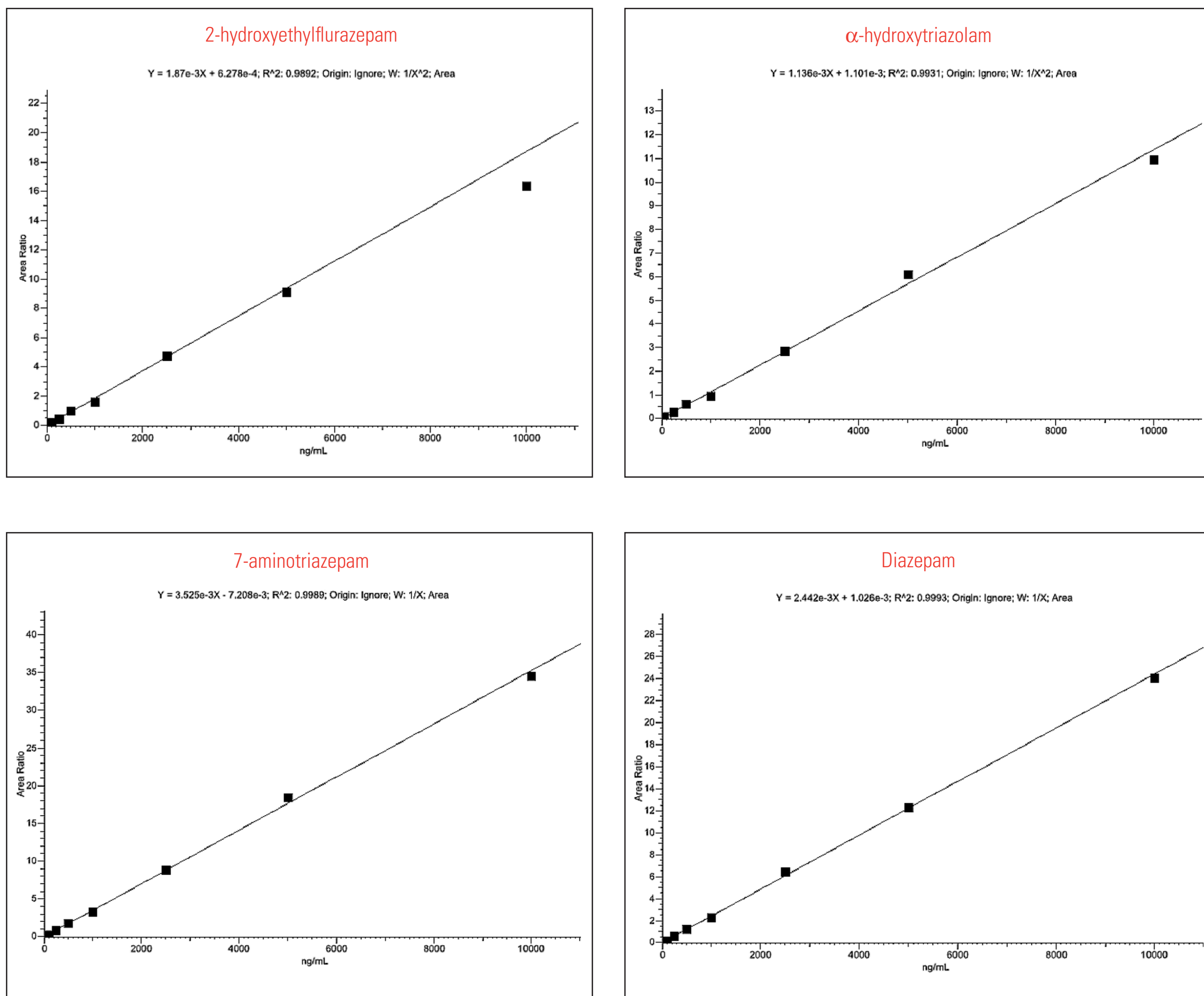

Figure 1. Representative calibration curves for some benzodiazepines showing linearity from 25-10,000 ng/mL in urine

Table 1. Percent recovery of 14 benzodiazepines in eight lots of urine

Compound	Lot A	Lot B	Lot C	Lot D	Lot E	Lot F	Lot G	Lot H
2-hydroxyethyl-flurazepam	83.6	94.7	113	106	131	107	101	102
7-amino-clonazepam	90.9	92.4	93.1	90.0	95.5	98.5	92.0	92.2
7-aminoflunitrzepam	97.1	98.0	100	101	97.6	108	94.9	96.5
7-aminonitrazepam	88.9	99.6	94.9	101	94.0	101	96.5	89.3
α-hydroxyalprazolam	107	104	90.9	112	105	106	113	99.3
α-hydroxytriazolam	95.5	107	101	96.9	87.5	90.7	109	107
alprazolam	108	101	107	110	107	98.9	92.7	95.5
desalkylflurazepam	108	89.3	104	97.6	103	98.9	105	103
diazepam	105	102	113	106	105	111	89.3	103
lorazepam	104	93.1	94.9	95.8	91.1	94.4	108	107
midazolam	113	111	110	101	104	107	105	95.6
nordiazepam	112	99.3	112	109	98.4	109	95.6	102
oxazepam	96.4	91.5	96.7	96.9	92.0	99.3	95.1	96.0
temazepam	105	98.2	99.1	95.5	101	99.1	98.2	101

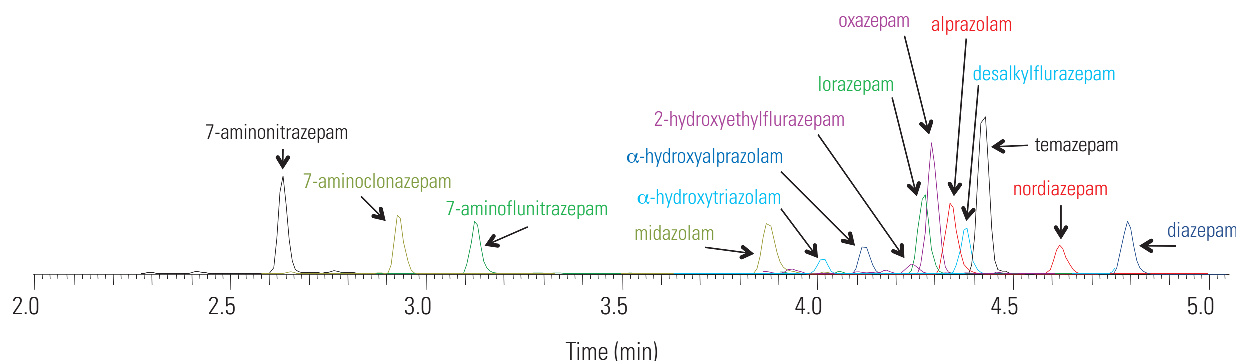

Figure 2. SRM chromatogram of 14 benzodiazepines and metabolites in urine at a concentration of 25 ng/mL

Conclusion

A robust dilute and shoot method with simple and easy sample preparation for the analysis of 14 benzodiazepines in 6.5 minutes was developed for forensic toxicology use. The data window and total run time make this method amenable to multiplexing with the Thermo Scientific Aria Transcend system. Multiplexing with the Transcend™ system would result in a run time of 3.5 minutes per sample.

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Application Note: 536

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Targeted Screening of Drugs of Abuse and Toxic Compounds with LC-MS/MS Using Triple Stage Quadrupole Technology

C. Gechtman, A. Masarin, A.O. Ospedale Niguarda Cà Granda, Milano, Italy
S. Scurati, Thermo Fisher Scientific, Milano, Italy
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For Forensic Toxicology Use Only.

Key Words

- TSQ Quantum Access MAX
- TraceFinder Software
- QED
- Forensic Toxicology

Introduction

Screening of biological samples for drugs of abuse and other toxic compounds is one of the main issues in forensic toxicology. The challenge is to provide rapid and accurate results despite the large number of targeted molecules and the complexity of biological matrices.

Here we present the workflow and results obtained by using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) timed selected reaction monitoring (T-SRM) method utilizing a triple stage quadrupole mass spectrometer. In a T-SRM experiment, the method is set to look for specific transitions only during the expected retention-time window. This increases the number of SRM transitions that can be monitored in a single experiment. It also increases the dwell time and duty cycle for monitoring individual compounds per experiment. Then, quantitation-enhanced data dependent (QED) MS/MS scan functions are used to trigger data dependent full scan MS/MS spectra from SRM transitions. When a particular SRM transition reaches a predefined intensity threshold, the instrument automatically triggers QED-MS/MS, using the reverse energy ramp (RER) scan function to increase the product ion sensitivity (Figure 1). Dynamic exclusion settings allow the maximum number of MS/MS collected for each compound to be specified, thus giving the ability to collect MS^2 spectra of coeluting molecules.

Goal

To evaluate a triple stage quadrupole mass spectrometer for targeted screening in human urine utilizing a LC-QED-MS/MS method for forensic toxicology laboratories. This screening technique is asked to be fast and reliable enabling high throughput screening.

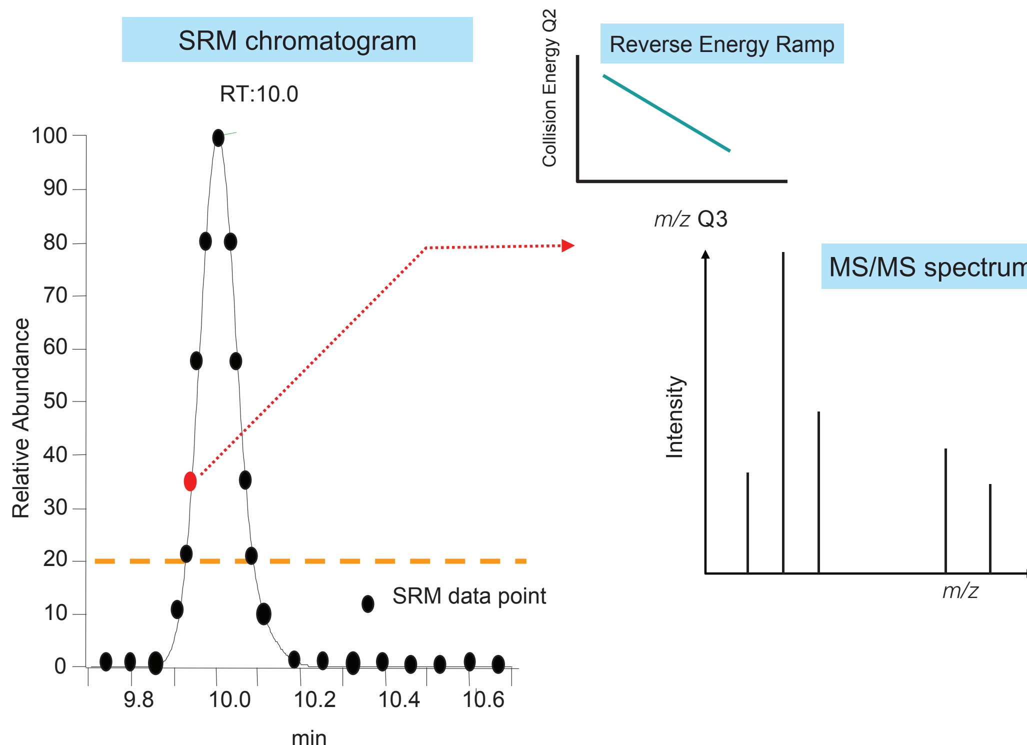

Figure 1: QED detection mode: when a monitored SRM transition reaches a targeted threshold, a full MS^2 spectrum is acquired using a Reverse Energy Ramp scan.

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Experimental Conditions

Sample Preparation

Urine was stored at -20 °C; for the analysis. After thawing, the urine was diluted 10 times with water. For the analysis, 10 µL of urine was directly injected into the LC-MS/MS.

Chromatography and Mass Spectrometry

A Thermo Scientific Hypersil GOLD PFP analytical column (50 x 2.1 mm, 5 µm) was used for separation of the compounds. A 15-minute gradient was set up using 10 mM ammonium formate and 0.1% formic acid in water for the mobile phase A and acetonitrile containing 0.1% formic acid for the mobile phase B.

The mass spectrometer was a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole with an Ion Max ion source. The instrument acquired SRM (Figure 2A) transitions of 294 compounds (drugs, toxic compounds, and metabolites) using T-SRM (Figure 2B). When an SRM transition reached 10,000 counts, QED detection was activated to collect full MS/MS spectra applying a ramp of collision energy from 15 to 35 eV (Figure 2C).

Data generated were processed with Thermo Scientific TraceFinder software for automated target screening. TraceFinder™ software can identify compounds based on their respective retention time, SRM transition, and full MS/MS spectra. The library contains 294 spectra of toxic and illicit compounds, and the corresponding SRM transitions are reported in the method.

Run Settings
MS Acquire Time (min): 10.00 | Experiment Type: QED MS
Chrom Filter Peak Width (s): 10.0 | Collision Gas Pressure (mTorr): 1.0

QED MS Settings
Q1 Peak Width (FWHM): 0.70 | Cycle Time(s): 1.000

#	Parent	Product	SRM Collision Energy	QED Start Energy	QED End Energy	Retention Time	Time Window	Polarity	Trigger	Reference	Name
281	340.200	128.170	42	15	35	6.80	3.00	+	10000	No	Propoxyphene
282	371.130	98.280	34	15	35	6.80	3.00	+	10000	No	Thioridazine
283	315.130	86.320	18	15	35	6.90	3.00	+	10000	No	Clomipramine
284	372.200	70.450	38	15	35	6.90	3.00	+	10000	No	Tamoxifen
285	345.190	327.180	15	15	35	7.00	3.00	+	10000	No	11-nor-9-carboxy-delta-9
286	438.180	143.180	30	15	35	7.00	3.00	+	10000	No	Fluphenazine
287	308.170	100.230	13	15	35	7.30	3.00	+	10000	No	Norpropoxyphene
288	444.180	221.060	63	15	35	7.30	3.00	+	10000	No	Thiothixene
289	369.200	167.080	19	15	35	7.60	3.00	+	10000	No	Cinnarizine
290	417.000	123.100	53	15	35	8.00	3.00	+	10000	No	Miconazole
291	472.250	454.310	22	15	35	8.30	3.00	+	10000	No	Terfenadine
292	355.170	280.080	30	15	35	9.00	3.00	+	10000	No	Vincamine
293	646.050	645.220	15	15	35	9.30	3.00	+	10000	No	Amiodarone
294	459.250	135.170	36	15	35	10.60	3.00	+	10000	No	Astemizole

A C B

Scan Parameters
Scan Time (s): 0.800 | Charge State: 1 | Q1 Peak Width (FWHM): 0.70

Advanced Data Dependent Settings And Activation
Dynamic Exclusion | Advanced Settings...

Figure 2: Method parameters used for LC-MS/MS screening of 294 compounds
Panel A: SRM transitions monitored
Panel B: Time segment used for Timed SRM
Panel C: When QED is activated an energy ramp from 15 to 35 eV is applied

Results and Discussion

The analysis time was 15 minutes. Figure 3A shows an example of an ion chromatogram of one of the monitored SRMs. Using QED-RER, the corresponding full MS^2 was recorded also (Figure 3B).

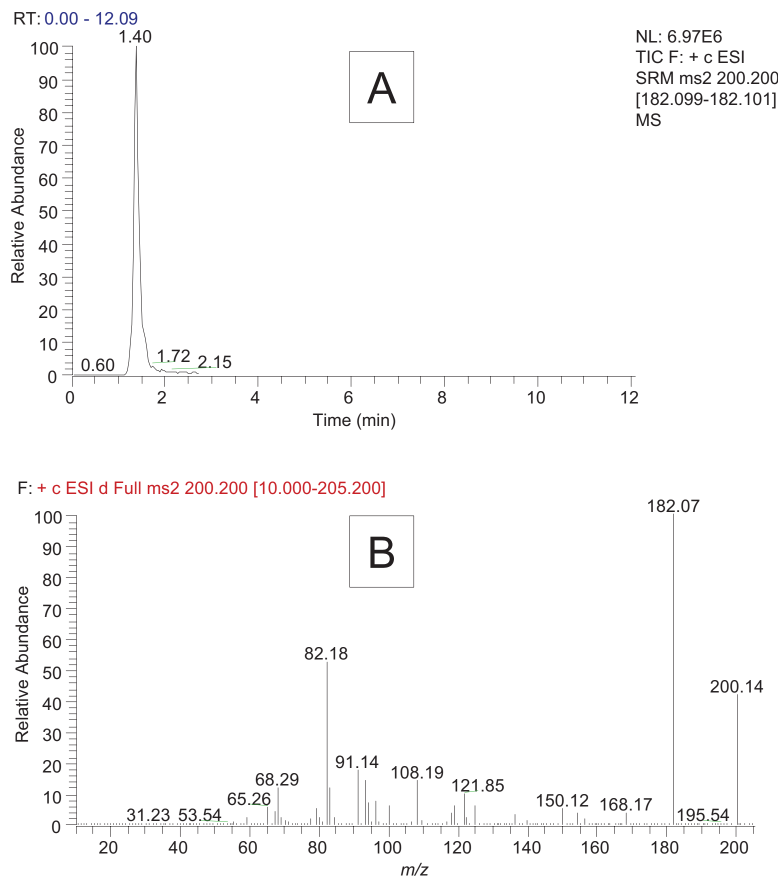


Figure 3: Example of ion chromatogram of transition 200 → 182 (A) and corresponding full MS^2 spectra collected (B)

Analyses were then processed with TraceFinder software using the Target Screening option (Figure 4), which allows the identification of target compounds present in the sample. Data obtained are highly specific and reliable because the identification of compounds is based on three parameters: retention time of the molecule, SRM transition, and MS/MS spectra.

Figure 5 shows an example of a summary report generated by TraceFinder software after the analysis of a urine sample that tested positive for cocaine. In addition to cocaine, *in vivo* metabolites such as benzoylecgonine, ecgonine methyl ester, and cocaethylene were also identified. The same sample was found positive for methadone – its metabolite, EDDP, was also identified.

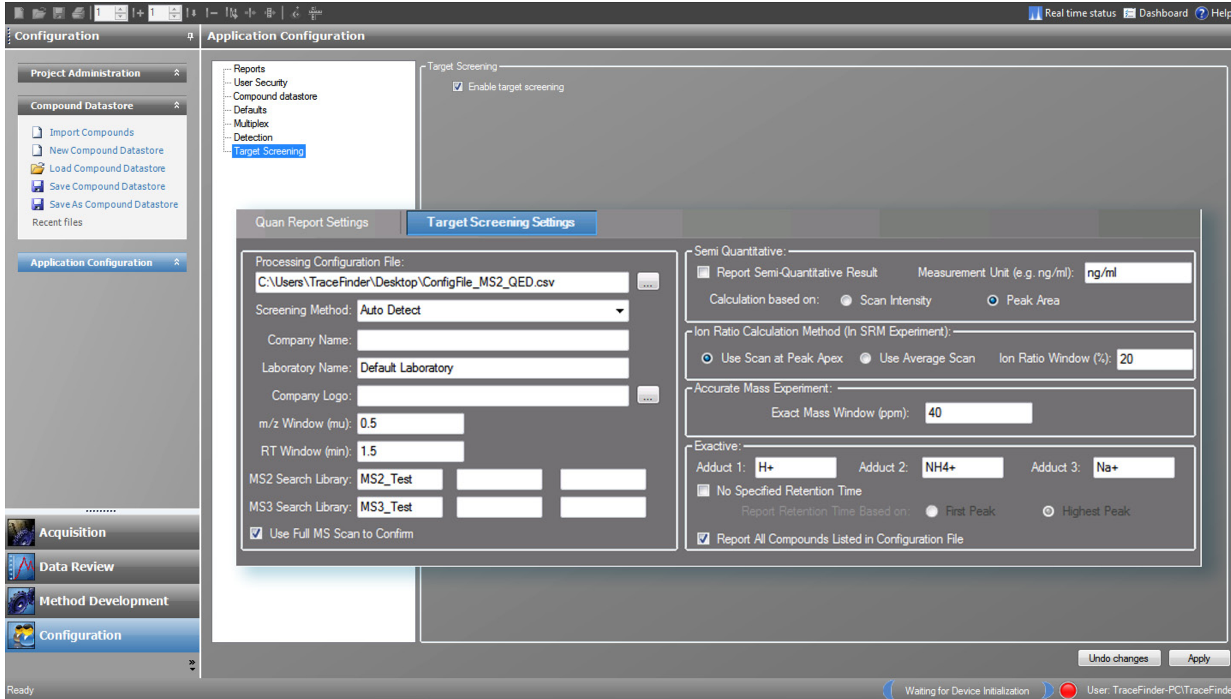
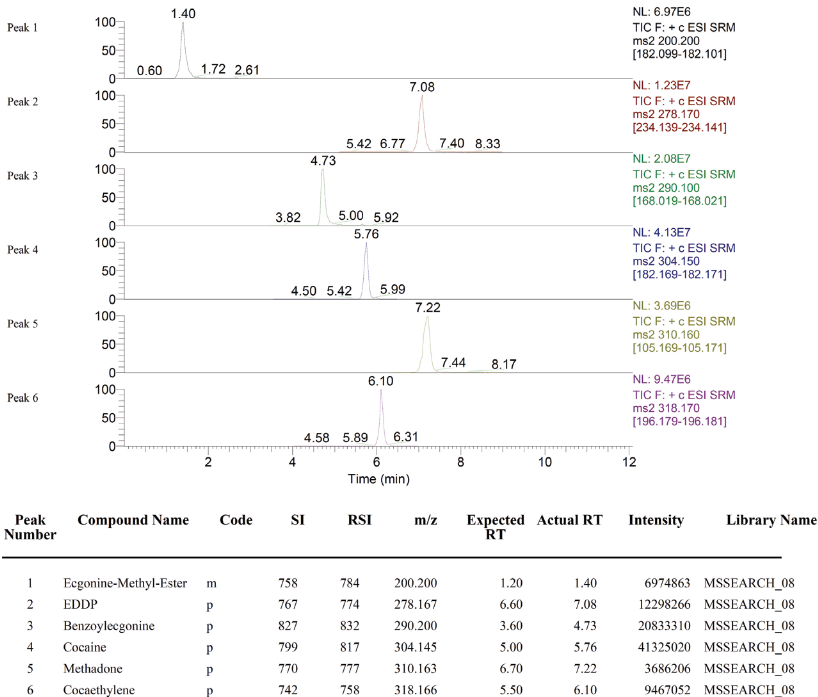


Figure 4. Selection of the Target Screening option in the configuration panel of TraceFinder software and settings used



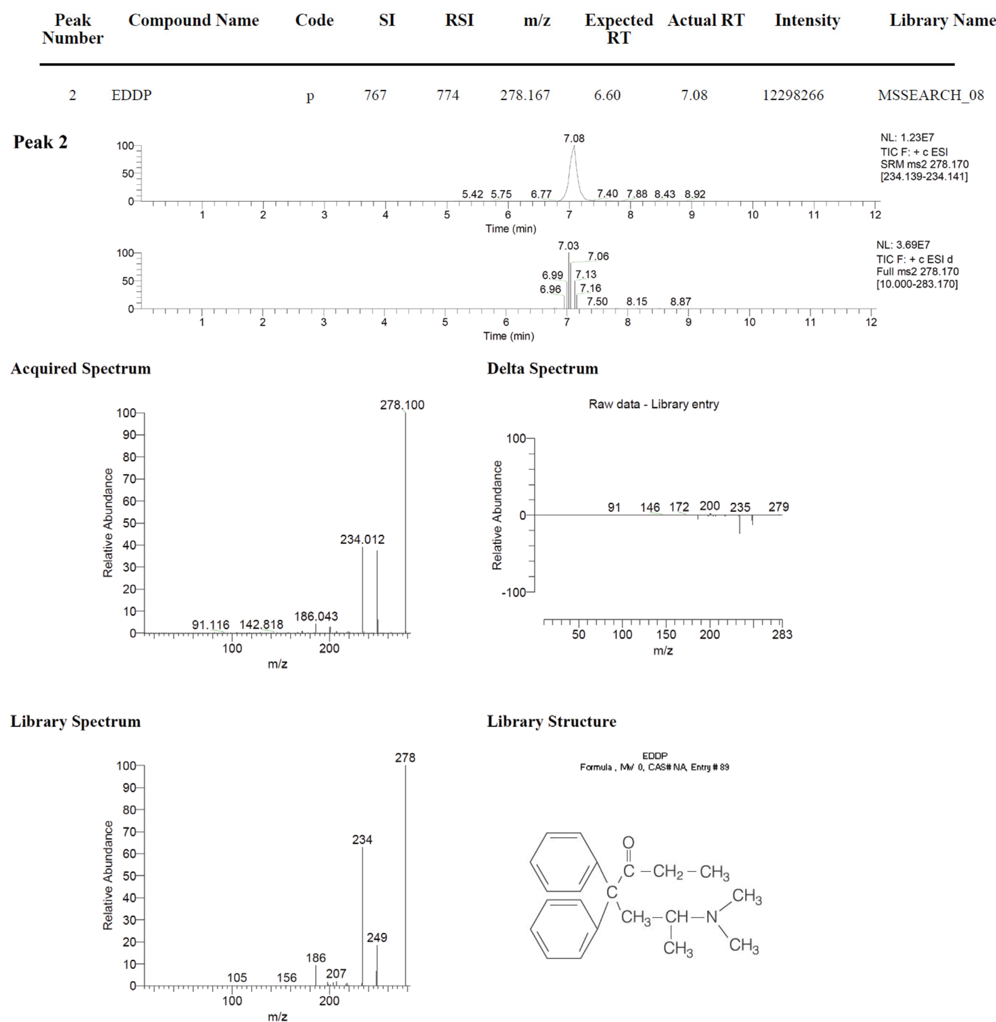
Peak Number	Compound Name	Code	SI	RSI	m/z	Expected RT	Actual RT	Intensity	Library Name
1	Egonine-Methyl-Ester	m	758	784	200.200	1.20	1.40	6974863	MSSEARCH_08
2	EDDP	p	767	774	278.167	6.60	7.08	12298266	MSSEARCH_08
3	Benzoylecgonine	p	827	832	290.200	3.60	4.73	20833310	MSSEARCH_08
4	Cocaine	p	799	817	304.145	5.00	5.76	41325020	MSSEARCH_08
5	Methadone	p	770	777	310.163	6.70	7.22	3686206	MSSEARCH_08
6	Cocaethylene	p	742	758	318.166	5.50	6.10	9467052	MSSEARCH_08

Figure 5: TraceFinder Target Screening Short Report showing ion chromatograms and a list of compounds detected in urine positive for cocaine and methadone

Figure 6 shows an extract of the long report generated by TraceFinder software, showing the comparison between experimental spectra and library spectra for each compound. All of the spectra showed a high matching score confirming the presence of cocaine, methadone, and their metabolites in the urine sample.

Conclusion

The TSQ Quantum Access MAX™ with T-SRM and QED-RER acquisition mode was used to screen toxic compounds and their metabolites in urine. This screening approach provides rapid sample preparation, ease-of-use, sensitivity, specificity, and a low cost per sample analysis for forensic toxicology laboratories.



Peak Number	Compound Name	Code	SI	RSI	m/z	Expected RT	Actual RT	Intensity	Library Name
2	EDDP	p	767	774	278.167	6.60	7.08	12298266	MSSEARCH_08

Figure 6. Extract of a TraceFinder Target Screening Long Report showing ion chromatograms and MS/MS spectra of EDDP detected in urine

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Application Note: 486b

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Simultaneous Analysis of Opiates and Benzodiazepines in Urine in Under 3 Minutes per Sample Using LC-MS/MS

Forensic Toxicology Use Only

Christopher L. Esposito, Matthew Berube, Francois Espourteille, Thermo Fisher Scientific, Franklin, MA

Key Words

- Transcend TLX-2 system
- TSQ Quantum Access Max
- Forensic Toxicology
- H-ESI
- SRM

Introduction

A two-channel liquid chromatography separation method has been developed for the simultaneous analysis of opiates and benzodiazepines in urine for forensic use. A Thermo Scientific Transcend TLX-2 system powered by multiplexing and automated online sample preparation technology was used to run two LC-MS/MS methods, one for each class of compounds. The multiplexing technology and data windowing of the system increase throughput with minimal operator intervention.

Experimental Conditions

Sample Preparation

Urine samples were spiked with a deuterated internal standard mix. Opiate samples were acidified to hydrolyze the metabolites, and then all samples were centrifuged.

HPLC

HPLC analysis was performed using the Transcend™ TLX-2 system. Samples were separated from the matrix using Thermo Scientific TurboFlow Cyclone-P polymer columns. Chromatographic separation was performed using a Thermo Scientific Hypersil GOLD C18 column (50 x 3 mm; 5 ! m) for benzodiazepines and a Hypersil GOLD™ PFP column (100 x 3 mm; 3 ! m) for opiates.

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer with a heated electrospray ionization source (H-ESI). The selective reaction monitoring (SRM) mode was used for mass spectrometry detection.

Results and Discussion

The analysis of directly-injected urine is accomplished for both drug classes. Seven benzodiazepines and internal standards and seven opiates and internal standards were analyzed. Figures 1 and 2 display data-windowed runs for selected benzodiazepines and opiates, respectively. Table 1 provides calibration curve statistics for several benzodiazepines and opiates.

Conclusion

The Transcend TLX-2 system with its unique multiplexing technology successfully runs two totally independent channels for forensic use. Limits of detection were 1 ng/mL (25 ng/mL for morphine). Quantitative analysis ranges were 5-5000 ng/mL for benzodiazepines and 50-25,000 ng/mL for opiates. Multiplexing both channels for analysis of benzodiazepines and opiates produces very significant time savings. The total MS data collection run times are efficiently reduced to less than 3 minutes per sample, inclusive of online sample preparation, thus resulting in more than 50% time savings versus running the analyses separately.

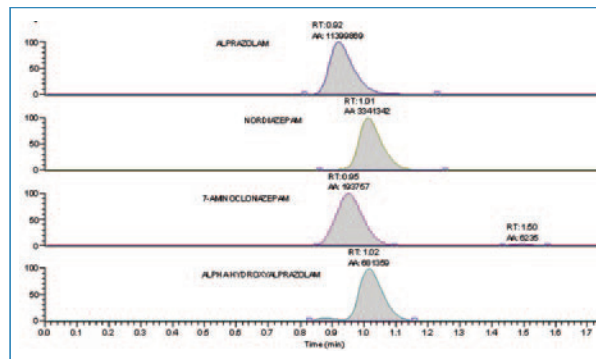

Figure 1: Data-windowed run for selected benzodiazepines

Assay performance summary

Target Analytes	Benzodiazepines	Opiates
Matrix	Urine	Urine
LOD	1 ng/mL	1 ng/mL (25 ng/mL morphine)
LOQ	5 ng/mL	50 ng/mL
Assay Linearity	1 ng/mL – 5 µg/mL	1 ng/mL – 25 µg/mL
Precision (%CV)	±15% (20% at LLOQ)	±15% (20% at LLOQ)
Sample Volume	10 µL	20 µL
Analysis Time	5.5 minutes, with a 2.5 minute data collection window	7 minutes, with a 3 minute data collection window

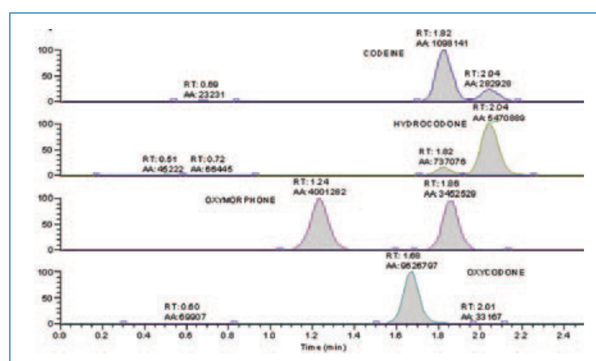

Figure 2: Data-windowed run for selected opiates

Table 1: Calibration curve statistics of 4 analytes

Analyte	R^2 (1/x weighing)	Range (ng/mL)	LOD (ng/mL)
Nordiazepam	0.9900	5-5000	1
Clonazepam	0.9960	5-5000	1
Oxymorphone	0.9903	50-25000	1
Hydromorphone	0.9950	50-25000	1

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Quantitation of Urinary Ethyl Glucuronide and Ethyl Sulfate Using Ultrahigh Resolution LC-MS

Forensic Toxicology Use Only

Kent Johnson, Fortes Laboratories, Portland, OR; Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Key Words

- Exactive
- Accela HPLC
- EtG / EtS
- Pain Management
- Forensic Toxicology

Introduction

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are sensitive and specific urinary biomarkers of recent alcohol intake that are of great interest in today's forensic toxicology laboratories.

Goal

To demonstrate the quantitation of EtG and EtS in urine using a liquid chromatography-mass spectrometry (LC-MS) method with ultrahigh resolution on the Thermo Scientific Exactive benchtop mass spectrometer.

Experimental

Calibration Standards and Sample Preparation

Calibration standards were prepared by spiking blank urine with EtG and EtS to final concentrations ranging from 25 ng/mL to 20,000 ng/mL.

Calibration standards and urine samples were spiked with internal standards (EtG-d5 and EtS-d5) and diluted 10 times with an LC mobile phase prior to injection onto the analytical column.

Commercial QC samples were used to obtain method accuracy and precision.

HPLC

HPLC analysis was performed using a Thermo Scientific Accela liquid chromatography system with a Thermo Scientific Hypersil GOLD C18 column (50 x 2.1 mm; 5 µm). A diluted sample of 20 µL was analyzed with a 6-minute gradient method.

Mass Spectrometry

MS analysis was carried out on the Exactive™ benchtop LC-MS instrument equipped with an electrospray ionization (ESI) source. Full scan data with resolution of 100,000 FWHM at *m/z* 200 was acquired.

Results and Discussion

Figure 1 shows the linear calibration curves for EtG (100-20,000 ng/mL) and EtS (100-20,000 ng/mL).

Figure 2 shows chromatograms of EtG and EtS at 25 ng/mL and the respective deuterated internal standards. Chromatograms for compound detection and quantitation are reconstructed with a mass tolerance of 5 ppm.

Conclusion

The Exactive benchtop LC-MS instrument provides excellent quantitative analysis of EtG and EtS in a 6-minute method. When applied to real samples, the method meets the demands of today's forensic toxicology laboratories with exceptional performance.

Method Performance Summary

Target Analytes	Ethyl glucuronide	Ethyl sulfate
Matrix	Urine	Urine
LOD	25 ng/mL	25 ng/mL or less
LOQ	100 ng/mL	100 ng/mL
Recovery	> 85%	> 85%
Precision	< 15%	< 15%
Assay Linearity	100 – 20,000 ng/mL	100 – 20,000 ng/mL
Carryover at LLOQ	< 1%	< 1%
Sample Volume	100 µL	100 µL
Analysis Time	6 minutes	6 minutes

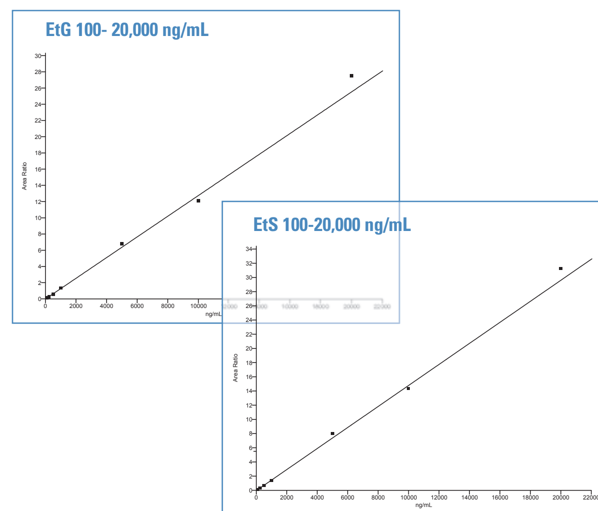

Figure 1: Linear calibration curves for EtG (100-20,000 ng/mL) and EtS (100-20,000 ng/mL).

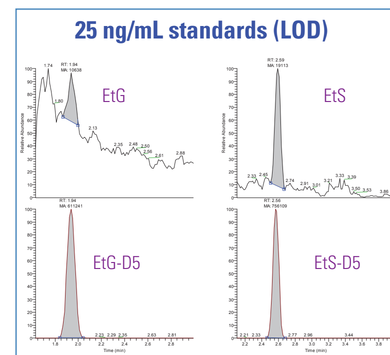

Figure 2: LOD chromatograms of EtG and EtS at 25 ng/mL with deuterated internal standards.

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Application Note: 489b

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Quantitation of 12 Benzodiazepines and Metabolites in Urine Using Ultrahigh Resolution LC-MS for Forensic Toxicology Use

Kent Johnson, Fortes Laboratories, Portland, OR; Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Key Words

- Exactive
- Accela HPLC
- Pain Management
- Forensic Toxicology

Goal

To demonstrate the quantitation of 12 benzodiazepines in urine using a liquid chromatography-mass spectrometry (LC-MS) method and ultrahigh resolution with the Thermo Scientific Exactive benchtop mass spectrometer for forensic analysis.

Experimental

Standards and Samples Preparation

Calibration standards were prepared by spiking blank urine with 12 benzodiazepines (lorazepam, nordiazepam, oxazepam, temazepam, hydroxytriazolam, 7-aminoclonazepam, 7-aminonitrazepam, hydroxyalprazolam, 7-aminoflunitrazepam, desalkylflurazepam, diazepam, and 2-hydroxyethylflurazepam) to final concentrations ranging from 10 ng/mL to 2,000 ng/mL.

Calibration standards and urine samples were spiked with internal standards (10 deuterated benzodiazepines), hydrolyzed and processed using a solid phase extraction (SPE) procedure.

Third party QC samples containing 6 benzodiazepines were processed and analyzed to obtain method accuracy and precision.

HPLC

HPLC analysis was performed using a Thermo Scientific Accela liquid chromatography system with a Thermo Scientific Hypersil GOLD PFP column (50 x 2.1 mm; 5 µm). A processed sample of 5 µL was analyzed with a 6-minute gradient method.

Mass Spectrometry

MS analysis was carried out on an Exactive™ benchtop LC-MS instrument with an electrospray ionization (ESI) source. Full scan data with resolution of 100,000 (FWHM) was acquired.

Results and Discussion

Figure 1 displays 6 of the 12 selected benzodiazepines at 10 ng/mL and internal standards. Chromatograms for compound detection and quantitation are reconstructed with a mass tolerance of 5 ppm.

Figure 2 shows the calibration curve for this set. Data results for the other six benzodiazepines are available upon request.

Conclusion

The Exactive benchtop LC-MS instrument provides excellent quantitative analysis of 12 benzodiazepines, from 10 ng/mL to 5000 ng/mL in urine, using ultrahigh resolution full scan data acquisition in a 6-minute method. The accuracy, precision, LOQ, and linearity range of the method meet the demands of today's forensic toxicology laboratories.

Method Performance Summary

Target Analytes	Benzodiazepines
Matrix	Urine
Limit of Quantitation (LOQ)	10 ng/mL
Recovery	> 85%
Assay Linearity	10 ng/mL – 5000 ng/mL
Precision (%CV)	< 4%
Carryover at Lower Limit of Quantitation (LLOQ)	< 1%
Sample Volume	2 mL
Analysis Time	6 minutes

Analyte	Mean Conc.(ng/mL)	% Recovery	%RSD
Oxazepam	248	99.3	1
Nordiazepam	234	93.5	1.4
Temazepam	218	87.1	4
Desalkylflurazepam	214	85.7	4
Lorazepam	227	90.8	0.4
Hydroxyalprazolam	255	102	0.4

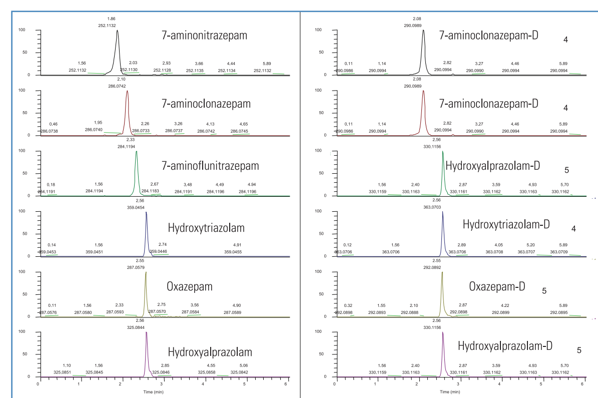

Figure 1: Chromatograms of 6 of the 12 selected benzodiazepines at 10 ng/mL and internal standards.

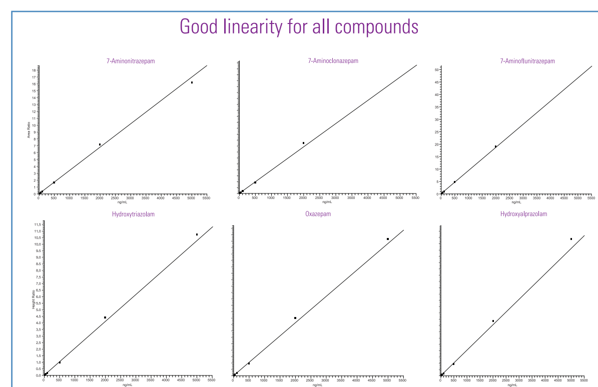

Figure 2: Calibration curves (10-5000 ng/mL) for all analytes

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Screening and Quantification of Multiple Drugs in Urine Using Automated Online Sample Preparation and Tandem Mass Spectrometry

Barbora Brazdova, Marta Kozak, Thermo Fisher Scientific, San Jose, CA

Key Words

- Transcend System
- TSQ Quantum Access MAX
- ToxID Software
- Clinical Research

Introduction

Liquid chromatography-mass spectrometry (LC-MS) is a sensitive, accurate, and precise technique applied in clinical research for the analysis of a large number of compounds and metabolites from various drug classes, such as antidepressants, hypnotics, stimulants, cardiacs, and antihistamines. Thermo Scientific Transcend system powered by TurboFlow™ technology provides an alternative separation technique for complex biomatrices, simplifying sample preparation, increasing LC-MS/MS sensitivity, and reducing ion suppression.

Goal

To develop a fast and efficient LC-MS/MS method using Thermo Scientific TurboFlow technology for the analysis of 30 drugs and metabolites in urine.

Experimental

Sample Preparation

Eight internal standards were used in the study for the corresponding compounds: nicotine-d4, cotinine-d4, midazolam-d4, diphenhydramine-d3, promethazine-d3, norlfuoxetine-d6, chlorpromazine-d3, and fluoxetine-d6. For the other compounds, the internal standard with the closest retention time was assigned.

Human urine samples (100 µL) were diluted with 100 µL of methanol containing the internal standards in concentrations of 100 ng/mL. The samples were vortexed and centrifuged. Then, 10 µL of the supernatant was injected onto the TurboFlow column.

HPLC

HPLC analysis was performed using the Transcend™ system with a TurboFlow Cyclone MAX column (0.5 x 50 mm) and a Thermo Scientific Hypersil GOLD PFP analytical column (100 x 2.1 mm; 5 µm). Total analysis time was 9 minutes.

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Quantum Access MAX triple stage quadrupole mass spectrometer equipped with a Thermo Scientific Ion Max source and an electrospray ionization (ESI) probe. Two selected reaction monitoring (SRM) transitions with scan times of 10 msec were collected for each analyte.

Results and Discussion

Quantitation of 30 drugs in urine was performed in 9 minutes with a calibration range of 1-1000 ng/mL for 14 compounds, 5-1000 ng/mL for 9 compounds, 10-1000 ng/mL for 5 compounds and 50-1000 ng/mL for 2 compounds. Figure 1 shows the chromatograms of the lowest calibration standard. Table 1 displays the calibration ranges and method precision for all analyzed drugs.

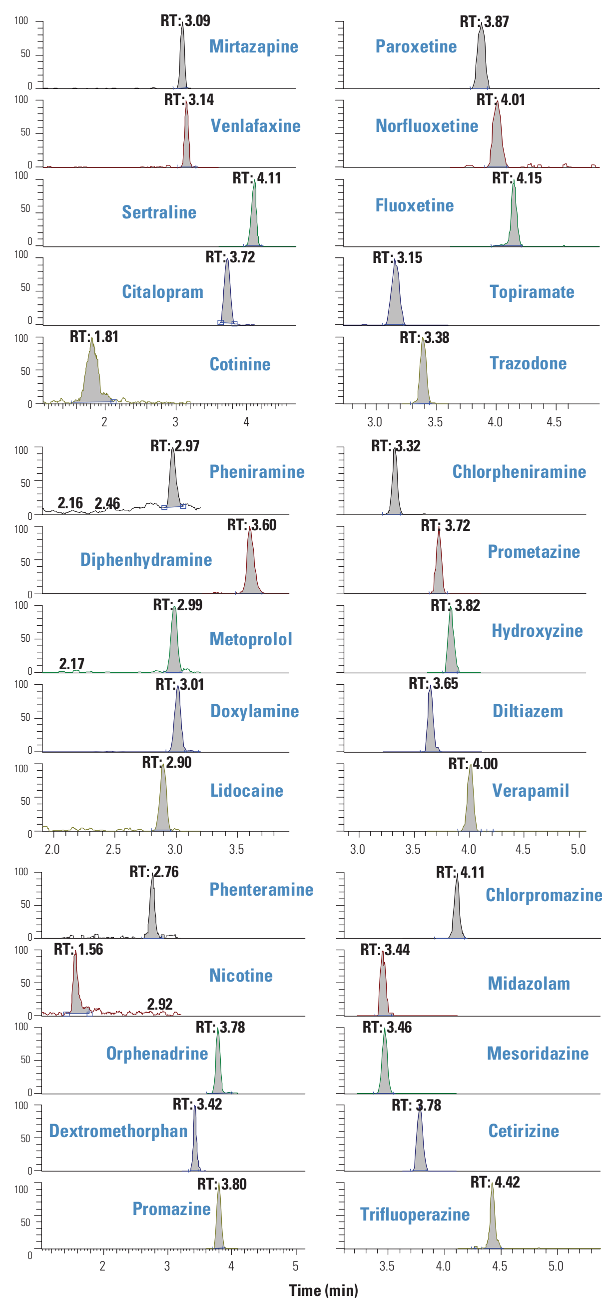

Figure 1: Chromatographs of the lowest calibration standard.

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Within-day and between-days precisions were determined with QC samples prepared by spiking blank urine to three concentrations: twice the lowest standard concentration (QC1), the middle of the calibration range concentration (QC2), and 80% of the highest standard concentration (QC3).

Table1: Calibration ranges, within-day precision, and between-days precision for the lowest QC sample.

Analyte	Calibration range (ng/mL)	Within-day (%RSD)	Between-days (%RSD)
Citalopram	1-1000	10.7	9.3
Fluoxetine	10-1000	10.4	9.1
Norfluoxetine	10-1000	16.4	11.0
Mirtazapine	1-1000	13.6	12.5
Paroxetine	10-1000	10.2	14.6
Sertraline	10-1000	8.0	15.7
Trazodone	1-1000	11.7	10.6
Venlaflaxine	1-1000	13.6	12.1
Diphenhydramine	1-1000	7.1	7.1
Chlorpheniramine	1-1000	8.2	7.2
Pheniramine	1-1000	7.6	5.6
Cetirizine	5-1000	15.4	15.1
Promethazine	50-1000	4.6	4.2
Nicotine	5-1000	10.7	7.1
Cotinine	5-1000	12.0	8.1
Dextromethorphan	1-1000	8.6	10.9
Topiramate	50-1000	13.2	10.4
Orphenadrine	1-1000	7.2	9.1
Lidocaine	1-1000	11.5	9.4
Phenteramine	10-1000	11.1	13.8
Mesoridazine	5-1000	3.4	4.4
Midazolam	1-1000	14.3	12.2
Chlorpromazine	5-1000	8.0	15.0
Promazine	5-1000	17.1	10.8
Trifluoperazine	5-1000	7.9	17.5
Diltiazem	1-1000	10.1	10.2
Metaprolol	5-1000	10.0	8.5
Verapamil	5-1000	8.7	9.2
Doxylamine	1-1000	14.4	11.7
Hydroxyzine	1-1000	17.9	14.0

Conclusion

An efficient (9 minute), sensitive (LOQ of 1-50 ng/mL), and precise LC-MS/MS method using TurboFlow technology was developed for the quantitation of 30 drugs in human urine. In clinical research, TurboFlow technology simplifies sample preparation and improves method robustness and sensitivity.

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Determination of LSD and Its Metabolites in Human Biological Samples by Liquid Chromatography–Tandem Mass Spectrometry

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Key Words

- TSQ Quantum
- Drugs of Abuse
- Forensic analysis
- LC-MS/MS
- LSD (Lysergic acid diethylamide
- LSD metabolites
- Toxicology

Introduction

Lysergic acid diethylamide (LSD) is a very potent hallucinogenic drug involving, particularly, behavioral disorders and is also extensively metabolized in man. Moreover, LSD and its major metabolites are present at low concentration in biological fluids, such as whole blood or urine. Identification and quantitation of such compounds for forensic use necessitate a sensitive and specific method. This study aims to describe a method using liquid chromatography/tandem mass spectrometry and permitting to quantify LSD and its metabolites at low concentrations.

Goal

The goal of this study was to identify and quantify LSD, iso-LSD, nor-LSD, nor-iso-LSD and 2-oxo-3-hydroxy-LSD in biological matrices. This report demonstrates the use of the TSQ Quantum for this application.

Experimental Conditions/Methods

Chemicals and Reagents

Lysergic acid diethylamide (LSD), d_3-LSD (internal standard), 2-oxo-3-hydroxy-LSD, iso-LSD, nor-LSD were purchased from Cerilliant (Austin, TX, USA). Ammonium formate and formic acid (>99 % pure) were purchased from Sigma. All reagents and solvents used in the extraction procedures were of analytical grade.

Sample Preparation

To 2 mL of serum, urine or whole blood content were added 100 µL of a 0.025 µg/mL aqueous solution of d_3-LSD (Internal Standard), 1 mL of a solution of pH 9.50 carbonate buffer and 8 mL of dichloromethane-isopropanol (95:5 by volume). The tubes were vortex-mixed and shaken on an oscillatory mixer. After centrifugation at 3,400 g for 5 min, the organic phase was poured in a conical glass tube and evaporated under a stream of nitrogen at 37°C. The dried extracts were reconstituted in 25 µL of acetonitrile : pH 3.0, 2 mmol/L ammonium formate (30:70 by volume) and 10 µL were injected into the chromatographic system.

Instrumentation Methods

HPLC Conditions

The chromatographic system consisted of a Shimadzu 10ADvp micro-flow rate, high-pressure gradient pumping system with a Rheodyne® Model 7725 injection valve equipped with a 5 µL internal loop. A C18, 5 µm (50×2.1 mm) column, maintained at 25°C, was used with a linear gradient of mobile phase A (pH 3.0, 2 mmol/L ammonium formate) and mobile phase B (acetonitrile:pH 3.0, 2 mmol/L ammonium formate [90:10; v/v]), flow rate of 200 µL/min, programmed as follows: 0-1.5 min, 5% B; 1.5-9 min, 5 to 50% B; 9-10 min, 50 to 90% B; 10-10.5 min, decrease from 90 to 5% B; 10.5-13 min, equilibration with 5% B.

MS Conditions

Mass Spectrometer: Thermo Scientific TSQ Quantum
Source: ESI mode
Ion Polarity: Positive
Spray Voltage: 4000 V
Sheath/Auxiliary gas: Nitrogen
Sheath gas pressure: 25 (arbitrary units)
Auxiliary gas pressure: 15 (arbitrary units)
Ion transfer tube temperature: 250°C
Scan type: SRM
Collision gas: Argon
Collision gas pressure: 1.5 mTorr

SRM Conditions

Settings were optimized by infusing at 5 µL/min a 1 µg/L solution containing the studied compound in acetonitrile: pH 3.0, 2 mmol/L ammonium formate (30:70, by volume). The structure of these compounds is shown in Figure 1.

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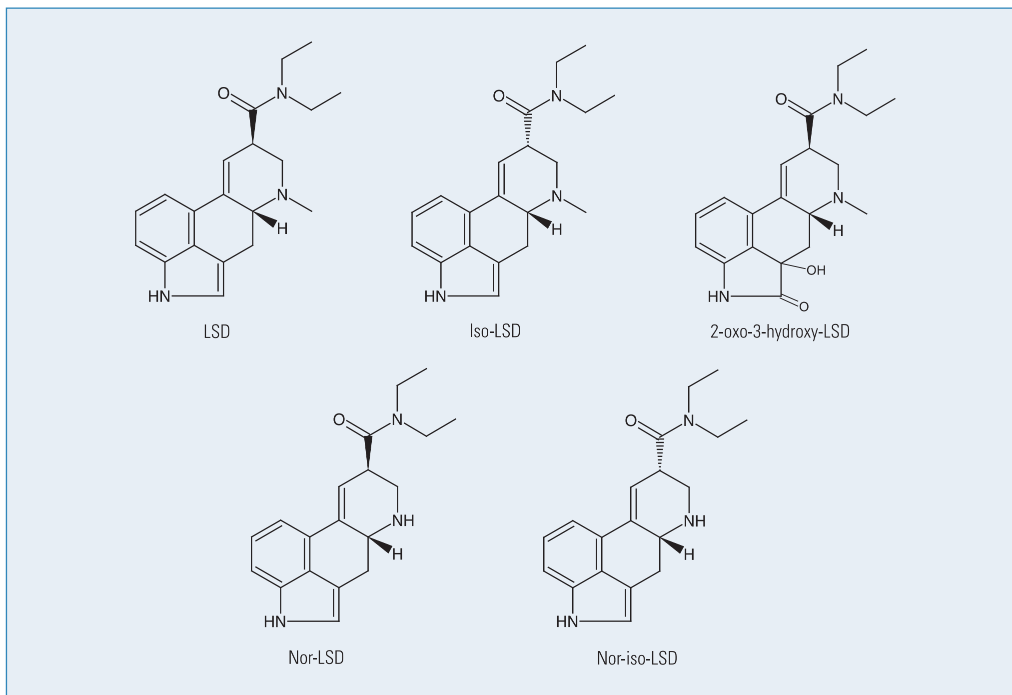

Figure 1: Structures of investigated compounds

Figure 2: Chromatogram of a urine spiked at 0.5 ng/mL

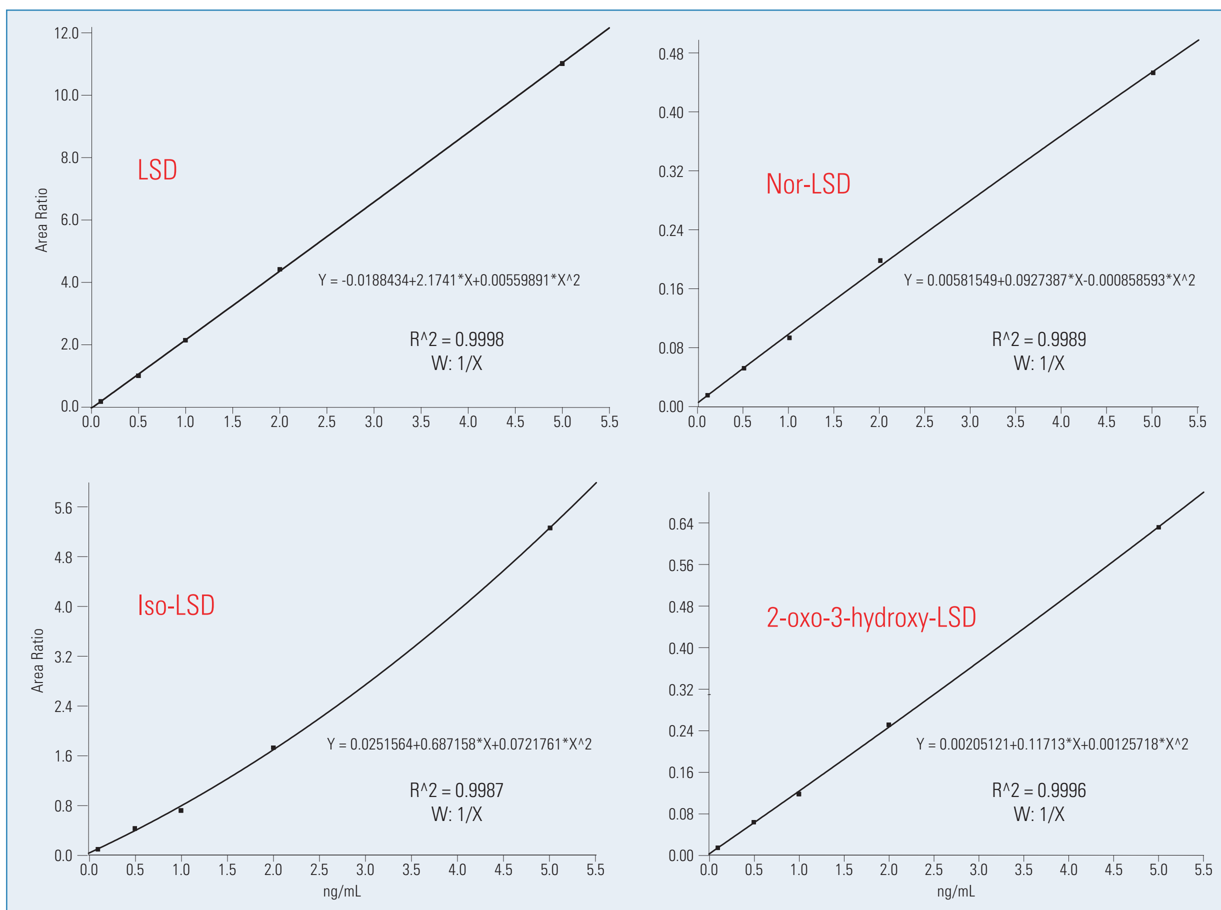

Figure 3: Representative calibration curves from standards spiked in urine

Compounds	Quantification transition	Collision energy	Confirmation transition	Tube lens voltage
LSD	324.0/223.1	30	324.01/207.1	50
Iso-LSD	324.0/223.1	30	324.01/207.1	50
Nor LSD	310.9/208.9	28	310.91/194.0	54
Nor-iso-LSD	310.9/208.9	28	310.91/194.0	54
2-oxo-3-hydroxy-LSD	356.0/236.6	30	356.01/222.0	36
d3-LSD	327.1/210.1	50	327.11/226.2	30

Results and Discussion

The LC-ESI/SRM chromatograms obtained for a blank urine spiked at 0.5 ng/mL are shown in Figure 2. As presented, LSD and iso-LSD are separated using the chromatographic conditions described previously. Identification of LSD is performed using two characteristic transitions and the retention time given by its deuterated internal standard.

Linearity

Calibration curves obtained for each compound spiked in urine samples are presented in Figure 3. Concentration ranges were comprised between 0.1 ng/mL and 5 ng/mL.

Conclusion

This application note described a sensitive, specific method developed for the quantitation of lysergide and metabolites in various biological matrices for forensic use.

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A Quantitative Test for Multiple Classes of Illicit Drugs and Their Primary Metabolites in Human Biological Fluids by LC-MS/MS for Forensic Use

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Key Words

- TSQ Quantum Discovery MAX
- Surveyor HPLC
- Forensic drugs of abuse testing
- SRM

Introduction

Currently, GC/MS is the method of choice for quantifying drugs of abuse. In recent years, however, many forensic labs have been switching to LC-MS/MS methods, which do not require time-consuming derivatization or extensive sample cleanup necessary in GC/MS analyses. Yet, many of the LC-MS/MS methods described in the literature either assay a limited number of illicit drug classes or do not include their primary metabolites of these illicit drugs (see table 1).[1-5] Herein is described a method to assay multiple drugs of abuse including opiates, stimulants, depressants, and the primary metabolites of these illicit drugs.

	Drug of Abuse	Parent *m/z*	Quantifier Product *m/z*	Qualifier Product *m/z*	Ion Ratio
A	Morphine	286	201	165	87
B	7-amino-nitrazepam	252	121	94	14.5
C	Ephedrine	166	115	133	95
D	Hydromorphone	286	185	157	56
E	Amphetamine	136	119	91	86
F	Codeine	300	165	215	97
G	7-amino-clonazepam	286	222	250	85
H	Noroxycodone	302	187	227	97
I	Methamphetamine	150	91	119	67
J	Oxycodone	316	241	256	65
K	MDA	180	135	105	92
L	6-MAM	328	165	211	68
M	Norketamine	224	125	179	43
N	Hydrocodone	300	199	171	28
O	Benzoylecgonine	290	168	105	24
P	7-amino-flunitrazepam	284	135	227	52
Q	MDMA	194	163	135	30
R	Ketamine	238	125	179	40
S	MDEA	208	163	135	32
T	Meperidine	248	220	174	55
U	Oxazepam	287	241	269	54
V	Nordiazepam	271	140	208	82
W	Cocaine	304	182	82	11.1
X	Lorazepam	321	275	229	25
Y	Nitrazepam	282	236	180	38
Z	Alprazolam	309	281	205	85
AA	Temazepam	301	255	177	11.8
BB	Clonazepam	316	270	214	28
CC	Diazepam	285	193	154	70
DD	Cocaethylene	318	196	82	15
EE	Flunitrazepam	314	268	239	34
FF	Methadone	310	265	105	18

Table 1: Summary of SRM transitions for 32 illicit drugs.

Goal

To apply a single LC-MS/MS method to screen for 32 illicit drugs of abuse and their metabolites in biological fluids.

Experimental Conditions

Sample Preparation

Whole blood or urine samples (0.1–0.4 mL) were spiked with 20 ng of isotopically labeled internal standards and purified by solid phase extraction (SPE). Extracted samples were reconstituted to yield solutions with the internal standards at 25 ng/mL.

HPLC

HPLC analysis was performed using the Thermo Scientific Surveyor HPLC System. Each 10 µL sample was injected directly onto a Thermo Scientific Hypersil GOLD PFP 50×2.1 mm, 3 µm analytical column. A gradient LC method used mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.3 mL/min.

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Quantum Discovery MAX triple stage quadrupole mass spectrometer with an electrospray ionization (ESI) probe. The MS conditions were as follows:

Ion source polarity: Positive ion mode
Ion transfer tube temperature: 370 °C
Scan Type: SRM
SRM scan time: 10 ms per transition
Q1, Q3 resolution: unit (0.7 Da FWHM)

Two SRM transitions were monitored for each component to provide ion ratio confirmations (IRC). Table 1 summarizes these SRM transitions.

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Results and Discussion

Figures 1 and 2 demonstrate the separation of 32 illicit drugs in less than 10 minutes. Using an SRM dwell time of 10 ms per transition yielded a minimum of 15 data points across an LC peak. The limits of quantitation (LOQs) were determined as either 0.5 ng/mL (lowest calibrator concentration used) or as the concentration where the percent relative errors and %CVs were less than 20% for five replicate injections.

As shown in Figure 3, most calibration curves were fit using linear regression. Some standards (for example, cocaine) yielded better statistical calibration curves using quadratic regression. In these select cases, the target compound used a structurally different isotopically labeled internal standard (for example, cocaine used D5-nordiazepam as internal standard).

The assay of biological sample extracts identified multiple drugs of abuse and related metabolites. Figures 4A and B demonstrate examples of urine and whole blood extracts assayed for the presence of illicit drugs with the developed LC-MS/MS method. Note that cocaine and benzoylecgonine were detected and qualified below the assay LOQs in a whole blood extract (Figure 4B), indicating that lower LOQs are possible for these compounds.

Conclusion

An LC-MS/MS method for assaying illicit drugs and their metabolites at an LOQ of 0.5–2.5 ng/mL in biological fluids for forensic use has been demonstrated. Confirmation of the drugs of abuse was achieved by monitoring two SRM transitions per compound and measuring their area ratios to within ±20%. Utilizing a low SRM dwell time of 10 ms per transition to achieve sufficient data points across a chromatographic peak had no adverse effects, such as SRM cross-talk, on the quantitation and confirmation of these illicit drugs. To authenticate this assay, extracts from biological fluids were analyzed, showing the presence of several drugs of abuse and their metabolites.

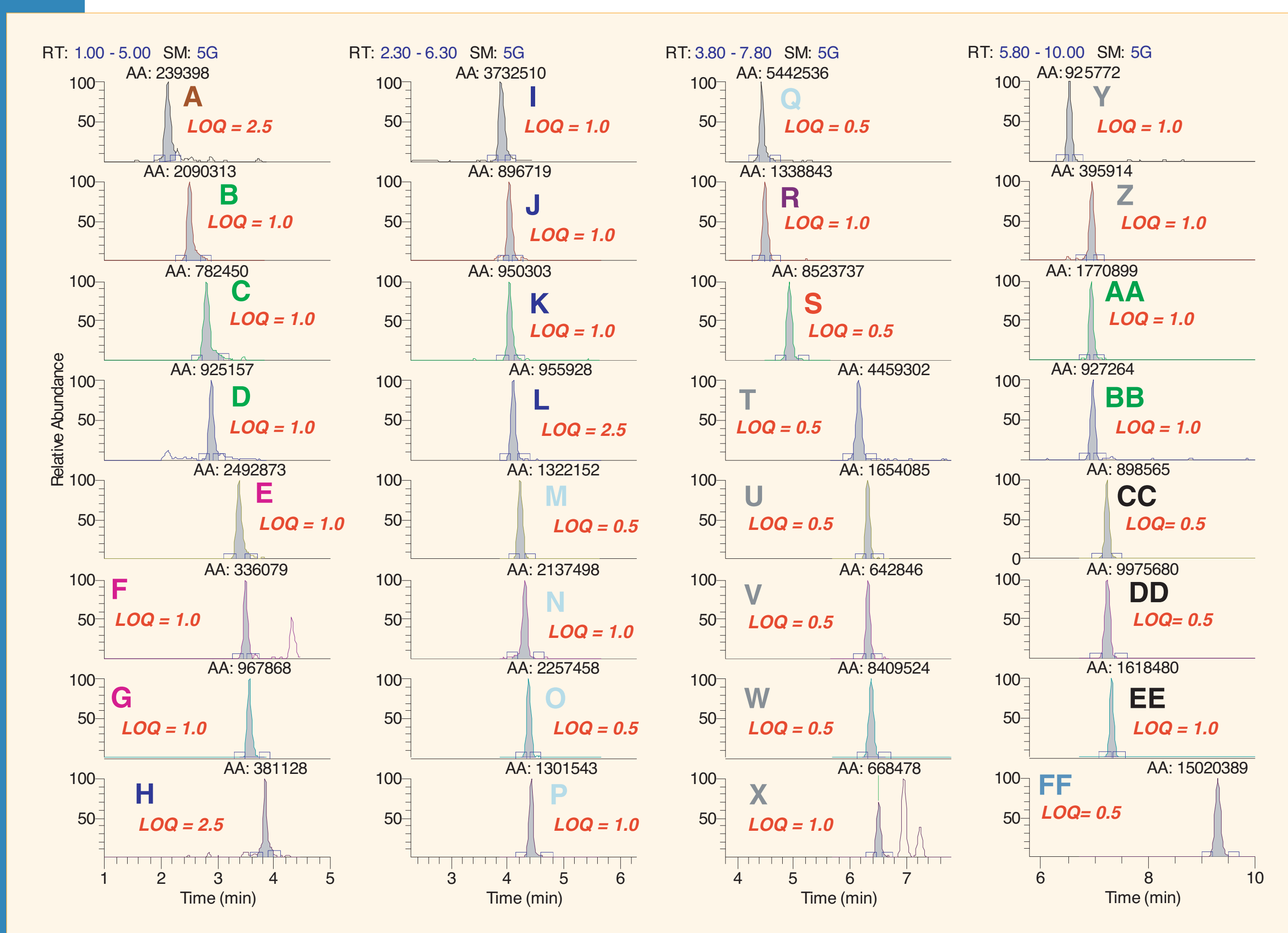

Figure 1: Quantifier SRM transitions for the 2.5 ng/mL standard. For the compound designators, refer to the legend in Table 1.

Figure 2: Qualifier SRM transitions for the 2.5 ng/mL standard. For the compound designators and the target ion ratio %, see Table 1.

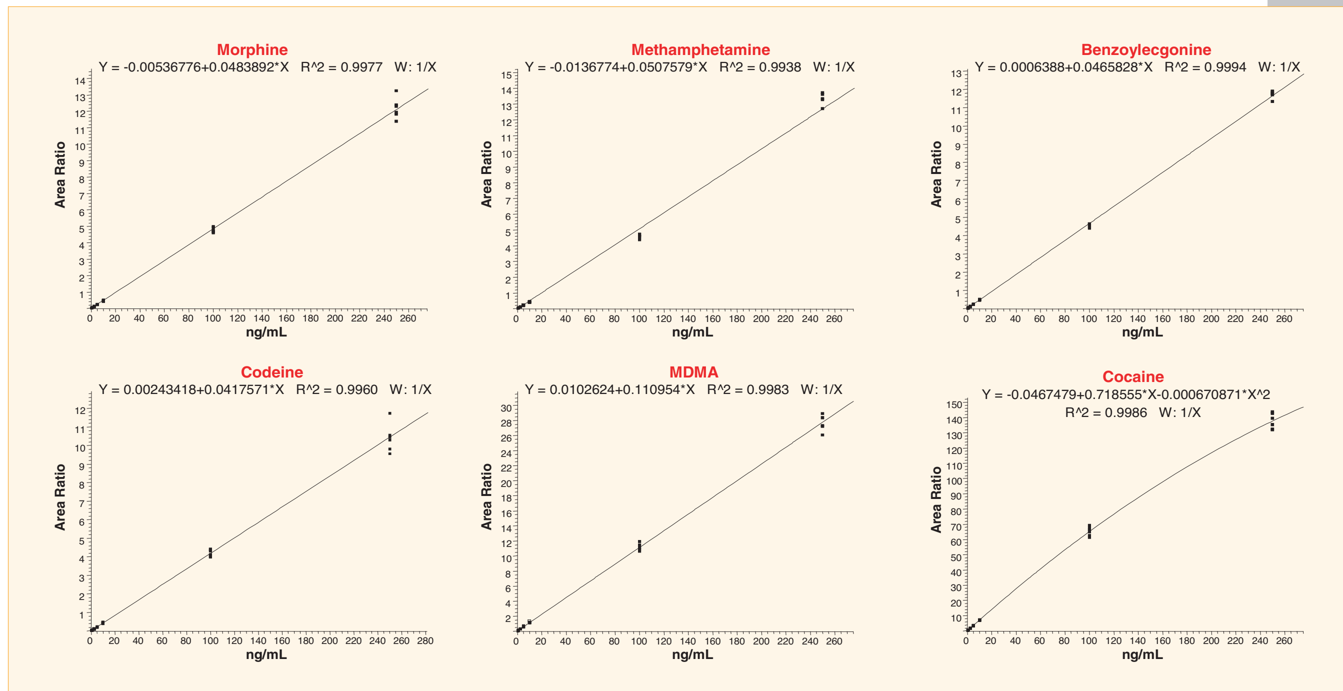

Figure 3: Calibration curves for select drugs of abuse. Regression curve fitting used 1/x weighting from five replicate injections, where R^2 > 0.993 for all standards.

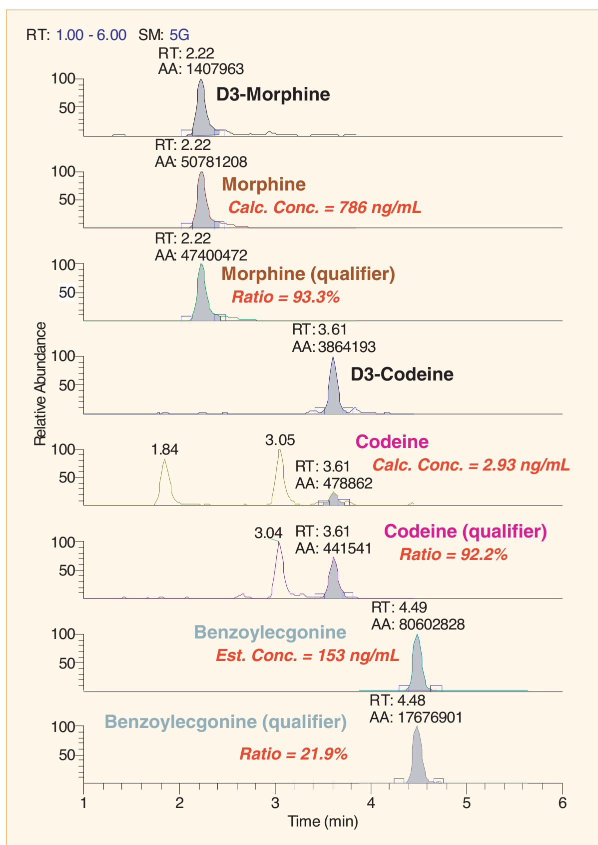

Figure 4A: Assay of urine extract (#423) targeting morphine and its metabolites. The concentration of benzoylecgonine is estimated because a labeled internal standard was not added to the sample extract.

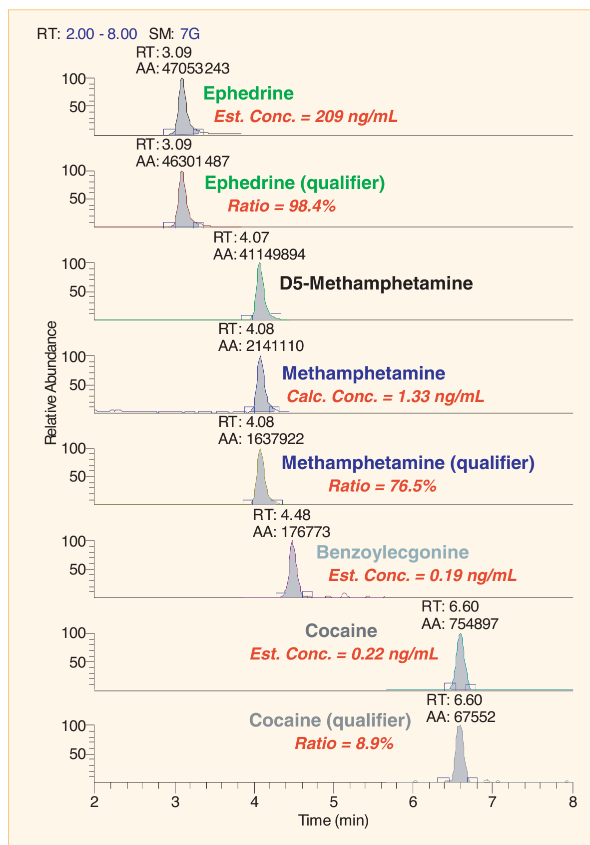

Figure 4B: Assay of whole blood extract (#473) targeting amphetamine and its metabolites. The concentrations of ephedrine, benzoylecgonine and cocaine are estimated because labeled internal standards were not added to sample extract.

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Quantitation of Fentanyl and Norfentanyl from Urine Using On-line High Throughput System

Francois A. Espourteille, Ph. D., Thermo Fisher Scientific, Franklin, MA

Key Words

- TurboFlow Technology
- TSQ Quantum Access
- Clinical Research
- Aria TLX-4

Introduction

The use of the Thermo Scientific Aria TLX-4 system with TurboFlow™ methods for automated on-line sample cleanup of a biological sample is well documented in the literature[1]. The Aria™ TLX-4 system enhances the sensitivity, specificity, and precision for mass spectrometric detection of fentanyl and norfentanyl. Increasing demand in clinical research laboratories for higher sample throughput has put the emphasis on automated methods and platforms that have the ability to quickly ramp up throughput to meet demand.

The Aria TLX-4 system extracts both fentanyl and its metabolite, norfentanyl, from the many interferences found in urine and chromatographically separates them from each other, before sending them to the mass spectrometer. TurboFlow extraction methods exclude both high molecular weight species and salts while the stationary phase coating retains the analyte(s) through reverse phase column chemistry. This results in fast, efficient, on-line separation of fentanyl and its metabolite prior to introduction into the mass spectrometer.

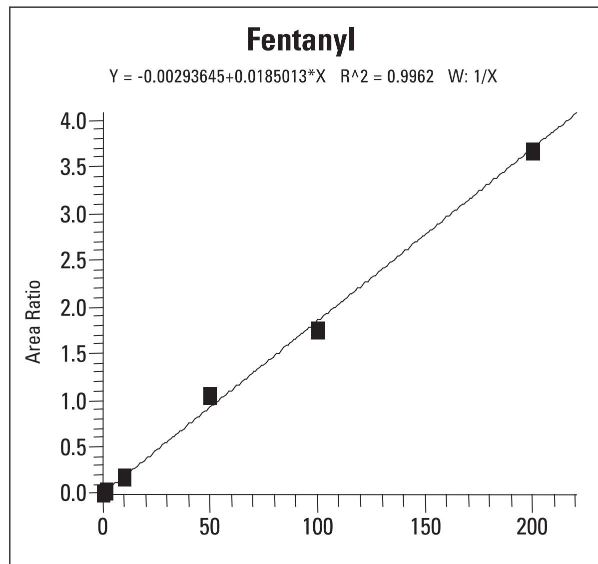

Figure 1: Calibration curves of Fentanyl from 4 channels of Aria TLX-4 System. Data courtesy of Dennis Crouch, Ameritox, LTD.

Goal

- Eliminate the need for SPE extraction of urine samples for fentanyl/norfentanyl assay
- Significantly increase sample throughput by running multiple samples simultaneously in front of one mass spectrometer
- Confirm the stability of the on-line assay

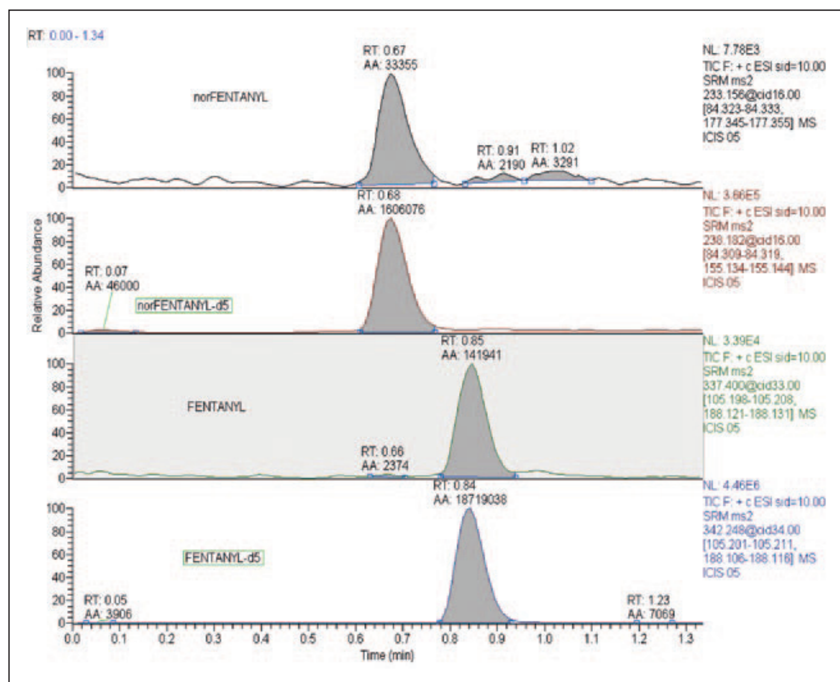

Figure 2: Excellent Signal/Noise at LOQ for (A) Norfentanyl and (B) Fentanyl at 0.5 ng/mL calibration. Data courtesy of Dennis Crouch, Ameritox, LTD.

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Methods

This method describes the analysis for the determination of fentanyl and its metabolite, norfentanyl, from a urine sample. Human urine was used as the test matrix. An LOQ of 0.5 ng/mL was seen in human urine, with an LOD below 0.1 ng/mL. Instrumentation used is identified in Table 1.

Table 1. Instrumentation used in this method

LS-MS/MS:	Aria TLX-4 with Thermo Scientific TSQ Quantum Access triple quadrupole mass spectrometer
Extraction column:	Thermo Scientific TurboFlow XL C18 P 0.5x50 mm
Analytical column:	Thermo Scientific Hypersyl GOLD aQ 3x50, 5 µm

Experimental Conditions:

A working solution containing fentanyl and norfentanyl at 1000 ng/mL was made. Subsequent dilutions yielded a curve from 200 ng/mL to 0.5 ng/mL. An internal standard solution containing both fentanyl-D5 and norfentanyl-D5 was added to all standards. Samples were vortexed and then centrifuged at 10,000 RCF for 5 minutes and analyzed immediately.

Results:

The data in Figure 1 shows linear regression for 0.5 ng/mL to 200 ng/mL, with 1/x weighing. Figure 2 demonstrates the limit of quantitation with excellent signal to noise ratio.

Conclusion:

The Aria TLX-4 system powered by TurboFlow technology provides a fast, efficient, and automated on-line separation technology for the extraction and analysis of fentanyl and its metabolite, norfentanyl. The ability to run 5.5 minute methods on four channels further decreases analysis time and increases the efficiency of the TSQ Quantum Access™ mass spectrometer. The Aria TLX-4 coupled with the TSQ Quantum Access can run one sample every 86 seconds with a 92.9% sample completion rate with 7.1% re-injection[2]. The method run time was 5.5 minutes. This system provides a reliable high throughput method of fentanyl and norfentanyl for clinical research labs.

References

1. Sauvage et al. 2006. Therapeutic Drug Monitoring 28(1), pp. 123-130.
2. Crouch, Dennis. *The Analysis of Fentanyl and Norfentanyl using TurboFlow Column Analyte Isolation and Multiplex-HPLC/MS/MS.* Oral presentation, AAFS, Washington DC February 17-20 2008.

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Forensic Analysis of Opiates in Whole Blood by LC-MS/MS Using Automated, Online Sample Preparation

Peter Ashton, Alex Allan, Bob Ardrey, Triple A Forensics Ltd., Oldham, UK
Shane McDonnell, Sarah Robinson, Thermo Fisher Scientific, Hemel Hempstead, UK

Key Words

- Transcend TLX-1
- TurboFlow Technology
- TSQ Quantum Ultra
- Forensic Toxicology

Introduction

Forensic use of free- and protein-bound opiate analytes in whole blood by LC-MS/MS traditionally requires rigorous sample cleanup via solid phase extraction (SPE) or liquid-liquid extraction (LLE). The method described here can be used in place of these laborious offline sample preparation methods.

Goal

The goal is to quantitate opiate compounds in whole blood by using a simple, fast, low-volume protein precipitation step followed by a Thermo Scientific TurboFlow method coupling automated, online sample preparation and chromatography with selective reaction monitoring (SRM) tandem mass spectrometry.

Experimental

Sample Preparation

Horse blood was spiked with a mixture of opiates [codeine, morphine, 6-monoacetyl morphine (6-MAM), morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), and d6-codeine (internal standard)] at concentrations ranging from 1 ng/mL to 500 ng/mL. A 150 µL sample of spiked whole blood was mixed with 200 µL acetonitrile, vortexed, and centrifuged for 10 minutes at 300 rpm. For analysis, 10 µL of supernatant was used.

HPLC

HPLC analysis was performed using the Thermo Scientific Transcend TLX-1 system. Whole blood supernatant samples were extracted using a TurboFlow™ Cyclone MAX column (0.5 x 50 mm). Chromatographic separation was performed using a Thermo Scientific Hypersil GOLD aQ column (50 x 2.1 mm, 5 µm).

Mass Spectrometry

MS analysis was carried out on a Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer with a heated electrospray ionization (H-ESI) source. The SRM mode was used for mass spectrometry detection.

Results and Discussion

The extracted ion chromatograms of the lowest concentration sample are presented in Figure 1. The calibration curves for morphine (Figure 2), codeine and M3G/M6G covered 10–500 ng/mL and the curve for the 6-MAM metabolite covered 1–50 ng/mL. All calibration curves were linear over the concentration range, and carryover was calculated at less than 1% for all analytes.

Conclusion

The use of a simple, rapid work-up followed by a TurboFlow method on the Transcend™ TLX-1 system followed by MS/MS analysis allowed the specific and sensitive analysis of various common opiates and their metabolites from a small volume of whole blood. The 4 minute method allows 15 samples per hour to be completed, and the throughput can be doubled or quadrupled with the use of multiplexing. Significant time is saved with the absence of SPE or LLE sample preparation.

The forensic toxicologist can use this method to assist with the determination of time of heroin injection (presence of 6-MAM) and the detection of M3G and M6G to determine prior use or accumulation following heavy use of opiates.

Assay performance summary

Target Analytes	codeine, morphine, 6-MAM, M3G, M6G
Matrix	whole blood
Assay Linearity	1 - 50 ng/mL (6-MAM) 10 - 500 ng/mL (all other analytes))
Carryover at LLOQ	< 1% for all analytes
Sample Volume	10 µL
Analysis Time	~ 4 minutes

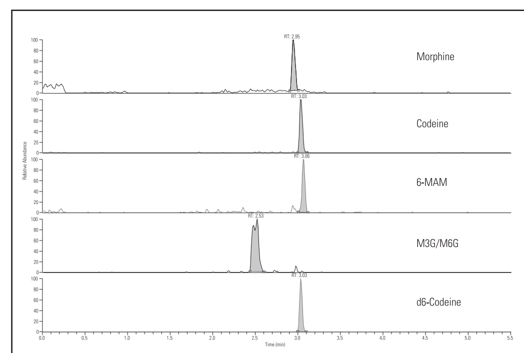

Figure 1: Extracted ion chromatogram for the lowest standard of each analyte

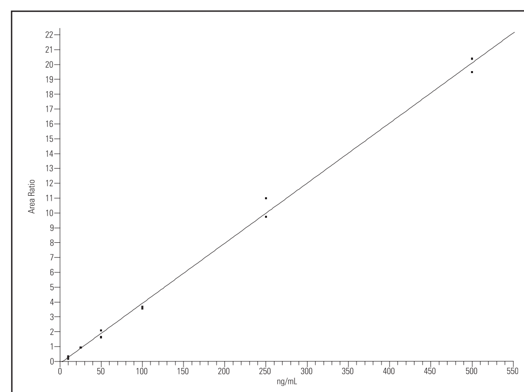

Figure 2: Calibration curve for the analyte morphine from 10–500 ng/mL

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A Complete Toxicology Screening Procedure for Drugs and Toxic Compounds in Urine and Plasma Using LC-MS/MS

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Key Words

- ToxSpec Analyzer
- ToxID Software
- LXQ Linear Ion Trap
- Clinical Toxicology
- General Unknown Screening

Introduction

Toxicology laboratories commonly use automated immunoassays, gas chromatography-mass spectrometry (GC-MS) and high pressure liquid chromatography-diode array detector (HPLC-DAD) techniques to perform toxicology screening analyses. None of these techniques are able to identify all the drugs and toxic compounds that are potentially present in a sample. Implementation of liquid chromatography-mass spectrometry (LC-MS) for toxicology screening provides specific and sensitive analysis of drugs and toxic substances. The benefits of the LC-MS/MS screening methodology include a simple sample preparation procedure, ease of adding new compounds to the screening method and fewer limitations based on compound volatility and thermal stability. In addition, Thermo Scientific ToxID automated toxicology screening software is able to automatically generate both Summary and Long Reports, avoiding the need for manual analysis of each sample chromatogram. This application note describes the use of the Thermo Scientific LXQ ion trap mass spectrometer equipped with an ESI source and HPLC for identification of unknown compounds in human urine and human plasma.

Goal

To develop a complete LC-MS/MS screening methodology which includes a sample preparation method, LC-MS method, spectra library, and data processing and reporting software.

Experimental Conditions

An MS/MS spectral library of 275 drugs and toxic compounds was created. Sample preparation of spiked human urine or human plasma was carried out using a solid-phase extraction (SPE) cartridge for basic, neutral and acidic compounds. A 13-minute LC method implementing a Perfluorophenyl (PFP) column was developed. Samples were analyzed using electrospray ionization (ESI) on an ion trap mass spectrometer in polarity switching scan dependent MS/MS experiments (see Figure 1), with retention time windows specified for each listed parent mass. The method allows acquisition of MS^2 spectra for co-eluting compounds and analysis of positively and negatively ionized compounds with a single run. Figure 2 shows the overall application workflow.

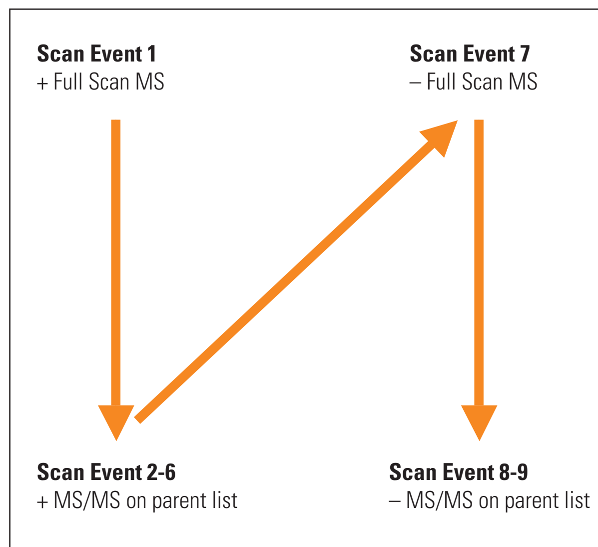

Figure 1: MS scan events

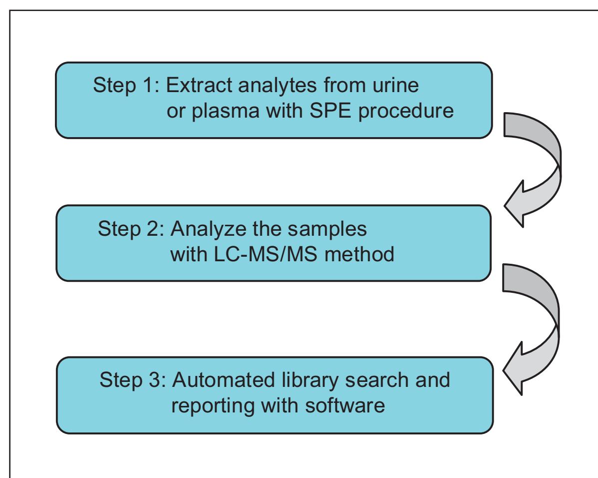

Figure 2: Step-by-step application workflow

Sample Preparation

Samples (1 mL of urine or 0.5 mL of plasma) were spiked with 0.1 mL of an internal standard solution at a concentration of 1 µg/mL (Chlorpromazine-D3, Haloperidol-D4 and Prazepam-D5) and diluted with 2 mL of 0.1 M phosphate buffer pH 6.0. The resulting mix was extracted with an SPE (Thermo Scientific Hypersep Verify-CX 200 mg mixed mode cartridges) procedure prior to injection onto LC-MS.

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Chromatography

HPLC separation was performed with a Thermo Scientific Accela pump using a Thermo Scientific Hypersil GOLD PFP column (50 x 2.1 mm; 5 µm particles). Flow rate was set to 200 µL/min. The gradient is summarized in Table 1 (solvent A = water/0.1% formic acid/10 mM ammonium formate, solvent B = acteonitrile/0.1% formic acid). Injection volume was 10 µL.

Table 1. Thirteen-minute LC method

Time (minutes)	%A	%B
0	95	5
0.5	95	5
5.5	5	95
8.5	5	95
8.6	5	95
13	95	5

MS Conditions

Instrument:	LXQ ion trap mass spectrometer
Ionization:	ESI, Thermo Scientific Ion Max source
Capillary temperature:	275 °C
Spray voltage:	5.0 kV
Sheath gas:	30
Aux gas:	8
Data acquisition mode:	Polarity switching scan dependent experiment
Microscans:	1
WideBand Activation™:	On
Stepped Normalized Collision Energy:	35% ± 10%

Method Validation and Results:

The method was prequalified by processing and analyzing urine samples spiked with 10 randomly selected compounds in concentrations of 10 ng/mL, 100 ng/mL and 1000 ng/mL. Table 2 lists the concentration at which each analyte in the toxicology screen for urine samples is identified. The presence of an analyte at 10, 100 or 1000 ng/mL implies that the limit of detection is likely below that value. Of the 275 compounds analyzed, 70% were detected at 10 ng/mL, 20% at 100 ng/mL, 8% at 1000 ng/mL and 2% were detected at a concentration above 1000 ng/mL.

Table 2. Results for spiked urine samples in toxicology screen by LC-MS/MS

LXQ – 13 min method Compound	**Concentration Tested (ng/mL)** 10	100	1000
All barbiturates require an APCI source for detection.	*P=Drug present.*	*N=Drug not present.*	
11-Hydroxy-delta-9-THC	N	N	>1000
11-nor-9-carboxy-Delta-9-THC	N	N	P
2-Bromo-Alpha-Ergocryptine	P	P	P
2-Hydroxyethylflunitrazepam	N	P	P
3-Hydroxystanozolol	N	N	>1000
4-Hydroxynordiazepam	N	P	P
6-Acetylcodeine	P	P	P
6-Acetylmorphine (6-MAM)	P	P	P
7-Amino-Clonazepam	P	P	P
7-Amino-Flunitrozepam	P	P	P
Acebutolol	P	P	P
a-Hydroxy-Alprazolam	P	P	P
a-Hydroxy-Triazolam	P	P	P
Albuterol	P	P	P
alpha-Hydroxymidazolam	N	P	P
Alprazolam	P	P	P
Alprenolol	P	P	P
Aminorex	N	P	P
Amiodarone	P	P	P
Amitriptyline	P	P	P
Amlodipine	N	N	P
Amobarbital	P	P	P
Amoxapine	P	P	P
Amphetamine	P	P	P
Anhydroecgonine MethylEster	N	P	P
Antipyrine	N	N	>1000
Apomorphine	N	N	>1000

LXQ – 13 min method	**Concentration Tested (ng/mL)**		
Compound	10	100	1000
Astemizole	N	P	P
Atenolol	P	P	P
Atropine	N	P	P
BDB	N	P	P
Benzocaine	N	N	P
Benzoylecgonine	N	P	P
Betaxolol	P	P	P
Bisacodyl	P	P	P
Bisoprolol	P	P	P
Bromazepam	P	P	P
Brompheniramine	P	P	P
Bupivocaine	P	P	P
Buprenorphine	P	P	P
Bupropion	P	P	P
Buspirone	P	P	P
Butalbital	N	P	P
Butorphanol	P	P	P
Cannabidiol	N	N	>1000
Cannabinol	N	N	>1000
Captopril	N	N	P
Carbamazepine	P	P	P
Carbinoxamine	N	P	P
Carisoprodol	N	N	P
Cathinone	N	N	P
Chlordiazepoxide	P	P	P
Chlorothiazide	N	P	P
Chlorpheniramine	P	P	P
Chlorpromazine	P	P	P
Chlorpromazine-D3	N	P	P
Chlorprothixene	N	N	>1000
Cinnarizine	P	P	P
cis-4-Methylaminorex	N	P	P
Cisapride	N	P	P
Citalopram	P	P	P
Clenbuterol	P	P	P
Clenbuterol	N	P	P
Clobazam	N	P	P
Clomipramine	P	P	P
Clonazepam	P	P	P
Clonidine	P	P	P
Clopidogrel	P	P	P
Clozapine	P	P	P
Cocaethylene	P	P	P
Cocaine	P	P	P
Codeine	P	P	P
Cyclobenzaprine	P	P	P
Delta9-THC	N	P	P
Desalkylflurazepam	N	P	P
Desipramine	N	P	P
Desmethyldoxepin	P	P	P
Dextromethorphan	P	P	P
Diazepam	P	P	P
Diflunisal	P	P	P
Digoxin	N	N	P
Dihydrocodeine	P	P	P
Dihydroergotamine	P	P	P
Diltiazem	P	P	P
Diphenhydramine	P	P	P
Dipyridamole	N	N	P
Disopyramide	P	P	P
Dothiepin	N	P	P
Doxepin	P	P	P
Doxylamine	P	P	P
Ecgonine-Methyl-Ester	N	N	P
EDDP	P	P	P
EMDP	P	P	P
Enalapril	P	P	P
Ephedrine	N	P	P

LXQ – 13 min method	Concentration Tested (ng/mL)		
Compound	10	100	1000
Ergotamine	P	P	P
Estazolam	N	P	P
Felcainide	P	P	P
Fendiline	P	P	P
Fenfluramine	P	P	P
Fentanyl	P	P	P
Fexofenadine	P	P	P
Flumethasone	N	N	P
Flunitrazepam	P	P	P
Flunixin	N	P	P
Fluoxetine	P	P	P
Fluoxymesterone	N	P	P
Fluphenazine	P	P	P
Flurazepam	P	P	P
Fluvoxamine	P	P	P
Furosemide	N	P	P
Gabapentin	N	N	P
Gliclazide	N	N	P
Glimepiride	N	P	P
Glipizide	P	P	P
Glyburide	P	P	P
Haloperidol	P	P	P
Haloperidol-D4	N	P	P
Heroin	P	P	P
HMMA	N	N	>1000
Hydrochlorothiazide	N	N	P
Hydrocodone	P	P	P
Hydromorphone	P	P	P
Hydroxyzine	N	P	P
Imipramine	P	P	P
Indomethacin	N	N	>1000
Isradipine	P	P	P
Ketamine	P	P	P
Ketoconazole	P	P	P
Ketoprofen	N	N	>1000
Ketorolac	N	N	>1000
Labetolol	N	P	P
Lamotrigine	P	P	P
LAMPA	P	P	P
Lidocaine	P	P	P
Lometazepam	N	P	P
Loratadine	P	P	P
Lorazepam	P	P	P
LSD	P	P	P
Maprotiline	P	P	P
MBDB	N	P	P
MDA	P	P	P
MDEA	N	P	P
MDMA	P	P	P
Melatonin	N	N	>1000
Meperidine	P	P	P
Mepivocaine	N	P	P
Meprobamate	N	P	P
Mescaline	P	P	P
Mesoridazine	P	P	P
Metaprolol	P	P	P
Methadienone	P	P	P
Methadone	P	P	P
Methamphetamine	P	P	P
Methaqualone	N	N	>1000
Methcathinone	N	N	P
Methenolone	P	P	P
Methohexital	P	P	P
Methoxyverapmil	P	P	P
Methylphenidate	P	P	P
Metoclopramide	P	P	P
Metronidazole	N	P	P
Mexiletine	N	N	>1000

LXQ – 13 min method
Compound
Mianserin
Miconazole
Midazolam
Mirtazapine
Molsidomine
Morphine
Morphine-3-b-glucuronide
Nalbuphine
Nalorphine
Naloxone
Naltrexone
NAPA
N-DemethylTrimipramine
N-Desmethyl-cis-tramadol
N-Desmethylflunitrazepam
N-Desmethylselegiline
N-DesmthylClomipramine
N-Ethylamphetamine
Nicardipine
Nicotine
Nitrazepam
Nitrendipine
Nizatidine
Norbenzoylecgonine
Norbuprenorphine
Norclomipramine
Norcocaethylene
Norcocaine
Norcodeine
Nordiazepam
Nordoxepin
Norethandrolone
Norfentanyl
Norfluoxetine
Norketamine
NOR-LSD
Normeperidine
Normorphine
Noroxycodone
Noroxymorphone
Norpropoxyphene
Nortriptyline
Noscapine
OH-LSD
Ondansetron
Opipramol
Oxazepam
Oxcarbazepine
Oxycodone
Oxymorphone
Papaverine
Paraxanthine
Paroxetine
PCP
Pentazocine
Pentobarbital
Perphenazine
Pheniramine
Phenobarbital
Phenolphthalein
Phentermine
Phenylbutazone
Phenyltoloxamine
Physostigmine
Pindolol
Piroxicam
PMA
PMMA

Concentration Tested (ng/mL)		
10	100	1000
P	P	P
P	P	P
P	P	P
P	P	P
N	N	>1000
N	P	P
N	N	>1000
P	P	P
P	P	P
P	P	P
P	P	P
P	P	P
P	P	P
N	N	P
N	P	P
N	P	P
N	P	P
N	P	P
P	P	P
P	P	P
N	N	>1000
P	P	P
N	N	P
N	N	>1000
N	N	>1000
P	P	P
P	P	P
P	P	P
N	P	P
P	P	P
P	P	P
N	P	P
N	P	P
P	P	P
N	P	P
P	P	P
P	P	P
N	N	P
N	P	P
N	N	>1000
P	P	P
P	P	P
P	P	P
N	P	P
P	P	P
P	P	P
P	P	P
N	N	P
P	P	P
N	P	P
P	P	P
N	N	>1000
N	P	P
P	P	P
P	P	P
P	P	P
P	P	P
N	P	P
P	P	P
P	P	P
N	N	P
N	N	P
N	N	P
P	P	P
N	N	P
P	P	P
N	N	P
N	P	P

LXQ – 13 min method	Concentration Tested (ng/mL)		
Compound	10	100	1000
Prazepam-D5	N	P	P
Prazosin	P	P	P
Prilocaine	N	N	P
Procainamide	N	P	P
Promazine	P	P	P
Promethazine	N	P	P
Prometryn	N	P	P
Propafenone	P	P	P
Propoxyphene	P	P	P
Propranolol	P	P	P
Protriptyline	P	P	P
Psilocin	N	P	P
Pyrilamine	P	P	P
Quetiapine	P	P	P
Quinidine	P	P	P
Quinine	N	P	P
Ranitidine	N	N	P
Risperidone	P	P	P
Scopolamine	P	P	P
Secobarbital	P	P	P
Selegiline	N	P	P
Sertraline	P	P	P
Sotalol	N	P	P
Spironolactone	N	P	P
Stanozolol	N	P	P
Telmisartan	P	P	P
Temazepam	P	P	P
Terfenadine	P	P	P
Tetracine	P	P	P
Thiamylal	N	P	P
Thiopental	P	P	P
Thioridazine	P	P	P
Thiothixene	P	P	P
Timolol	P	P	P
Topiramate	P	P	P
Trazodone	P	P	P
Triazolam	P	P	P
Trimethoprim	P	P	P
Trimipramine	P	P	P
Venlafaxine	P	P	P
Verapamil	P	P	P
Vincristine	P	P	P
Warfarin	P	P	P
Zimelidine	P	P	P
Zolpidem	P	P	P
Zopiclone	N	N	P

All barbiturates require an APCI source for detection. *P=Drug present.* *N=Drug not present.*

Table 3. Results for spiked plasma samples in toxicology screen by LC-MS/MS

LXQ – 13 min method Compound	Concentration Tested (ng/mL) 10	 100	 1000
BDB	N	P	P
Benzocaine	N	P	P
Benzoylecgonine	P	P	P
Betaxolol	P	P	P
Bisacodyl	P	P	P
Bisoprolol	P	P	P
Bromazepam	N	P	P
Brompheniramine	N	P	P
Bufotenine	N	P	P
Bupivocaine	P	P	P
Buprenorphine	P	P	P
Bupropion	N	P	P
Buspirone	P	P	P
Butorphanol	P	P	P
Cannabidol	N	P	P
Cannabinol	N	P	P
Captopril	N	N	>1000
Estazolam	N	P	P
Carbamazepine	P	P	P
Carbinoxamine	P	P	P
Carisoprodol	N	P	P
Cathinone	N	N	>1000
Chlordiazepoxide	N	P	P
Chloroquine	N	P	P
Chlorpheniramine	P	P	P
Chlorpromazine	N	P	P
Chlorprotixene	P	P	P
Clozapine N-Oxide	N	P	P

All barbiturates require an APCI source for detection. P=Drug present. N=Drug not present.

For selected sets of compounds the method was also prequalified by processing and analyzing spiked plasma samples. Table 3 lists the concentration at which each analyte in the toxicology screen for plasma samples is identified. In general, detection limits for urine and plasma are comparable.

In addition, the assay performance was verified by analyzing patient urine samples obtained from the Johns Hopkins University Hospital Clinical Laboratory and data were compared to the results from established LC-UV and immunoassay analytical techniques. The result is shown in Table 4. The LC-MS/MS method has consistently identified more analytes present in the sample than either LC-UV or immunoassays.

Table 4. Urine sample analyzed with LC-MS/MS, LC-UV and Immunoassay methods

LC-MS	LC-UV	Immunoassay
Nortriptyline	Nortriptyline	Barbiturates
Amitriptyline	Amitriptyline	Benzodiazepines
Benzoylecgonine	Benzoylecgonine	Cocaine
Cocaine	Cocaine	Opiates
Norcocaethylene	Cocaethylene	THC
Norbenzoylecgonine	-	-
Morphine	-	-
Norcocaine	-	-
Quinidine/Quinine	-	-
Hydroxyzine	-	-
Noskapine	-	-
Diltiazem	-	-
Morphine-3-beta-Glucuronide	-	-

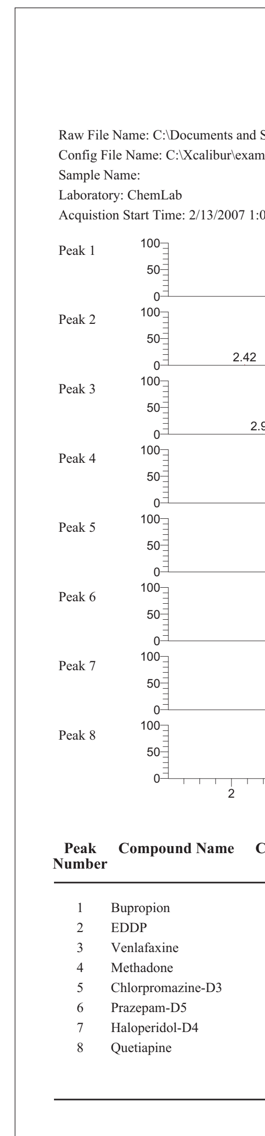

Figure 3: The ToxID Summary Re

Company Name
ToxID Summary Report

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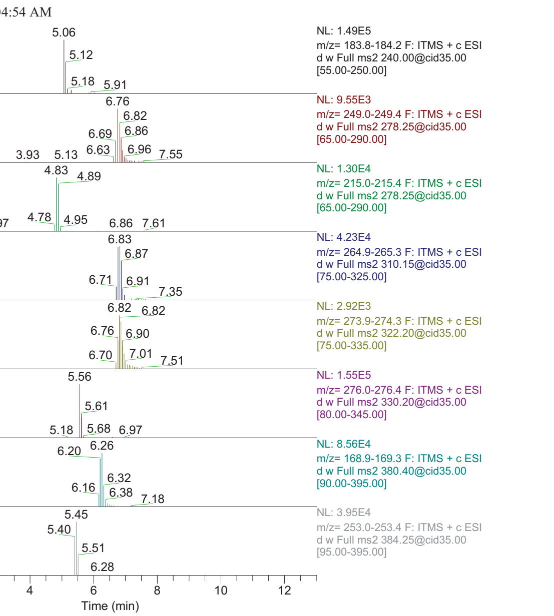

ode	SI	RSI	m/z	Expected RT	Real RT	Intensity	Library Name
p	909	909	240.0	5.20	5.06	148721	Tox_Library
p	857	873	278.2	6.60	6.76	9549	Tox_Library
p	816	837	278.2	4.90	4.83	12964	Tox_Library
p	932	932	310.2	6.70	6.83	42262	Tox_Library
i	859	859	322.2	6.80	6.82	2924	Tox_Library
i	969	974	330.2	5.60	5.56	154827	Tox_Library
i	830	837	380.4	6.20	6.26	85589	Tox_Library
p	870	871	384.2	5.40	5.45	39512	Tox_Library

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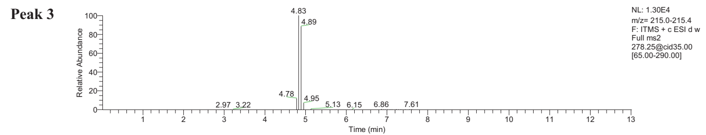
port is designed for a quick synopsis of the data.

Company Name ToxID Long Report

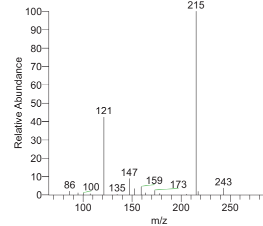
Raw File Name: C:\Documents and Settings\marta.kozak\Desktop\Desktop\Application_Notes\ToxID\2J.RAW
Config File Name: C:\Xcalibur\examples\ToxID\ToxID_config_13min.csv
Sample Name:
Laboratory: ChemLab
Acquistion Start Time: 2/13/2007 1:04:54 AM

Peak Number	Compound Name	Code	SI	RSI	m/z	Expected RT	Real RT	Intensity	Library Name
3	Venlafaxine	p	816	837	278.2	4.90	4.83	12964	Tox_Library

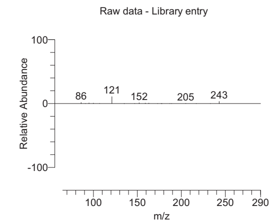
Peak 3



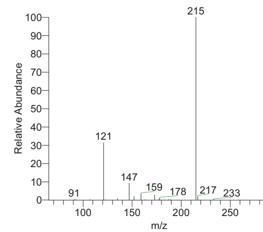
Acquired Spectrum



Delta Spectrum



Library Spectrum



Library Structure

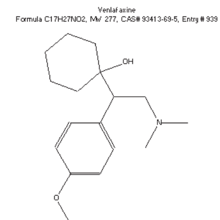


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Figure 4: The ToxID Long Report is designed for a more thorough examination of the data.

Table 5. Simple workflow for adding new analytes

STEP 1: Directly infuse analyte to obtain MS^2 spectra, then add spectra to the library	10 Minutes
STEP 2: Run analyte on column to obtain retention times	13 Minutes
STEP 3: Update Parent Mass Table in instrument method with parent masses and retention times	2 Minutes
STEP 4: Update ToxID with name, parent masses, the most intense product ion and retention times	2 Minutes

ToxID™ Software Automates Reporting, Reduces Manual Analysis

ToxID software identifies compounds present in the sample based on MS/MS spectra and retention times. Positive hits are automatically reported via ToxID software. Reports are automatically generated, reducing the time necessary for manual analysis of each sample chromatogram. An example of a Summary Report is shown in Figure 3. A Long Report with one page per detected compound is shown in Figure 4.

Adding New Compounds to the Application

This LC-MS/MS workflow allows the user to quickly and easily add new analytes to the screening method. This feature is very important for toxicology screening because new target compounds are continually being added to the target list. As shown in Table 5, new compounds can typically be added in less than 1 hour.

Conclusion

The comprehensive, turn-key toxicology screening methodology described in this application note utilizes an LXQ ion trap, and includes an SPE procedure and LC method that enables the identification of 275 compounds in human urine and human plasma. Accompanying ToxID software performs automatic data analysis and reporting. This eliminates the need for manual data interpretation and increases confidence in compound identification. It is worth noting that when compared to other screening methods, the LC-MS/MS screening methodology identifies more analytes.

Mass Spectrometers are general purpose laboratory instruments. They have not been cleared or approved by the United States Food and Drug Administration, the European IVD Directive or any other agency for diagnostic, clinical or other medical use.

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Rapid Analysis of Opiates from Low Volume Whole Blood Samples by LC-MS/MS Utilizing TurboFlow Methods

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Key Words

- Transcend TLX-1
- TurboFlow Technology
- TSQ Quantum Ultra
- Whole Blood
- Opiates

Introduction

The opiate morphine, and its derivatives, are medicines often used for pain-relief, cough-relief and as anti-diarrhoeals. For example, codeine and dihydrocodeine (morphine derivatives) are available in over-the-counter preparations in combination with paracetamol (acetaminophen) and are slowly metabolized to morphine and dihydromorphine respectively. However, the semi-synthetic opiate diacetylmorphine (heroin) is subject to wide abuse and has become such a major social problem that it is responsible for almost half of the drug-related deaths in the UK.[1]

Heroin is deacetylated very rapidly (half-life ca. 3 mins in plasma) to its major active metabolite 6-monoacetylmorphine (6-MAM), which readily penetrates the blood-brain barrier to produce the desired euphoric effects.[2] 6-MAM also has a short plasma half-life of about 38 minutes (producing morphine), and thus, its detection in blood is very important to the forensic toxicologist in establishing the recent use of heroin.[3] As a product of heroin metabolism, via 6-MAM, or from its own administration, morphine also undergoes further metabolism. The conjugation step produces inactive morphine-3-glucuronide (M3G) and the potently active morphine-6-glucuronide (M6G) along with other minor ones, including diglucuronides.

The forensic toxicologist is often asked to interpret results and possibly account for time of death in opiate (especially heroin) abuse cases. This task can be made easier if it is possible to identify and quantify the components such as 6-MAM, morphine, codeine, dihydrocodeine and the glucuronides in whole blood rather than urine. The volume of a human whole blood sample, however, may often only be available in the low microlitre range, thus presenting sample preparation and analysis sensitivity issues.

The analysis of free- and protein-bound opiate analytes in human whole blood by LC-MS/MS is routinely done after rigorous sample cleanup via solid phase extraction or liquid-liquid extraction in order to minimize ion suppression in the ionization source of the mass spectrometer. These clean up steps can be lengthy, laborious and expensive. Here we present a method to quantitatively analyze opiate compounds present in whole blood utilizing a simple, fast, low-volume extraction procedure followed by a Thermo Scientific TurboFlow method, an online extraction and chromatography coupled with selected reaction monitoring tandem mass spectrometry.

Goal

To replace laborious off line sample preparation with TurboFlow™ methodology and tandem mass spectrometry for the analysis of opiates in acetonitrile extracts from low volume whole blood samples.

Experimental

Sample Preparation

Horse blood was spiked with a mixture of opiates (codeine, morphine, 6-MAM, M3G, M6G and d6-codeine) at concentrations ranging from 1 ng/mL to 500 ng/mL. 150 µL spiked whole blood was mixed with 200 µL acetonitrile and vortexed. The resulting sample was then centrifuged for 10 min at 300 rpm. The supernatant was placed into a 96-well microtitre plate and 10 µL of the supernatant was used for the analysis.

TurboFlow Methodology

Thermo Scientific Transcend TLX-1 system	
Column:	Thermo Scientific TurboFlow Cyclone MAX 0.5 x 50 mm
Mobile phase A:	0.1% formic acid
Mobile phase B:	0.1% formic acid in acetonitrile
Mobile phase C:	10 mM ammonium bicarbonate pH 9
Mobile phase D:	10 mM ammonium acetate pH 6

Analytical LC

Column:	Thermo Scientific Hypersil GOLD aQ 50 x 2.1 mm, 1.9 µm
Mobile phase A:	0.1% formic acid
Mobile phase B:	0.1% formic acid in acetonitrile

The eluent gradients for both pumps are shown in Table 1.

			TurboFlow Method								Analytical			
Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	00:00	30	1.50	Step	-	-	100	-	====	out	0.30	Step	100	0
2	00:30	60	0.20	Step	100	-	-	-	T	in	0.10	Step	100	0
3	01:30	60	1.50	Step	-	-	-	100	====	in	0.30	Ramp	5	95
4	02:30	120	1.50	Step	99	1	-	-	====	in	0.30	Step	5	95
5	04:30	60	1.50	Step	-	-	100	-	====	out	0.30	Step	100	0

Table 1: Thermo Scientific Aria operating software gradient programs for the Transcend™ TLX-1 system with TurboFlow method and analytical LC method. Flow rate is reported as mL/min.

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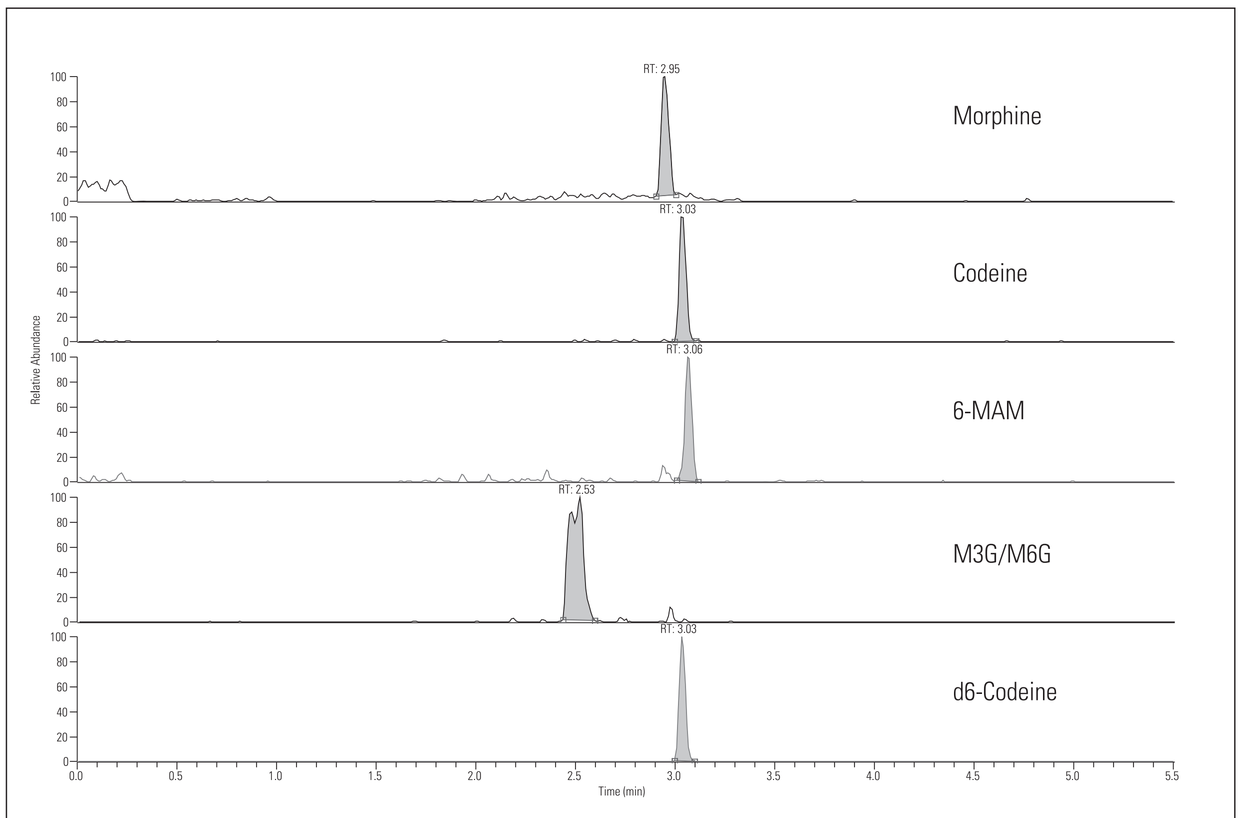

Figure 1: Extracted ion chromatogram for the lowest standard of each analyte

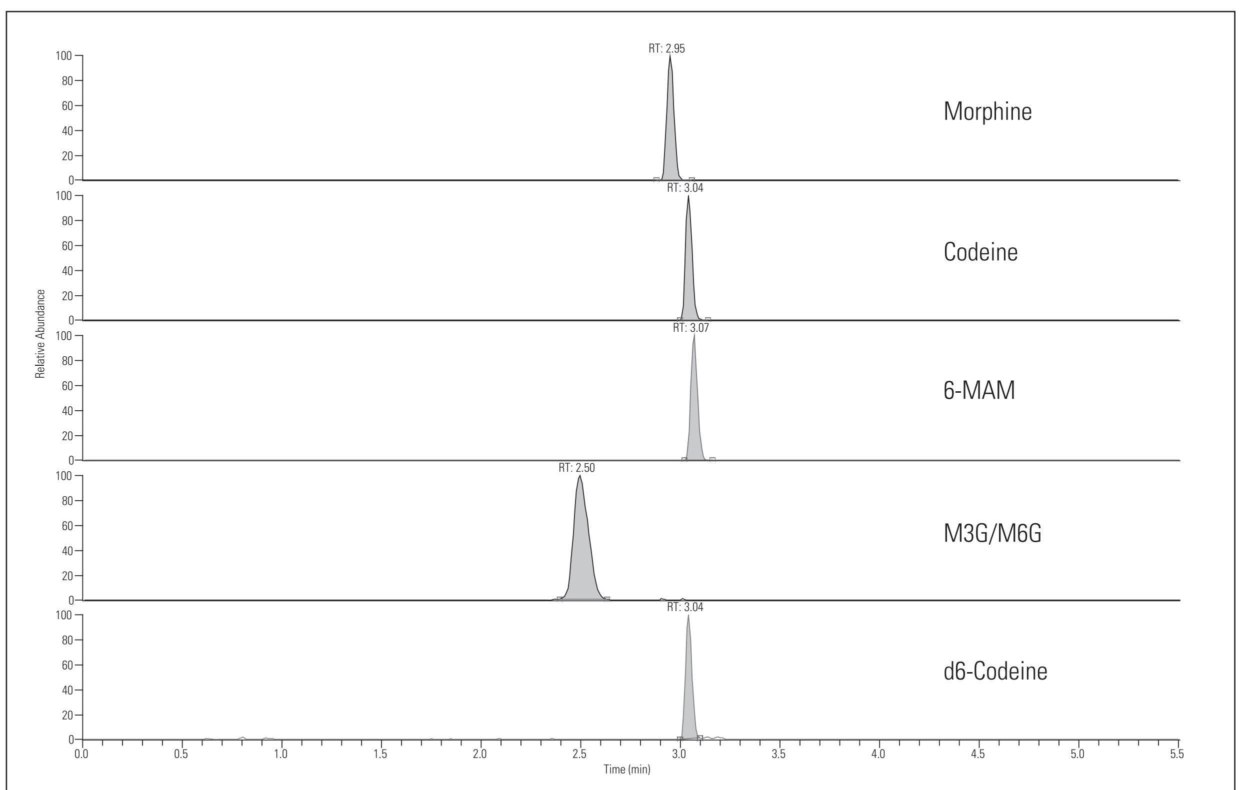

Figure 2: Extracted ion chromatogram for the highest standard of each analyte

Mass Spectrometry

Thermo Scientific TSQ Quantum Ultra	
Ion Source & polarity:	HESI, positive ion mode
Spray Voltage:	4750 V
Vaporizer Temperature:	450 °C
Sheath Gas:	50 units
Ion Sweep Gas:	5 units
Auxillary Gas:	60 units
Capillary Temperature:	200 °C
Collision Gas Pressure:	1.5 mTorr

The SRM transitions used for this experiment are presented in Table 2.

Analyte	Parent	Product	Scan Time	Collision Energy	Tube Lens
Morphine	286.13	165 201	5 ms 5 ms	39 25	133 133
Codeine	300.14	165 215	5 ms 5 ms	38 26	148 148
6-MAM	328.13	165 211	5 ms 5 ms	38 25	145 145
M3G/M6G	462.16	286	5 ms	31	155

Table 2: SRM transitions monitored in the experiment

Results and Discussion

Prior to the analysis of spiked whole blood samples, opiate analytes were spiked into 100% acetonitrile and analyzed by the TurboFlow and LC-MS/MS method in order to demonstrate that the high organic content of the sample did not affect peak shape (peak splitting, etc.). The extracted, spiked whole blood samples were analyzed using the same TurboFlow method. Samples were run from low to high concentration with a solvent blank sample submitted after the highest concentration sample to calculate carryover. In all analyses, 10 µL of the extracted sample was injected and replicated to generate a calibration curve.

The extracted ion chromatograms of the lowest concentration sample and highest concentration sample are presented in Figures 1 and 2 respectively. The calibration curves for morphine, codeine and M3G/M6G covered 10–500 ng/mL (Figure 3, 4 and 6) and for the 6-MAM metabolite the curve covered 1–50 ng/mL (Figure 5). The isotopically labeled internal standard (d6-codeine) was spiked into each sample at 50 ng/mL. The concentration data for each analyte are provided as blood equivalents, i.e. the concentration in the blood before extraction. For example, 1 ng/mL blood equivalent was actual 0.43 ng/mL in the sample vial (150 µL diluted with 350 µL acetonitrile). Therefore, the equivalent on column amount of the lowest 6-MAM standard was 4.3 pg.

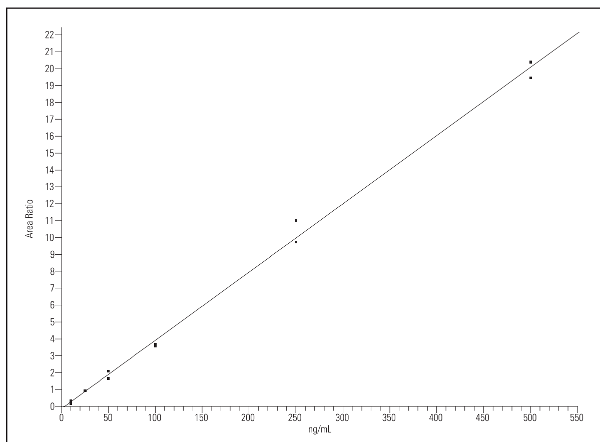

Figure 3: Calibration curve for the analyte morphine from 10–500 ng/mL

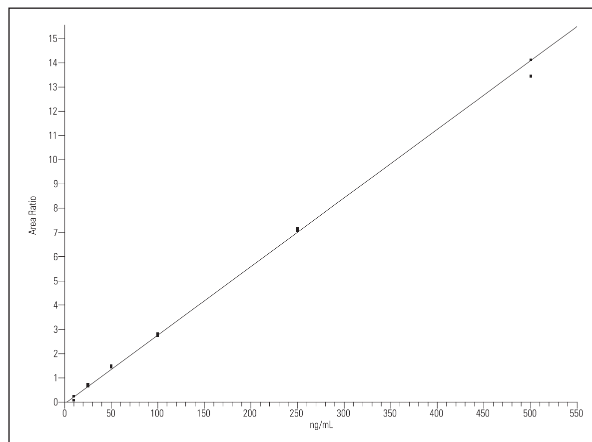

Figure 4: Calibration curve for the analyte codeine from 10–500 ng/mL

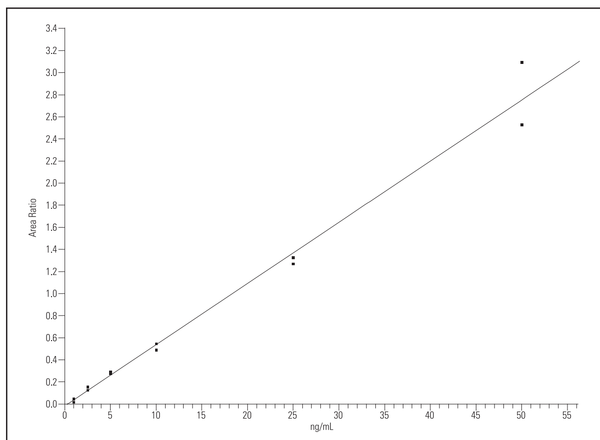

Figure 5: Calibration curve for the analyte 6-MAM from 1–50 ng/mL

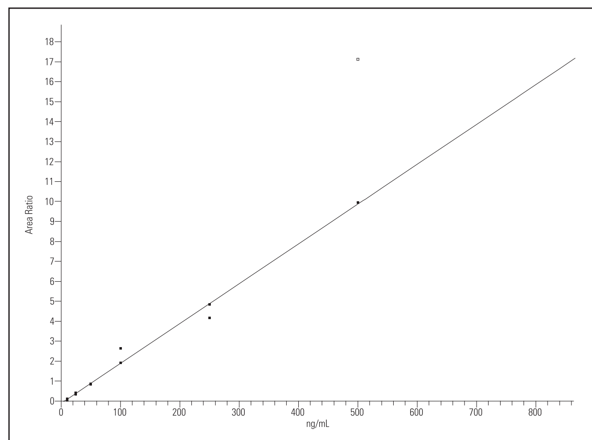

Figure 6: Calibration curve for the analyte M3G/M6G from 10–500 ng/mL

Conclusion

The use of a simple rapid acetonitrile work-up followed by a TurboFlow method (online extraction and chromatography) on the Thermo Scientific Transcend TLX-1 system with tandem MS/MS allowed the specific and sensitive analysis of various common opiates and their metabolites from a small volume of whole blood. Moreover, a limited portion of the acetonitrile extract volume was utilized in the analysis, thus, the method presents potential to scale down to a volume of blood achievable from a finger prick (5–10 µL). The calibration curves for all analytes analyzed were linear over the concentration range and carryover was calculated at less than 1% for all analytes. Since the method is ~ 4 minutes, 15 samples per hour may be completed, or indeed, doubled/quadrupled with the use of multiplexing. Significant time is saved in the absence of SPE sample preparation.

The method enables the forensic toxicologist to produce a full picture of the opiates and metabolites in blood to assist with the determination of time of injection (presence of 6-MAM) and the detection of M3G and M6G to determine prior use or accumulation following heavy use.

References and Acknowledgements

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Forensic Toxicology Screening with LC-MS/MS and Automated Online Sample Preparation

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Forensic Toxicology Use Only.

Key Words

- ToxSpec Analyzer
- ToxID Software
- LXQ Linear Ion Trap
- TurboFlow Technology
- Forensic Toxicology

Introduction

The quality of liquid chromatography-mass spectrometry (LC-MS) data collected in forensic drug screening applications is largely affected by sample preparation methods. Offline solid phase extraction (SPE) and liquid-liquid extraction (LLE) are the most commonly used methods. Automated online sample preparation using Thermo Scientific TurboFlow technology provides a robust front end platform for forensic drug screening, which is convenient and labor-saving.

Goal

The goal is to evaluate the performance of three sample preparation techniques – TurboFlow™ technology, SPE, and LLE – to screen 300 basic, neutral, and acidic drug compounds for forensic toxicology use.

Experimental

SPE – Mixed-mode Thermo Scientific HyperSep Verify-CX SPE cartridges (200 mg; 6 mL) were used for offline SPE. Samples of 1 mL of urine were spiked to final concentrations of 10, 100 and 1000 ng/mL with analytes of interest, as well as 100 ng/mL of three deuterated internal standards, and loaded on the SPE column. Basic, acidic, and neutral fractions were collected, combined, evaporated to dryness, reconstituted in 100 µL, and injected onto the LC column.

LLE – Toxi-Tubes® A & B (Varian) were used for offline LLE. Samples of 1 mL of urine were spiked to final concentrations of 10, 100 and 1000 ng/mL with analytes of interest, as well as 100 ng/mL of three deuterated internal standards, and then applied to the Toxi-Tube. The organic layers were transferred, evaporated to dryness, reconstituted in 100 µL, and injected onto the LC-MS.

TurboFlow Method – Urine samples were diluted in ratio 1:1 v/v with 50% MeOH containing internal standards. Fifty (50) µL of diluted sample was injected onto the TurboFlow columns. Two different chemistry TurboFlow columns were used to extract chemically diverse compounds.

A 12-minute LC method was developed for TurboFlow and LLE samples. Samples were injected onto a Thermo Scientific Hypersil GOLD PFP 100 x 30 mm, 3 µm column. A gradient method was employed with flow rates of 600 µL/min. For offline SPE samples, a 13-minute LC gradient was used with a Thermo Scientific Hypersil GOLD PFP analytical column (50 x 2.1 mm, 5 µm) and a 200 µL/min flow rate.

Mass Spectrometry

All samples were analyzed on the Thermo Scientific ToxSpec Analyzer system equipped with a Thermo Scientific LXQ linear ion trap mass spectrometer and an electrospray ionization (ESI) source using a scan-dependent, polarity-switching method. Reports were automatically produced with Thermo Scientific ToxID automated forensic toxicology screening software, including lists of identified compounds and their matching MS/MS spectrum.

Results and Discussion

Table 1 shows limits of identification for representative compounds from the SPE, LLE, and TurboFlow methods. The lowest concentration validated was 10 ng/mL. All three methods showed comparable limits of identification. In addition, with the automated TurboFlow method, the sample quantity loaded on the column was one-quarter of that in the SPE method and one-eighth of that in the LLE method.

Table 1. Comparison of limits of identification for selected compounds

Compound	TurboFlow Method (ng/mL in urine)	SPE Method (ng/mL in urine)	LLE Method (ng/mL in urine)
Codeine	10	10	10
Hydrocodone	10	10	10
Cocaine	10	10	10
Amphetamine	10	10	1000
Stanozolol	100	100	10
Diazepam	10	10	10

Figure 1 shows the results of the identification limits of 300 drugs with the three sample preparation methods. Compared to traditional sample preparation methods, the automated TurboFlow method provides competent performance with automated online sample preparation.

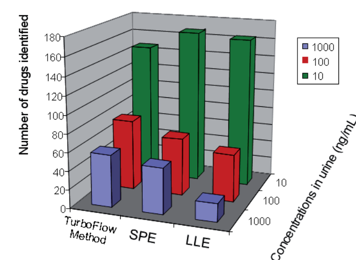

Figure 1: Limits of identification of 300 compounds

Conclusion

The TurboFlow method with the ToxSpec™ Analyzer allows for the identification of 300 drugs, with limits of detection (LODs) ranging from less than 10 ng to greater than 1000 ng per milliliter of urine. It provides an automated online sample preparation platform for forensic toxicology screening with competent performance and limits of identifications. The TurboFlow method is easier, faster, and cost efficient in comparison to traditionally used SPE and LLE methods.

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Screening for Drugs and Toxic Compounds: Comparison between LC-MS/MS, HPLC-DAD, and Immunoassay

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Key Words

- **ToxID software**
- **LXQ Linear Ion Trap**
- **Drugs of Abuse**
- **Toxicology Screening**

Introduction

Screening of biological samples for drugs of abuse and other toxic compounds is a critical feature of forensic toxicology laboratories. The main challenge is to provide rapid and accurate results despite the large number of target molecules and the complexity of biological matrices. The classical approach is based on immunoassay or high pressure liquid chromatography-diode-array detection (HPLC-DAD). However, the advent of newer and more effective liquid chromatography-tandem mass spectrometry (LC-MS/MS) technologies can lead to a significant improvement in non-targeted screening.

Goal

Evaluate the Thermo Scientific ToxSpec Analyzer, LC-MS solution for forensic toxicology screening, for non-targeted screening of several compounds in human urine. LC-MS technology is used to increase the confidence of identification and to simplify the workflow in a forensic toxicology laboratory when compared with the classical screening approaches.

Experimental

Sample Preparation

Urine was stored at -20 °C for the analysis. After thawing, the sample was diluted 1:10 with water. For the analysis, 20 µL of diluted urine were directly injected.

Chromatography and Mass Spectrometry

The ToxSpec™ Analyzer was used for the analysis. Briefly, for the LC separation a Thermo Scientific Hypersil GOLD PFP analytical column (50 x 2.1, 5 µm) was used, with mobile phase A (10 mM ammonium formate in 0.1% formic acid) and B (ACN containing 0.1% formic acid). The gradient was from 95% A to 95% B in about 5 minutes with a flow rate of 200 µL/min. For the MS analysis, a Thermo Scientific LXQ linear ion trap mass spectrometer equipped with an electrospray ionization (ESI) source utilizing polarity switching was employed. A data dependent scan collected MS/MS spectra of all the compounds eluted. Data generated were processed with Thermo Scientific ToxID automated screening software.

ToxID™ software identifies compounds on the basis of retention time, precursor ion, and MS/MS spectrum. Samples screened by LC-MS/MS were previously analyzed also with immunoassay or HPLC-DAD, allowing a comparison between methods.

Results and Discussion

The ToxSpec Analyzer is able to process a sample in about 15 minutes, which allows the performance of routine screening analysis. Data obtained are highly specific and reliable because the identification of compounds is based on three peculiar characteristics of the molecules: retention time, precursor ion, and MS/MS spectrum. Figure 1 shows a report generated by ToxID software after the analysis of a urine sample that tested positive for LSD.

The comparison of results obtained by analyzing the same urine samples with different screening approaches has given interesting results (see Table 1). The ToxSpec Analyzer confirmed, for the most part (Urine 1-4), the results obtained with HPLC-DAD or an immunoassay, but also identified additional compounds, such as metabolites or other minor components that were not recognized with other screening approaches.

Surprisingly, in Urine 5, the results are clearly not in agreement. Particularly, the immunoassay identified amphetamines, while the ToxSpec Analyzer method identified ranitidine and metoclopramide, two therapeutics drugs often used in combination. To better understand the difference between the techniques, we compared the MS/MS spectra of the molecules detected in Urine 5 with those present in the library.

Table 1. Comparison of results obtained analyzing the same urine samples using different screening techniques.

Sample	HPLC-DAD or Immunoassay	ToxSpec Analyzer	Results Comparison
Urine 1	Cocaine	Cocaine, Benzoylecgonine, Cocaethylene, Nicotine	√
Urine 2	Ketamine	Ketamine, Norketamine	√
Urine 3	Quetiapine	Lidocaine, Quetiapine	√
Urine 4	LSD	OH-LSD	√
Urine 5	Amphetamines	Ranitidine, Metoclopramide	X

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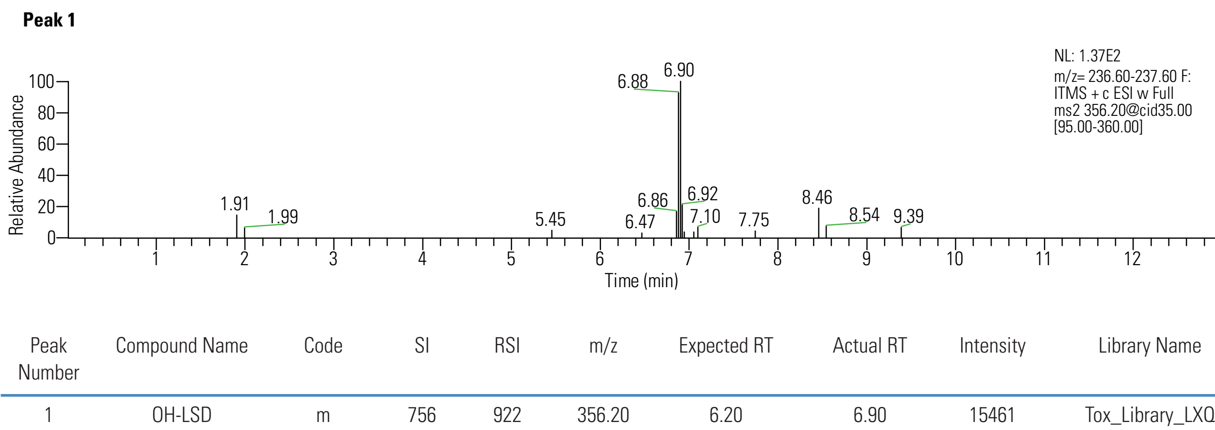


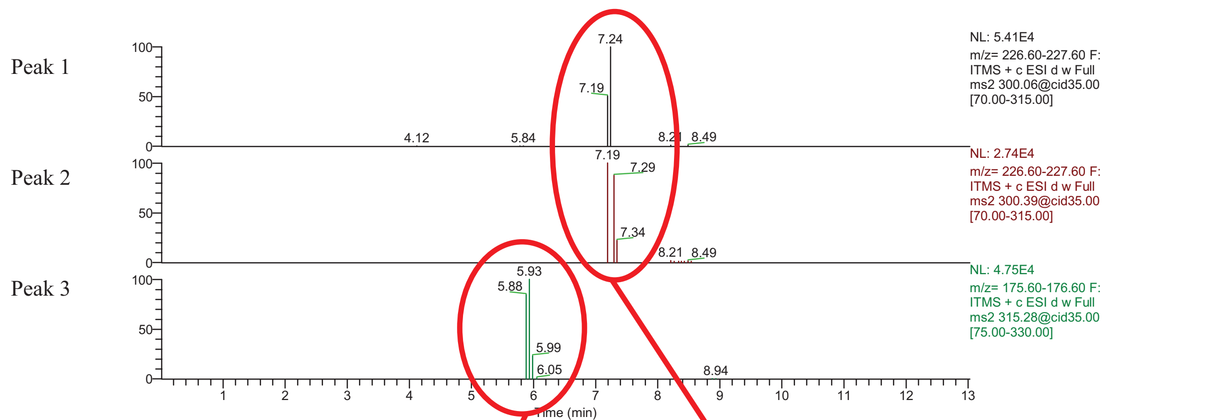
Peak Number	Compound Name	Code	SI	RSI	m/z	Expected RT	Actual RT	Intensity	Library Name
1	OH-LSD	m	756	922	356.20	6.20	6.90	15461	Tox_Library_LXQ

Figure 1. Example ToxID software short report showing ion chromatogram and a compound detected in urine positive for LSD.

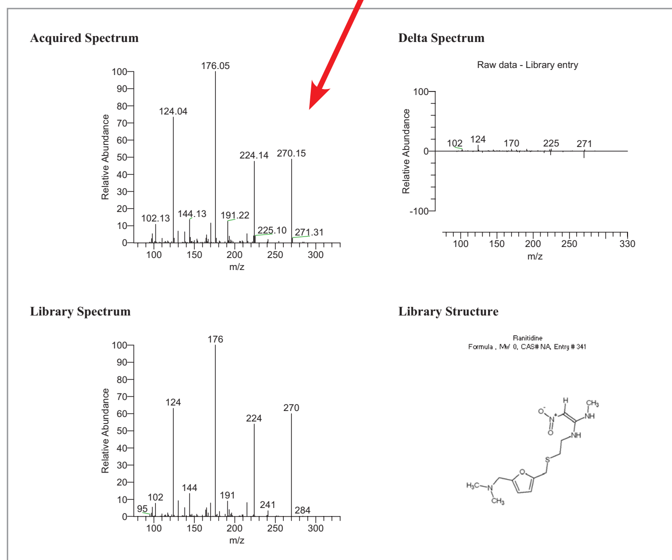
We confirmed the presence of ranitidine and metoclopramide through the mass spectra, which are very similar to that present in the library and the measured retention times are very similar to the expected (Figure 2). Moreover, some immunoassays are known to give cross-reactivity between ranitidine and amphetamines. As a consequence, we established that a false positive was found by the immunoassay and the ToxSpec Analyzer that identified the cross-reacting molecule as ranitidine.

Conclusion

The ToxSpec Analyzer was used to screen toxic compounds and their metabolites in urine based on LC-MS/MS. This method has been compared with other classical screening techniques such as HPLC-DAD and an immunoassay. LC-MS/MS demonstrated more reliable results than other techniques. In conclusion, the LC-MS/MS method provides rapid sample preparation, ease-of-use, sensitivity, specificity and a low cost per sample analysis, making the ToxSpec Analyzer an appropriate tool for non-targeted screening in a forensic toxicology laboratory.



Peak Number	Compound Name	Code	SI	RSI	*m/z*	Expected RT	Real RT	Intensity	Library Name
1	Metoclopramide	p	958	958	300.20	7.20	7.24	54117	Tox_Library_LXQ
2	Metoclopramide	p	984	984	300.20	7.20	7.19	27361	Tox_Library_LXQ
3	Ranitidine	p	792	802	315.20	5.45	5.93	47525	Tox_Library_LXQ



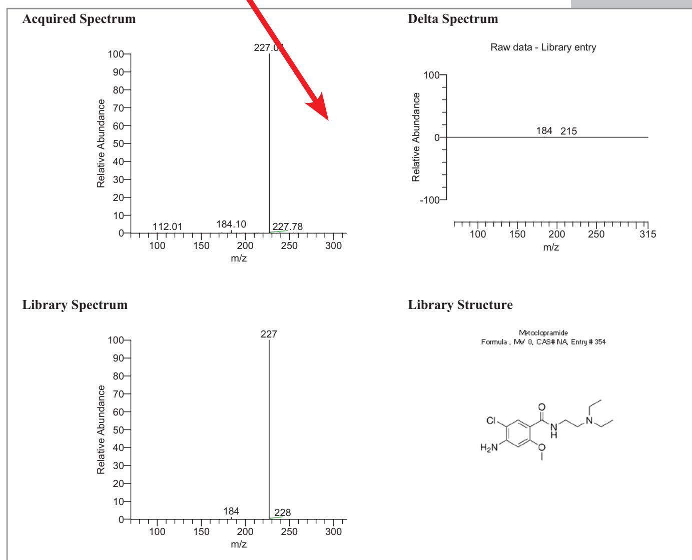


Figure 2. ToxID software long report showing ion chromatograms and MS/MS spectra of compounds detected in Urine 5. Mass spectra recorded for ranitidine and metoclopramide show a perfect match when compared with spectra from the database.

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Screening of 20 Benzodiazepines and Four Metabolites in Whole Blood using UHPLC-MS/MS

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Key Words

- **Psychoactive drugs**
- **TSQ Quantum Ultra**
- **T-SRM method**
- **Accela UHPLC System**
- **Forensic Toxicology**

Introduction

Benzodiazepines have a broad range of therapeutic use and are widely prescribed as safe drugs with relatively few side effects for the treatment of insomnia, anxiety and epilepsy. However, they are also abused in cases of crime, suicide, and drug-facilitated sexual assault. These molecules are active at very low concentrations and some of them have very short half lives. For this reason, the analytical methods must show extensive specificity and sensitivity for forensic purposes. We have developed and validated a method for 20 benzodiazepines and four metabolites in whole blood using liquid chromatography-tandem mass spectrometry (LC-MS/MS) coupled with ultrahigh pressure liquid chromatography (UHPLC) pumps.

Goal

To present a rapid and quantitative forensic screening approach for the analysis of benzodiazepines in blood matrix using UHPLC conditions.

Experimental

Sample Preparation

Extraction was performed using a liquid-liquid extraction (LLE) procedure. After the extraction, the sample was evaporated to dryness and reconstituted with 100 µL of a mixture containing acetonitrile/5 mM ammonium formate pH3 (30/70).

HPLC Conditions

Chromatographic analyses were performed using the Thermo Scientific Accela UHPLC system. The chromatographic conditions were as follows:

Column:	Thermo Scientific Hypersil GOLD 1.9 µm, 50 x 2.1 mm
Flow rate:	0.6 mL/min
Mobile phase A:	Water containing 5 mM ammonium formate, pH3
Mobile phase B:	Acetonitrile containing 0.1% formic acid

A gradient was performed starting from 95% of A to 95% of B in 6 minutes. The injection volume was 10 µL.

MS Conditions

Mass Spectrometer:	Thermo Scientific TSQ Quantum Ultra triple stage quadrupole mass spectrometer
Source:	Heated electrospray ionization (HESI) mode
Ion Polarity:	Positive mode
Spray Voltage:	3000 V
Sheath/Auxiliary gas:	Nitrogen
Sheath gas pressure:	50 (arbitrary units)
Auxiliary gas pressure:	40 (arbitrary units)
Capillary temperature:	300 °C
Scan Type:	Selected reaction monitoring (SRM)
Q1, Q3 resolution:	Unit (0.7 Da FWHM)

Two SRM transitions were monitored for each component to provide ion ratio confirmations (IRC).

Results and Discussion

We validated a timed SRM (T-SRM) method for screening and quantifying 20 benzodiazepines and four metabolites. The run time was less than eight minutes, although most compounds eluted before four minutes. The T-SRM method allows the acquisition of an SRM transition only during a specified time window, not the entire run time. T-SRM divides the task into smaller batches by programming the instrument to look for each SRM only when it is expected to enter the instrument from an upstream LC system. Each time period is then optimized for the retention time of each compound. More time per transition results in better signal-to-noise (S/N) ratios or more scans per peak, allowing better quantitative data.

Standard spiking solutions of the analytes in porcine whole blood at concentrations of 5, 10, 50, 100, 300 and 500 ng/mL were prepared. All benzodiazepine calibration curves were evaluated using linear regression. Excellent linearity with a correlation coefficient of $R^2 > 0.99$ was obtained for each molecule. Seventeen were linear on the entire concentration range from 5 to 500 ng/mL. Six were linear from 10 to 500 ng/mL, and 3 were validated under linear conditions from 5 to 300 ng/mL. In all cases, the concentration range covered the therapeutic ranges.

Intra-method variability was calculated by processing five replicates of four calibration levels: the LOQ (limit of quantitation), two intermediate concentrations, and the maximum concentration. (%CV = coefficient of variance).

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Inter-method variability was determined by processing five replicates of four calibration levels in four different batches run on four different days. All values were below 15% and therefore within the guidelines set for a validated LC-MS/MS method.

Extraction efficiency also was evaluated and calculated at three concentration levels: 10 ng/mL, 100 ng/mL and 300 ng/mL. Values were between 50% and 100%, except for 7 amino-clonazepam which was around 30%.

The lower limit of quantitation (LLOQ) and the limit of detection (LOD) of the compounds were determined based on the calibration curve of S/N ratio versus concentration and the definitions of LOQ and LOD using S/N = 10 and 3. LLOQs were between 0.1 and 3 ng/mL for all molecules. Figure 1 shows the chromatogram obtained from a real sample acquired using the developed UHPLC-MS/MS method.

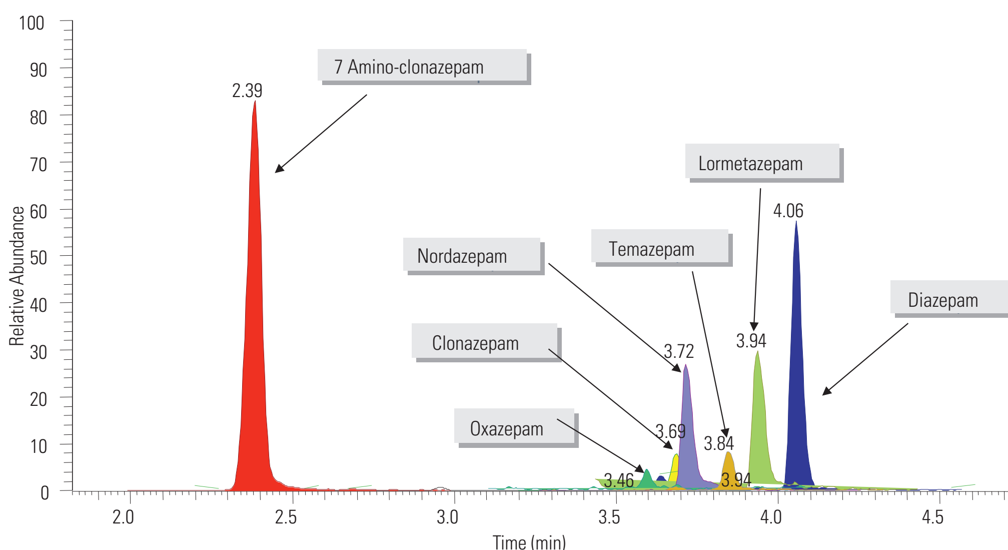

Figure 1. Chromatogram obtained from a real sample acquired using the T-SRM UHPLC-MS/MS method

Conclusion

A rapid UHPLC-MS/MS method for quantifying benzodiazepines in whole blood samples was developed for forensic toxicology. The precision of the analysis meets current consensus guidelines. A T-SRM method was used to increase the acquisition time per compound and achieve better signal-to-noise ratios for the analytes.

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Quantitative LC-MS Analysis of 14 Benzodiazepines in Urine Using TraceFinder 1.1 Software and High Resolution Accurate Mass

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Key Words

- TraceFinder Software
- Exactive
- Clinical Research

Introduction

Thermo Scientific TraceFinder 1.1 software is developed for quantitative analysis for clinical research laboratories. The software is designed for routine data acquisition, quantitation, qualitative screening and reporting on all Thermo Scientific liquid chromatography mass spectrometry (LC-MS) systems, including high resolution accurate mass (HRAM) instruments, with fully integrated support for the Thermo Scientific Transcend multiplexing system.

TraceFinder™ 1.1 quantitative software simplifies routine analysis for the operator by executing a stepwise workflow from batch creation to reporting. For clinical research laboratories employing multiple types of LC-MS systems, TraceFinder 1.1 software eliminates the need to learn and maintain multiple software programs.

TraceFinder 1.1 software provides many easy approaches to execute workflow routines for operators and lab managers. The work presented here demonstrates the workflow used by lab managers during method development and includes processing method creation using the compound data store (CDS). The operator's workflow includes batch submission, real time monitoring, data review and report generation.

Goal

To demonstrate a new, easy-to-use workflow-driven quantitative method for 14 benzodiazepines in urine using the Thermo Scientific Exactive high performance benchtop mass spectrometer and TraceFinder 1.1 routine quantitative software.

Methods

Sample Preparation

Urine was spiked with internal standards and hydrolyzed with beta-glucuronidase. Acetonitrile was added to the hydrolyzed sample and the resulting mixture was centrifuged. Supernatant was further diluted and subjected to LC-MS analysis.

LC-MS/MS conditions

LC-MS analysis was performed on an Exactive™ mass spectrometer with a heated electrospray ionization (HESI) source coupled with a Transcend™ TLX system used in LX mode. Full scan mass spectrometry analysis was done with resolution of 100,000 (FWHM at *m/z* 200) with a mass isolation window of 3 ppm. Exact mass was used for compound identification. High performance liquid chromatography (HPLC) was carried out on a Thermo Scientific Hypersil GOLD PFP column (100 × 2.1 mm, 5 µm particle size) at room temperature.

The MS conditions were as follows:

Ionization	HESI-II
Polarity	Positive
Vaporizer temp (°C)	350
Capillary temp (°C)	350
Spray voltage (V)	3500
Sheath gas (AU)	40
Auxillary gas (AU)	10
Data acquisition mode	Full scan
AGC target	1.00E+06
Lock mass *(m/z)*	279.2591
Scan range *(m/z)*	135-600
Max injection time (ms)	100
Resolution	100,000

Software

Method development, data acquisition, data processing and report generation were all executed in TraceFinder 1.1 routine quantitation software.

Results and Discussion

Streamlined Workflow:

The entire workflow in TraceFinder 1.1 software is easy to set up and is summarized in Figure 1.

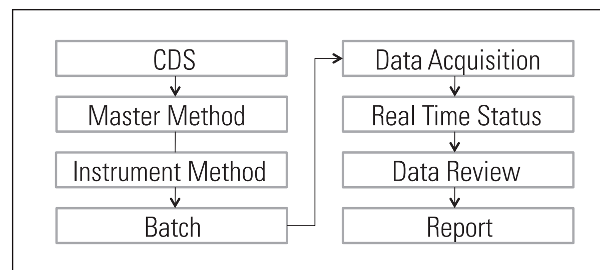

Figure 1. TraceFinder 1.1 workflow for quantitative analysis

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Main Tabs in TraceFinder 1.1

Figure 2 shows the four main tabs: Configuration, Method Development, Data Review and Acquisition.

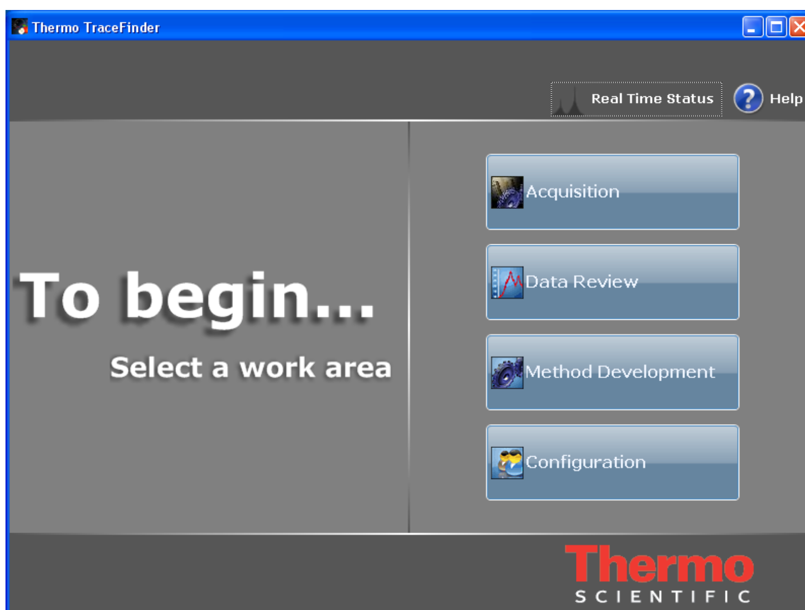


Figure 2. TraceFinder 1.1 welcome screen

Compound Data Store (CDS)

Figure 3 shows the CDS for this benzodiazepines application. Entries in this CDS are built based on the accurate masses. CDS can be later updated with retention times of analytes.

	Compound Name	ExperimentType	Category	Ionization
1	2-Hydroxyethylflurazepam	XIC	Benzo	ESI
2	2-Hydroxyethylflurazepa...	XIC	Benzo	ESI
3	7-Aminoclonazepam	XIC	Benzo	ESI
4	7-Aminoclonazepam-D4	XIC	Benzo	ESI
5	7-Aminoflunitrazepam	XIC	Benzo	ESI
6	7-Aminoflunitrazepam-D7	XIC	Benzo	ESI
7	7-Aminonitrrazepam	XIC	Benzo	ESI
8	a-Hydroxyalprazolam	XIC	Benzo	ESI
9	a-Hydroxyalprazolam-D5	XIC	Benzo	ESI

Compound Name	Mass	RT (min)	Window (sec)
a-Hydroxyalprazolam-D5	330.1160	5.420	240.00

	Compound Name	ExperimentType	Category	Ionization
10	a-Hydroxytriazolam	XIC	Benzo	ESI

Compound Name	Mass	RT (min)	Window (sec)
a-Hydroxytriazolam	359.0460	5.330	240.00

	Compound Name	ExperimentType	Category	Ionization
11	a-Hydroxytriazolam-D4	XIC	Benzo	ESI

Compound Name	Mass	RT (min)	Window (sec)
a-Hydroxytriazolam-D4	363.0710	5.330	240.00

Figure 3. Compound Data Store for benzodiazepines application

Master Method

The "Master Method" contains information on data acquisition (including instrument method), data processing, and analysis. In detail, it contains settings for 5 main categories: General (including method type, injection volume, instrument method, etc), Compound (acquisition list selected from CDS, detection, calibration, etc), Flags, Groups and Reports. Selected tabs in "General" and "Compound" are shown in Figure 4. To complete the master method setup, settings in "Flags", and "Reports" can also be customized. TraceFinder software provides 50 predefined report templates.

Instrument Method

The instrument method is comprised of individual LC, autosampler and MS portions. The software allows for optimization of chromatography and customizable autosampler programming.

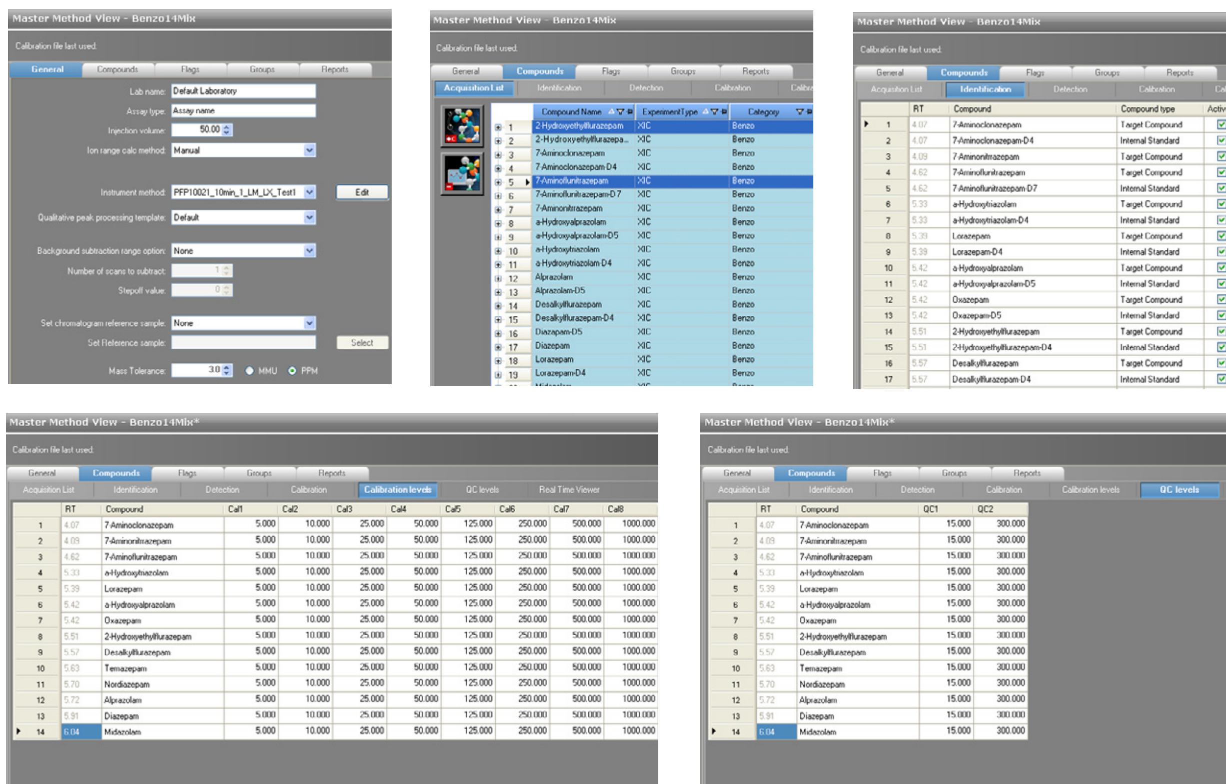
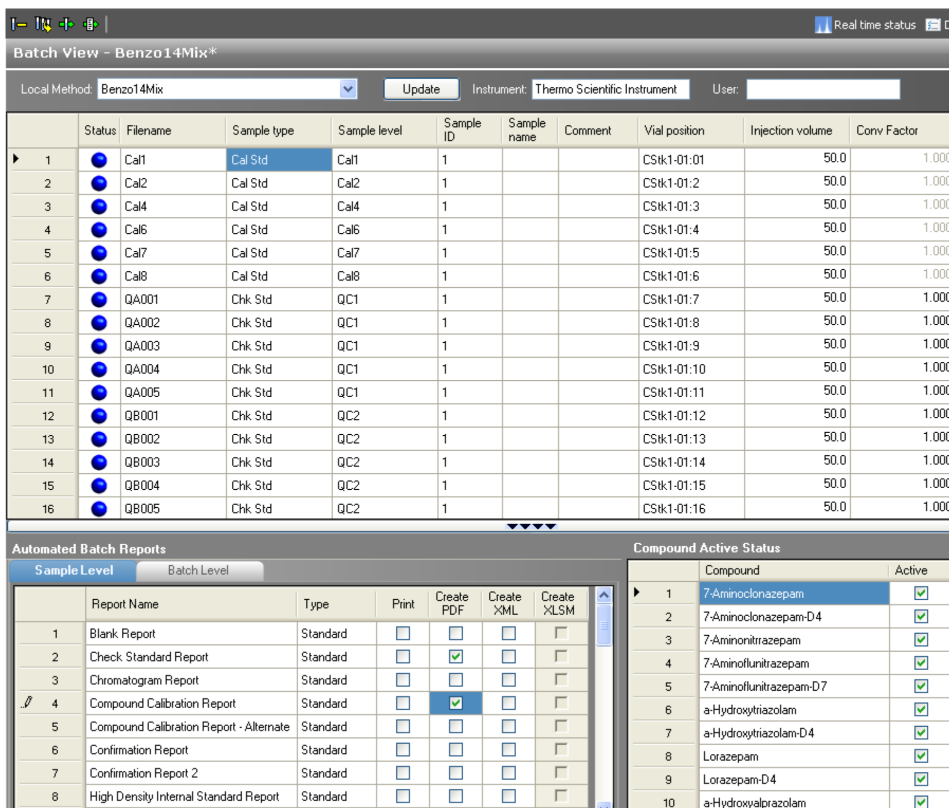


Figure 4. Master Method creation process (selected tabs)

Batch

After creation of the master method, a new sample batch can be created for data acquisition. Creating a batch involves assigning a project, linking to the master method, building a run-sequence and submitting. Figure 5 shows an exemplary batch view containing six calibrators and two levels of “Check Standards” (or QCs, n=5).



Batch View - Benzo14Mix*

Local Method: Benzo14Mix | Update | Instrument: Thermo Scientific Instrument | User:

	Status	Filename	Sample type	Sample level	Sample ID	Sample name	Comment	Vial position	Injection volume	Conv Factor
1		Cal1	Cal Std	Cal1	1			CStk1-01:01	50.0	1.000
2		Cal2	Cal Std	Cal2	1			CStk1-01:2	50.0	1.000
3		Cal4	Cal Std	Cal4	1			CStk1-01:3	50.0	1.000
4		Cal6	Cal Std	Cal6	1			CStk1-01:4	50.0	1.000
5		Cal7	Cal Std	Cal7	1			CStk1-01:5	50.0	1.000
6		Cal8	Cal Std	Cal8	1			CStk1-01:6	50.0	1.000
7		QA001	Chk Std	QC1	1			CStk1-01:7	50.0	1.000
8		QA002	Chk Std	QC1	1			CStk1-01:8	50.0	1.000
9		QA003	Chk Std	QC1	1			CStk1-01:9	50.0	1.000
10		QA004	Chk Std	QC1	1			CStk1-01:10	50.0	1.000
11		QA005	Chk Std	QC1	1			CStk1-01:11	50.0	1.000
12		QB001	Chk Std	QC2	1			CStk1-01:12	50.0	1.000
13		QB002	Chk Std	QC2	1			CStk1-01:13	50.0	1.000
14		QB003	Chk Std	QC2	1			CStk1-01:14	50.0	1.000
15		QB004	Chk Std	QC2	1			CStk1-01:15	50.0	1.000
16		QB005	Chk Std	QC2	1			CStk1-01:16	50.0	1.000

Automated Batch Reports — Sample Level | Batch Level

	Report Name	Type	Print	Create PDF	Create XML	Create XLSM
1	Blank Report	Standard	☐	☐	☐	☐
2	Check Standard Report	Standard	☐	☑	☐	☐
3	Chromatogram Report	Standard	☐	☐	☐	☐
4	Compound Calibration Report	Standard	☐	☑	☐	☐
5	Compound Calibration Report - Alternate	Standard	☐	☐	☐	☐
6	Confirmation Report	Standard	☐	☐	☐	☐
7	Confirmation Report 2	Standard	☐	☐	☐	☐
8	High Density Internal Standard Report	Standard	☐	☐	☐	☐

Compound Active Status

	Compound	Active
1	7-Aminoclonazepam	☑
2	7-Aminoclonazepam-D4	☑
3	7-Aminonitrazepam	☑
4	7-Aminoflunitrazepam	☑
5	7-Aminoflunitrazepam-D7	☑
6	a-Hydroxytriazolam	☑
7	a-Hydroxytriazolam-D4	☑
8	Lorazepam	☑
9	Lorazepam-D4	☑
10	a-Hydroxyalprazolam	☑

Figure 5. Acquisition Batch view

Data Acquisition and Real Time Status

After batch submission, data will be acquired and real time chromatograms can be shown in customizable ways (Figure 6). Status of acquisition (pressure profile, event log), device status, and sample queue can all be monitored in Real Time Status. TraceFinder software allows for multiple batches submission prioritization.

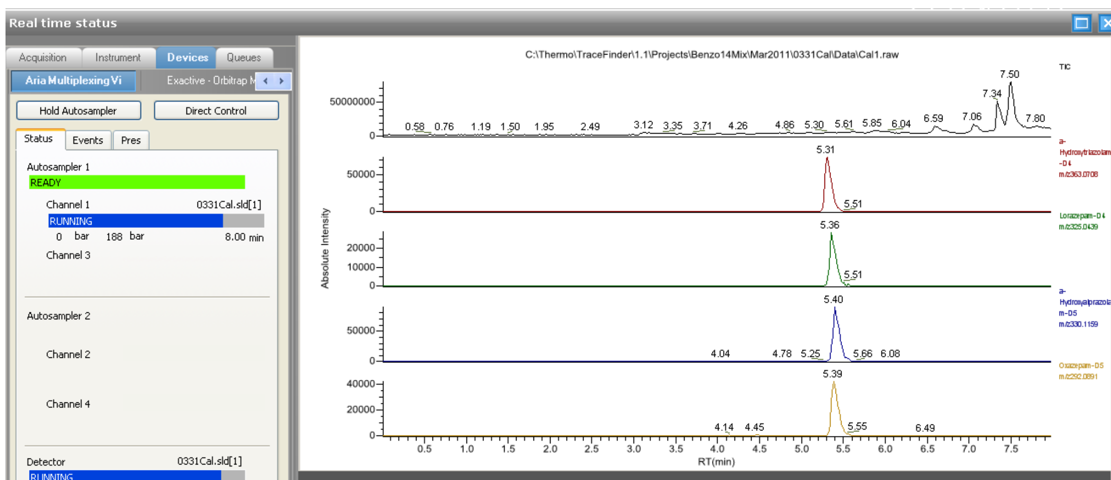


Figure 6. Real Time Status view

Data Review

Data Review (Figure 7) allows for flagging for any items that require attention (retention time drift, limit of quantitation, ion ratio discrepancy, etc.).

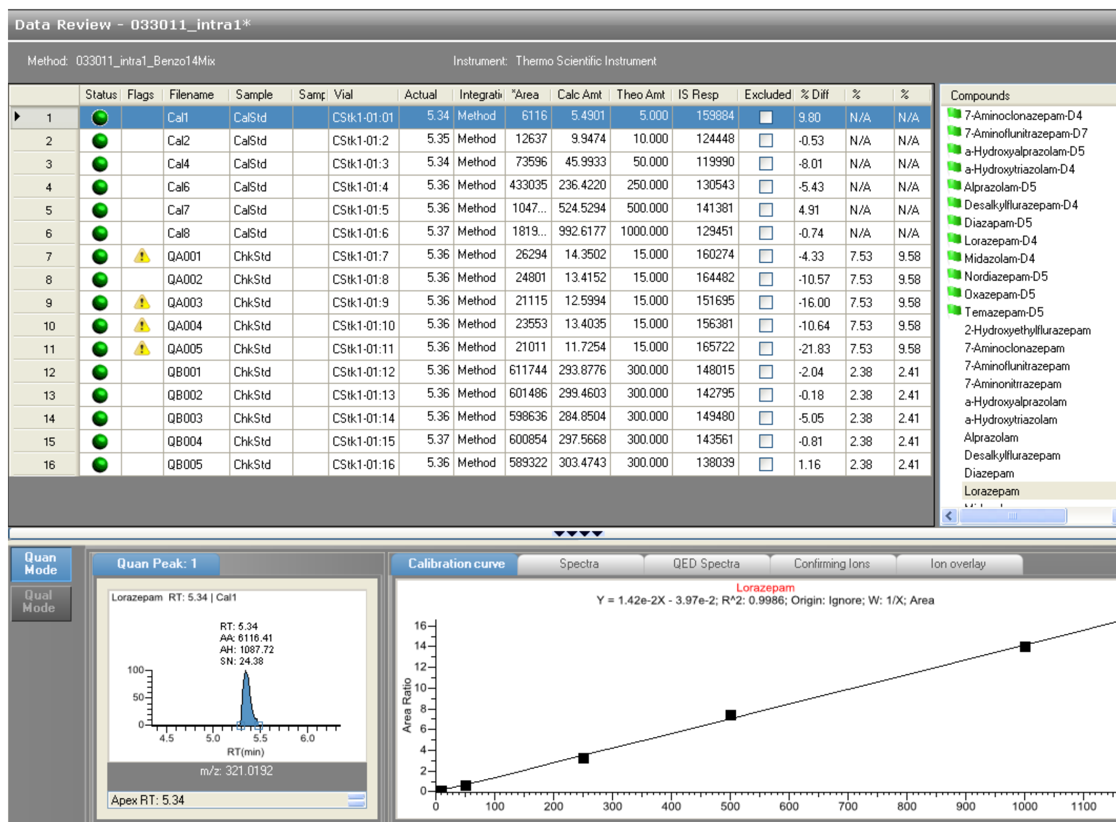
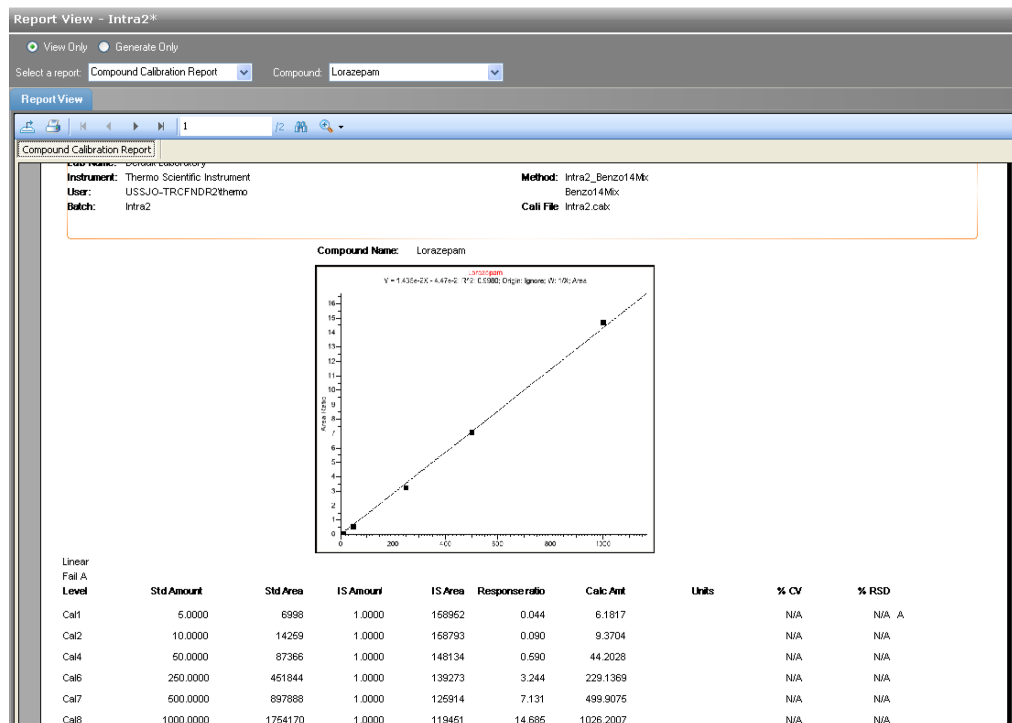


Figure 7. Data Review view for lorazepam, one of the 14 benzodiazepines

Reporting

Figures 8 and 9 are two examples (compound calibration and check standard/quality control) of the Report View.



Report View - Intra2*

View Only / Generate Only

Select a report: Compound Calibration Report — Compound: Lorazepam

Instrument: Thermo Scientific Instrument — Method: Intra2_Benzo14Mix Benzo14Mix

User: USSJO-TRCFNDR2\thermo

Batch: Intra2 — Cali File: Intra2.calx

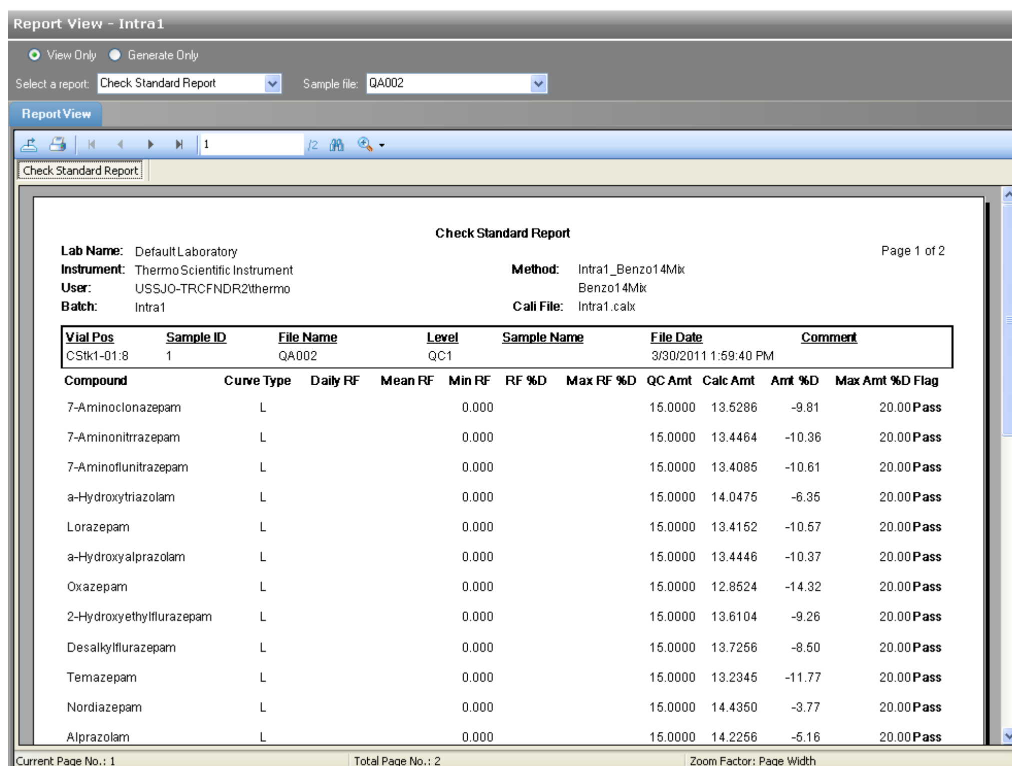
Compound Name: Lorazepam

Linear

Fail A

Level	Std Amount	Std Area	IS Amount	IS Area	Response ratio	Calc Amt	Units	% CV	% RSD
Cal1	5.0000	6998	1.0000	158952	0.044	6.1817		N/A	N/A A
Cal2	10.0000	14259	1.0000	158793	0.090	9.3704		N/A	N/A
Cal4	50.0000	87366	1.0000	148134	0.590	44.2028		N/A	N/A
Cal6	250.0000	451844	1.0000	139273	3.244	229.1369		N/A	N/A
Cal7	500.0000	897888	1.0000	125914	7.131	499.9075		N/A	N/A
Cal8	1000.0000	1754170	1.0000	119451	14.685	1026.2007		N/A	N/A

Figure 8. Compound Calibration Report for lorazepam



Report View - Intra1

View Only / Generate Only

Select a report: Check Standard Report — Sample file: QA002

Check Standard Report

Lab Name: Default Laboratory — Page 1 of 2

Instrument: Thermo Scientific Instrument — Method: Intra1_Benzo14Mix Benzo14Mix

User: USSJO-TRCFNDR2\thermo

Batch: Intra1 — Cali File: Intra1.calx

Vial Pos	Sample ID	File Name	Level	Sample Name	File Date	Comment
CStk1-01:8	1	QA002	QC1		3/30/2011 1:59:40 PM	

Compound	Curve Type	Daily RF	Mean RF	Min RF	RF %D	Max RF %D	QC Amt	Calc Amt	Amt %D	Max Amt %D	Flag
7-Aminoclonazepam	L			0.000			15.0000	13.5286	-9.81	20.00	Pass
7-Aminonitrrazepam	L			0.000			15.0000	13.4464	-10.36	20.00	Pass
7-Aminoflunitrazepam	L			0.000			15.0000	13.4085	-10.61	20.00	Pass
a-Hydroxytriazolam	L			0.000			15.0000	14.0475	-6.35	20.00	Pass
Lorazepam	L			0.000			15.0000	13.4152	-10.57	20.00	Pass
a-Hydroxyalprazolam	L			0.000			15.0000	13.4446	-10.37	20.00	Pass
Oxazepam	L			0.000			15.0000	12.8524	-14.32	20.00	Pass
2-Hydroxyethylflurazepam	L			0.000			15.0000	13.6104	-9.26	20.00	Pass
Desalkylflurazepam	L			0.000			15.0000	13.7256	-8.50	20.00	Pass
Temazepam	L			0.000			15.0000	13.2345	-11.77	20.00	Pass
Nordiazepam	L			0.000			15.0000	14.4350	-3.77	20.00	Pass
Alprazolam	L			0.000			15.0000	14.2256	-5.16	20.00	Pass

Current Page No.: 1 — Total Page No.: 2 — Zoom Factor: Page Width

Figure 9. Check Standard (QC) Report for one QC sample

Method Performance

Sample preparation for urine analysis of benzodiazepines was previously done with solid phase extraction (SPE). Here we tested a simple urine dilution strategy. The absolute recovery of deuterated benzodiazepine internal standards was tested with several lots of human urine. It was determined that the absolute recoveries of the internal standards ranged from 83.0% to 100.5% at 100 ng/mL from all lots of urine tested (data not shown).

This method was linear from 5 to 1000 ng/mL for all 14 benzodiazepines with an accuracy of 85.4%-106.0%. Inter- (n=15) and intra-batch (n=5) coefficients of variation (CV) at two different concentration levels ranged from 0.5% to 11.7%. The method has a lower limit of quantitation (LLOQ) of 5 ng/mL for all 14 benzodiazepines tested. The method performance is summarized in Table 1. Figure 10 shows the extracted ion chromatograms (XICs) with 3 ppm mass isolation window of all 14 benzodiazepines at their LLOQ (5 ng/mL).

Table 1. Method performance for 14 benzodiazepines in urine

Name	m/z	QC level 1: 15 ng/mL		QC level 2: 300 ng/mL		Linear Range (ng/mL)	LLOQ (ng/mL)
		% Precision	% Accuracy	% Precision	% Accuracy		
7-Aminonitrazepam	252.1131	2.9	88.7	2.9	106.0	5 - 1000	5
Nordiazepam	271.0633	5.7	89.6	2.9	100.9	5 - 1000	5
7-Aminoflunitrazepam	284.1194	3.4	91.2	4.0	100.9	5 - 1000	5
Diazepam	285.0789	8.8	96.0	2.6	99.7	5 - 1000	5
7-Aminoclonazepam	286.0742	2.0	89.1	2.1	99.4	5 - 1000	5
Oxazepam	287.0582	5.0	85.6	3.5	98.4	5 - 1000	5
Desalkylflurazepam	289.0539	5.5	88.5	2.9	98.6	5 - 1000	5
Temazepam	301.0738	3.4	89.1	2.7	97.6	5 - 1000	5
Alprazolam	309.0902	3.1	90.0	3.2	101.5	5 - 1000	5
Lorazepam	321.0192	7.6	85.4	3.4	95.3	5 - 1000	5
α-Hydroxyalprazolam	325.0851	3.0	87.0	1.8	97.3	5 - 1000	5
Midazolam	326.0855	3.6	91.3	2.6	101.2	5 - 1000	5
2-Hydroxyethylflurazepam	333.0801	3.7	89.0	2.5	99.7	5 - 1000	5
α-Hydroxytriazolam	359.0461	5.9	86.9	2.8	97.5	5 - 1000	5

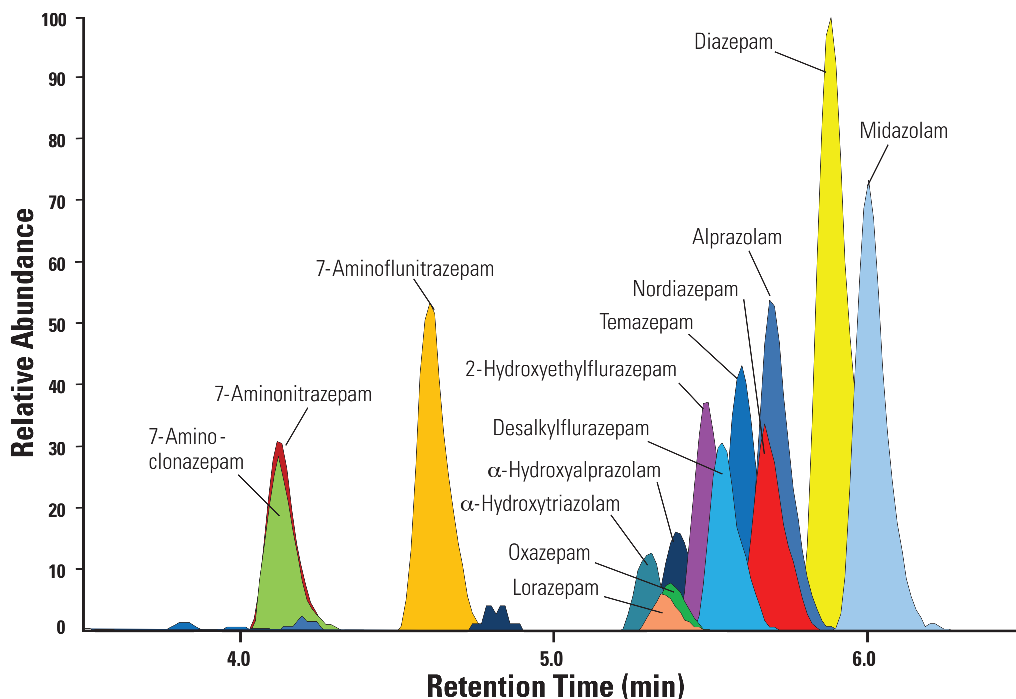


Figure 10. Extracted ion chromatograms of 14 benzodiazepines in urine at their LLOQ (5 ng/mL, mass isolation window=3 ppm)

Conclusion

We have developed a fast and sensitive LC-MS method for 14 benzodiazepines in urine using a benchtop Exactive mass spectrometer with TraceFinder 1.1 software. TraceFinder 1.1 software is easy to use and effective in performing quick routine quantitative analysis of benzodiazepines in urine. The software enables easy method development, batch creation, submission and real time monitoring for clinical research laboratories. The data review functionality was very useful in quick review and verification of the calibration accuracy and linearity. The report templates make selecting and generating reports with all the necessary information easy and quick.

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Application Note: 524

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A Fully Automated LC-MS Screening System using Automated Online Sample Preparation for Forensic Toxicology

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[3]Thermo Fisher Scientific, Franklin, USA

Key Words

- **Transcend TLX-1 system**
- **TurboFlow Technology**
- **LXQ Linear Ion Trap**
- **Illicit Drugs**
- **Forensic Toxicology**

Introduction

Liquid chromatography-mass spectrometry (LC-MS) is a powerful tool widely used for forensic targeted drug screening. However, the quality of the results is highly affected by the sample preparation. Offline solid phase extraction (SPE) and liquid-liquid extraction (LLE) are widely used, but these methods are often time-consuming and costly. To provide a fast and sensitive approach, an automated online sample preparation method using Thermo Scientific Transcend TLX-1 system powered by TurboFlow™ technology for the forensic toxicological screening of more than 400 acidic, neutral, and basic drugs in urine with LC/MS^n has been developed.

Goal

To evaluate the performance of an automated online sample preparation method for an LC/MS^n screening approach.

Experimental

Sample preparation was performed by an online sample extraction method utilizing Thermo Scientific TurboFlow technology. Two TurboFlow columns (Cyclone, C18XL) were connected in series and used for sample extraction. Urine samples were run both natively and after enzymatic hydrolysis. The eluent was then transferred to the LC column (Thermo Scientific Betasil Phenyl-Hexyl, 100 x 3 mm, 3 µm) for separation.

A 30-minute gradient from 1% to 98% organic was employed for separation of the analyte with flow rates of 300 µL/min. All samples were then analyzed on a Thermo Scientific LXQ linear ion trap mass spectrometer with the atmospheric pressure chemical ionization (APCI) source. A data-dependent polarity switching method was used for data acquisition. MS^2 and MS^3 spectra were acquired. Since polarity switching was used, a single injection of a sample containing unknown compounds was sufficient to detect both substances ionizing in negative and positive mode. The data was automatically processed, post-acquisition, by Thermo Scientific ToxID automated screening software.

Results and Discussion

The method using online extraction has been fully validated. A minor matrix effect (suppression < 5%) was observed for over 98% of the compounds. A recovery of more than 90% was seen in 90% of the substances. The limit of identification (LOI) was below 10 ng/mL for 60% of the substances and 90% could be identified at a concentration of 100 ng/mL. The 400-compound library contains both MS^2 and MS^3 spectra. MS^3 spectra bring an additional level of specificity, although in most cases, the analytes can be easily identified by using only the MS^2 spectra. However, some analytes may have the same molecular weight, very similar MS^2 spectra, and a very close retention time. For these reasons, MS^3 data have to be used for the identification. One example is the isobaric compounds O-desmethylvenlafaxine and tramadol. The two analytes have the same molecular weight, very close retention times (see details in Table 1), and the same MS^2 spectra (Figure 1). Therefore, by running only MS^2 experiments, it is impossible to properly differentiate the two analytes. When MS^3 spectra are recorded, tramadol does not fragment ions while O-desmethylvenlafaxine gives a specific spectrum (Figure 1). Therefore, the analytes can be properly identified. Total run time of the analysis is 30 minutes. An example of a chromatogram obtained from a sample is presented in Figure 2.

Table 1. Tramadol and O-desmethylvenlafaxine information

	O-Desmethylvenlafaxine	Tramadol
Precursor mass	264.3	264.3
MS^2 Fragment	246.3	246.3
Retention Time	10.6 min	10.3 min

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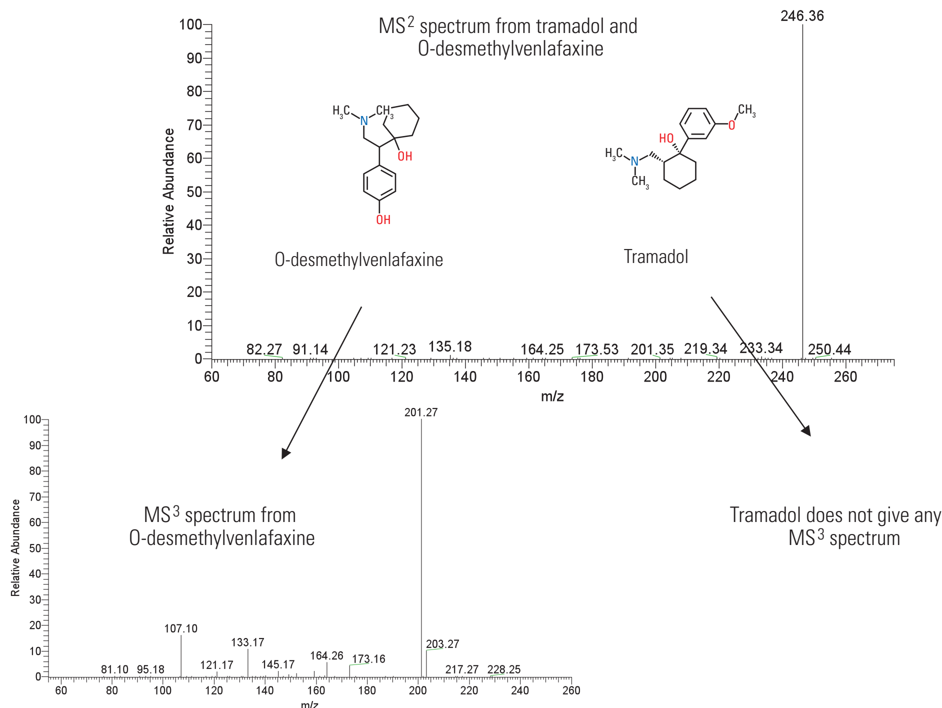

Figure 1. Fragmentation of tramadol and O-desmethylvenlafaxine in MS^2 and MS^3

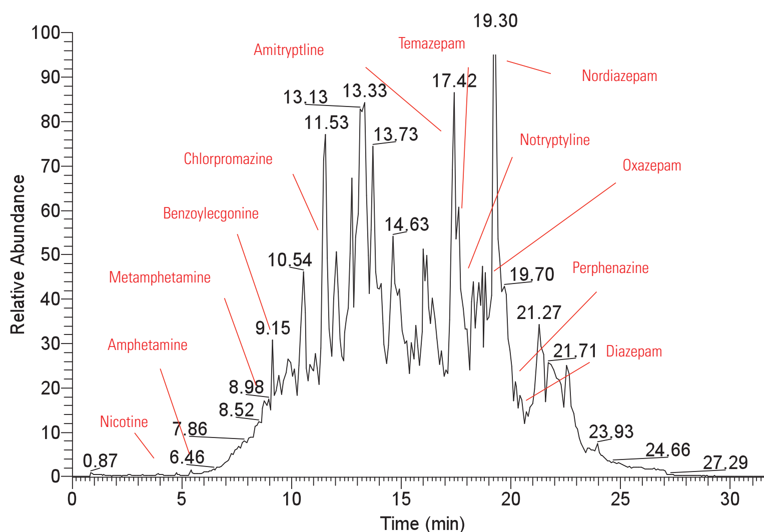

Figure 2. Full scan MS chromatogram of a sample containing 12 different analytes

Conclusion

The automated online TurboFlow method with the LXQ™ linear ion trap mass spectrometer allows a fast and specific approach for the identification of a broad range of compounds in positive and negative mode in a single run. The sample preparation time is 15 minutes with this method as compared to 2 hours with an offline approach. The LOIs are below 100 ng/mL for more than 90% of the analytes. MS^3 spectra acquisition brings an additional level of specificity for forensic toxicology laboratories.

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Analysis of Multiple Illicit Drugs, Methadone, and their Metabolites in Oral Fluid Using a Linear Ion Trap Mass Spectrometer

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Key Words

- LXQ
- Surveyor Plus
- Drugs of Abuse Forensics
- Hypersil GOLD Columns
- MS³ Quantification

Introduction

Traditionally, the analysis of urine samples has been the major approach for the detecting of drugs of abuse.[1] However, a common risk for this type of analysis is adulteration or manipulation of the sample at the point of collection. As an alternative, the analysis of oral fluid provides an easy method of sample collection and has the advantage of providing a relatively clean matrix. Because of the reduced sample volume, this technique requires a high sensitivity and robust analytical method to make an attractive alternative to conventional methods.

In this report, a rapid and rugged LC-MS/MS method using the Thermo Scientific LXQ is described for analyzing a mixture of twenty drugs and their metabolites using intelligent automated mass spectrometry (INTAMS). The detection limits for the mixture of drugs and dynamic range are superior to results reported previously.[2] In addition, this method provides for the simultaneous identification and quantification of drugs and their metabolites.

Experimental Conditions

Sample Preparation:

Ten milliliters of oral fluid collected from a volunteer were protein precipitated using 30 mL acetonitrile. The sample was vortexed and then centrifuged at 5,000 rpm for 10 minutes. The supernatant was evaporated to dryness under nitrogen and reconstituted in 5 mL water. Table 1 provides a list of 20 drugs along with the parent and product ion masses. For quantification experiments, known amounts of a stock solution of the 20 drug mixture were spiked into the treated oral fluid to prepare the standards in concentrations ranging from 50 fg/µL to 1 ng/µL.

Compound	Parent ion *m/z*	Product ions *m/z*
EEE[a]	214.3	196.2
Normorphine	272.3	201.0
AEM[b]	182.3	150.1, 122.1
Morphine	286.3	229.1, 211.2
Norcodeine	286.3	243.3, 225.3, 215.0
Codeine	300.3	175.0, 225.3
6-Acetylmorphine	328.3	268.3, 193.2
m-Hydroxybenzoylecgonine	306.2	168.2
Benzoylnorecgonine	276.2	154.1
Benzoylecgonine	290.3	168.2
Acetylcodeine	342.3	282.3, 225.2
Heroin	370.3	310.2, 328.2, 268.3
Cocaine	304.3	182.1
Norcocaine	290.2	168.1, 136.2
Cocaethylene	318.3	196.2
Norcocaethylene	304.2	182.1, 136.1
Methadol	312.3	223.1, 249.2, 171.2
EDDP[c]	278.0	249.2
Propoxyphene	340.1	266.1
Methadone	310.9	266.2

Table 1: List of 20 drugs and metabolites with their respective parent and product ion masses. EEE: ecgonine ethyl ester; AEM: anhydroecgonine methyl ester; EDDP: 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium

HPLC:

LC System: Thermo Scientific Surveyor Plus

Column: Thermo Scientific Hypersil GOLD™ (20 × 2.1 mm, 1.9 µm particle size)

Mobile phase:

(A) water with 0.1% formic acid and 10 mM ammonium acetate

(B) acetonitrile with 0.1% formic acid

Flow rate: 400 µL/min

Injection volume: 10 µL

Gradient:

t (min)	A%	B%
0.00	95	5
0.10	95	5
1.00	85	15
4.20	50	50
4.21	95	5
7.00	95	5

Mass Spectrometer:

The LXQ linear ion trap mass spectrometer was operated in positive atmospheric pressure chemical ionization (APCI) mode. The corona discharge needle voltage was 4.5 kV and the vaporizer temperature was 400 °C. The capillary temperature was 220 °C and the sheath gas flow was 25 units. All scan events were acquired with one micro scan. No internal standard was used. The set up of the acquisition method using INTAMS is shown in Figure 1.

Results and Discussions

INTAMS data acquisition software was used for the simultaneous identification of 20 drugs in oral fluid. The extracted ion chromatogram is shown in Figure 2. INTAMS software enables the maximum number of scans to be acquired under a given chromatographic peak by obtaining MS/MS spectra on only the masses identified within a specified time window which helps facilitate a faster duty cycle.

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In addition, the excellent ion statistics and the fast cycle time of the LXQ linear ion trap mass spectrometer enabled the simultaneous quantification and identification of these analytes. Calibration curves based on MS/MS spectra were generated using the standards for the drug mixture spiked in oral fluid over a concentration range from 50 fg/µL to 1.0 ng/µL. Figure 3 shows calibration curves for 8 of the 20 compounds analyzed simultaneously. The R^2 values of these curves are better than 0.996 and they exhibit linear dynamic range over 3 to 4 orders of magnitude. The detection limits (LOD and LOQ) for each analyte in oral fluid are listed in Table 2 along with the linear dynamic ranges. Compared with data published previously[2], the LXQ linear ion trap provided up to 10 times lower detection limits and an increased linear dynamic range.

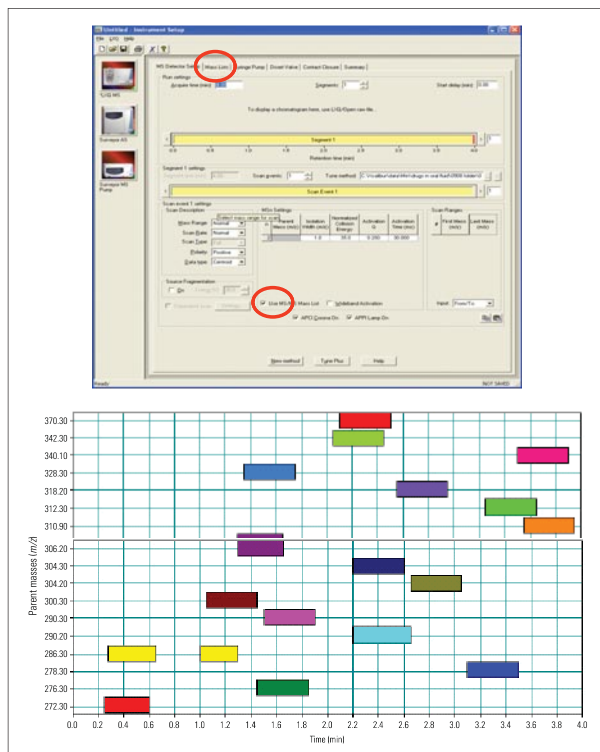

Figure 1: INTAMS (Intelligent Automated Mass Spectrometry) data acquisition software setup for simultaneous analysis of 20 compounds

Further confirmatory information and higher specificity results were also easily generated by performing quantification based on MS^3 data. The use of MS^3 quantification is demonstrated for the ecogonine ethyl ester sample (EEE) which undergoes a neutral loss of water molecule upon ion activation. When spiked in oral fluid, interference from the matrix masked the analyte peak. This was overcome as shown in Figure 4. The signal-to-noise ratio (S/N) of the extracted ion chromatogram obtained from MS^3 data (top chromatogram) is dramatically higher than that obtained from the MS/MS data. The high quality of the MS^n spectra obtained using the LXQ also results in greater sensitivity over a wider linear dynamic range (Figure 4b and 4c).

The quantitative study was completed by analyzing two QC oral fluid samples, each containing a mixture of ten drugs. The results shown in Table 3 demonstrate a high level of quantification accuracy, with a deviation of less than 10% for all the analytes. In addition, excellent reproducibility was demonstrated with the %RSD being less than 9% for all the compounds within five injections.

Data Analysis

Mass Frontier™ software includes a number of tools for structure identification. The powerful search features and database management make it valuable for identifying drugs, metabolites and related compounds. A library of target drugs can be easily searched. As an example, the MS/MS spectrum obtained from 6-acetylmorphine in oral fluid was searched against an NIST library using Mass Frontier software. In addition to being the top hit (Figure 5), the chromatographic elution time and the mass of the precursor ion provide added degrees of confidence for identification.

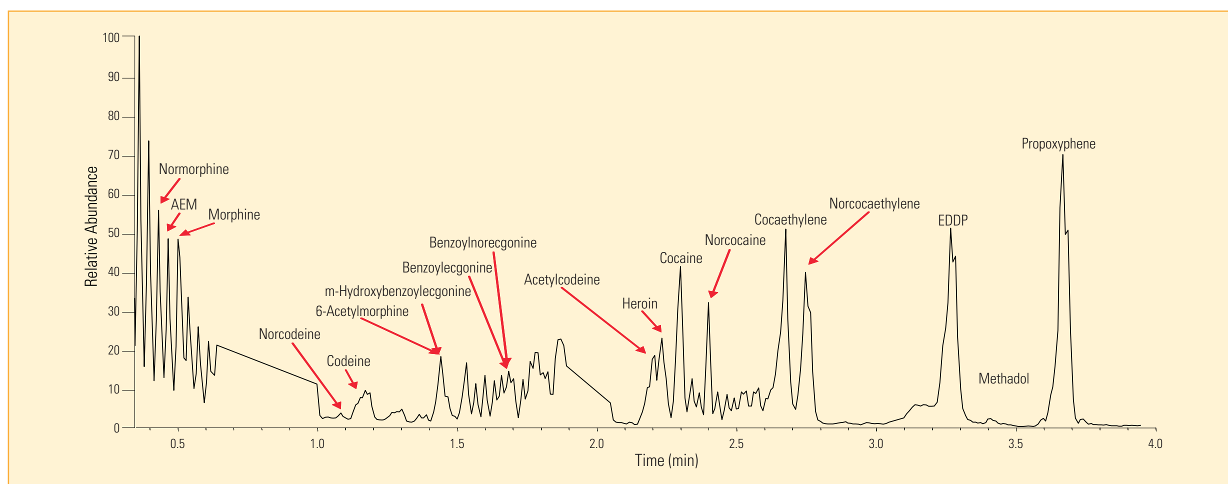

Figure 2: Chromatogram of the drugs and metabolites in oral fluid using LC-MS/MS with INTAMS data acquisition software

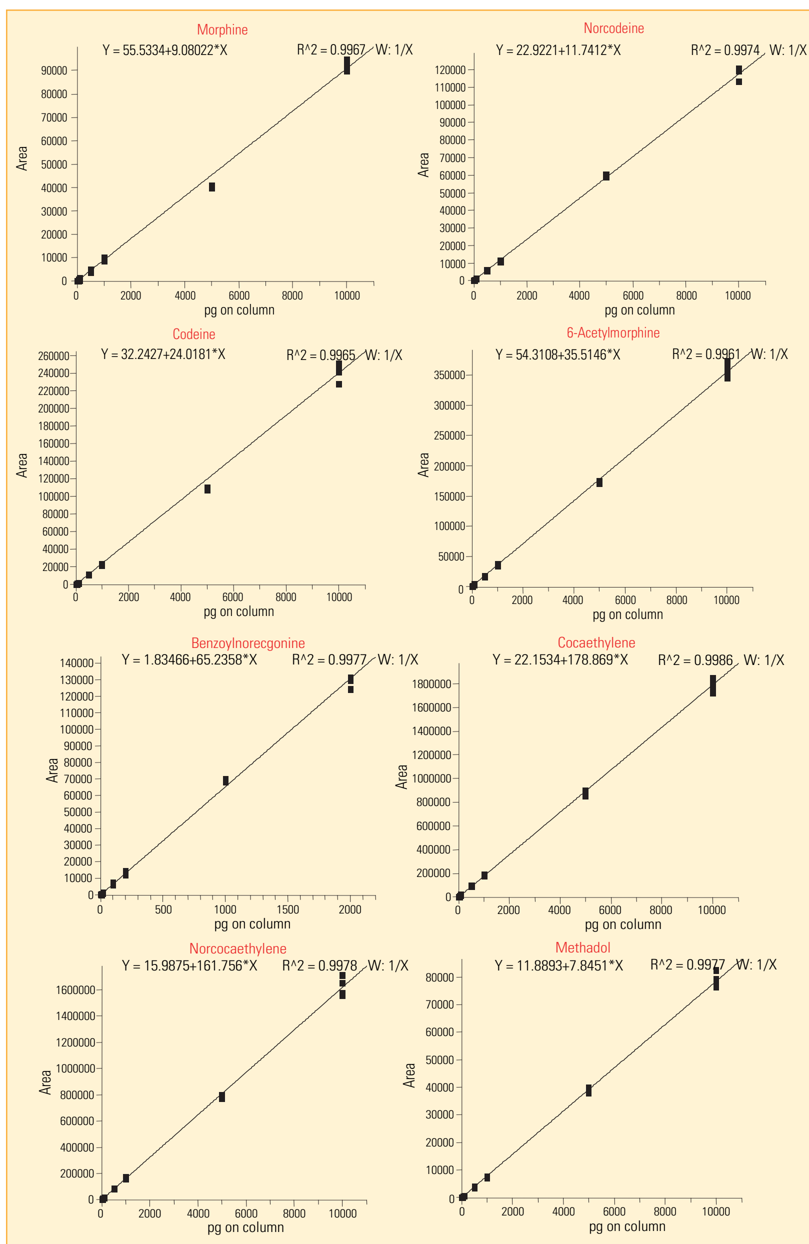

Figure 3: Representative calibration curves for eight drugs in oral fluid

Conclusions

Rigorous simultaneous characterization and quantification of a large number of drugs and their metabolites in a biological matrix can be performed in a fast and robust LC-MS/MS method using an LXQ linear ion trap mass spectrometer. The superior sensitivity and faster cycle time of the LXQ makes this possible in a single chromatographic run, resulting in high throughput analyses. High specificity quantification was done using MS^3 data which can reduce overall chemical noise even if there is a co-eluting isobaric interfering ion. Additional compound confirmation was obtained using Mass Frontier software, where a high match score to a library search provided enhanced confidence in the compound identification.

Acknowledgements

The authors would like to thank Dr. C. Murphy for her assistance and technical discussions. C. Yang and R. Chen are acknowledged for suggestion and advice.

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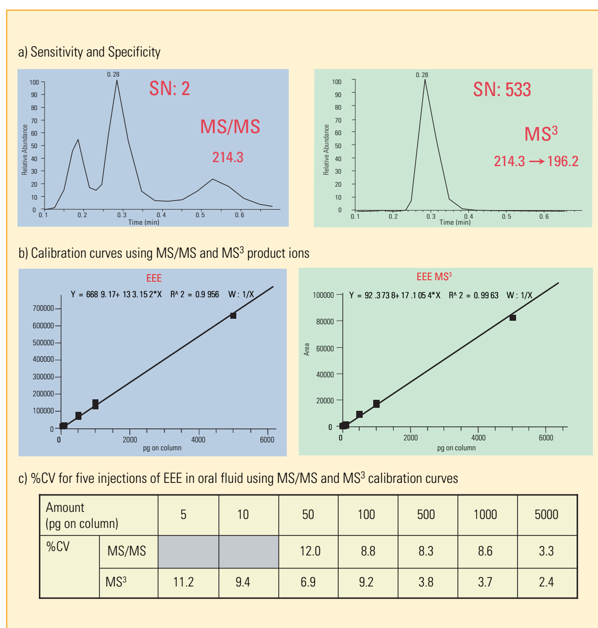
[2] Dams, R.; Murphy, C.M.; Choo, R.E.; Lambert, W.E.; De Leenheer, A.P.; Huestis, M.A. *Anal. Chem.* 2003, 75, 798-804

Compound	LOD (pg)	LOQ (pg)	Linear dynamic range (pg)
EEE	1	5	5-5000
Normorphine	5	10	10-10000
AEM	5	10	10-10000
Morphine	5	10	10-10000
Norcodeine	5	10	10-10000
Codeine	1	5	5-10000
6-Acetylmorphine	1	5	5-10000
m-Hydroxybenzoylecgonine	0.2	1	1-2000
Benzoylnorecgonine	0.2	1	1-2000
Benzoylecgonine	0.5	1	1-10000

Compound	LOD (pg)	LOQ (pg)	Linear dynamic range (pg)
Acetylcodeine	0.5	1	1-10000
Heroin	0.5	1	1-10000
Cocaine	0.5	1	1-10000
Norcocaine	0.5	1	1-10000
Cocaethylene	0.5	1	1-10000
Norcocaethylene	0.5	1	1-10000
Methadol	1	5	1-10000
EDDP	0.5	1	1-10000
Propoxyphene	1	5	5-10000
Methadone	0.5	1	1-10000

Table 2: LOD (limit of detection), LOQ (limit of quantification) and linear dynamic range for analysis of 20 drugs and metabolites in oral fluid using the LXQ linear ion trap mass spectrometer



Amount (pg on column)		5	10	50	100	500	1000	5000
%CV	MS/MS			12.0	8.8	8.3	8.6	3.3
	MS3	11.2	9.4	6.9	9.2	3.8	3.7	2.4

Figure 4: Analysis of EEE (Ecgonine Ethyl Ester) in oral fluid using MS/MS and MS3 spectra product ions

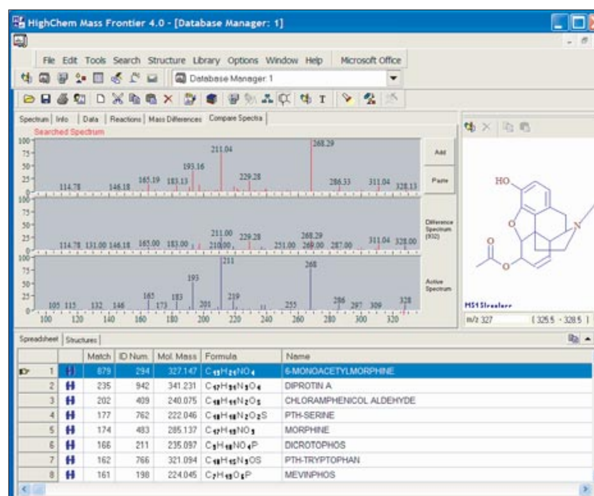


Figure 5: Library search results for 6-acetylmorphine using Mass Frontier software. High match score is highlighted

Compound	QC Sample I (5 injections)				QC Sample II (5 injections)			
	Conc (pg)	Calc. conc. (pg)	% Diff	% RSD	Conc (pg)	Calc. conc. (pg)	% Diff	% RSD
EEE[a]	200.0	183.2	-8.4	4.6	40.0	37.7	-5.7	5.6
Morphine	200.0	189.2	-5.4	7.6	40.0	40.4	1.0	8.9
Norcodeine	200.0	190.8	-4.6	5.5	40.0	40.1	0.3	7.8
6-Acetylmorphine	200.0	182.2	-8.9	8.1	40.0	41.0	2.6	8.4
Cocaethylene	133.3	120.1	-9.7	7.4	26.7	26.3	-1.5	1.6
Norcocaethylene	200.0	190.6	-4.7	5.5	40.0	42.0	4.9	7.4
Methadol	200.0	184.6	-7.7	9.6	40.0	37.6	-6.1	3.8
EDDP	133.3	121.4	-8.9	4.9	26.7	24.8	-7.1	4.4
Propoxyphene	200.0	190.4	-4.7	4.0	40.0	42.4	6.3	5.8
Methadone	133.3	122.5	-9.5	7.2	26.7	24.9	-6.8	3.9

Table 3: Quantification results for the analysis of unknown levels of drugs in oral fluid. [a] *based on MS3 results*

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Screening Drugs and Toxic Compounds with LC-MS/MS: An Alternative to LC-UV for Research Toxicology Labs

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Key Words

- ToxSpec Analyzer
- ToxID software
- LXQ Linear Ion Trap
- Accela UHPLC System

Introduction

Screening for drugs of abuse and other toxic compounds in biological samples has quickly become a routine assay conducted in many research toxicology laboratories. The main challenge is to get rapid and accurate results amidst the generally large number of potential analytes to be identified within complex biological matrices. One of the techniques widely used in this area is high pressure liquid chromatography (HPLC) combined with photo diode array detection (DAD) or ultra-violet (UV) detection. The most popular LC-UV platform has been the Bio-Rad® REMEDi™ HS drug profiling system. When this platform was recently discontinued, a significant technological gap became apparent. Now this gap is rapidly being filled by newer, more effective high pressure liquid chromatography - mass spectrometry (HPLC-MS) technologies.

Here we present the workflow and results obtained by using the Thermo Scientific ToxSpec Analyzer, a new UHPLC-MS system based on ultra-high pressure liquid chromatography and linear ion trap mass spectrometry technology.

Goal

Evaluate an LC-MS/MS method for screening and semi-quantitation of drugs and toxic compounds in serum and urine matrices to determine if this approach can provide an alternative to REMEDi technology for research toxicology.

Experimental

The ToxSpec™ Analyzer combines hardware, software, and screening methodologies designed to significantly simplify and improve the screening assay workflow. LC-MS² data is acquired by using a pre-configured instrument method, and the data is automatically processed, post-acquisition, by Thermo Scientific ToxID automated drug screening software.

The LC-MS screening was performed on Thermo Scientific instrumentation including an LXQ™ linear ion trap mass spectrometer coupled to an Accela™ UHPLC system using a polarity-switching and scan-dependent MS/MS experiment (Figure 1). The MS² spectra generated were processed through ToxID™ software. Using a novel screening algorithm, the software program identifies target analytes through a MS² library search against a large spectral library of known analytes as well as expected retention times. Semi-quantitative data results can also be generated concurrently from the MS² spectral intensity ratios between the target analyte and the corresponding internal standard.

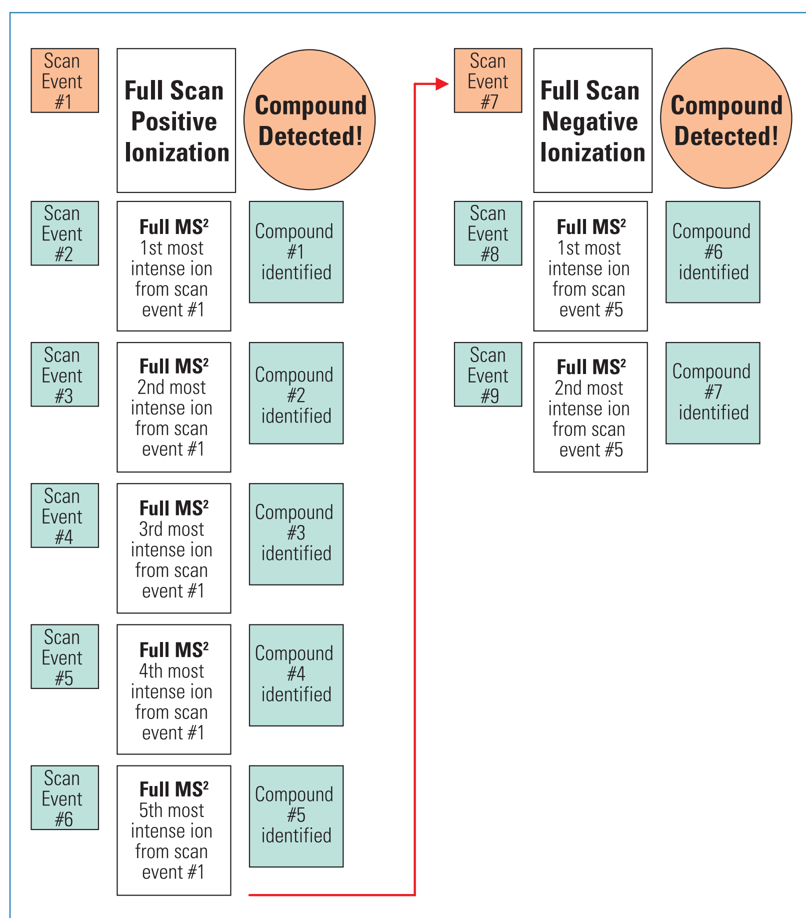

Figure 1: Polarity-switching and scan-dependent MS/MS experiment

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The ToxSpec Analyzer includes a diverse and easily-expandable MS/MS library of 300 compounds that it screens using a single pre-configured method. In our laboratory, we have expanded the library by more than 50 entries to date.

Sample preparation

The extraction procedure was performed by using liquid/liquid extraction (LLE) with Toxi-Tube A® (Varian, les Ulis, France). Details of the procedure are described below.

- Vortex the Toxi-Tube A for 10 seconds.
- Add 1 mL of serum or urine into the Toxi-Tube A.
- Add 200 µL of a solution of internal standard [haloperidol-d4, chlorpromazine-d3, and prazepam-d5 at the following concentrations: 100 ng/mL, 1 µg/mL and 100 ng/mL, respectively, in 70/30 of A/B (A: water containing 10 mM ammonium acetate and 0.1% formic acid; B: acetonitrile containing 0.1% formic acid)].
- Add 5 mL of water.
- Vortex for 10 seconds.
- Mix for 5 minutes.
- Centrifuge for 5 minutes at 2700 rpm.
- Transfer the upper layer to a tube and evaporate to dryness at 40 °C.
- Reconstitute the sample in 200 µL of 70/30 of A/B.

HPLC Conditions

Chromatographic analyses were performed using the Thermo Scientific Accela UHPLC system. The chromatographic conditions were as follows:

Column:	Thermo Scientific Hypersil GOLD PFP 5 µm, 150 x 2.1 mm		
Flow rate:	0.2 mL/min		
Mobile phase:	A: water containing 10 mM ammonium acetate and 0.1% formic acid; B: acetonitrile containing 0.1% formic acid		
Injection volume:	10 µL		
Gradient:	T (min)	A (%)	B (%)
	0.0	95	5
	5.0	55	45
	18.0	30	70
	20.0	5	95
	27.0	5	95
	27.1	95	5
	32.0	95	5

Mass Spectrometry Conditions

MS analysis was carried out on a our LXQ linear ion trap mass spectrometer with an electrospray ionization (ESI) source. The MS conditions were as follows:

Ion polarity:	Polarity-switching scan-dependent experiment
Spray voltage:	5000 V
Sheath gas (N_2) pressure:	30 (arbitrary units)
Auxiliary gas (N_2) pressure:	8 (arbitrary units)
Capillary temperature:	275 °C
Microscan:	1
Wideband Activation™:	Activated
Stepped normalized collision energy:	35% ± 10%

Results and Discussion

More than 150 real laboratory samples (serum and urine) have been analyzed. Table 1 reports some of the data obtained from both the REMEDi HS LC/UV system and the ToxSpec Analyzer UHPLC/MS system. Among the 12 samples reported here, 22 compounds have been identified using both the REMEDi HS and the ToxSpec Analyzer. Notably however, the ToxSpec Analyzer system identified 24 additional compounds that were not detected with the REMEDi HS due in most cases to a lack of sensitivity, specificity, and coelution capability.

The ToxSpec Analyzer also provided a better response for some classes of compounds, like benzodiazepines. With the REMEDi HS system, the retention time for this class of compounds was close to the dead volume of the column. For that reason, the signals that interfered with matrix components were rather difficult to identify. It was also observed that haloperidol (sample #5) and paroxetine (sample #10) gave a much better signal with the ToxSpec Analyzer.

Sample #	Compounds identified using ToxSpec Analyzer	Compounds identified using REMEDi HS
1	Acetaminophen	**Not detected**
	Nortriptyline	**Not detected**
	Amitriptyline	Amitriptyline
	Oxazepam	**Not detected**
2	Nordiazepam	Nordiazepam
	Alprazolam	**Not detected**
	Cyamemazine	Cyamemazine
3	Acetaminophen	**Not detected**
	Nordiazepam	Nordiazepam
	Venlafaxine	Venlafaxine
	Oxazepam	Oxazepam
	Alprazolam	**Not detected**
4	Nordiazepam	**Not detected**
	Diazepam	Diazepam
	Oxazepam	**Not detected**
	Temazepam	**Not detected**
	Levomepromazine	Levomepromazine
	Zopiclone	Zopiclone
5	Oxazepam	**Not detected**
	Clomipramine	Clomipramine
	Quinidine	Quinine
	Haloperidol	**Not detected**
	Clonazepam	**Not detected**
6	Acetaminophen	**Not detected**
	Bisoprolol	Bisoprolol
7	Venlafaxine	Venlafaxine
	Risperidone	**Not detected**
8	Quinine	Quinine
	Hydromorphone	Hydromorphone
	Morphine	Morphine
9	Lidocaine	**Not detected**
	Nortriptyline	**Not detected**
	Mirtazapine	**Not detected**
	Amitriptyline	Amitriptyline
	Cyamemazine	Cyamemazine
	Levomepromazine	Levomepromazine
	Zopiclone	**Not detected**
10	Bromazepam	Bromazepam
	Paroxetine	**Not detected**
11	Sertraline	**Not detected**
	Hydrocortisone	**Not detected**
12	Acetaminophen	**Not detected**
	Alprazolam	Alprazolam
	Prednisolone	**Not detected**
	Hydroxyzine	Hydroxyzine
	Fexofenadine	**Not detected**
TOTAL	46 Molecules	22 molecules

Table 1: List of psychoactive molecules identified in real laboratory samples using the ToxSpec Analyzer compared to the REMEDi HS system

Our aim was to quickly and confidently identify toxic compounds in the samples by spectral library searching while performing a semi-quantification calculation for identified compounds. To perform the semi-quantification, a response factor that correlated the intensity of the MS^2 spectra to a concentration was calculated for each molecule present in the library using internal standards. The semi-quantification result was automatically calculated using ToxID software. An example of the automatically-generated report can be seen in Figure 2. The report includes a list of compounds identified in a real laboratory sample and their respective calculated concentrations.

One important aspect of this method is the ability to reprocess data retrospectively from the MS spectra. The ToxID report is based on MS^2 spectra library searching. This means that if the entry corresponding to the compound is not currently available in the library, ToxID will not be able to identify the analyte. However, as data are acquired in MS mode, it is possible to reprocess the MS trace and check that all major ions have been identified by ToxID. If not, it is then possible to re-inject the sample and perform MS^2 acquisition on specific ions.

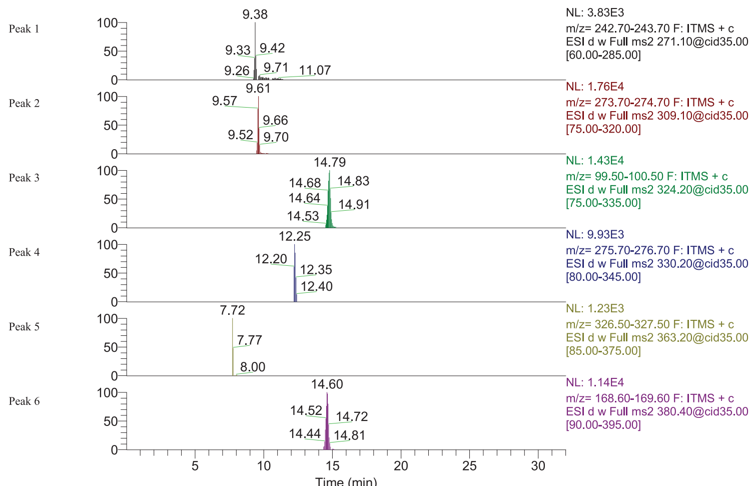
Conclusion

The ToxSpec Analyzer is a good replacement for the REMEDi HS system in research toxicology laboratories because it offers increased sensitivity, greater specificity, and lower cost-per sample analysis.

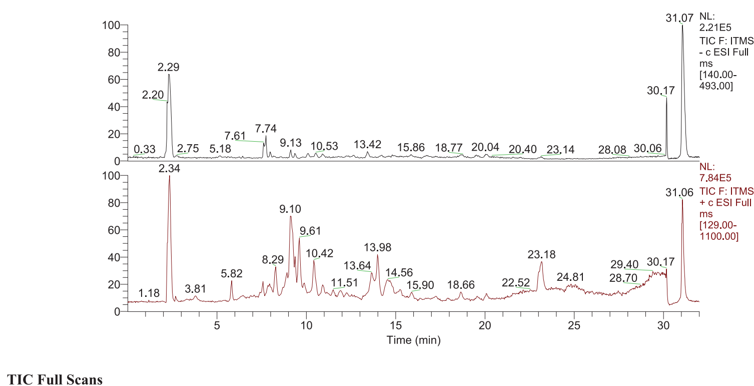
Centre Hospitalier Lyon Sud

Summary Report

Raw File Name: C:\Xcalibur\Validation REMEDI\Data\Real samples\090107\375489.RAW
Config File Name: C:\Xcalibur\Validation REMEDI\CSV fi...\LXQ_GUS_config_30min_TOXID Semi-quanti.csv
Sample Name: Laboratory: Laboratoire de Toxicologie
Acquistion Start Time: 1/7/2009 1:23:18 PM
Screening Conditions: Based on Full MS2 scans. m/z window (amu): 0.50, RT window (min): 2.00, MS2 Search libraries: Tox_Library_LXQ,Use full MS scan to confirm.



Peak Number	Compound Name	Code	SI	RSI	m/z	Expected RT	Actual RT	Concentration mg/l	Library Name
1	Nordiazepam	p	700	708	271.10	8.60	9.38	1.05	Tox_Library_LXQ
2	Alprazolam	p	811	814	309.10	9.10	9.61	0.84	Tox_Library_LXQ
3	Cyamemazine	p	818	819	324.20	15.00	14.79	0.25	Tox_Library_LXQ
4	Prazepam-D5	i	890	894	330.20	11.70	12.25	0.87	Tox_Library_LXQ
5	Hydrocortisone	p	863	865	363.20	7.50	7.72	0.11	Tox_Library_LXQ
6	Haloperidol-D4	i	845	861	380.40	14.90	14.60	1.00	Tox_Library_LXQ



TIC Full Scans

Figure 2: ToxID report – short summary style

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Forensic Toxicology Poster Notes

PN64316: Direct Analysis Using Paper-Spray Mass Spectrometry: Method Development for the Rapid Screening of Drugs of Abuse for Forensic Toxicology

PN64226: Confirmatory Determination of Buprenorphine and Norbuprenorphine in Urine Using A High-Throughput LC-HRAM-MS/MS Forensic Methodology

PN64230: Evaluating New ToxFinder Data Processing Software in Targeted Screening Applications Implemented on Orbitrap Ultra High Resolution Mass Spectrometers and Triple Quadrupole Mass Spectrometers

PN64231: Quantitation of 47 Forensic Compounds in Urine by HPLC-MS

PN64227: Multiplexing Multiple Methods to Maximize Workflow Efficiency in LC-MS Laboratories

PN64075: Verification of an LC-MS/MS Research Method for 14 Anti Depressants Utilizing Dried Blood Spots

PN64077: Verification of an LC-MS/MS Forensic Method for 19 Opioids, Opiates, and Their Metabolites in Human Urine without Hydrolysis

MSACL 14 PN: Advantages of Ultra-High-Resolution Q Exactive Mass Spectrometer in Analysis of Unlimited Number of Compounds in Urine Quantitative Screening Application for Forensics

ASMS 2013 PN: Quantitation of Seven Designer Cathinones in Urine Using Q Exactive Mass Spectrometer

ASMS 2013 PN: High-Resolution, Accurate-Mass Forensic Toxicology Screening in Blood Samples Using a Q Exactive Mass Spectrometer

ASMS 2013 PN: Verification of the Simultaneous Analysis of Heroin Addiction Treatment Compounds Using LC/MS/MS with a New Prelude SPLC™ System

ASMS 2013 PN: Quantitative Confirmatory Analysis of the NIDA 5 Panel Using Prelude SPLC System and TSQ Quantum Ultra MS

PN63786: Quantitative Analysis of THC and Main Metabolites in Whole Blood Using Tandem Mass Spectrometry and Automated Online Sample Preparation

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Direct Analysis using Paper-Spray Mass Spectrometry: Method Development for the Rapid Screening of Drugs of Abuse for Forensic Toxicology

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[2]Department of Chemistry, Indiana University-Purdue University Indianapolis, IN

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Overview

Purpose: Method development for the rapid and semi-quantitative screening of drugs of abuse in forensic toxicology using paper spray mass spectrometry.

Methods: Bovine blood spiked with common drugs of abuse and analyzed as dried blood spots by paper spray ionization/Orbitrap mass spectrometry. High resolution and accurate mass used in full MS, MS^2 and All Ion Fragmentation experiments for the identification and confirmation of drugs from dried blood spot samples. Thermo Scientific™ TraceFinder™ 3.0 software used for data analysis.

Results: Able to identify six drugs of abuse from dried blood spots at a 100 ng/mL level with outstanding signal to noise. Limit of detection from dried blood spots with this technique is 1-10 ng/mL, compound dependent. Paper spray is easy to use, requires no sample preparation and no prior chromatography, making for a quick technique with the potential to identify compounds in seconds. The Thermo Scientific™ Orbitrap™ Exactive™ family of mass spectrometers are ideally suited for coupling to paper spray ionization.

Introduction

Paper spray is a direct ionization technique that simplifies the mass spectrometric analysis of dried blood spots (DBS). Paper-spray technology is therefore attractive for forensic toxicology screening for drugs of abuse. The sample collection and storage of DBS in a simple paper cassette make shipment of samples to the forensic toxicology lab safe and convenient. Both qualitative and quantitative analysis of small molecules from complex matrices such as blood or other biological fluids is possible without time consuming sample preparation and chromatography.

Quantitation of DBS samples with paper-spray MS is fairly well established even though a commercial product is not yet available (1). While previous work used a Thermo Scientific triple quadrupole mass spectrometer and monitored specific MS/MS transitions, full-MS instruments with Orbitrap analyzers are ideally suited as rapid screening tools. Orbitrap analyzers provide high resolution, accurate mass (HR/AM) analysis for high confidence identification, allow for unlimited number of analytes in the method and retrospective data analysis is possible because a full MS spectrum is recorded in addition to All Ion Fragmentation (AIF) or Data Dependent (DD) MS/MS.

In this work, the ability of paper spray coupled to a very sensitive and fast Orbitrap analyzer is explored for its potential as a forensic toxicology screening tool.

Methods

Sample Preparation

- Mixtures of drugs (Cerilliant, TX) were spiked in blood (bovine blood, Lampire Biologicals, New Jersey) stabilized with K2-EDTA. Blood sample integrity maintained by not exceeding 5% of solvent in blood (v/v).
- Twelve microliters of spiked blood sample were loaded to paper cartridges, dried under a nitrogen gas flow for 20 min and loaded into stackers that hold up to 40 cassettes.
- Solvent is automatically dispensed to the DBS before analysis and an applied high voltage (3-5 kV) induces electrospray from the sharp tip of the paper (Figure 1).
- The extraction solvent used in this work is 95/5 (v/v) methanol/water with 100 ppm acetic acid (pH 4.5).

Mass Spectrometry

- The paper-spray source was coupled to either a Thermo Scientific™ Exactive Plus™ or a Thermo Scientific™ Q Exactive™ Orbitrap mass spectrometer.
- An automated experiment for drug screening consisted of 30 sec data collection, switching between full scan and AIF experiments (Exactive Plus MS) or full scan and Data Dependent Higher Collision Dissociation (HCD) MS/MS (Q Exactive MS).
- For maximum specificity and sensitivity, both full scan and fragmentation data were acquired at 140,000 resolving power (FWHM at m/z 200). Normalized collision energy was 40 eV.
- All data acquisition used the Thermo Scientific™ Xcalibur™ sequences and contact closure trigger from the paper spray source.

Data Analysis

- Thermo Scientific™ QualBrowser™ software from the Xcalibur platform was used for spectra visualization. TraceFinder 3.0 software was used for the automated identification and confirmation in the targeted screening of drugs.

FIGURE 1. Prototype paper spray ion source (Prosolia, Inc., IN) showing, clockwise from top left: paper spray ion source, mechanism for dispensing solvent to the sample, paper cassette indicating sample deposition and DBS-spotted paper cassette electrospraying into mass spectrometer inlet.

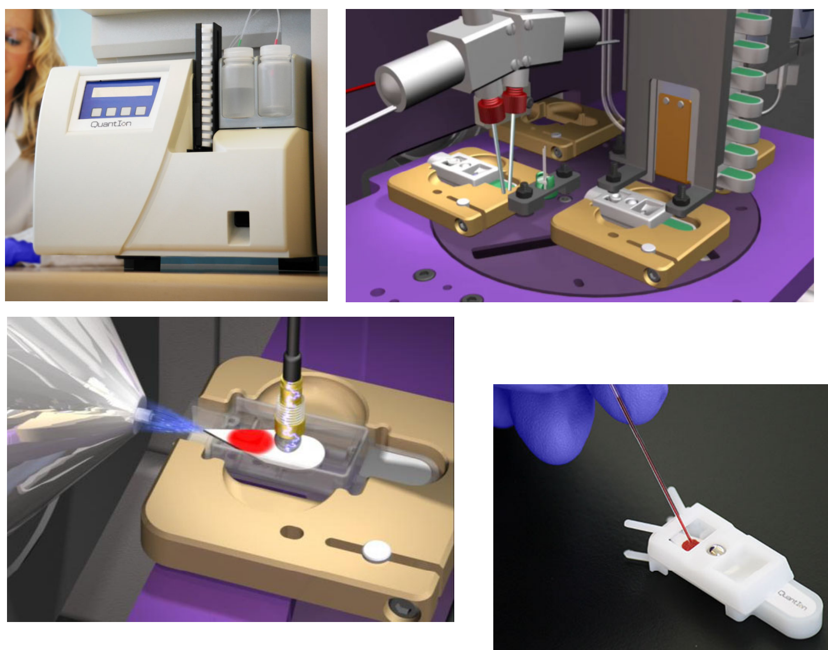
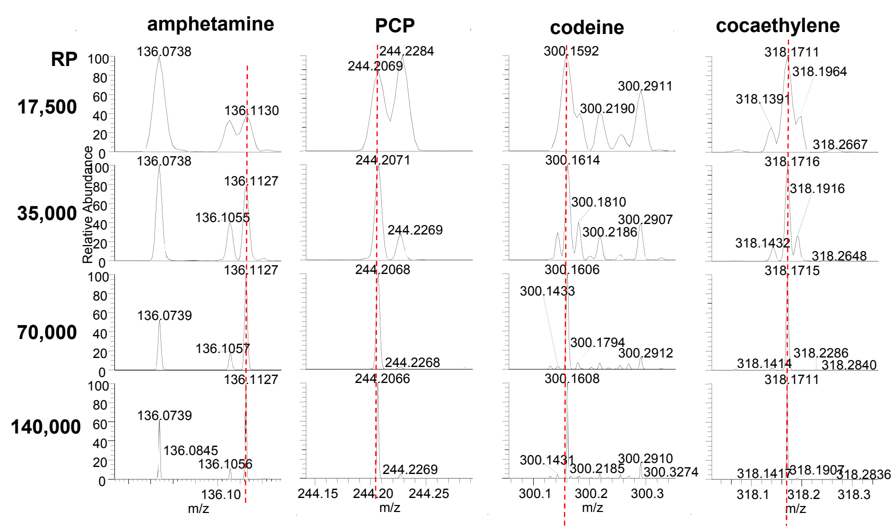


FIGURE 2. Full scan MS experiments - highest resolving powers, *e.g.*, 70,000 and 140,000 (FWHM at *m/z* 200), are required for the identification of drugs from DBS due to matrix interference. Bovine blood spiked with six drugs, four drugs shown below. Resolving power from 17,500, 35,000, 70,000 and 140,000 top to bottom. The [M+H]$^+$ ion is highlighted by a red line. Mass accuracies 1-2 ppm.



Results

Screening for drugs of abuse: resolving power, accurate mass for compound identification

- Figure 2 shows that high and ultrahigh resolving powers (70,000 and 140,000 FWHM at *m/z* 200) are required when evaluating samples from complex matrices with no sample preparation and no prior chromatographic separation. Mass accuracies 1-2 ppm at the higher resolving powers (70,000 and 140,000, FWHM at m/z 200).
- Results from TraceFinder software, which is effectively used for targeted or unknown screening analysis, are neatly summarized in Figure 3. All six drugs are positively identified from a dry blood spot sample.

Screening for drugs of abuse at various concentrations

- A drug mixture of six compounds was analyzed at 100, 500, 1000 and 2500 ng/mL for forensic toxicology screening. Amphetamine, methamphetamine, cocaine, cocaethylene, codeine and PCP are shown in this work.
- This group of samples were detected by full scan MS down to 100 ng/mL levels (Figure 4) (140,000 resolving power; FWHM at m/z 200).

Fragmentation and isotopic pattern matching for compound confirmation

- Accurate mass m/z values were used for identification of screened drugs. Isotopic pattern matching and two fragments from the AIF experiment were used for drug confirmation (TraceFinder table Fig. 3). Alternatively, DD MS/MS from a Q Exactive mass spectrometer can be used.
- Figure 5 shows accurate mass fragmentation spectra by targeted DD MS/MS for a DBS sample containing a mixture of 6 drugs. DD MS/MS is acquired at ultra high resolution for enhanced signal to noise. Please note that at the higher resolution, the signal to noise is exceptional thus allowing much lower limits of detection than demonstrated.

Quantitation

- Amitriptyline-spiked in blood (10–5,000 ng/mL) yielded limits of quantitation (LOQ) of 25 ng/mL using amitriptyline-d_3 as internal standard (Figure 6).
- Variability in terms of %RSD (Std Dev/Mean*100) is between <1 to 16% for drug in blood. Figure 6 displays amitriptyline data for dried blood spots.

FIGURE 3. TraceFinder 3.0 software results shown below. Data processed in targeted screening analysis mode. All analytes in the mix are positively identified by exact m/z values and confirmed by isotopic pattern and the presence of two fragments from the AIF experiment (see Table).

Data collected with the Exactive Plus mass spectrometer.

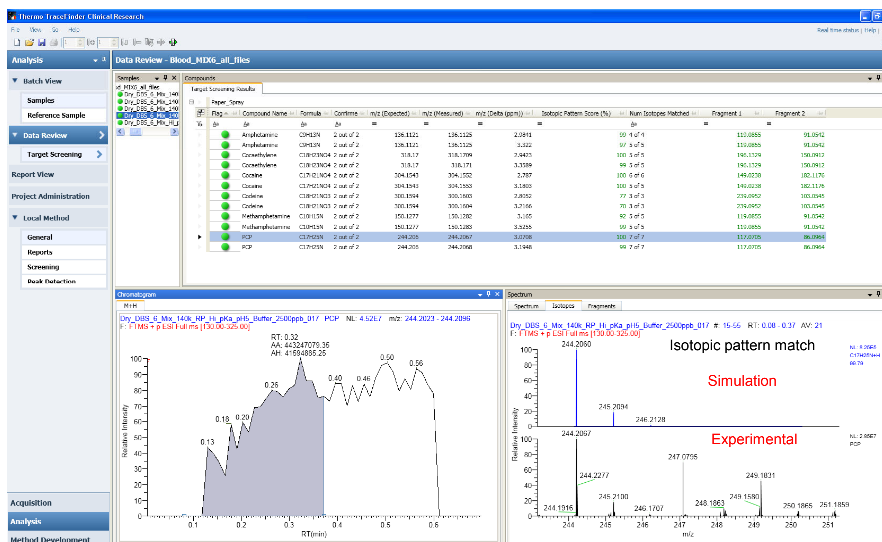


FIGURE 4. Accurate mass (3-4 ppm) MS spectra at 140,000 resolving power (FWHM at m/z 200) showing drugs detected down to 100 ng/mL. Sample contained six drugs analyzed from DBS.

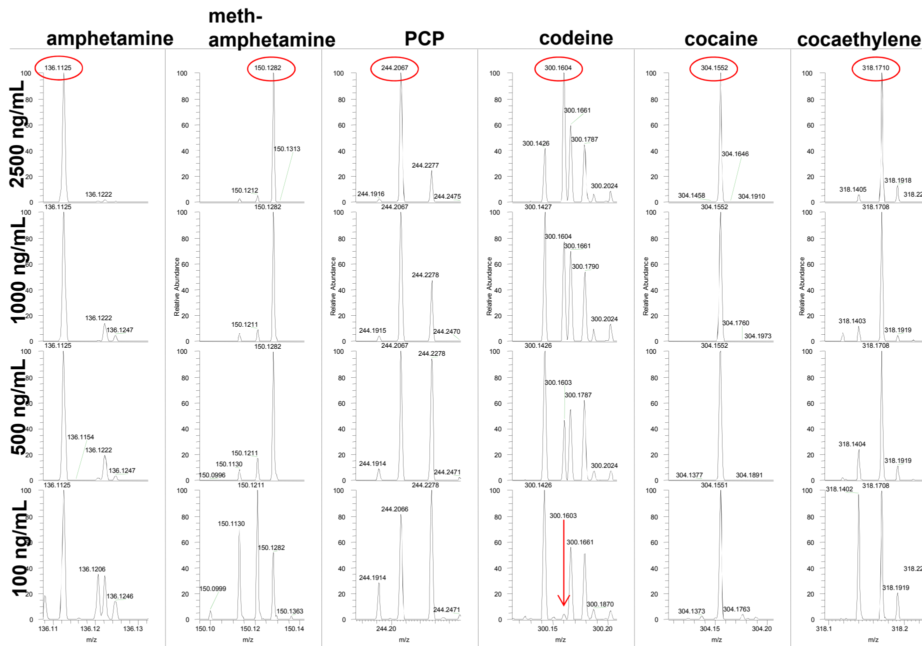


FIGURE 5. DD MS/MS fragmentation at the highest resolving power of 140,000 (FWHM at m/z 200) in the Q Exactive allows for sensitive detection. An enhanced signal to noise ratio (as compared to MS/MS at 17,500 resolving power, data not shown) is observed. Accurate mass on both precursor and fragments (4-5 ppm and 1-3 ppm, respectively) are used for the identification of compounds in screening applications.

Sample: mixture of six drugs analyzed from DBS, four shown below. Concentrations noted in each panel.

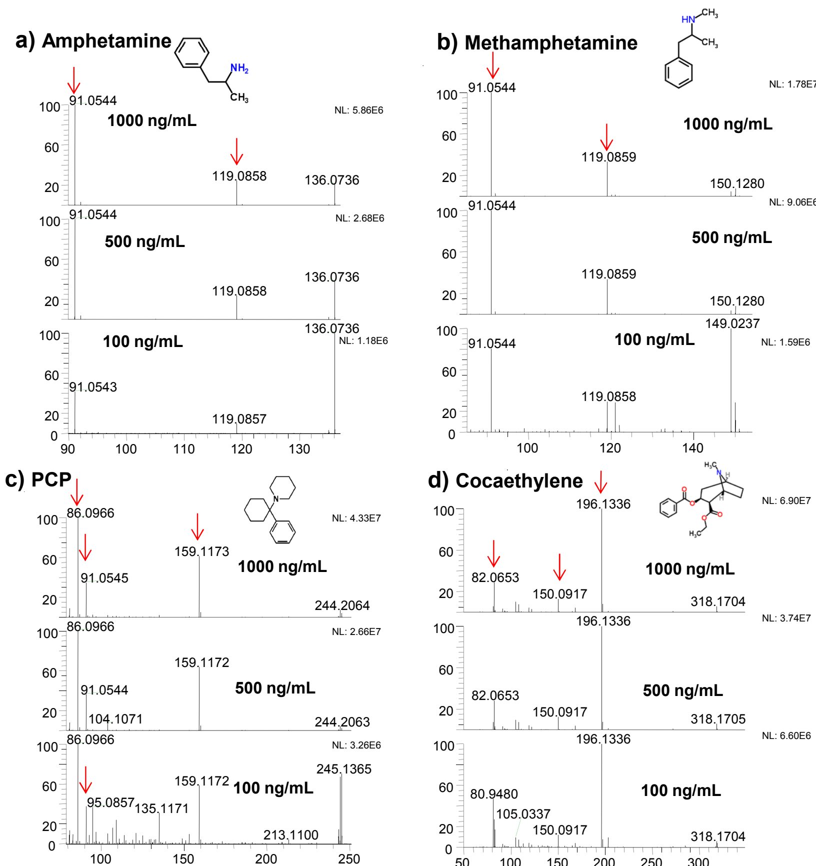


FIGURE 6a. Full scan MS spectra for the $[M+H]^+$ ion of amitriptyline at various concentrations from DBS samples. Acquired at 70,000 resolving power.

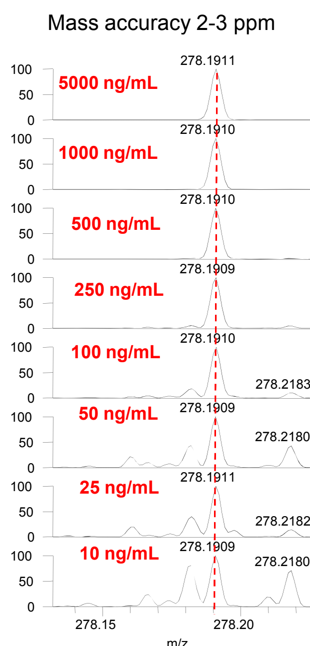
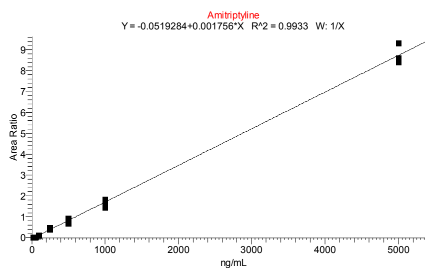


FIGURE 6b. Quantitative results for amitriptyline normalized by internal standard from DBS samples. Calibration curve and %RSD variability (n=3) shown.



Level (ng/mL)	% RSD
5000	5.4
1000	11.9
500	15.6
250	9.7
50	13.2
25	0.3

Conclusion

- We have shown an easy to use technique (no sample preparation, no chromatography) that shows extraordinary potential for the semi-quantitative screening of drugs of abuse in forensic toxicology.
- Any combination of user required experiments, *e.g.*, MS, AIF and Data Dependent MS/MS, are allowed for the best hit confirmation in a single experiment.
- Accurate mass fragments (from AIF or DD MS/MS experiment) and isotopic pattern matching are required to confirm drugs identified solely by accurate mass (Fig. 3).
- We have demonstrated feasibility for rapid blood analysis for intoxication cases where expected concentrations are high (≥100 ng/mL, Fig. 4). DD MS^2 data (Fig. 5) indicates lower levels can be achieved and this is part of ongoing investigations.
- High resolution and accurate mass are crucial techniques for analyzing complex samples by MS and nicely complement the paper spray technique in the screening of drugs from dried blood spots.
- Data collected in this screening application allows for retrospective analysis as a full scan MS event is always acquired.
- The paper spray technique coupled with automated data processing using TraceFinder 3.0 software provides a complete solution for drug screening in forensic toxicology.

References

1. Manicke, N.; Yang, Q.; Wang, H.; Oradu, S.; Ouyang, Z.; Cooks, R.G. Assessment of Paper Spray Ionization for Quantitation of Pharmaceuticals in Blood Spots. *IJMS* **2011**, *300*, 123-129.

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Confirmatory Determination of Buprenorphine and Norbuprenorphine in Urine Using A High-Throughput LC-HRAM-MS/MS Forensic Methodology

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Overview

Purpose: To demonstrate that the use of a two-channel UHPLC system with a high resolution accurate mass (HRAM) MS equipped with segmented quadrupole allowed for high specificity, sensitivity, and sample throughput.

Methods: The LC-HRAM-MS/MS method was validated by injection of 8-point calibration curves, QCs, and donor samples in staggered fashion across two LC channels and three days.

Results: This methodology resulted in achieving a sample cycle time of 1.07 minutes and dynamic linearity range of 5 – 2000ng/mL.

Introduction

Forensic toxicology labs monitor levels of buprenorphine (BUP) and its major active metabolite, norbuprenorphine (NBUP), in urine for law enforcement purposes. LC-MS/MS is currently the most popular method used for confirmation. Here we demonstrate that the use of a two-channel UHPLC system with a high resolution accurate mass (HRAM) MS equipped with segmented quadrupole (Figure 1 – system view) allowed for highest specificity, sensitivity, and sample throughput as compared to standard LC-MS/MS.

Methods

Sample Preparation

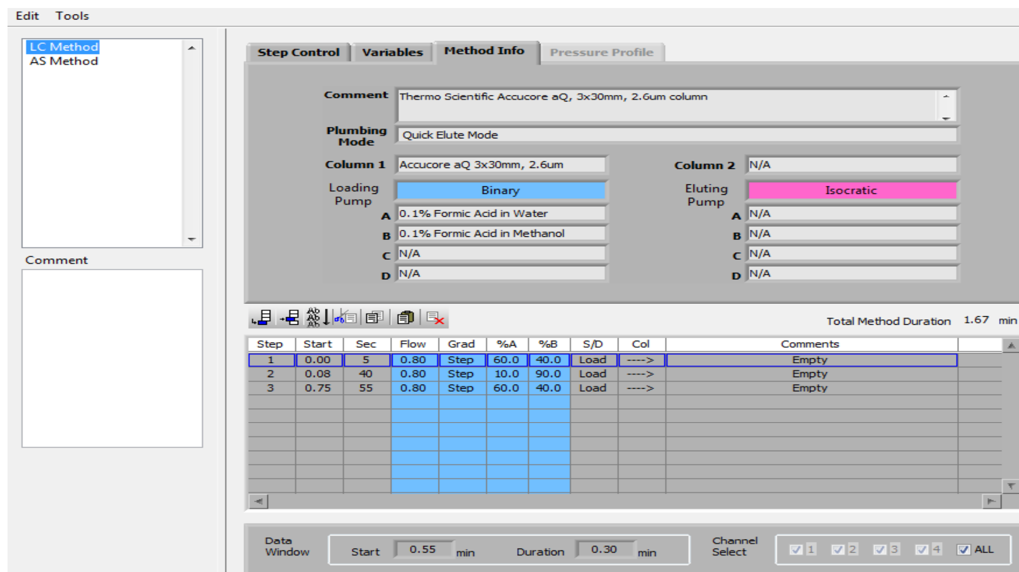
A synthetic urine (Surine™, DYNA-TEK Industries, Lenexa, KS) was used for calibrator and QC preparations. Six drug-free donor urines and an HPLC-grade water sample (in triplicate) *were each prepared* at 100 ng/mL and used for matrix study. BUP, NBUP, their deuterated analogs, BUP-d4 and NBUP-d3, and NBUP glucuronide (NBUP-Gluc) were purchased from Cerilliant Corp., Round Rock, TX. A beta-glucuronidase (Type L-II from *Patella vulgata,* Sigma-Aldrich Corp., St. Louis, MO) was used for hydrolysis of glucuronides. A sample containing the NBUP-Gluc was prepared at 500ng/mL in Surine™ to monitor as a hydrolysis control. Sample prep: To 200uL of sample (calibrant or QC) was added 100uL of the enzyme prepared in 1M Acetate buffer, pH4, and 10uL of an internal standard mix. This was then hydrolyzed @ 60^{0}C for 2 hours. The reaction was stopped with addition of 200uL of methanol and cooled with refrigeration for 10 minutes. Centrifugation was subsequently performed @ 10k rpm for 10 minutes and the supernatant diluted 10X with water. LC-MS/MS analysis was performed with 75-uL injections volumes.

FIGURE 1. Transcend II LX-2 with Q Exactive Plus Hybrid Quadrupole-Orbitrap High-Throughput LC-MS/MS system.

Liquid Chromatography

A Thermo Scientific™ Transcend™ II LX-2 UHPLC system was configured to evaluate performance of the LC method. This 1.67-minute method and its details are seen in a screen shot of the Thermo Scientific™ Aria ™ MX 2.1 LC control software (Figure 2). An 8-point calibration curve, QCs, and donor samples were injected in staggered fashion across the two LC channels of this multi-channel LC system. This workflow was repeated over each of three days.

FIGURE 2. Aria MX LC method view with details – column, mobile phases, and gradient..



Mass Spectrometry

MS/MS analysis was performed using a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap MS operating in a targeted-MS2 (t-MS2) heated electrospray ionization (HESI) mode at a resolution of 17,500 and positive ionization mode. Table 1 shows the exact mass inclusion list settings for BUP, NBUP, and their respective IS used to collect t-MS2 data. Tables 2 & 3 highlight the MS/MS HESI source and method parameters, respectively, used.

Table 1. Q Exactive Plus t-MS2 exact mass inclusion list of settings.

Analyte	Mass [m/z]	Formula	Species
BUP	468.31084	C29H41NO4	+H
NBUP	414.26389	C25H35NO4	+H
BUP-d4	472.33594	C29H37NO4	+H
NBUP-d3	417.28272	C25H32D3NO4	+H

Table 2. Q Exactive Plus HESI II source parameters used.

HESI II Source Parameters	
Sheath Gas*	60
Aux Gas*	15
Sweep Gas*	0
Spray Voltage	3.5 kV
Capillary Temp.	350^0C
Aux Gas Heater Temp.	450^0C
S-Lens RF Level	70.0

*Arbitrary Units

Table 3. Q Exactive Plus Targeted-MS2 method properties used.

Targeted-MS2 Properties	
Polarity	Positive
Runtime	18 secs
Resolution	17,500
AGC Target	$1e^5$
Max. IT	60ms
MSX count	1
Isolation window	2.0 m/z
Isolation offset	0.5 m/z
NCE	50

Data Analysis

Two major t-MS2 transitions per analyte were extracted from the MS raw files and data processing performed using Thermo Scientific™ TraceFinder ™ Clinical Research version 3.1 quantitation software. Exact mass quantifier and qualifier ions are as noted in Table 4. Ion ratios of these were used for confirmation.

Table 4. Extracted t-MS2 transitions used for quantitation and confirmation.

Analyte, [M+H]+ Formula	Parent Exact Mass (m/z)	Quantifier Ion Exact Mass (m/z)	Qualifier Ion Exact Mass (m/z)
BUP, $C_{29}H_{42}NO_4$	468.31084	396.21693	187.07536
NBUP, $C_{25}H_{36}NO_4$	414.26389	187.07536	211.07536
BUP-d4, $C_{29}H_{38}{}^2H_4NO_4$	472.33594	400.24204	187.07536
NBUP-d3, $C_{25}H_{33}{}^2H_3NO_4$	417.28272	187.07536	211.07536

Results

Running this LC method across multi-channels using one MS resulted in a sample cycle time of 1.07 minutes. The method was linear from 5 to 2000 ng/mL for both BUP and NBUP with correlation of coefficients (R2) > 0.996 (1/x weighting; origin ignored) and a limit of quantitation (LOQ) of 5 ng/mL. Figure 3 shows a representative extracted ion chromatogram (XIC) for the analytes and their respective IS with a mass accuracy of 5 ppm at their LOQ (5 ng/mL). Table 5 contains a 3-day summary of precision and accuracy in the QCs across two LC channels.

Matrix Effects

The absolute recoveries of BUP and NBUP were tested in urine obtained from six different drug-free human donors as compared to the average in the water samples (n=3). Absolute recoveries of BUP in these samples ranged from 30.0% to 84.1% and for NBUP from 28.9% to 57.5% at 100 ng/mL. The relative recoveries of BUP and NBUP with their respective IS in these samples ranged from 90.2% to 111%.

Hydrolysis Control

As a result of enzymatic hydrolysis demonstrated here between 98.7% and 113% of expected NBUP was recovered from its glucuronide standard in the control after hydrolysis and LC/MS/MS analysis based on the ratio of their molecular weights (0.701).

Figure 3. Representative chromatogram of quan and qual XICs for NBUP and its IS at left and for BUP and its IS at right at LOQ (5 ng/mL).

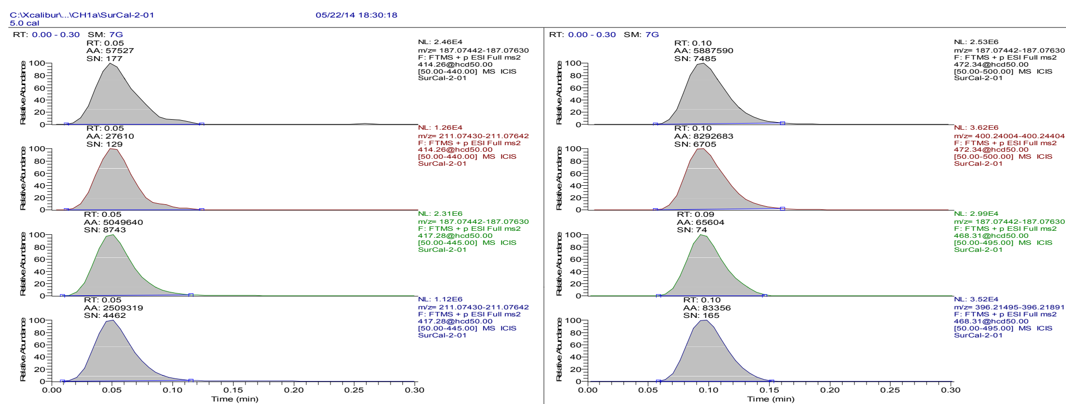


Table 5. Inter-Assay (3-days) Precision (%CV, n=5) and Accuracy (%Diff, n=5) obtained by staggered injection across Channel 1 (CH1) and Channel 2 (CH2) of a Multi-Channel LC.

Analyte	QC Level (ng/mL)	CH1 %CV	CH1 %Diff	CH2 %CV	CH2 %Diff
BUP	10	8.7	7.4	9.7	6.7
	800	3.3	3.3	4.2	4.4
	1600	2.9	2.8	3.9	3.8
NBUP	10	9.5	7.3	10.8	7.8
	800	4.7	4.8	2.7	5.1
	1600	4.6	3.5	3.4	5.6

Conclusions

- Use of a Transcend II UHPLC system with high-throughput, multi-channeling LC configured with a Q Exactive Plus Hybrid Quadrupole-Orbitrap MS using HRAM and a Targeted-MS2 method resulted in a fast, sensitive, and highly specific forensic assay in urine for BUP and NBUP.
- The excellent relative recoveries of BUP and NBUP with their respective IS in the donor urine samples demonstrates that use of appropriate IS can overcome matrix interferences.
- It is expected that the two-hour beta-glucuronidase hydrolysis as described herein would be sufficient for analysis of donor urine containing these exogenous drugs.
- Future work will focus on ways of eliminating matrix interferences which caused absolute analyte recoveries in donor samples to be significantly reduced.

References

1. Yuan et al., Quantification of Buprenorphine, Norbuprenorphine and 6-Monoacetylmorphine in Urine by Liquid Chromatography-Tandem Mass Spectrometry, *J Chromat Separation Techniq* **2013**, 4:3.

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Evaluating New ToxFinder Data Processing Software in Targeted Screening Applications Implemented on Orbitrap Ultra High Resolution Mass Spectrometers and Triple Quadrupole Mass Spectrometers

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Overview

Purpose: To evaluate new data processing software supporting two forensic screening applications on an ultra high resolution mass spectrometer and one screening application on triple quadrupole mass spectrometer.

Methods: Urine samples were spiked with specified concentrations of 300 analytes in groups of 50 and analyzed using screening applications. Data were processed with Thermo Scientific™ ToxFinder™ software and evaluated for presence of analytes and semi-quantitative values.

Results: All tested screening workflows returned 100% accurate results. Software was easy to use. Data review window provided comprehensive information and at the same time allowed for fast data review.

Introduction

Targeted screening applications are commonly used in forensic toxicology laboratories. Screening applications utilize all kinds of mass spectrometers, each with advantages and limitations. User friendly software that fully utilizes screening data across instrument types, simplifies data interpretation and provides user specific data output is critical component.

Methods

Sample preparation

Urine samples were spiked with mixture of internal standards and diluted 30 fold with water.

Liquid chromatography

•Column: Thermo Scientific™ Hypersil™ GOLD PFP 5 µm, 50 x 2.1 mm

•Mobile phase:

•A: 10 mM NH4Ac in DI water

•B: 10 mM NH4Ac in MeOH

•LC Gradient

Step	Start (min)	Time (s)	Flow (mL/min)	%A	%B
1	0.00	30	0.5	100.0	0
2	0.5	30	0.5	80	20
3	1.0	180	0.5	30	70
4	3	120	0.5	0	100
5	5	6	1.0	0	100
6	5.01	114	1	-	100.0
7	7.0	6	1	100	0
8	7.01	54	1	100	0

Mass Spectrometry

Method #1 on Thermo Scientific™ Q Exactive™ MS (Full Scan AIF)

•4 scan events: Full Scan and All-Ion-Fragmentation (AIF) in both positive and negative ionization modes.
•Compounds are identified base on accurate m/z, retention time, fragments in AIF spectrum and isotopic pattern.

Method #2 on Q Exactive MS (Full Scan Data Dependent)

•4 scan events: 2 in positive and 2 in negative ionization mode
•Data dependent MS2 spectrum (ddMS2).
•Compounds are identified base on accurate m/z, retention time, MS2 spectra and isotopic pattern.

Method #3 on Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer (SRM)

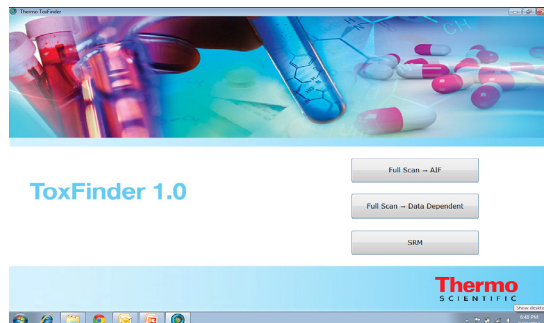
•2 SRM transitions for analytes and 1 SRM transition for internal standards.
•Compounds are identified base on SRM transitions, retention time and ion ratio.

Data Analysis

Data collected with each of the above screening applications were processed using ToxFinder software (Figure 1).

The software was developed with special attention to provide intuitive workflow and ease of use. Software allows analysis of data collected on both the Q Exactive benchtop Orbitrap MS and triple quadrupole MS instruments.

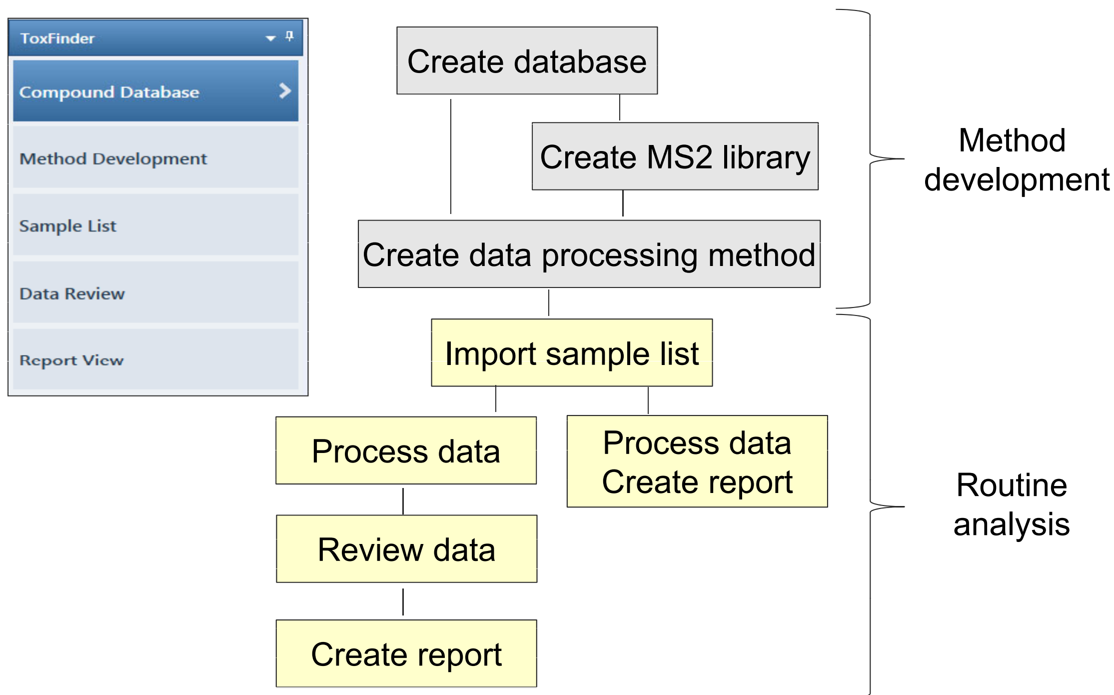
FIGURE 1. ToxFinder home page allowing user to select which screening application to use.



Data processing workflow

ToxFinder software provides rapid method set up and intuitive data processing workflow (Figure 2). The workflow begins with database creation followed by simple method set up. Samples processing can be followed by immediate report generation or by data review and then report creation.

FIGURE 2. ToxFinder data processing workflow



The ToxFinder compound database is a spreadsheet-like worksheet allowing fast and easy data management. It can be exported to and imported from a *.csv file. The database stores information required for both compound identification and acceptance criteria. Figure 3 shows an example of a database for a Q Exactive screening application collecting full scan and AIF spectra. Compounds are identified based on accurate m/z, retention time, compound specified fragments in AIF spectra, isotopic pattern and, optionally, MS2 spectra library search, if applicable.

FIGURE 3. ToxFinder database for Q Exactive screening application collecting full scan and AIF spectra.

Compound Database - FullScan_AIF_Database [Full Scan - AIF]

	Compound Name	Compound Type	Molecular Formula	Extracted m/z	Polarity	Adduct	Charge State	RT (min)	RT Window (sec)	Relative RT	Internal Standard
1	13C6-Zonisamide_pc	Internal Standa	[13]C6C2H8N2O3S	219.05297	Positive	Hydrogen	1	2.17	30.00		
2	6-MAM	Analyte	C19H21NO4	328.15433	Positive	Hydrogen	1	3.03	30.00	1.10989011	Tolbutamide_pos
3	7-Aminoclonazepam	Analyte	C15H12ClN3O	286.07417	Positive	Hydrogen	1	2.34	30.00	1.07834101	13C6-Zonisamide
4	a-Hydroxyalprazolam	Analyte	C17H13ClN4O	325.08507	Positive	Hydrogen	1	3.13	30.00	1.14652014	Tolbutamide_pos
5	Alprazolam	Analyte	C17H13ClN4	309.09015	Positive	Hydrogen	1	3.35	30.00	1.22710622	Tolbutamide_pos

more columns

_AIF_Database [Full Scan - AIF]

	Compound Name	Internal Standard	Area Threshold	Height Threshold	Isotope Score	Fragment 1	Fragment 2	Fragment 3	Library SI Score	Library RSI Score
1	13C6-Zonisamide_pc		5000	5000	70	156.0749	100.0662	109.0665	500	700
2	6-MAM	Tolbutamide_pos	5000000	5000	70	165.0695	211.0751	58.066	500	700
3	7-Aminoclonazepam	13C6-Zonisamide_pc	5000	5000	70	121.0761	222.1022	250.0971	500	700
4	a-Hydroxyalprazolam	Tolbutamide_pos	5000	5000	70	297.0657	205.0757	216.0805	500	700
5	Alprazolam	Tolbutamide_pos	5000	5000	70	281.0707	205.0756	274.1206	500	700

more columns

_AIF_Database [Full Scan - AIF]

	Compound Name	ment 3	Library SI Score	Library RSI Score	Concentration Threshold	Internal Standard Concentration	Concentration Area Response Factor	Concentration Height Response Factor	Concentration Units
1	13C6-Zonisamide_pc	0665	500	700	1000	250			ng/mL
2	6-MAM	66	500	700	1000		113.7	137.7	ng/mL
3	7-Aminoclonazepam	0971	500	700	1000		57.1	63	ng/mL
4	a-Hydroxyalprazolam	0805	500	700	1000		445	436.7	ng/mL
5	Alprazolam	1206	500	700	1000		146.8	150.7	ng/mL

ToxFinder MS2 spectra library

ToxFinder uses NIST application to store MS2 spectra.

ToxFinder Method Editor

Method editor is screening application specific and consists of 3 pages :

1. Method Settings to specify compound identification parameters
2. Peak Detection to specify peak detection parameters
3. Reports to select reports

ToxFinder processing method provides semi-quantitative results which can be calculated based either on a single point calibrator using analyte/internal standard peaks ratio (area or height) or on internal standard amount in the sample. An example of the method editor for Full Scan –Data Dependent workflow is shown in Figure 4. In this method compounds are identified base on exact m/z, relative retention time, MS2 spectra and isotopic pattern. Semi-quantitative calculations will be performed using quantification coefficient calculated based on analyte peak area/internal standard peak area for known concentration. ToxFinder software provides the tool for automated concentration coefficient calculations and csv file output allowing easy transfer of results to ToxFinder database.

FIGURE 4. ToxFinder method editor showing an example of processing method for application collecting Full scan and dd MS2 spectra.

ToxFinder data review

User may chose to report only identified compounds or all analytes listed in the method. Figures 5 and 6 presents data review screen for Full scan-dd MS2 method with the status of all analytes in the method shown, sorted alphabetically.

Flags

Confirmation Flag

Green: compound detected and confirmed

Yellow: compound detected but not confirmed

Red: compound not detected

Identification criteria specific flags: green=pass, red=fail

PK: Peak detection flag

IP: Isotopic pattern flag

LS: Library search flag

IR: Ion ratio flag

FR: Fragments detection flag

FIGURE 5. ToxFinder data review page: Compound results table with compound chromatogram, library search and isotopic match results.

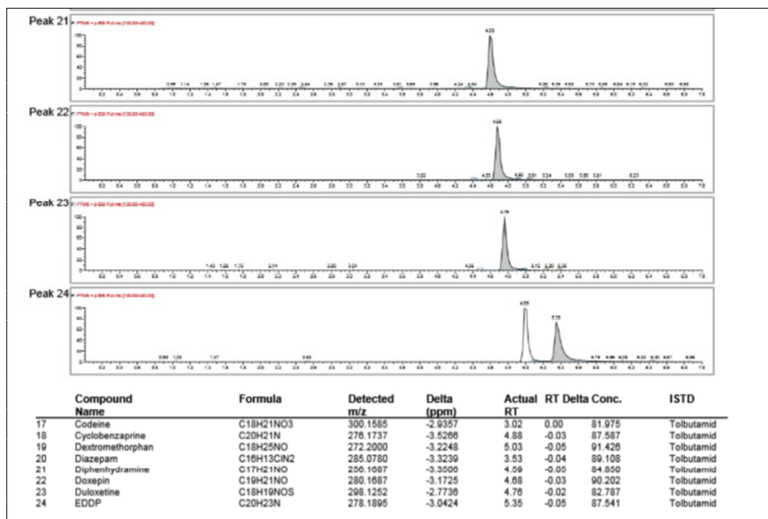
Figure 6. ToxFinder semi-quantitative calculations for urine spiked at a concentration of 100 ng/mL.

Compound Results

	Compound	RT Delta	Relative RT	Isotopic Pattern Score (%	Num Isotopes Matched	Height	Area	Calculated Amt	Concentration Units	Response Ratio	ISTD Respo
12	Carisoprodol	-0.02	1.124	82	4 of 5	4900288	15126838	92.898	ng/mL	0.626	24166369.6
13	Clomipramine	-0.07	1.845	85	5 of 8	9251038	17930381	99.860	ng/mL	0.742	24166369.6
14	Clonazepam	-0.03	1.197	90	5 of 7	3579546	12101195	108.575	ng/mL	0.501	24166369.6
15	Codeine	0.00	1.094	97	3 of 4	3077230	12886594	81.975	ng/mL	0.533	24166369.6
16	Cyclobenzaprine	-0.03	1.768	79	3 of 4	7902111	31237491	87.587	ng/mL	1.293	24166369.6
17	Dextromethorphan	-0.05	1.822	88	4 of 4	10330878	32957828	91.426	ng/mL	1.364	24166369.6
18	Diazepam	-0.04	1.277	75	5 of 8	7328627	19804901	89.108	ng/mL	0.820	24166369.6
19	Diphenhydramine	-0.05	1.664	83	4 of 5	5762978	25426382	84.850	ng/mL	1.052	24166369.6
20	Doxepin	-0.03	1.693	89	4 of 4	8128637	31499128	90.202	ng/mL	1.303	24166369.6
21	Duloxetine	-0.02	1.724	100	5 of 7	1949547	6732224	82.787	ng/mL	0.279	24166369.6
22	EDDP	-0.05	1.937	93	4 of 4	5914384	34043054	87.541	ng/mL	1.409	24166369.6
23	Fentanyl	-0.02	1.617	90	4 of 4	6810613	22704738	85.793	ng/mL	0.940	24166369.6

Reporting

Designed report templates in user selected pdf, Excel, csv format (Figure 7). Export of data review table into csv file. Utility allowing custom reports development.


Peak 21

Peak 22

Peak 23

Peak 24

	Compound Name	Formula	Detected m/z	Delta (ppm)	Actual RT	RT Delta	Conc.	ISTD
17	Codeine	$C_{18}H_{21}NO_3$	300.1585	-2.9357	3.02	0.00	81.975	Tolbutamid
18	Cyclobenzaprine	$C_{20}H_{21}N$	276.1737	-3.5266	4.88	-0.03	87.587	Tolbutamid
19	Dextromethorphan	$C_{18}H_{25}NO$	272.2000	-3.2248	5.03	-0.05	91.426	Tolbutamid
20	Diazepam	$C_{16}H_{13}ClN_2$	285.0780	-3.3239	3.53	-0.04	89.108	Tolbutamid
21	Diphenhydramine	$C_{17}H_{21}NO$	256.1687	-3.3506	4.59	-0.05	84.850	Tolbutamid
22	Doxepin	$C_{19}H_{21}NO$	280.1687	-3.1725	4.68	-0.03	90.202	Tolbutamid
23	Duloxetine	$C_{18}H_{19}NOS$	298.1252	-2.7736	4.76	-0.02	82.787	Tolbutamid
24	EDDP	$C_{20}H_{23}N$	278.1895	-3.0424	5.35	-0.05	87.541	Tolbutamid

FIGURE 7. Portion of ToxFinder data summary report

Conclusion

ToxFinder software evaluation showed:

- Intuitive workflow and ease of use.
- Fast data processing.
- Easy data review.
- Accuracy in forensic compound identification.
- Accurate, semi-quantitative calculations.

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Quantitation of 47 Forensic Compounds in Urine by HPLC-MS

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Overview

Purpose: To demonstrate analytical workflow for robust, fast and cost efficient analysis of large panel of drugs in urine samples.

Methods: Hydrolyzed and diluted urine samples were analyzed with 8 minute LC-MS method on a dual channel LC system coupled to triple quadrupole mass spectrometer.

Results: We demonstrated simple and cost efficient method producing data which meet forensic toxicology lab requirements for sensitivity and robustness.

Introduction

Forensic toxicologists face an ever-expanding list of compounds for analysis. The need to reliably quantitate large-panel assays with ion ratio confirmation is continually increasing. Large panel assays are required in order to speed sample analysis time, lower analytical costs and obtain results quicker while keeping good data quality.

Methods

Sample Preparation

- Take a 200-μL aliquot of urine sample.
- Add 100 μL β-glucuronidase and incubate 2 hours at 60 C.
- Cool samples and add 100 μL of Internal Standard spiking solution in methanol.
- Vortex and then centrifuge.
- Dilute sample 15 fold with water to minimize matrix effects.
- Inject 10 μL onto analytical column.

Note: Deuterated analogs of each analyte were used as internal standards.

Liquid Chromatography

- Column: Thermo Scientific ™Accucore™ PFP 2.6 μm , 5 0x 2.1 mm
- Mobile phase:
  - A: 10 mM ammonium formate, 0.1% formic acid in water (Fisher Scientific™Optima™ LC/MS)
  - B: 10 mM ammonium formate, 0.1% formic acid in methanol water ((Fisher Scientific™ Optima™ LC/MS)
- Ambient temperature
- LC Gradient (Table 1)

Table 1. Liquid chromatography program for the method, including solvent composition, flow rate, and timing

Step	Start (min)	Time (s)	Flow (mL/min)	%A	%B
1	0.00	30	0.5	100.0	0
2	0.5	30	0.5	80	20
3	1.0	180	0.5	30	70
4	3	120	0.5	0	100
5	5	6	1.0	0	100
6	5.01	114	1	-	100.0
7	7.0	6	1	100	0
8	7.01	54	1	100	0

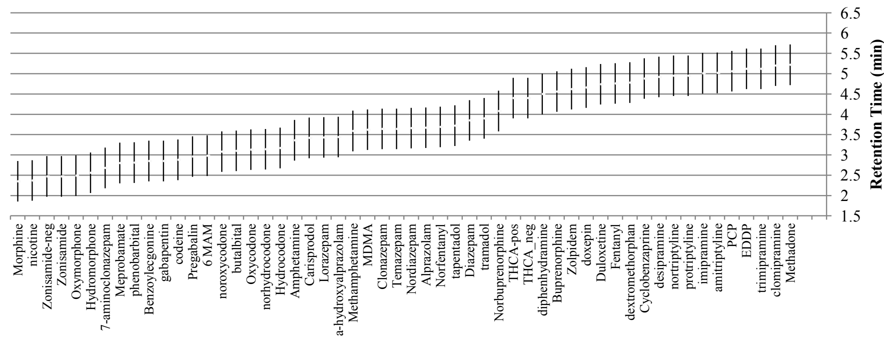
Mass Spectrometry

A Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer with a HESI ionization probe in polarity switch mode was used as the detector. Two SRM transitions were collected for each analyte, and one SRM transition was collected for each internal standard. The SRM transitions collection time scheme is presented in Figure 1.

Data Analysis

All data acquisition and quantification for this method was performed using Thermo Scientific ™ TraceFinder™ software version 3.2.

Figure 1. SRM transitions acquisition scheme in mass spectrometer method. Deuterated internal standards for each analyte (not listed at the graph) were analyzed with the same analyte specific time windows.



Method Performance Evaluation

Calibration standards and QC samples were prepared in artificial urine.

Concentration of the highest calibration standard was limited by concentration of individual analytes stock solutions (1 mg/mL) purchased from Cerillant.

The following method performance parameters were obtained:

•**LOQ:** precision within 20% and ion ratio within specified range

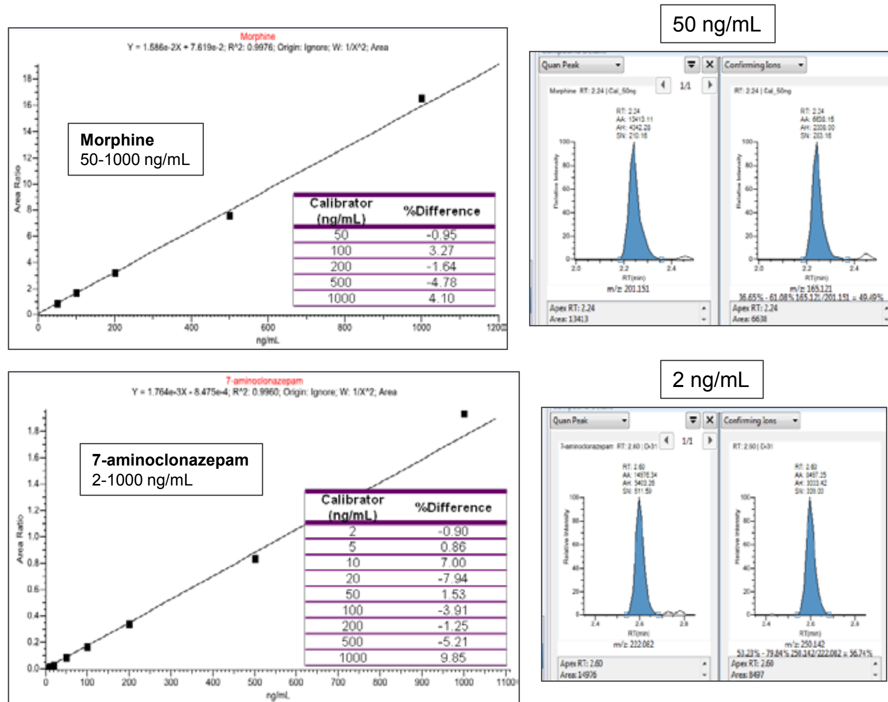
•**Linearity range**: all calibrators accuracy 15%

•**Precision:** 5 replicates of QC samples

•**Matrix effects**: Internal standard peak area %Recovery in donor samples (n=40) calculated against internal standard peak area in water.

Results

Figure 1. Calibration curves and chromatograms of the lowest calibration standards showing quantifier and qualifier ions for selected analytes.



Calibrator (ng/mL)	%Difference
50	-0.95
100	3.27
200	-1.64
500	-4.78
1000	4.10

Calibrator (ng/mL)	%Difference
2	-0.90
5	0.86
10	7.00
20	-7.94
50	1.53
100	-3.91
200	-1.25
500	-5.21
1000	9.85

Figure 1. (continued)

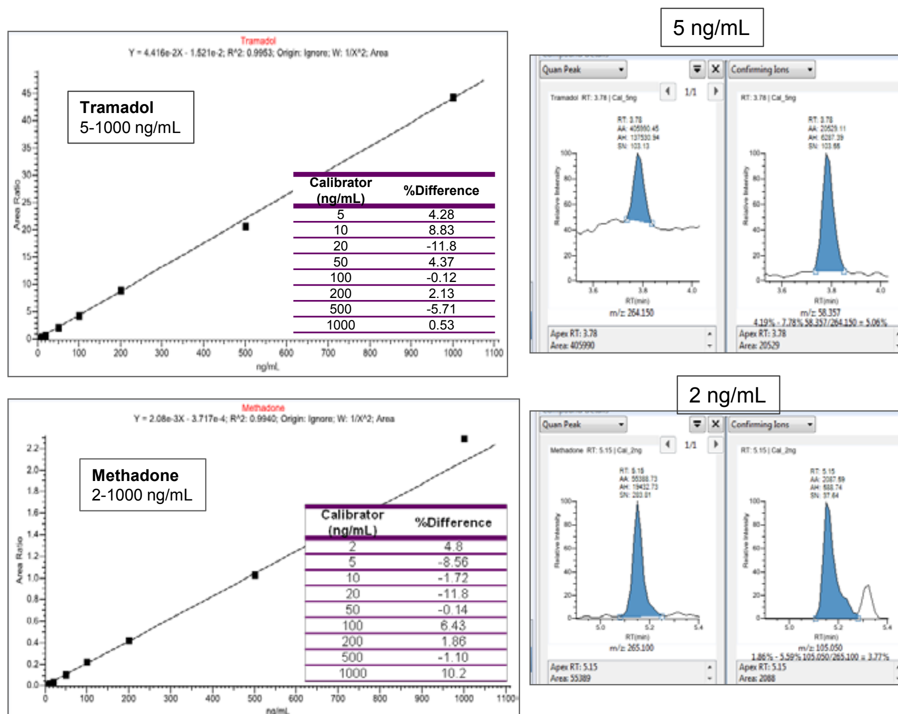


TABLE 2. Limits of quantitation and intra-assay precision for the lowest QC sample with concentration either 10, 50 or 200 ng/mL according to LOQ.

Analytes in this table are listed in order of retention time.

Analyte	LOQ (ng/ml)	Precision %RSD (n=5)	Analyte	LOQ (ng/ml)	Precision %RSD (n=5)
Morphine	50	7.14	Alprazolam	2	10.2
Nicotine	10	10.1	Norfentanyl	5	14.3
Zonisamide	50	5.60	Tapentadol	2	4.82
Oxymorphone	5	7.08	Diazepam	20	5.47
Hydromorphone	20	3.36	Tramadol	5	5.81
7-Aminoclonazepam	2	13.5	Diphenhydramine	2	3.72
Meprobamate	1	12.6	Buprenorphine	20	9.27
Benzoylecgonine	2	4.32	Zolpidem	1	1.81
Codeine	20	4.11	Doxepin	20	10.3
Pregabalin	100	4.62	Duloxetine	20	5.46
6-MAM	20	8.94	Fentanyl	1	4.90
Noroxycodone	20	9.85	Dextromethorphan	2	5.69
Oxycodone	10	9.42	Cyclobenzaprine	2	5.40
Norhydrocodone	50	8.21	Desipramine	50	3.98
Hydrocodone	20	5.95	Nortriptyline	2	6.24
Amphetamine	5	1.90	Protriptyline	50	13.3
Carisoprodol	2	9.55	Imipramine	10	7.28
Lorazepam	50	11.0	Amitriptyline	5	4.63
a-Hydroxyalprazolam	100	5.10	PCP	1	2.47
Methamphetamine	1	6.10	EDDP	2	3.49
MDMA	2	5.63	Trimipramine	10	4.33
Temazepam	10	13.9	Clomipramine	50	3.66
Clonazepam	50	11.4	Methadone	2	1.99
Nordiazepam	50	17.9			

Table 4. Matrix effects: Mean internal standard %Recovery for 50 donor urine samples.

Analyte	%Rec	Analyte	%Rec
Morphine-d3	64.3	Alprazolam-d5	83.8
Nicotine-d4	90.2	Norfentanyl-d5	90.7
Zonisamide-13C6	102	Tapentadol-d3	88.7
Oxymorphone-d3	85.7	Diazepam-d5	83.2
Hydromorphone-d6	78.3	Tramadol-d3	89.6
7-Aminoclonazepam-d4	62.9	Diphenhydramine-d3	92.2
Meprobamate-d7	94.5	Buprenorphine-d4	99.1
Benzoylecgonine-d3	78.4	Zolpidem-d6	107
Codeine	92.2	Doxepin-d3	95.2
Pregabalin-d6	105	Duloxetine-d3	82.1
6-MAM-d3	97.3	Fentanyl-d5	104
Noroxycodone-d3	84.4	Dextromethorphan-d3	99.3
Oxycodone-d3	85.4	Cyclobenzaprine-d3	97.4
Norhydrocodone-d3	83.4	Desipramine-d3	97.9
Hydrocodone-d6	83.8	Nortriptyline-d3	101
Amphetamine-d3	72.7	Protriptyline-d3	95.9
Carisoprodol-d7	92.9	Imipramine-d3	99.5
Lorazepam-d4	Not used	Amitriptyline-d3	105
a-Hydroxyalprazolam-d5	98.8	PCP-d5	102
Methamphetamine-d5	88.6	EDDP-d3	107
MDMA-d5	89.9	Trimipramine-d3	101
Temazepam-d5	95.0	Clomipramine-d3	98.4
Clonazepam-d4	89.4	Methadone-d3	100
Nordiazepam-d5	87.9		

Figure 2. Matrix effects: Internal standard %Recovery in 50 donor samples for some of the least and the most affected analytes.

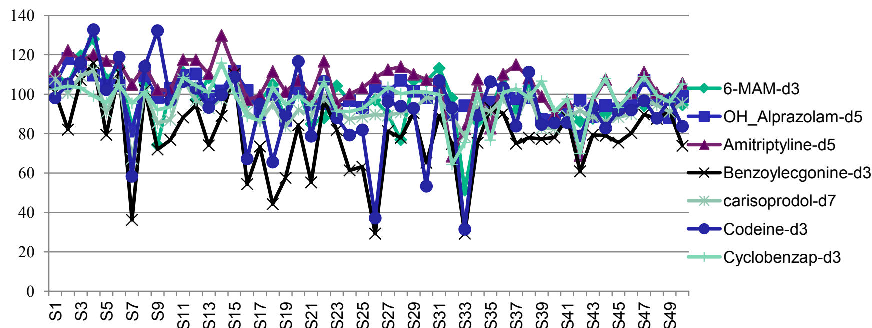


Figure 3. Chromatograms from donor urine samples. Chromatograms selected are those with lowest analyzed concentration for a given compound.

Fentanyl 64.6 ng/mL | Norfentanyl 675 ng/mL

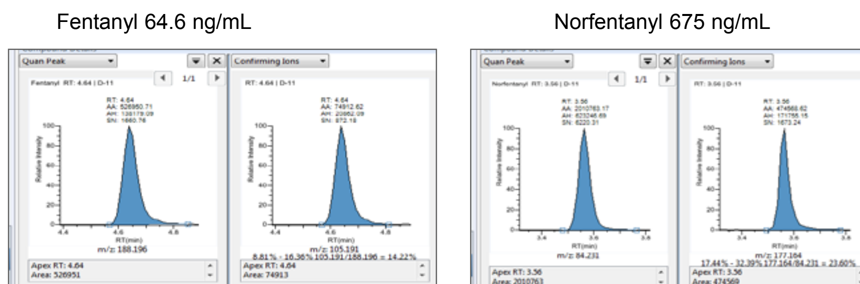


Figure 3. (continued)

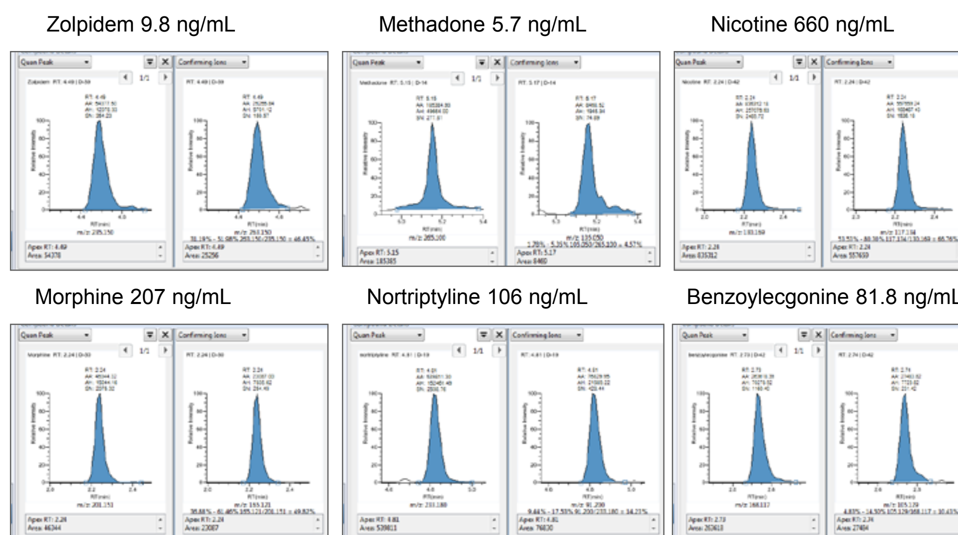
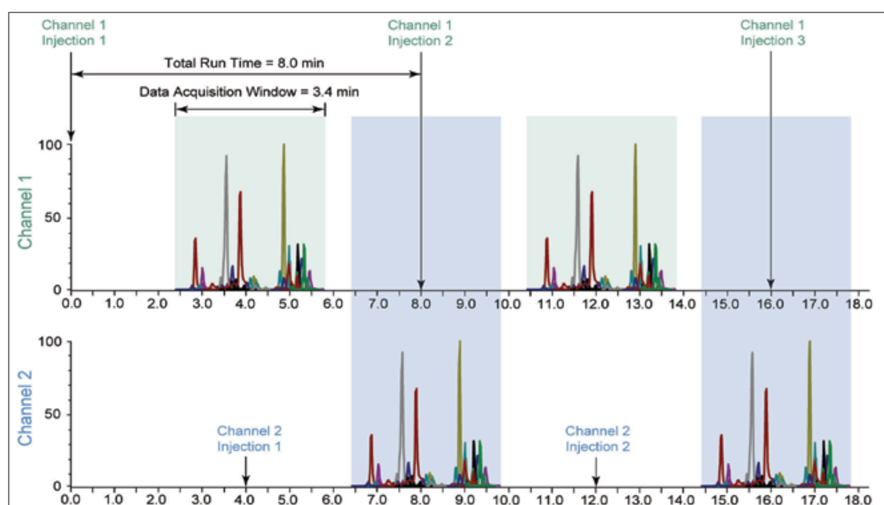


Figure 4. The method executed in multiplexing mode on dual channel Transcend II LC system: one injection every 4 minutes.



Conclusion

- We developed fast, cost efficient method for quantitative analysis of 47 forensic compounds in urine.
- Method meets laboratory requirements for limit of quantitation.
- Matrix effects are observed for some analytes and thus deuterated internal standards are required.
- Short 3.8 minutes acquisition window in 8 minutes method allows to double throughput on dual channel LC system resulting in analysis of 15 samples per hour.
- Analysis of THC-COOH, Norbuprenorphine, Gabapentin, Butalbital, Phenobarbital were evaluated in this method. Results are not reported because either required LOQ was not achieved or significant matrix effects were observed.

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Multiplexing Multiple Methods to Maximize Workflow Efficiency in LC-MS Laboratories

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Overview

Purpose: To demonstrate the performance of research and forensic methods running simultaneously across most or all channels of a multichannel ultra-high performance liquid chromatography (UHPLC) system interfaced to a tandem mass spectrometer (MS/MS) in order to maximize sample throughput and workflow efficiency.

Methods: Reversed-phase liquid chromatography of analytes with corresponding stable-isotope internal standards eluting from up to four UHPLC channels into a common ion source of a triple-quadrupole mass spectrometer were used to measure blood serum levels of the following compounds for research purposes:

> ***25-OH-Vitamins D2 & D3*** after protein precipitation and ***Methylmalonic Acid*** after protein precipitation and butylation, eluting to an atmospheric-pressure chemical ionization (APCI) source;

or to measure urine levels of the following forensic compounds:

> ***Buprenorphine & Norbuprenorphine*** after hydrolysis and ***Ethyl-Glucuronide & Ethyl Sulfate*** after dilution, eluting into a heated electro-spray ionization (HESI) source.

Results: Desired quantitation ranges, accuracy and repeatability criteria were achieved for each application when various specimen batches ran on any of the channels of the 4-channel UHPLC system. Typically, internal standard (IS) peak area counts showed less than 20% coefficient of variability (CV) among calibrators, QCs and specimens (n = 20) on any and across all 4 channels. Retention time variations through these batches were less than 3% CV. Calculated amounts were within +/- 15% of theoretical amounts.

Introduction

Many laboratories run several different LC-MS methods in series on a single channel LC-MS system. If the methods involve different ion sources, columns and mobile phases, the changeover is time consuming, labor intensive and increases the risk of mistakes and contamination. A four-channel UHPLC system multiplexed into one mass spectrometer permits parallel batches of up to four different methods utilizing a common ion source and unique columns and mobile phases to be completed in a fraction of the time and effort.

Methods

Sample Preparation:

"Neat" specimens were prepared in HPLC-grade solvents - acetonitrile, methanol, water - using standards purchased from Cerilliant (Round Rock, TX).

Blood serum specimens and corresponding calibrators and quality controls (QCs) were subjected to protein precipitation by mixing 1:2 with acetonitrile containing internal standard (IS) - 25-OH-VitD3-d6 or d3-methylmalonic acid. After centrifugation, 50 uL of supernatants from the 25-OH-Vitamin D (VitD) batches were injected directly into the UHPLC system. From the methylmalonic acid (MMA) batches, 100 uL of supernatants were evaporated to dryness by heated nitrogen flow. The residues were derivatized by 100 uL of 10% acetyl chloride in butanol for 15 minutes. After evaporation to dryness and reconstitution with 100 uL of 50% methanol in water, 10 uL injections of each sample preparation were made into the UHPLC system.

Urine specimens and corresponding calibrators and QCs to be analyzed for buprenorphine & norbuprenorphine (Bup/Norbup) were hydrolyzed by incubating a mixture of 150 uL of β-glucuronidase solution (10,000 U/mL, pH 5) with 200 uL of specimen, and 50 uL of IS solution containing - buprenorphine-d3 & norbuprenorphine-d4 for 1.5 hours at 60°C. Each preparation was then mixed with 200 uL of cold methanol and refrigerated for 10 minutes before centrifugation. 20 uL injections of supernatants from each preparation were made into the UHPLC system.

Urine specimens and corresponding calibrators and QCs to be analyzed for ethyl-glucuronide & ethyl-sulfate (EtG/EtS) were diluted 1:10 with water and then spiked with 50 uL of IS solution containing EtG-d3 & EtS-d3 before making 20 uL injections into the UHPLC system.

Note: β-glucuronidase powder was purchased from Sigma-Aldrich. All other reagents and consumables were from Thermo Fisher Scientific.

Liquid Chromatography: The UHPLC system was a Thermo Scientific™ Transcend™ II LX4 equipped with binary-solvent pumps and a dual-arm autosampler configuration. The columns and mobile phase conditions for each method are described with the results.

Mass Spectrometry

The Thermo Scientific™ TSQ Endura™ triple-quadrupole mass spectrometer was used with APCI when multiplexing VitDs with MMA batches or HESI when multiplexing Bup/Norbup with EtG/EtS batches. Ion source and MS/MS conditions are described with the results.

System Control & Data Analysis

Thermo Scientific™ TraceFinder™ with Aria™ MX software was used to control the Transcend II LX4 and Endura MS/MS systems, submit batches to desired channels as well as for analyzing data and reporting results.

Results

Multi-channeling batches of 25-OH-VitDs and MMA

FIGURE 1. Common APCI Source Conditions

Ion Source Type: **APCI**
Current LC Flow (µL/min): **0**
Sheath Gas (Arb): **20**
Aux Gas (Arb): **5**
Sweep Gas (Arb): **0**
Ion Transfer Tube Temp (°C): **300**
Vaporizer Temp (°C): **400**
Pos Ion Discharge Current (µA): **4**

Cycle Time (secs): **.25**
Use Calibrated RF Lens: **False**
RF Lens (V): **70**
Q1 Resolution (FWHM): **0.7**
Q3 Resolution (FWHM): **0.7**
CID Gas (mTorr): **1.5**
Source Fragmentation (V): **10**
Chrom Filter (secs): **5**

FIGURE 2. MS/MS & LC Conditions for 25-OH-VitDs

SRM Table

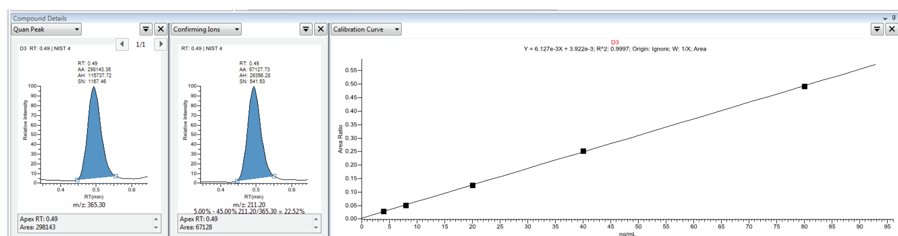
Compound	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
D3	0	1	Positive	383.35	211.15	30	112
D3	0	1	Positive	383.35	365.3	20	112
IS	0	1	Positive	389.4	263.25	23	115
IS	0	1	Positive	389.4	371.35	20	115
D2	0	1	Positive	395.35	269.1	25	115
D2	0	1	Positive	395.35	377.4	20	115

Start data 1.1 min   Data window: 1.0 min   Total run time: 4.0 min

Column 1	Accucore RP-MS, 2.6um, 50x2.1mm
Loading Pump	Binary
A	Water + 0.1% Formic Acid
B	Methanol + 0.1% Formic Acid

Step	Start	Sec	Flow	Grad	%A	%B	Comments
1	0.00	15	0.50	Step	25.0	75.0	Load sample
2	0.25	45	0.50	Ramp	5.0	95.0	Separate analytes
3	1.00	60	0.50	Step	5.0	95.0	Elute analytes
4	2.00	5	0.50	Ramp	25.0	75.0	Ramp to initial conditions
5	2.08	115	0.50	Step	25.0	75.0	Equilibrate column

FIGURE 3. Typical results for 25-OH-VitDs
Desired quantitation range from 4 to 80 ng/mL achieved



25-OH-VitDs batches submitted to one or two channels have throughputs of 15 or 28 injections per hour, respectively. Large batches submitted across 4 channels typically have throughputs around 58 injections per hour.

FIGURE 4. Multi-channeling 25-OH-VitDs

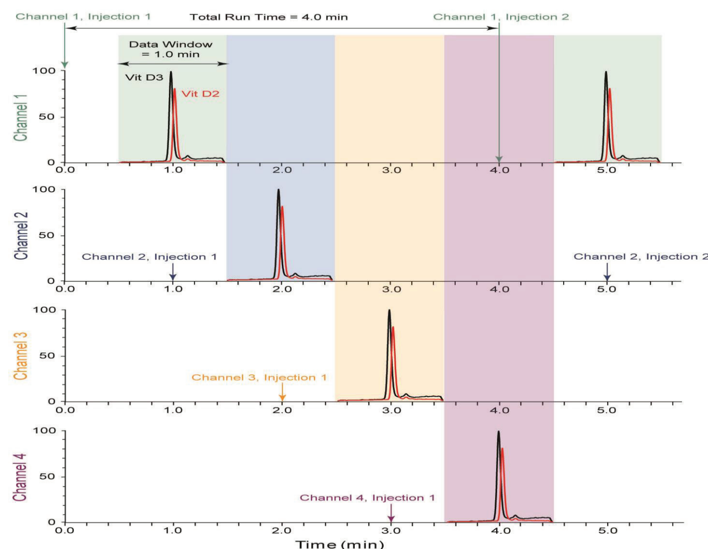


FIGURE 5. MS/MS & LC Conditions for Butyl-MMA

SRM Table

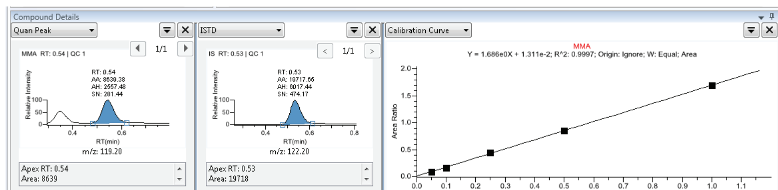
Compound	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)
Butyl-MMA	0	1	Positive	231.15	101.15	23
Butyl-MMA	0	1	Positive	231.15	119.3	16
IS	0	1	Positive	234.2	104	23
IS	0	1	Positive	234.2	122.2	16

Start data 1.75 min   Data window: 1.0 min   Total run time: 5.0 min

Column 1	Accucore C8, 2.6um, 50x2.1mm
Loading Pump	Binary
A	Water + 0.1% Formic Acid
B	Methanol + 0.1% Formic Acid

Step	Start	Sec	Flow	Grad	%A	%B	Comments
1	0.00	10	0.50	Step	45.0	55.0	Load sample
2	0.17	30	0.50	Ramp	35.0	65.0	Separate analytes
3	0.67	90	0.50	Ramp	30.0	70.0	Elute analytes
4	2.17	60	0.50	Step	-	100.0	Wash column
5	3.17	110	0.50	Step	45.0	55.0	Equilibrate column

FIGURE 6. Typical results for MMA (butylated) Desired quantitation range from 0.05 to 1.00 uM achieved



MMA batches submitted to one or two channels have throughputs of 12 or 23 injections per hour, respectively. Since demand for MMA is much less than for 25-OH-VitDs, one channel is used while the other three are used for the VitDs. Thus, 8 injections from MMA and 36 injections of 25-OH-VitDs are completed in one hour.

Internal standard peak areas among blood serum specimens varied greatly due to ion suppression by co-eluting interferences or by sample matrix components that interfere with the butylation reaction. The cause(s) of this variability is being investigated. However, the calculated amounts of MMA concentrations in the QCs and specimens measured agreed with theoretical values within +/- 15%.

Multi-channeling batches of Bup/Norbub and EtG/EtS

FIGURE 7. Common HESI Source Conditions

Ion Source Type: **HESI**
Spray Voltage:
Positive Ion (V): **3500**
Negative Ion (V): **1000**
Current LC Flow (µL/min): **0**
Sheath Gas (Arb): **50**
Aux Gas (Arb): **15**
Sweep Gas (Arb): **2**
Ion Transfer Tube Temp (°C): **350**
Vaporizer Temp (°C): **400**

Cycle Time (secs): **.5**
Use Calibrated RF Lens: **False**
Q1 Resolution (FWHM): **0.7**
Q3 Resolution (FWHM): **0.7**
CID Gas (mTorr): **2**
Source Fragmentation (V): **10**
Chrom Filter (secs): **3**

FIGURE 8. MS/MS & LC Conditions for Bup/Norbup

SRM Table

Compound	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
NorBup	0	1.5	Positive	414.3	243.1	30	150
NorBup	0	1.5	Positive	414.3	340.2	30	150
NorBup-d3	0	1.5	Positive	417.3	246.1	30	150
NorBup-d3	0	1.5	Positive	417.3	343.2	30	150
Bup	0	1.5	Positive	468.35	396.3	40	170
Bup	0	1.5	Positive	468.35	414.3	35	170
Bup-d4	0	1.5	Positive	472.35	243.05	40	170
Bup-d4	0	1.5	Positive	472.35	400.2	40	170

Start data 0.5 min Data window: 1.5 min Total run time: 4.0 min

Column 1	Accucore RP-MS, 2.6um, 50x2.1mm
Loading Pump	Binary
A	Water + 0.1% Formic Acid
B	Methanol + 0.1% Formic Acid

Step	Start	Sec	Flow	Grad	%A	%B	Comments
1	0.00	60	0.50	Step	60.0	40.0	Load sample
2	1.00	60	0.50	Ramp	-	100.0	Elute analytes
3	2.00	30	0.50	Step	-	100.0	Wash column
4	2.50	90	0.50	Step	60.0	40.0	Equilibrate column

FIGURE 9. Typical results for Bup/NorBup
Desired quantitation range from 5 to 500 ng/mL achieved.

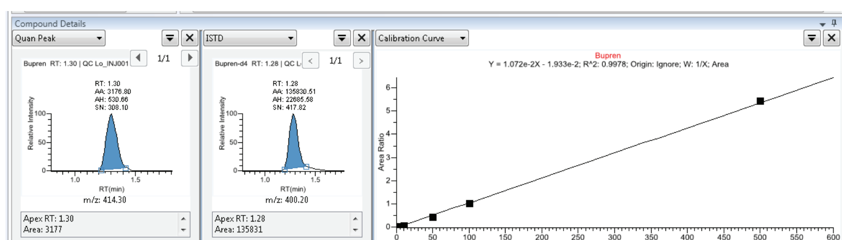


FIGURE 10. MS/MS & LC Conditions for EtG/EtS

SRM Table

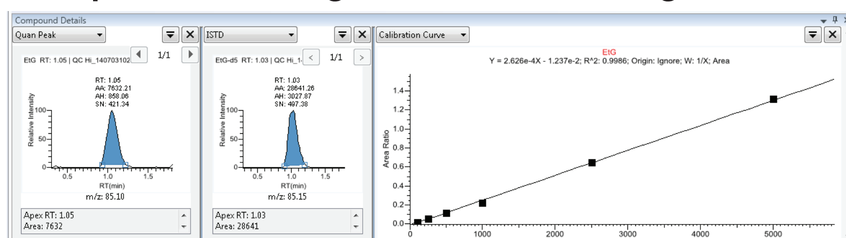
Compound	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
EtS	0	1.5	Negative	125.1	80.1	35	60
EtS	0	1.5	Negative	125.1	97.05	15	60
EtS-d5	0	1.5	Negative	130.1	80.1	35	65
EtS-d5	0	1.5	Negative	130.1	98.05	15	65
EtG	0	1.5	Negative	221.1	75.2	15	75
EtG	0	1.5	Negative	221.1	85.1	15	75
EtG-d5	0	1.5	Negative	226.1	75.2	15	75
EtG-d5	0	1.5	Negative	226.1	85.1	15	75

Start data 0.1 min   Data window: 1.5 min   Total run time: 4.0 min

Column 1	Syncronis aQ 5um, 50x3mm
Loading Pump	Binary
A	Water + 0.1% Formic Acid
B	Methanol

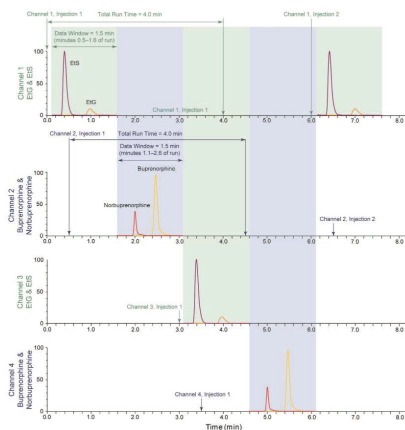
Step	Start	Sec	Flow	Grad	%A	%B	Comments
1	0.00	60	0.70	Step	100.0	-	Load sample
2	1.00	30	0.70	Ramp	-	100.0	Elute analytes
3	1.50	60	0.70	Step	-	100.0	Wash column
4	2.50	90	0.70	Step	100.0	-	Equilibrate column

FIGURE 11. Typical results for EtG/EtS
Desired quantitation range from 100 to 5000 ng/mL achieved.



Bup/Norbup batches and EtG/EtS batches submitted to one or two channels have throughputs of 15 or 28 injections per hour.

FIGURE 12. Multi-channeling Bup/Norbup & EtG/EtS Batches



Multi-channeling Bup/Norbup across 2 channels while EtG/EtS runs on one allows 26 Bup/Norbup and 13 EtG/EtS injections/hour.

Using 2 channels for each does not increase throughput but ensures completion of all batches in case one channel stops because of leakage or over-pressurization.

Conclusion

- Multi-channeling LC-MS research and forensic methods improves efficiency and throughput
- Multi-channeling also increases the cost-effectiveness of your mass spectrometer

Acknowledgements

We thank Dr. Hashim Othman of BioReference Laboratories (Elmwood Park, NJ) for supplying QCs and specimens for our tests and advice on desired quantitation ranges, accuracy and repeatability criteria.

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LC/MS/MS Research Method for 14 Antidepressants Utilizing Dried Blood Spots

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Overview

Purpose: To develop an LC-MS/MS research method for measuring the concentration of antidepressants from dried blood spots (DBS).

Methods: An LC-MS/MS method was developed to extract 14 antidepressants from dried blood spots for quantitation.

Results: The analytes of interest were extracted successful from dried blood spots showing good linearity, accuracy, and precision.

Introduction

Important factors in the analysis of drugs in whole blood are accurate measurements, storage capabilities, small sample volume, and easy extraction. Dried blood spots are becoming an adopted clinical research technique for the analysis of drugs in biological matrices. Due to the complexity of the solution resulting from dissolving the blood spot, the sample must undergo further cleanup by chromatographic separation before introduction into the mass spectrometer. A research application is demonstrated using the Thermo Scientific™ Prelude SPLC™ system and the Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer (Figure 1) to quantitatively analyze 14 antidepressant drugs collected from dried blood spots.

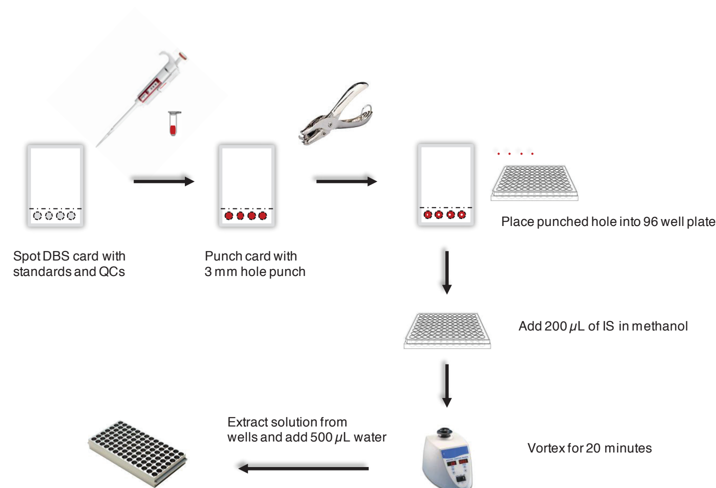
FIGURE 1. Prelude SPLC system with TSQ Endura MS

Methods

Sample Preparation

The analytes of interest were spiked into human whole blood at various concentrations to make calibrators and controls. The samples were spotted at a fixed volume onto Whatman® paper. Then, the analyte was extracted by solvent containing isotopically labeled internal standards and transferred into clean vials for LC-MS/MS analysis. The entire workflow is depicted in Figure 2.

FIGURE 2. Workflow of preparing the dried blood spot for LC/MS



Instrumentation

A Prelude SPLC system was used in TX mode and equipped with a Thermo Scientific™ TurboFlow™ Fluoro XL 0.5 x 50 mm cleanup column and a 2.1 x 50 mm, 2.6 µm particle size Thermo Scientific™ Accucore™ aQ analytical column. The detector for the system was a TSQ Endura triple quadrupole mass spectrometer with a heated electrospray ionization (HESI-II) probe in positive mode. Thermo Scientific™ TraceFinder™ software version 3.1 was used for quantitation. The liquid chromatography flow path is found in Figure 3.

Method Parameters

Mobile phases were (A) 10 mM ammonium formate, 0.05% formic acid in water; (B) 10 mM ammonium formate, 0.05% formic acid in methanol; and (C) 45/45/10 acetonitrile/isopropanol/acetone. The LC method is shown in Table 1. The mass spectrometer quantifier and qualifier selected-reaction monitoring (SRM) transitions are shown in Table 2. The method range for all the analytes was 10–750 ng/mL.

FIGURE 3. Online sample cleanup and analytic separation flow path

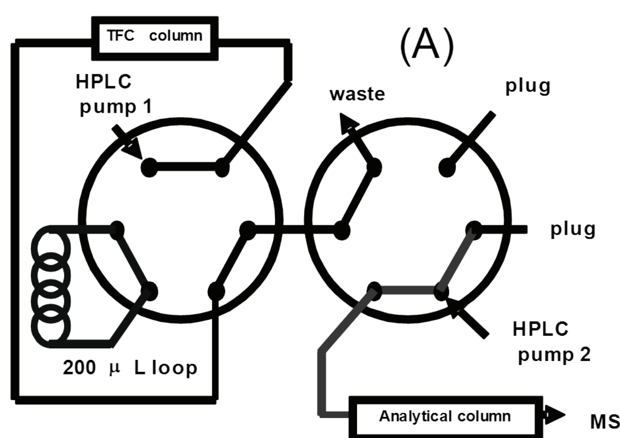


TABLE 1. LC method parameters

Step	Start	Sec	Flow	%A	%B	%C	Tee	Loop	Flow	Grad	%A	%B
1	0.00	40	1.5	100.0	-	-	====	out	0.50		100	-
2	0.67	65	0.20	-	100	-	T	in	0.50		100	-
3	1.75	5	0.20	-	100	-	====	in	0.50		40	60
4	1.83	120	1.20	-	-	100	====	out	0.50		25	75
5	3.83	30	1.10	-	-	100	====	out	0.50		-	100
6	4.33	60	1.50	100	-	-	====	out	0.50		100	-

TABLE 2. SRM transitions

Analyte	Precursor Ion (Q1)	Product Ions (Q3)	Collision Energy	S-lens
Amitriptyline	278.1	233.2	15	61
		191.1	15	61
Doxepin	280.2	235.0	26	61
Imipramine	281.0	86.3	25	88
		208.0	25	88
Fluvoxamine	319.1	200.0	19	70
		228.0	19	70
Clomipramine	314.9	86.2	20	55
		242.1	20	55
Fluoxetine	310.1	117.1	10	83
		148.0	10	83
Paroxetine	330.1	192.0	28	94
		151.0	28	94
Citalopram	325.1	109.1	25	120
		262.0	25	120
Nortiptyline	264.1	233.0	20	50
		191.0	20	50
Desipramine	267.1	208.1	20	48
		236.1	20	48
Venlafaxine	278.2	147.1	15	61
		121.1	15	61
Sertraline	306.0	275.0	13	80
		158.9	13	80
Duloxetine	298.0	154.0	8	55
		-		
Bupropion	241.0	167.0	15	61
		185.0	15	61
Amitriptyline-d_3	281.1	233.2	24	90
Doxepin-d_3	283.2	235.1	24	106
Fluoxetine-d_6	316.1	154.0	16	79
Paroxetine-d_6	336.1	198.1	20	125
Nortiptyline-d_3	267.1	233.1	18	97
Sertraline-d_3	309.0	275.0	12	69

Results

One day of accuracy and precision measurements were performed for system verification on each of the following analytes: amitriptyline, doxepin, imipramine, fluvoxamine, clomipramine, fluoxetine, paroxetine, citalopram, nortriptyline, desipramine, venlafaxine, sertraline, duloxeine, and bupropion. The inter-day and intra-day accuracy and precision were tested from 10–750 ng/mL for each analyte. A summary of the results is shown in Table 3. The assay precision had RSD values that were less than 15.0% for all compounds tested; LOQ compounds had values less than 20.0%. Additionally, accuracy was ±15.0% of the theoretical value for all assays. The correlation coefficient values for all compounds ranged from 0.9900 to 0.9950, showing linearity throughout all concentrations and analytes. All analytes passed carryover, recovery, and selectivity criteria, as well as benchtop and autosampler stability criteria. Recoveries were all above 90.0% including matrix effects. Example SRM chromatograms at the LOQ are shown in Figure 4. Examples of the calibration curves are shown in Figure 5.

TABLE 3. Quality control accuracy and precision summary

Analyte		Expected Conc		Amitriptylin	Doxepin	Impiramine	Fluvoxamine	Clomipramine	Fluoxetine	Paroxetine	Citalopram	Nortriptyline	Desipramine	Venlafaxine	Sertaline	Duloxetine	Bupropion
Level	Low	20	AVG	17.6	18.0	19.9	19.6	19.3	17.6	18.2	18.9	18.4	19.4	21.4	19.1	18.9	17.3
			%RSD	12.25	10.25	0.20	2.00	3.70	12.25	9.28	5.38	7.88	3.20	7.00	4.75	5.70	13.70
	Mid	110	AVG	113	98	115	125	116	118	115	115	116	111	114	17	117	118
			%RSD	3.14	11.18	4.29	8.87	5.82	7.09	4.18	4.73	5.64	0.91	4.00	6.00	1.91	2.44
	High	380	AVG	399	336	409	423	411	418	394	391	409	391	401	415	389	375
			%RSD	4.95	11.36	7.58	11.26	8.05	10.05	3.68	2.89	7.58	2.95	5.42	9.16	2.42	1.27

FIGURE 4. Representative chromatograms at the LOQ

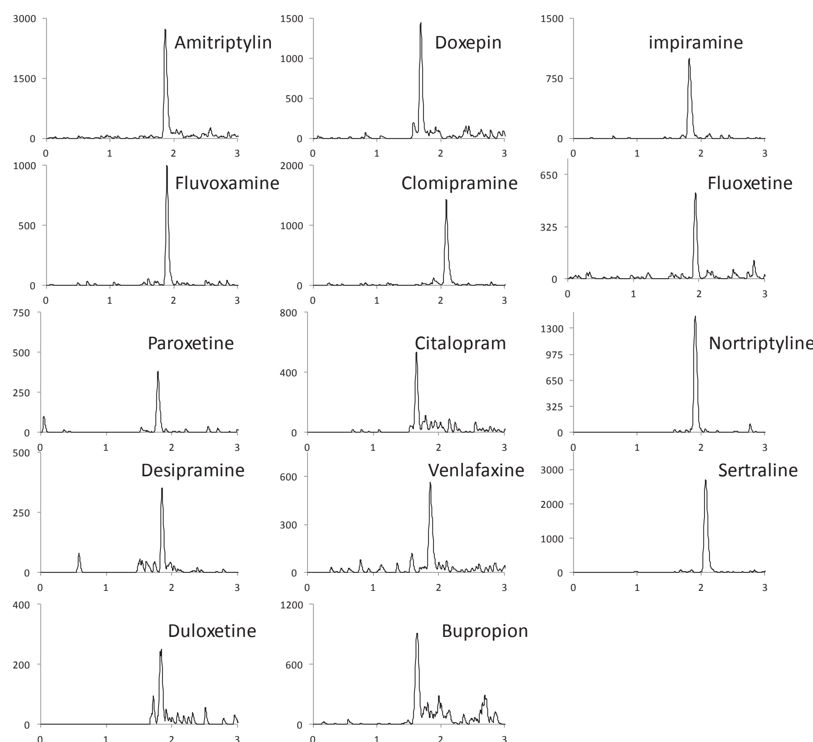
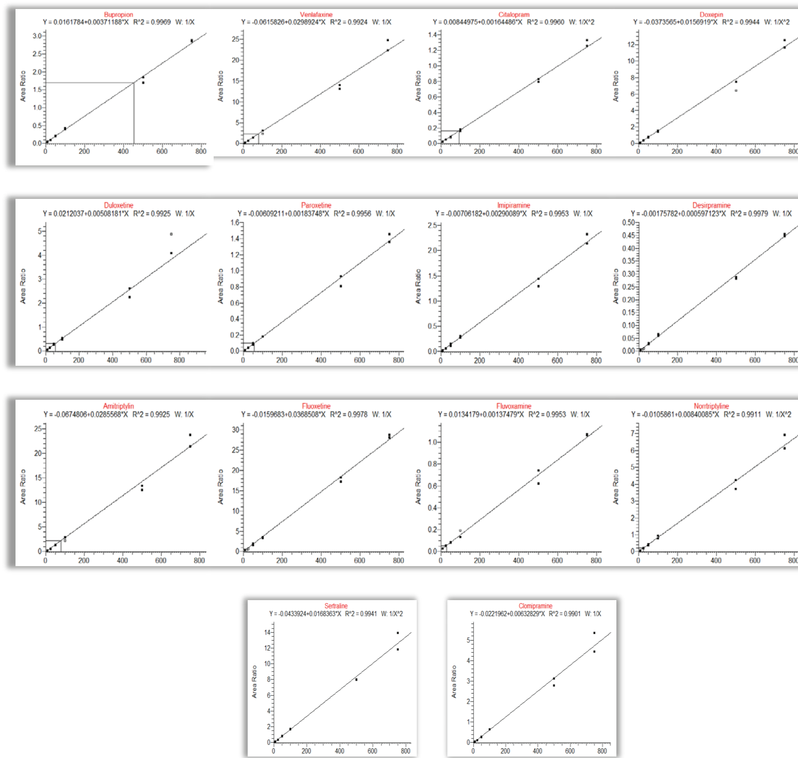


FIGURE 5. Representative calibration curves



Conclusion

- An LC-MC/MS research method has been successfully developed and verified for the quantification of 14 antidepressant drugs collected from dried blood spots using a Prelude SPLC system and a TSQ Endura triple quadrupole mass spectrometer.
- Online sample cleanup of the matrix resulting from dried blood spot collection reduced the complexity of the LC-MS/MS workflow.
- Due to the use of online sample preparation, this research method is more accurate, easier to perform, takes less time, is more robust, and is less costly then traditional offline sample preparation such as SPE plates.

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Verification of an LC-MS/MS Forensic Method for 19 Opioids, Opiates, and Their Metabolites in Human Urine Without Hydrolysis

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Overview

Purpose: This is the verification of a forensic method containing a panel of 19 opiates without the labor-intensive step of hydrolysis. These compounds were analyzed using a Thermo Scientific™ Prelude SPLC™ system for chromatographic separation coupled with a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer.

Methods: All compounds were spiked into human urine, diluted, and injected onto the Prelude SPLC system equipped with a Thermo Scientific™ Accucore™ aQ analytical column. After elution from the analytical column, the compounds were simultaneously analyzed using the TSQ Endura MS with a heated electrospray ionization (HESI-II) probe in positive mode. The total method time was approximately four minutes, allowing for rapid quantification of all analytes and 10 internal standards.

Results: All 19 opiates, opioids, and their metabolites were verified successfully and passed all acceptance criteria. These compounds were analyzed without hydrolysis, drastically reducing the total sample preparation time.

Introduction

It is currently common practice in many forensic laboratories to analyze opiates and opioids using hydrolysis during sample preparation. This process can take up to 24 hours to complete so that all conjugated metabolites are converted back to their parent molecules. We introduce a method that alleviates these time constraints, allowing for an efficient, sensitive analysis of the intact metabolites. Such metabolites (for example, morphine glucuronides) have a reputation for being difficult to chromatograph and detect at low levels. The Prelude SPLC system equipped with an Accucore aQ column offers great retention of all analytes and resolution of isobars. The highly sensitive TSQ Endura MS reproducibly detects the low end of the calibration range for even the least responsive analytes and provides accurate quantification of these somewhat problematic compounds.

Methods

Sample Preparation

The analytes of interest were spiked into human urine at various concentrations to make calibrators and controls. Each sample was divided into two parts; one set was hydrolyzed prior to analysis and the second set was analyzed directly. Hydrolysis was performed by adding 1 M ammonium acetate buffer containing β-glucuronidase to samples and incubating overnight at 60 °C. The nonhydrolyzed samples had the same volume of ammonium acetate buffer added but without any β-glucuronidase present and no incubation. Isotopically labeled internal standards were then added to all the samples. The samples were vortexed and centrifuged. The supernatant was removed from the pellet and transferred into clean vials for LC-MS/MS analysis.

Liquid Chromatography

The LC method is shown in Table 1. A Prelude SPLC system, seen in Figure 1, was used in LX mode, equipped with a 2.1 x 100 mm, 2.6 μm particle size Accucore aQ analytical column. The mobile phases consisted of 0.1% formic acid in (A) water and (B) methanol. Using less than 2 mL of solvent, per injection, the LC system was able to successfully resolve all isobaric compounds within this method. The total method time of 4.25 min, when multiplexed, allows for results every 2 min for all 19 compounds.

TABLE 1. Liquid chromatography program for the method, including solvent composition, flow rate, and timing

Step	Start (min)	Time (s)	Flow (mL/min)	Grad	%A	%B
1	0.00	20	0.40	Step	100.0	-
2	0.33	5	0.40	Step	92.0	8.0
3	0.42	50	0.40	Step	92.0	8.0
4	1.25	5	0.40	Step	75.0	25.0
5	1.33	130	0.40	Ramp	65.0	35.0
6	3.50	45	0.40	Step	-	100.0
7	4.25	100	0.40	Step	100.0	-

Mass Spectrometry

A TSQ Endura triple quadrupole mass spectrometer with a HESI-II ionization probe in positive mode was used as the detector.

Data Analysis

All data acquisition and quantification for this method was performed using Thermo Scientific™ Aria™ MX software version 2.1 and Thermo Scientific™ TraceFinder™ software version 3.1.

FIGURE 1. Prelude SPLC system (left) and TSQ Endura MS (right)

Results

Calibration standards containing all 19 compounds at a concentration range of 5–500 ng/mL were prepared in human urine. Quality control (QC) samples were also prepared in urine at three levels: 12, 225, and 400 ng/mL. Accuracy and precision were tested by using five replicates of three levels of quality controls over four days and quantitating them using calibration curves at the beginning and end of the batch run. Carryover was calculated by dividing the total analyte signal of the lower limit of quantitation (LLOQ) by the total analyte signal found in the matrix blank after the upper limit of quantitation (ULOQ). This number could not exceed 20% of the total LLOQ signal. Additionally, autosampler stability (24 hours at 4 °C) was determined by running QC samples that were refrigerated overnight in the autosampler and comparing them to a freshly prepared calibration curve the following day.

The assay precision had %RSD values that were within 15.0% for all QC and calibration standard levels. Additionally, accuracy was within 15.0% for all QC and calibrations standard levels. All of these results are shown in Table 2. All of the analytes passed acceptance criteria for carryover and autosampler stability. Example chromatograms at the lower limit of quantitation for each of the compounds are shown in Figure 2. Additionally, representative calibration curves are shown for two glucuronides in Figure 3. The R^2 value for morphine-6β-glucuronide was 0.9982 and for oxymorphone-3β-glucuronide was 0.9978. For all compounds within this method, $R^2 > 0.9900$.

Lastly, all mass spectrometer transitions and parameters are shown in Table 3 Both the Thermo Scientific™ TSQ Vantage™ MS and TSQ Endura MS used the same transitions and parameters. Switching between these two different quadrupole mass spectrometers required no method changes, allowing for ease of use and quick analysis.

TABLE 2. Accuracy and precision data for all 19 compounds

Analyte	Accuracy	Precision (%RSD)	
		Intra-Assay	Inter-Assay
Normorphine	94.6	<14.3	<5.7
Dihydromorphine	102	<14.1	<8.2
Morphine	99.2	<8.8	<4.8
Oxymorphone	103	<10.3	<3.5
Hydromorphone	102	<14.1	<5.8
Norcodeine	98.6	<9.6	<4.1
Dihydrocodeine	99.5	<11.1	<5.3
Codeine	99.2	<13.6	<5.7
Norhydrocodone	98.2	<13.5	<9.2
Oxycodone	99.4	<14.1	<5.8
Noroxycodone	100	<11.6	<10.4
Hydrocodone	95.2	<7.4	<5.0
6-Acetylmorphine	103	<9.7	<4.4
Codeine-6β-glucuronide	102	<8.5	<4.1
Oxymorphone-3β-glucuronide	100	<14.4	<4.4
Hydromorphone-3β-glucuronide	108	<7.9	<5.7
Morphine-3β-glucuronide	98.5	<14.9	<4.1
Morphine-6β-glucuronide	99.0	<10.8	<3.7
6-Acetylcodeine	102	<6.1	<6.9

FIGURE 2. Lower limit of quantitation chromatograms for all 19 compounds

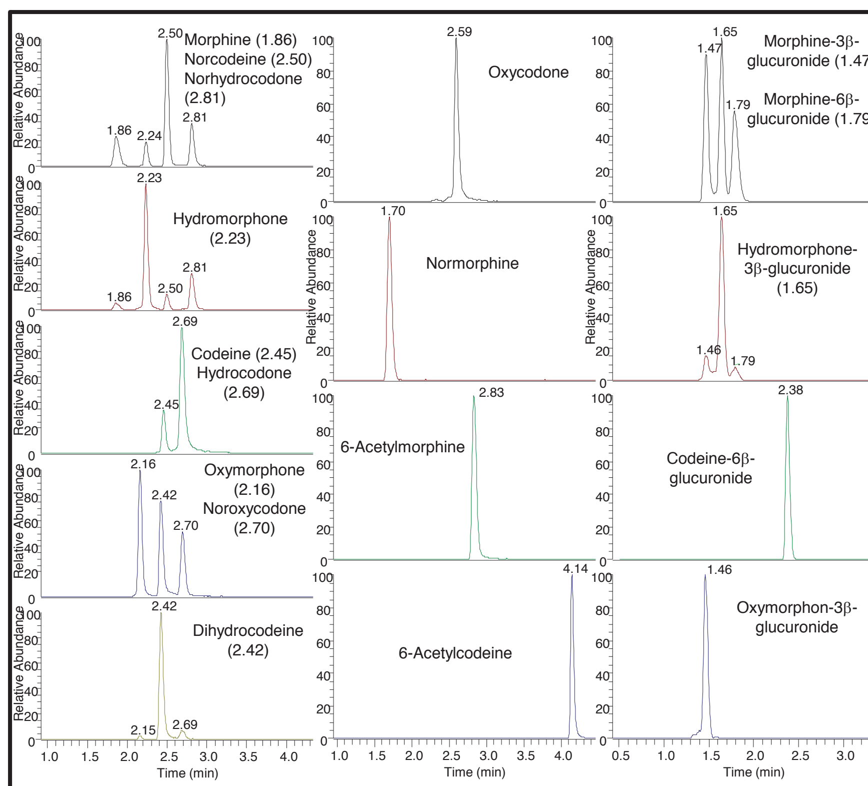


FIGURE 3. Representative calibration curves for the intact glucuronides

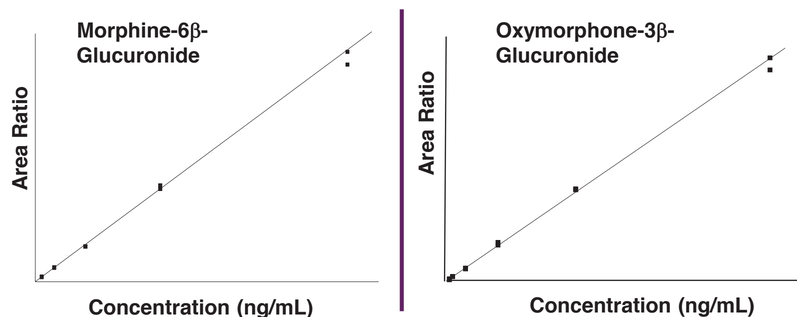


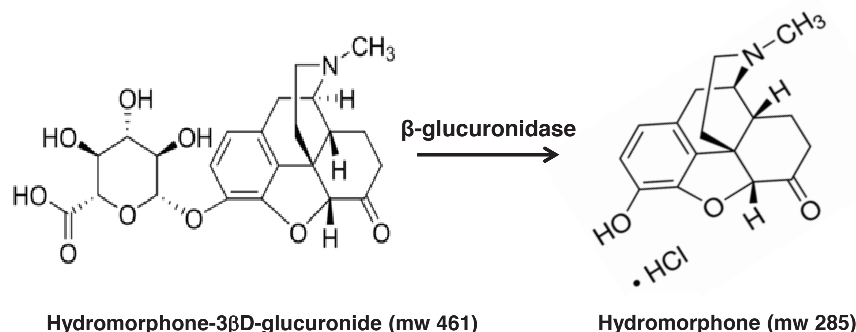
TABLE 3. Transitions and MS parameters for all compounds

Analyte	Precursor Ion (Q1)	Product Ions (Q3)	CE (V)	S-lens (V)
Normorphine	272.0	165.0	59	95
		209.0	40	95
Morphine-3β-glucuronide	462.1	286.1	52	148
		185.2	58	139
Oxymorphone-3β-glucuronide	478.1	284.1	47	147
		302.1	42	147
Hydromorphone-3β-glucuronide	462.1	185.2	58	139
		286.1	52	148
Morphine-6β-glucuronide	462.1	286.1	52	148
		185.2	58	139
Codeine-6β-glucuronide	476.2	300.2	31	114
		215.2	39	114
6-Acetylmorphine	328.1	165.0	58	112
		211.0	39	112
6-Acetylcodeine	342.1	225.1	27	109
		165.1	47	109
Dihydromorphine	288.1	185.1	48	95
		165.0	59	95
Morphine	286.1	165.1	64	90
		185.0	44	119
Oxymorphone	302.0	227.0	40	116
		199.1	55	116
Hydromorphone	286.1	185.0	44	119
		165.1	64	90
Codeine	300.0	171.0	40	119
		199.1	43	119
Dihydrocodeine	302.0	201.1	42	93
		199.0	52	93
Norcodeine	286.1	165.1	64	90
		181.6	49	90
Oxycodone	316.0	241.1	41	119
		256.0	40	119
Noroxycodone	302.1	227.0	41	116
		187.0	40	116
Norhydrocodone	286.1	199.0	39	119
		241.1	35	119
Hydrocodone	300.0	171.1	40	119
		181.1	51	94
Noroxycodone-d_3	305.1	190.1	25	116
Norhydrocodone-d_3	289.1	152.1	62	116
6-Acetylmorphine-d_6	334.1	165.1	38	116
Morphine-6β-glucuronide-d_3	465.1	289.1	32	140
Morphine-d_3	289.1	152.1	61	116
Dihydrocodeine-d_6	308.1	202.1	34	116
Codeine-d_6	306.1	165.1	43	116
Hydromorphone-d_6	292.1	185.1	32	116
Morphine-3β-glucuronide-d_3	465.1	289.1	31	140
Oxycodone-d_6	322.1	218.1	43	116

Figure 4 displays molecular structures for hydromorphone glucuronide and hydromorphone. The starting structure shows the sugar attached to the parent molecule, which exists before hydrolysis is performed. The method in this poster analyzes this structure as a whole, instead of converting it back to the parent.

Table 4 shows the results of a comparison done between hydrolyzed and nonhydrolyzed samples. Analyzing the intact metabolite gives equally as accurate results as does analyzing the parent.

FIGURE 4. Molecular structures of hydromorphone glucuronide (H3G) and hydromorphone (H)



Hydromorphone-3βD-glucuronide (mw 461) **Hydromorphone (mw 285)**

TABLE 4. Comparison of hydromorphone glucuronide (H3G) and hydromorphone (H) concentration from hydrolyzed and nonhydrolyzed samples

	H3G	H3G	H	H
Prepared concentration (ng/mL)	20	100	20	100
Measured concentration prior to hydrolysis (ng/mL)	18.6	112.4	0	0
Measured concentration after hydrolysis (ng/mL)	0	0	12.4	64.2
Expected % converted to parent based on molecular weight (285/461=62)	62		62	
Actual % measured based on results	66		57	
% Difference	6.3		8.4	

Conclusion

- This quantitative method shows that accurate, efficient analysis of opioids, opiates, and their metabolites without hydrolysis is possible.
- Verification of a forensic method for opioids, opiates, and their metabolites on a Prelude SPLC system and a TSQ Endura MS shows that the LC and MS systems can analyze these problematic compounds reproducibly.
- This forensic method is more accurate, easier to perform, takes less time, and is less costly then those that require hydrolysis because the sample preparation is eliminated from the workflow.
- The Prelude SPLC system with an Accucore aQ analytical column provides the necessary chromatography, while the TSQ Endura MS allows for sensitive detection of all compounds.

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Advantages of Ultra-High-Resolution Q Exactive Mass Spectrometer in Analysis of Unlimited Number of Compounds in Urine Quantitative Screening Application for Forensics

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Introduction

Implementation of ultra-high-resolution mass spectrometers for quantitative forensic toxicology allows for unlimited number of analytes, short acquisition times and simple sample preparation. At the same time, ultra-high-resolution mass spectrometry provides high confidence in reported hits. Quantitative screening in forensic toxicology applications is important because it allows reporting of only those compounds with concentrations above specified threshold, reinjection of samples following those with concentration above carry-over limit, and appropriate sample dilution, if required, for confirmatory quantitative analysis.

Instrumentation

Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system

Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap MS

Methods

Sample Preparation

Enzymatic hydrolysis followed by liquid-liquid extraction.

A 1 mL aliquot of urine (spiked calibrator, QC or donor sample) was spiked with internal standard (Tolbutamide), and incubated with 10,000 U/mL beta-glucuronidase enzyme in pH 5.5 buffer for 60 minutes at 60 °C. The resulting mixture was basified with sodium carbonate and extracted with ethylacetate:hexane (1:1). The organic supernatant was evaporated to dryness under nitrogen at 37 °C. The residue was reconstituted in 100 µL of 20% methanol and 10 µL of the sample was analyzed by LC-MS.

LC Method

The column used was a Thermo Scientific™Hypersil™ GOLD PFP 100 x 2.1 mm, 5 µm. Mobile phase was 10 mM ammonium acetate in water (A) and methanol (B). Both solvents were Fisher Scientific™ Optima™ grade. The LC gradient was as follows:

Time (min)	%A	%B	Flow rate (mL/min)
0	95	5	0.75
0.5	95	5	0.75
3.6	60	40	0.75
6.1	5	95	0.75
6.15	0	100	0.85
7.1	0	100	0.85
7.15	95	5	0.85
9.0	95	5	0.85

Mass Spectrometry Method

The Q Exactive benchtop orbitrap mass spectrometer was equipped with a HESI source and operated in positive ionization mode. The MS method consisted of 2 scan events: Full scan from 130–472 *m/z*+ (R = 70K) and all ion fragmentation (AIF) scan from 50–472 *m/z*+ (R = 70K). The AIF spectra were collected with stepped collision energy of 70 ± 50%

Method Validation

The method was validated for 37 representative compounds from different drug classes (Table 1). Calibration standards (0.05–1000 ng/mL) and QC samples (2, 10, 50 ng/mL) were prepared in pooled negative urine.

Matrix effects were evaluated by spiking urine from 15 different donors at concentrations of 10 ng/mL (opioids) , 20 ng/mL (benzodiazepines) or 100 ng/mL (amphetamines) and then processing the samples as described in sample preparation above. Percent recovery was calculated against samples at the same concentrations prepared in water instead of urine.

Data Analysis

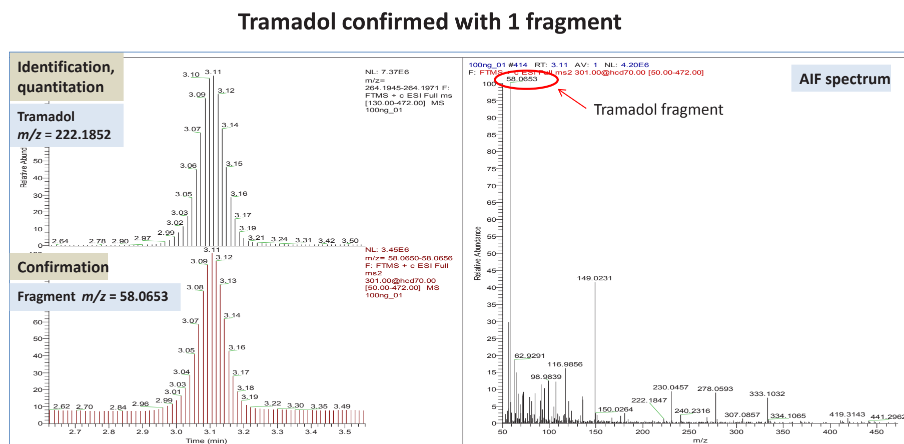
Data was acquired and processed with Thermo Scientific™ TraceFinder™ software version 3.1. Full-scan data (chromatograms reconstructed with *m/z* accuracy of 5 ppm) were used for analyte detection and quantification. AIF spectra were used for compound confirmation. Calibration ranges and LOQ's were evaluated based on concentration accuracy and data precision. Back-calculated concentration had to be within 30% for the LOQ.

Results

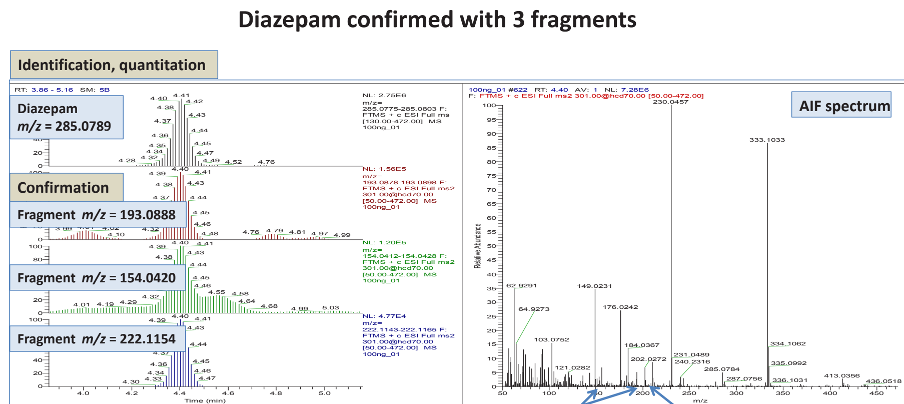
- Examples of data collected with the method are presented in Figure 1.
- Representative chromatograms for the lowest calibration standards of selected analytes are presented in Figure 2.
- Linearity ranges, method precision and % recovery in spiked urine samples from 15 different donors are presented in Table 1.
- Examples of calibration curves are shown in Figure 3.

FIGURE 1. Representative chromatograms and spectra of tramadol and diazepam in pooled urine spiked at 100 ng/mL. Shown are the full-scan chromatogram of parent compound for quantitation, chromatogram of fragments from AIF spectra reconstructed with *m/z* accuracy of 5 ppm, and AIF spectra for the scan collected at the apex of the peak. Chromatograms are reconstructed in stick mode to demonstrate sufficient number of scans across the peak.

Tramadol confirmed with 1 fragment



Diazepam confirmed with 3 fragments



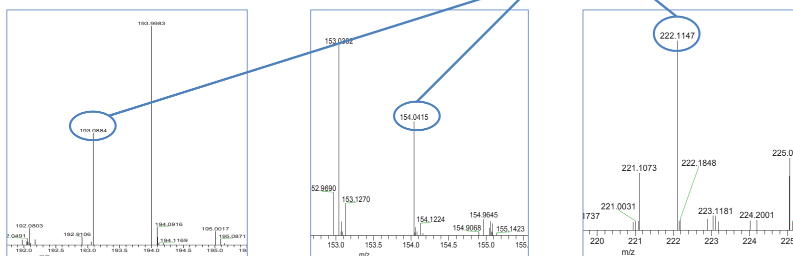


FIGURE 2. **Chromatograms of the lowest calibration standards for selected analytes as displayed in TraceFinder software.**

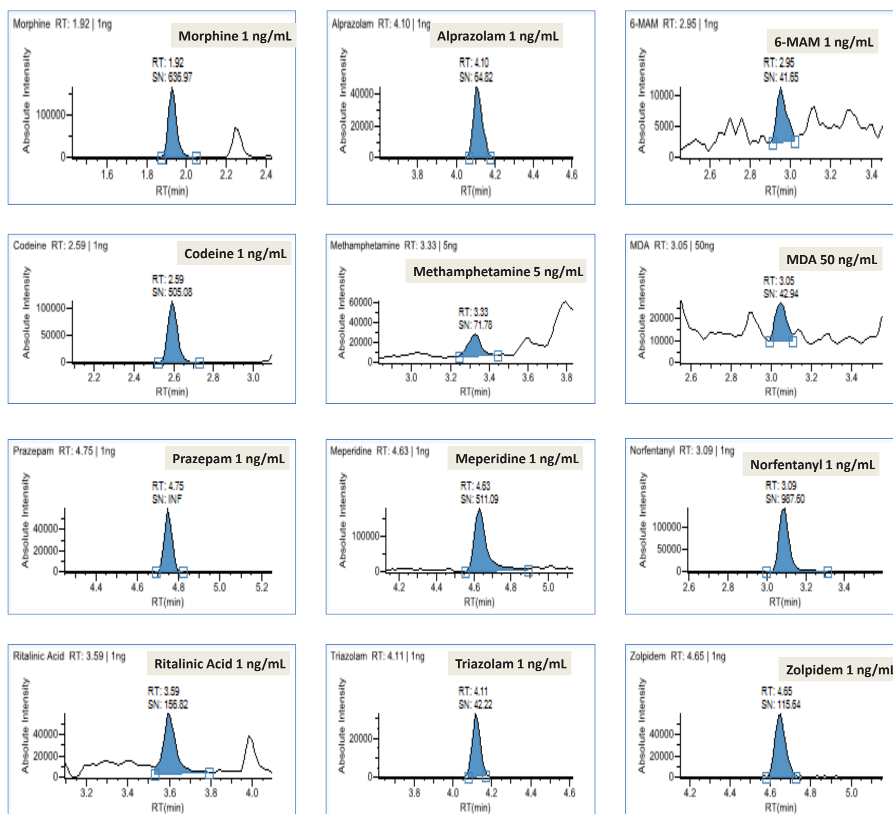
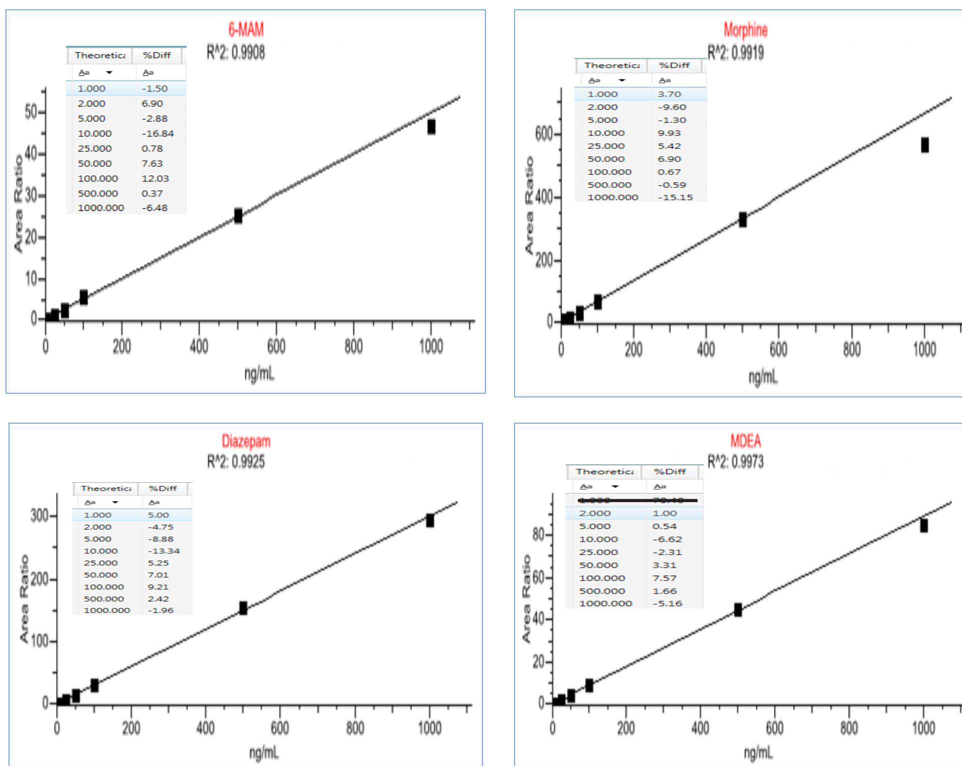


Table1. Linearity ranges, method precision and % recovery in spiked urine samples from 15 different samples.

Compound	Calibration range (ng/ml)	Precision 2 ng/mL (%RSD)	Precision 10 ng/mL (%RSD)	Spike Recovery	Compound	Calibration range (ng/ml)	Precision 2 ng/mL (%RSD)	Precision 10 ng/mL (%RSD)	Spike Recovery
6-MAM	1–1000	10	9.6	97.6–127	Midazolam	1–1000	20.8	9.9	104–126
7-aminoclonazepam	1–1000	8.1	9.1	87.0–118	Morphine	1–1000	16.2	10.6	99.2–126
Alprazolam	1–1000	10.5	8.9	96.5–110	Nordiazepam	1–1000	16.8	8.7	92.4–110
Clonazepam	1–1000	13	6.8	91.4–118	Norfentanyl	1–1000	13.1	10.1	96.8–119
Clorazepate	1–1000	22	8.7	92.4–107	Norhydrocodone	1–1000	14	9.6	97.5–118
Codeine	1–1000	13.6	7.7	96.4–113	Normeperidine	1–1000	8.5	8.9	105–123
Diazepam	1–1000	11.8	8	98.9–116	Noroxycodone	1–1000	15.7	7	95.6–128
Dihydrocodeine	1–1000	13.6	9.7	97.7–112	Desmethyltramadol	1–1000	12.4	8.7	95.6–121
Flurazepam	1–1000	9.5	12.9	92.7–111	Oxazepam	1–1000	10.5	9.2	90.2–124
Hydrocodone	1–1000	8.4	8.1	94.0–117	Oxycodone	1–1000	19.3	12.2	99.0–126
Hydromorphone	1–1000	13.6	7.7	96.8–121	Oxymorphone	1–1000	12.8	8.4	93.2–117
Lorazepam	2–1000	18.2	12.7	86.5–122	Prazepam	1–1000	8.5	6.7	96.1–114
MDA	50–1000	NA	11.1*	88.7–112	Ritalinic Acid	1–1000	7.1	10	98.0–122
MDEA	2–1000	21.5	13.8	106–128	Tapentadol	1–1000	11.9	11.1	98.5–116
MDMA	25–1000	NA	16.9*	106–136	Temazepam	1–1000	10.5	9.8	91.5–114
Meperidine	1–1000	13.7	13.2	103–125	Tramadol	1–1000	12.1	11.9	107–129
Methadone	5–1000	NA	15.6	80.4–128	Triazolam	1–1000	8.5	8.9	96.6–110
Methamphetamine	5–1000	NA	18.1	104–138	Zolpidem	1–1000	14	12.2	99.4–116
Methylphenidate	1–1000	8.5	13.3	110–123					

* Precision obtained for QC sample at concentration of 50 ng/mL

FIGURE 3. **Example of calibration curves and calibration standard accuracy tables for selected compounds. Note: tolbutamide was used as internal standard for all analytes.**



Conclusion

- A method was developed for quantitative screening which can be used for analysis of a virtually unlimited number of compounds in forensic toxicology.
- Method linearity, precision and accuracy meet requirements for quantitative screening in forensic toxicology laboratories.
- Method is robust: limited matrix effects were observed.
- Method can be multiplexed on dual channel LC system resulting in an analytical time of less than 5 minutes.

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Quantitation of Seven Designer Cathinones in Urine Using Q Exactive Mass Spectrometer

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Overview

Purpose: To evaluate various scan modes available through high-resolution, accurate-mass analysis to determine suitability for *in vitro* plasma protein binding assay analysis.

Methods: An *in vitro* plasma protein binding assay was analyzed using various scan modes available to a high-resolution, accurate-mass analysis LC-MS system and the results compared to data obtained using a triple quadrupole mass spectrometer.

Results: The lower limit of detection was found to be between 5 nM and 50 nM in full scan mode. The 5 nM was detected for a majority of the samples analyzed using full scan mode. The signal response was determined to be linear across 3 orders of magnitude for most test compound calibration curves. The results for the calculated amount of the free fraction remaining (% Free) for the binding assay demonstrated a good correlation between the results for the high-resolution, accurate-mass analysis and the results collected using LC-MS/MS analysis. Sample analysis performed using SIM mode provided a lower limit of detection of 5 nM for all compounds in the assay calibration curve demonstrating an improvement in sensitivity for several compounds in the more targeted scan mode.

Introduction

High-resolution mass spectrometers are becoming increasingly more powerful and capable of sophisticated scanning experiments that offer new solutions to complex challenges. Additionally, assays that fall into a well defined and routine workspace, such as *in vitro* screening assay in early drug discovery, will also benefit from the ease of use and high performance of high-resolution mass spectrometric analysis but do not require all available scan capabilities needed for more complex applications. In this evaluation several different full scan and SIM analyses were used to analyze a protein plasma binding assay with an Thermo Scientific™ Orbitrap™ mass analyzer and the results compared to previous analysis performed using traditional LC-MS/MS on a triple quadrupole mass spectrometer.

Methods

Sample Preparation

A set of 24 of commercially available drug compounds was selected based on reported binding properties and molecular weight and incubated in an *in vitro* plasma protein binding assay in triplicate at a concentration of 10 µM. Samples were incubated for 6.5 hours in a dialysis block followed by protein precipitation. Protein precipitation was performed by first adding 150 mL of acetonitrile containing internal standard compound (Alprenolol) to a 96-well 340-mL V-bottomed storage plate followed by addition of 50 mL of each of the assay samples. Calibration curves were also generated for each compound. A working stock solution of 50 mM in DMSO was first made for each compound. A five-point standard curve at concentrations of 5, 50, 500, 1000 and 2000 nM was prepared for each compound by serial dilution from the working stock solution into a blank mixed matrix using an eight channel pipette[1].

Liquid Chromatography

Gradient elution was accomplished using water (A) + 0.1% Formic Acid (v/v) and Acetonitrile (B) + 0.1% Formic Acid (v/v). The gradient was held at 98% aqueous for 0.25 minutes, ramped to 98% B over 0.35 minutes, and held at 98% B for 0.2 minutes before returning to the starting conditions at 2% B for a 0.4 minute equilibration time.

Chromatographic separation was performed using a C18, 2.1 x 30 mm, 3µm column with 5uL injections made for each sample. All injections were completed using a Thermo Scientific™ Accela™ Open system with DLW (Dynamic Load and Wash) and with Thermo Scientific™ Accela™ 1250 pumps at a flow rate of 900 µL/min.

Mass Spectrometry

Samples were analyzed using both a Thermo Scientific™ Exactive™ Plus mass spectrometer in Full Scan mode (*m/z* 220 – 900) and a Thermo Scientific™ Q Exactive™ mass spectrometer in both Full Scan (*m/z* 220 – 900) and SIM mode with each using a resolution setting of 35,000 (FWHM) at *m/z 200* and a spectral speed of 7 Hz. Generic ion source conditions were used for all sample collection including vaporizer temperature (350 °C), capillary temperature (300 °C), sheath gas of 45 arbitrary units, and an auxiliary gas of 10 arbitrary units. The instrument was calibrated in positive ion mode before sample acquisition using Thermo Scientific™ Pierce™ LTQ Velos™ ESI Positive Ion Calibration Solution.

Data Analysis

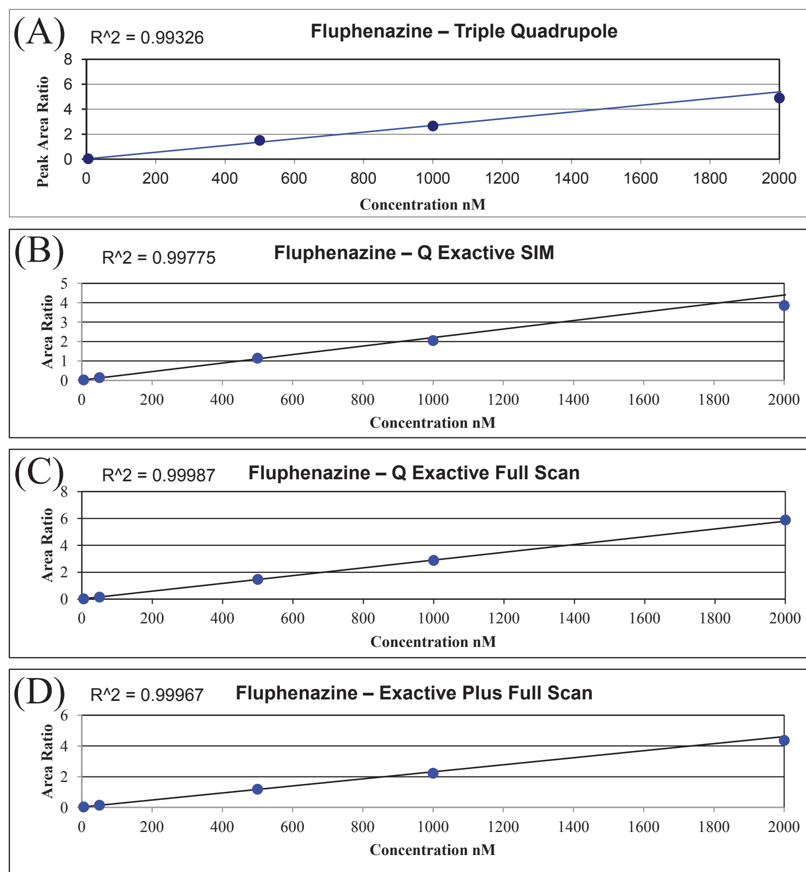
Data was acquired using Thermo Scientific™ Xcalibur™ 2.2 and Exactive Tune 2.1 software. Chromatographic data review and calibration curve generation was performed and reported using Thermo Scientific™ QuickCalc software (powered by Gubbs Inc., GMSU Gubbs™ Mass Spec Utilities, Atlanta, GA). Peak area measurements in the buffer chamber of the dialysis plate were compared to the peak area measurement in the serum chamber of the dialysis plate to calculate the percent of unbound compound (% Free) at assay equilibrium[1]. The average % Free for each compound replicate was reported for each analysis scan type and compared to values obtained using a triple quadrupole mass spectrometer. The coefficient of variation of the % Free values for each scan mode was also calculated for each compound analyzed.

Results

Scan Mode Signal Response

Each compound analyzed in the plasma protein binding (PPB) assay was evaluated in a concentration curve to evaluate overall sensitivity and linear dynamic range. All compounds were serially diluted using PPB matrix blank solution with concentrations ranging from 5 nM to 2000 nM concentration and analyzed using full scan and SIM analysis. The calibration curves for all compounds were generated using a linear regression and $1/x^2$ weighting. Individual calibration points exceeding a % difference of more than 20% of the regression line fit were excluded from the calibration curve. The majority of the compounds analyzed in full scan and SIM mode analysis exhibit the required sensitivity and linear dynamic range across the full range of the serial dilution and correlate well to the results collected using MS/MS analysis with a triple quadrupole mass spectrometer. Example calibration curves for each evaluated scan mode is displayed below for Fluphenazine (Figure 1).

FIGURE 1. Calibration curve of Fluphenazine in each scan mode. (A) MS/MS analysis, (B) Q Exactive SIM analysis, (C) Q Exactive Full Scan Analysis, (D) Exactive Plus Full Scan Analysis



The calibration curves for twenty-three of the twenty-four compounds analyzed using MS/MS analysis were linear across the full range of the calibration curve. One compound calibration curve in the MS/MS analysis required the exclusion of the 2000 nM calibration point due to signal saturation. Six of the twenty-four compounds analyzed using full scan and SIM mode analysis required the exclusion of the 2000 nM calibration point due to signal saturation (Figure 2). High-resolution analysis using an Orbitrap mass analyzer enables a user-definable parameter for the amount of target ions collected for each scan during analysis. An increase in the amount of ions collected during each scan should limit the effects of signal saturation for future analysis. Due to sample volume limitations, optimization of the ion collection target could not be performed for this experiment. Full scan analysis of the compound calibration curves demonstrated adequate sensitivity for the analysis of the calibration curves for twenty of twenty-four compounds or 83%. One compound demonstrated improved sensitivity in full scan mode using the Q Exactive Orbitrap MS, while all other calibration curve signal responses were consistent for full scan analysis across both high-resolution platforms.

FIGURE 2. Heat map display of compound calibration curve points included and excluded for each scan mode used for analysis. Calibration points with a % Difference greater than 20% were excluded from the linear regression. Excluded calibration points common to 3 scan modes are labeled in yellow. Excluded calibration points in 2 or fewer scan modes are labeled in red.

	Exactive Plus Full					Q Exactive Full				
Compound	5 nM	50 nM	500 nM	1000 nM	2000 nM	5 nM	50 nM	500 nM	1000 nM	2000 nM
Propranolol										
Diltiazem										
Imipramine										
Halperidol										
Carbamazpine										
Chlorpheniramine										
Phentolamine										
Buspirone										
Verapamil										
Desipramine										
Clozapine										
Acebutolol										
Retonavir										
Thioridazine										
Nefazadone										
Timolol										
Minaprine										
Fluphenazine										
Metoprolol										
Ticlopidine										
Compound A										
Erythromycin										
Clomipramin										
Bendamustine										

	Q Exactive SIM					Triple Quadrupole				
Compound	5 nM	50 nM	500 nM	1000 nM	2000 nM	5 nM	50 nM	500 nM	1000 nM	2000 nM
Propranolol										
Diltiazem										
Imipramine										
Halperidol										
Carbamazpine										
Chlorpheniramine										
Phentolamine										
Buspirone										
Verapamil										
Desipramine										
Clozapine										
Acebutolol										
Retonavir										
Thioridazine										
Nefazadone										
Timolol										
Minaprine										
Fluphenazine										
Metoprolol										
Ticlopidine										
Compound A										
Erythromycin										
Clomipramin										
Bendamustine										

Incuded in Curve

Excluded from curve %Diff > 20%

Excluded from curve %Diff > 20% and observed in 2 or fewer of the scan modes

Analysis in SIM mode using the Q Exactive MS provided adequate sensitivity for all compounds analyzed and provided a sensitivity improvement for some compounds over full scan analysis (Figure 2).

PPB % Free Calculation

Percent free or unbound amount of compound in the protein binding assay was calculated for each scan mode used for analysis[1]. The coefficient of variation of the % Free across each scan mode was calculated for each compound and the results were listed in a table and sorted from lowest to highest by %CV (Table 1).

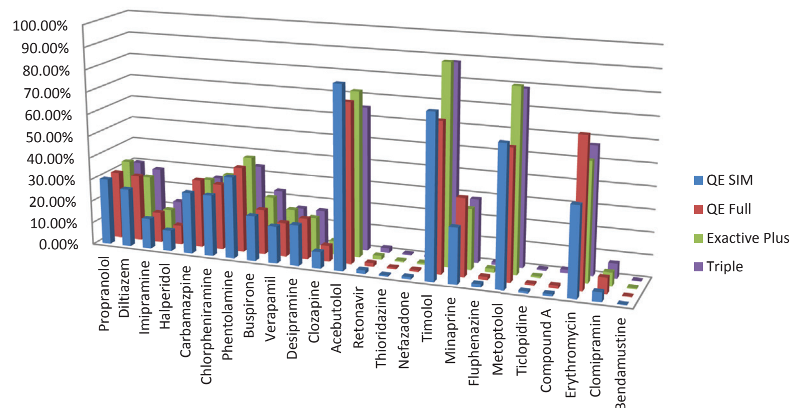
Cells highlighted in red in Table 1 denote a scan mode that did not provide sufficient signal for a specific compound to generate a % Free value and were excluded from the %CV calculation for the respective compound.

Table 1. % Free for analyzed compounds in each scan mode and %CV across scan modes. Cells highlighted in red denote scan modes with no results due to lack of analyte signal.

Q Exactive SIM	QE SIM	QE Full	E Plus Full	Triple			
Compound	% Free	% Free	% Free	% Free	Avg(%)	StdDev(%)	% CV
Propranolol	30.03	30.45	33.01	30.00	30.87	1.44	**4.66**
Diltiazem	26.36	29.80	26.72	27.70	27.64	1.54	**5.58**
Imipramine	13.73	13.67	12.11	13.10	13.15	0.75	**5.69**
Halperidol	9.38	8.73	10.04	9.50	9.97	1.56	**5.70**
Carbamazpine	27.75	30.74	28.28	26.40	28.29	1.81	**6.40**
Chlorpheniramine	27.60	29.79	31.40	27.00	25.06	6.26	**7.00**
Phentolamine	36.84	38.43	40.39	33.80	37.36	2.79	**7.46**
Buspirone	20.33	20.22	23.04	23.30	20.12	2.73	**7.71**
Verapamil	16.66	15.29	18.43	16.20	16.64	1.32	**7.92**
Desipramine	18.37	18.32	15.76	16.10	17.14	1.40	**8.18**
Clozapine	7.45	7.10	6.10	6.70	6.88	0.58	**8.42**
Acebutolol	82.00	72.00	74.52	65.10	73.41	6.98	**9.51**
Retonavir	1.61	1.59	1.70	2.00	1.58	0.36	**11.01**
Thioridazine	0.60	0.69	0.58	0.50	0.64	0.13	**12.93**
Nefazadone	1.00	0.80	0.73	0.90	0.86	0.12	**13.73**
Timolol	73.40	67.20	90.13	88.10	79.71	11.19	**14.03**
Minaprine	25.05	34.90	27.30	28.60	21.97	7.32	**14.57**
Fluphenazine	1.53	1.38	1.70	1.20	1.51	0.31	**14.64**
Metoptolol	62.92	58.52	82.10	79.00	70.64	11.66	**16.51**
Ticlopidine	0.91	0.75	0.73	0.60	0.75	0.13	**16.74**
Compound A	1.00	1.20		1.50	1.24	0.25	**20.22**
Erythromycin	40.00	66.49	53.13	57.10	54.18	10.99	**20.28**
Clomipramin	4.31	7.10	6.08	6.70	5.66	1.99	**20.38**
Bendamustine	0.18	0.19		0.20	0.26	0.13	**5.41**

The calculated % Free values for each compound were plotted in a bar chart to illustrate differences in the % Free values across each scan mode for the PPB analysis.(Figure 3).

Figure 3. % Free for individual compounds across each scan mode used for assay analysis. Twenty-two of twenty-four compounds analyzed demonstrate a %CV of less than 25% across the various scan modes while providing adequate sensitivity for assay analysis across all scan modes.



Twenty-two of the twenty-four compounds analyzed in the protein binding assay provide a %CV of less than 25% across the various scan modes while providing adequate sensitivity for analyte analysis in the binding assay. Although four compounds did not provide enough signal in the calibration curve analysis only two did not provide enough signal for % Free calculation in the PPB assay itself. 92% of the compounds analyzed provided sufficient signal in both full scan and SIM mode with a %CV of less than 25%. The two compounds that did not provide enough sensitivity to generate a % Free value in the binding assay were challenging in full scan on the Exactive Plus only and not on the Q Exactive. One explanation for this observation maybe due to the generic mass spec and chromatographic conditions used for data analysis. Although both instruments collected data in full scan mode, the Q Exactive filters all ions outside of the specified full scan mass range. While the Exactive Plus does filter some ions at the s-lens, additional ions outside the specified mass range are also collected and injected into the Orbitrap Mass Analyzer. Further optimization of the ion target amount collected per scan in the mass spec method along with optimized chromatographic clean up of the assay samples in the generic method may improve signal response in full scan mode in the absence of true ion filtering with a quadrupole and will be evaluated in future work.

Conclusion

- 83% of compounds analyzed met the assay calibration curve LOQ of 5nM.
- 92% of the compounds provided sufficient signal in the assay for calculation of the % Free in all scan modes evaluated.
- Full scan analysis using high resolution accurate mass provided adequate signal response and linear dynamic range to accurately measure 92% compounds analyzed in the PPB assay.
- Additional sensitivity and linear dynamic range may be achieved through further method optimization.

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High-Resolution, Accurate-Mass Forensic Toxicology Screening in Blood Samples Using a Q Exactive Mass Spectrometer

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Overview

Purpose: To evaluate the Thermo Scientific™ Q Exactive™ High-Resolution Mass Spectrometer in Forensic Toxicology Screening for whole blood analysis and make a comparison with Targeted Screening on a Triple Quadrupole MS using the SRM (Selected Reaction Monitoring) mode and also UPLC/Diode Array Detection (DAD).

Methods: Blood samples were spiked with internal standards and extracted with TOXI-TUBES™ A (Agilent Technologies, Santa Clara, CA). LC separation was performed with a 30 minute gradient. Mass spectrometry data were acquired in Full Scan and MS^2 mode using the Q Exactive MS.

Results: Data collected show benefits of high-resolution screening over both the triple quadrupole approach and DAD detection.

Introduction

Forensic scientists and forensic toxicologists need to identify an unlimited number of compounds in complex matrixes with the capability of retrospective data analysis for quick and confident analysis. The major challenge is to separate the analytes of interest from the matrix and accurately identify them. Here we evaluated the Q Exactive MS, a bench-top quadrupole-Orbitrap™ ultra-high resolution mass spectrometer routinely capable of better than 5 ppm mass accuracy and 140,000 FWHM resolution, with Thermo Scientific™ ExactFinder™ data processing software, for forensic toxicology screening in blood samples. We will also compare the results with those obtained by forensic targeted screening using an SRM approach and DAD detection.

Methods

Sample Preparation

500 µl of each blood sample was spiked with 20 µl of an internal standard solution (Flurazepam at1 mg/L) and extracted with TOXI-TUBES A™ (Agilent Technologies). The organic layers were transferred, evaporated to dryness, reconstituted in 2.5 ml of a mixture containing 70% of mobile phase A and 30% of mobile phase B, and injected onto the Q Exactive MS. For triple quadrupole analysis and DAD detection, the sample was reconstituted in 500 µl and 100 µl, respectively, of the mixture described above.

Liquid Chromatography

The U-HPLC comprises Thermo Scientific™ Accela™ 1250 pumps with an Accela Autosampler. Mobile phases are 10 mM Ammonium formate and 0.1% Formic acid in water (A) and 0.1% Formic acid in Acetonitrile (B). The LC separation was performed on a Thermo Scientific™ Hypersil™ GOLD PFP column 150 x 2.1 mm 3µm.

FIGURE 1. HPLC Gradient Method

Start (min)	Flow (mL/min)	%A	%B
0.00	0.2	95	5
5	0.2	55	45
18	0.2	30	70
20	0.2	5	95
27	0.2	5	95
27.1	0.2	95	5
32	0.2	95	5

Mass Spectrometry

Compounds are detected on a Q Exactive mass spectrometer equipped with an Orbitrap mass analyzer. A schematic diagram of the Q Exactive MS is illustrated in Figure 2. A Heated Electrospray Source Ionization (HESI) probe was used as an ion source. The instrument was operating in alternating positive and negative full scan mode. Each Full Scan was followed by 8 high-resolution MS^2 scans in positive mode and 3 high-resolution MS^2 scans in negative mode. Precursor selection was done in the data-dependent operation mode where the most intense ion of the previous scan was selected for fragmentation. Resolution was set to 70,000 FWHM for each full scan mode and 17,500 FWHM for MS^2 scan acquisition.

MS² spectra were acquired with a Normalized Collision Energy (NCE) of 70. Relevant scan and source parameters are shown in Figures 3 and 4.

DAD Detection

Data have been acquired on a UPLC-Acquity™ (Waters Corporation, Milford, MA) equipped with a DAD detector. The library contains 612 molecules. Acquisition is performed using a 15 minute LC gradient.

Triple Quadrupole Detection

Six different targeted LC/MSMS methods have been used to acquire data in SRM (Selected Reaction Monitoring) mode. This method includes 97 molecules.

FIGURE 2. Schematic diagram of the Q Exactive High-Resolution, Accurate-Mass Instrument.

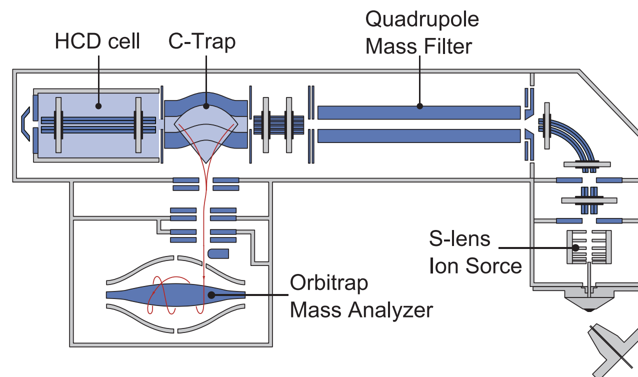


FIGURE 3. Scan Parameters for Q Exactive Mass Spectrometer

Parameter	Value
Full MS	
Microscans	1
Resolution (FWHM)	70,000
AGC Target	1e6
Maximum IT	250 msec
Scan Range	150-800 m/z
MS² Experiments	
Microscans	1
Resolution	17,500
AGC Target	1e5
Maximum IT	250 msec
NCE	70.0

FIGURE 4. Source Parameters for HESI Probe.

Parameter	Value
Sheath Gas	30
Aux gas	15
Spray voltage (V)	3500
Capillary temp (°C)	320
Vaporizer Temp (°C)	350

* Parameters are the same for positive and negative modes

Data Analysis

All MS data have been processed using ExactFinder 2.0 software. Identification of the analytes is performed using the exact mass of the precursor, the retention time, the isotopic distribution and the fragment exact masses.

Results

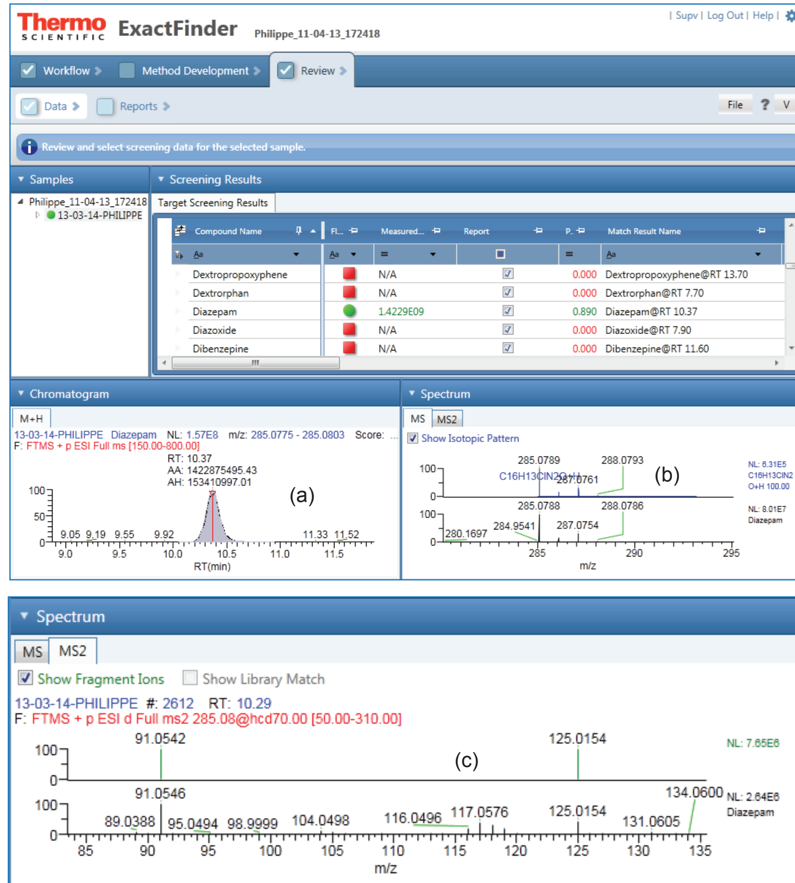
Data Processing

Chromatograms were reconstructed with a 5 ppm mass accuracy. The method was set to identify compounds based on the exact mass of the parent and the retention time. Confirmation was performed using the isotopic pattern and up to 5 fragment ions obtained from each precursor. A database containing up to 650 analytes was selected for processing. Figure 5 shows an example of the results page showing the XIC (extracted ion chromatogram) for Nordiazepam reconstructed with 5 ppm mass accuracy (a), isotopic pattern (b) and fragment ion confirmation (c).

Results are reported using flags of different colors :

- (green circle): When the sample/compound/peak combination is identified and fully confirmed.
- (yellow triangle): When the sample/compound/peak combination is identified but not fully confirmed.
- (red square): When the sample/compound/peak combination is not identified.

FIGURE 5. ExactFinder results page showing XIC chromatogram for Diazepam reconstructed with 5 ppm mass window (a), isotopic pattern (b) and fragment ion confirmation (c).



Metabolite Identification

In addition to compound identification, it is possible to confirm the results by identifying potential metabolites present in the sample. The approach is simple. As the acquisition is performed in Full Scan mode, identification of metabolites can be realized with the same HR-MS analysis by only extracting therorетical *m/z* values for predicted biotransformations. Figure 6 shows an example of metabolites identified from a single sample. The main compound identified is methadone and we have also been able to identify two major metabolites: EDDP and EMDP.

FIGURE 6. ExactFinder results page showing XIC chromatogram for EDDP (a), EMDP (b) and Methadone (c) reconstructed with 5 ppm mass window.

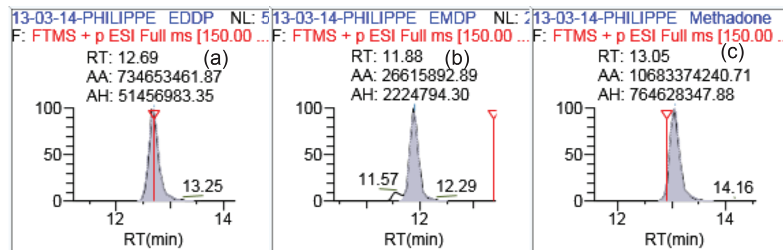
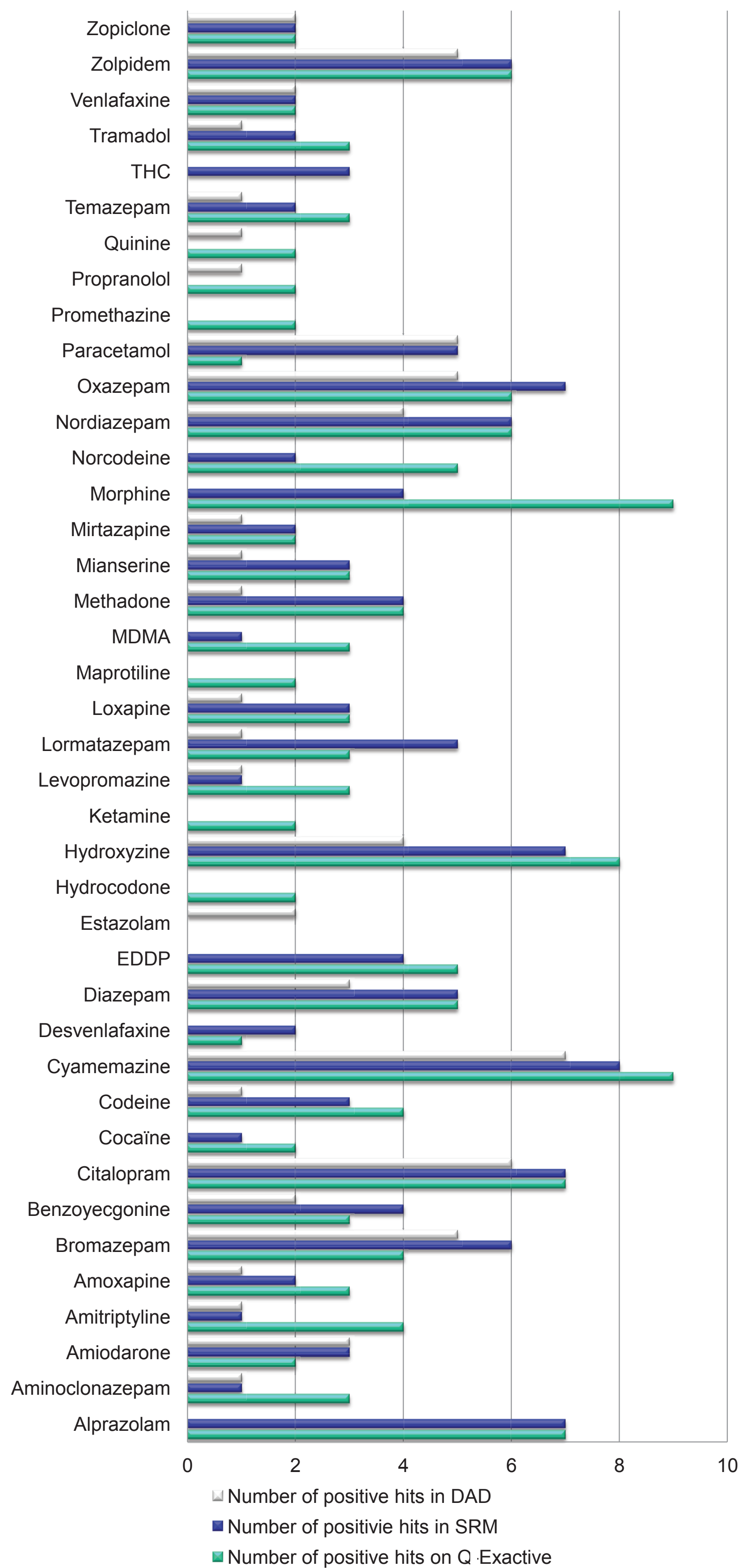


FIGURE 7. List of analytes that have been identified among 39 samples and confirmed using the 3 approaches: targeted screening in SRM, DAD and Q Exactive screening.



Comparison between the different approaches: DAD detection, targeted screening using a triple quadrupole, HRAM screening using the Orbitrap technology

We've analyzed and compared 39 samples using the 3 different technologies. Overall, the HRAM approach allowed identification of a higher number of analytes than the other approaches. We have been able to identify 143 compounds with the HRAM approach, 121 with the six targeted forensic screening methods performed on the triple quadrupole MS and 69 compounds using the DAD. Some of the results are reported in Figure 7 where we compare for 40 analytes (among the 77 identified) the number of positive hits obtained for each approach.

DAD Approach

Fewer analytes have been identified using this approach despite the size of the library (612 analytes). Sensitivity is certainly the main concern with this technique. Moreover, DAD may provide in some cases some false positive results. For example estazolam has been identified in DAD but not confirmed using the MS technologies. This approach is well known for its poor sensitivity in benzodiazepines analysis. As reported in Figure 7, alprazolam is not detected with DAD but is confirmed using the other two approaches.

Triple Quadrupole Approach Using the Six Targeted SRM Methods.

This approach gives good results in terms of positive hits identified. THC was identified using this approach as the sample preparation was done in acidic conditions unlike the other approaches where basic conditions were used. There are still some limitations. The identification is confirmed using six different SRM methods which means that we may have to inject the same sample several times. Moreover these six methods contain only 97 analytes. The run is performed in SRM mode and for this reason there is no capability for retrospective analysis and potential metabolite identification.

HRAM Approach Using the Q Exactive MS

This approach is able to identify the largest number of analytes with the 650 analytes library. But there are still some limitations to overcome. Precursor selection was done in the data-dependent operation mode where the most intense ion of the previous scan was selected for fragmentation. So we may, in some cases, have to add the compounds in the inclusion list in order to not miss the MS^2 acquisition. Some of the analytes listed are isomers (eg: maprotiline, paroxetine and EDDP). As they have exactly the same exact mass, we have to make sure they present different fragment ions in MS^2 or elute at different retention times. All data have been processed though ExactFinder 2.0 software with a 5 ppm mass accuracy. In this version of the software, the mass accuracy is set and can't be adjusted. For this reason, low mass fragments like the one we have with paracetamol at *m/z* 110.0595 are in some cases not properly identified with an accuracy of 5 ppm. This limitation is nevertheless going to be overcome with the launch of Thermo Scientific™ TraceFinder™ 3.0 where the mass accuracy is set by the user and can be expressed in ppm or milli-amu.

Conclusion

- The Q Exactive MS provides high confidence with high-resolution capabilities (up to 140,000 FWHM) for forensic screening.
- Data processing is performed using ExactFinder 2.0 software. Compounds are identified and confirmed using the exact mass of the precursor, the isotopic distribution, the retention time and the exact mass of up to 5 fragment ions.
- HRAM LC/MSMS method identified more compounds for forensic toxicology than Diode Array Detection and Triple Quadrupole Targeted SRMs methods.
- Additional information such as metabolites identification can be easily obtained by extracting the theoretical *m/z* values for predicted biotransformations
- This HRAM method also allows for retrospective data analysis.
- A new HRAM database (https://www.mzcloud.org/) will soon be available to perform targeted and also unknown identification.

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Verification of the Simultaneous Analysis of Heroin Addiction Treatment Compounds Using LC/MS/MS with a New Prelude SPLC™ System

Sarah Fair, Joseph Herman, and Kerry Hassell
Thermofisher Scientific, Franklin, MA

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Overview

Purpose: There are several compounds used for the treatment of heroin addiction. These compounds include methadone, buprenorphine, norbuprenorphine, naloxone, naltrexone , and their metabolites. The metabolites of interest are 2-ethylidine-1,5 dimethyl-3,3-diphenylpyrrolidine (methadone metabolite, aka EDDP), buprenorphine glucuronide (buprenorphine metabolite), and norbuprenorphine glucuronide (norbuprenorphine metabolite). All total, the analysis of these compounds for research includes 8 analytes with 4 internal standards, that are commonly used in the treatment of heroin addiction.

Methods: Samples for this analysis were prepared in human urine. After the addition of internal standard, they were injected for analysis using the Thermo Scientific™ Prelude™ SPLC sample preparation-liquid chromatography system. This system was fitted with a Thermo Scientific™ Accucore™ 100x3.0, 2.6 μm particle size column for separation. Additionally, a Thermo Scientific™ TSQ Vantage™ mass spectrometer in positive ion mode was used for analyte detection.

Results: All 8 compounds were simultaneously verified using the Prelude SPLC system and the TSQ Vantage MS. The resulting chromatography, correlation coefficients, standard curve linearity, quality control data, and analyte transitions are explained in the following sections to illustrate the success of this analysis.

Introduction

Several different compounds are currently used in the treatment of heroin addiction. These compounds and their metabolites were analyzed for research using the new Prelude SPLC system and a TSQ Vantage MS. This workflow takes advantage of a low system volume to decrease solvent consumption and successfully quantify methadone, 2-ethylidine-1,5 dimethyl-3,3-diphenylpyrrolidine (EDDP), buprenorphine, buprenorphine glucuronide, norbuprenorphine, norbuprenorphine glucuronide, naloxone, and naltrexone. This work verifies a heroin treatment panel method performed on the Prelude SPLC system. In order for this system to be evaluated, it must fall within certain acceptance criteria. These set parameters are designed to determine the success or failure of a particular LC-MS/MS workflow. These parameters include, but are not limited to:

1) The lower limit of quantitation (LLOQ) and low quality control need to be ±20% of the expected concentration.
2) All of the remaining calibrators and controls need to be ±15% in order for the instrument to be successfully validated.
3) All of these requirements must be met for three consecutive days so that interday and intraday accuracy and precision can be determined.
4) The signal in the blank following the highest standard may not exceed 20% of the LLOQ signal. This factor is often called carryover.

Methods

Sample Preparation

Human urine was spiked with all 8 analytes and then serial diluted into a calibration curve. Buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide had an analytical measurement range of 1.0 ng/mL to 100 ng/mL. Methadone, EDDP, naloxone, and naltrexone had an analytical measurement range of 5.0 ng/mL to 500 ng/mL. Quality controls were also prepared in human urine at three different levels. The urine aliquots were diluted with a combination of water and methanol that contained internal standards. These samples are then injected onto the system for analysis.

Liquid Chromatography

Chromatographic separations of all compounds were performed using Prelude SPLC system, seen in Figure 1, equipped with an Accucore 100x3.0mm C18 analytical column with 2.6 μm particle size. The system mobile phases consisted of 10mM ammonium formate, 0.05% formic acid in water and 10mM ammonium formate, 0.05% formic acid in methanol. The system needle washes were 60% water, 40% methanol, and 0.5% formic (aqueous) and 45% isopropanol, 45% acetonitrile, and 10% acetone (organic).

Mass Spectrometry and Data Analysis

The detector was a TSQ Vantage triple-stage quadrupole mass spectrometer with HESI-II ionization probe in positive ion mode. Quantitation of results was performed using Thermo Scientific™ LCQUAN™ software.

FIGURE 1. Prelude SPLC system

Results

Analyte result summary

Buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide were all prepared at a range of 1.0 ng/mL to 100 ng/mL with quality control concentrations at 3.0, 40.0, and 80.0 ng/mL. Methadone, EDDP, naloxone, and naltrexone were prepared at a range of 5.0 ng/mL to 500 ng/mL with quality control concentrations of 15.0, 200, and 500 ng/mL. Deuterated internal standards were used for each analyte. Methadone-d9 was used for the quantitation of methadone and EDDP. Naloxone-d5 was used for the quantitation of naloxone and naltrexone. Buprenorphine-d4 was used for buprenorphine and buprenorphine glucuronide, and norbuprenorphine-d3 for norbuprenorphine and norbuprenorphine glucuronide. The transitions used for the analytes and internal standards can be seen in Table 1.

Table 1. Analyte Transitions

Compound	Transition
methadone	310→265
EDDP	278→219
naloxone	328→212
naltrexone	342→270
buprenorphine	468→396
norbuprenorphine	414→187
buprenorphine glucuronide	644→468
norbuprenorphine glucuronide	590→414
methadone-d9	319→268
naloxone-d5	333→212
buprenorphine-d4	472→400
norbuprenorphine-d3	417→187

All analytes had linear calibration curves which are illustrated in Figure 2. The x-axis of each block is the area ratio of the analyte to the internal standard. The y-axis is the concentration in ng/mL. Additionally, near the top of each block the correlation coefficient values are posted. These values are also summarized in Table 2 for easier viewing. These r^2 values range from 0.9924 to 0.9995 for all compounds.

Figure 3 show the lower limit of quantitation (LLOQ) chromatograms for each of the 8 analytes. Buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide all have an LLOQ of 1.0 ng/mL while methadone, EDDP, naloxone, and naltrexone have an LLOQ of 5.0 ng/mL.

Figure 2. Calibration curve linearity for all analytes.

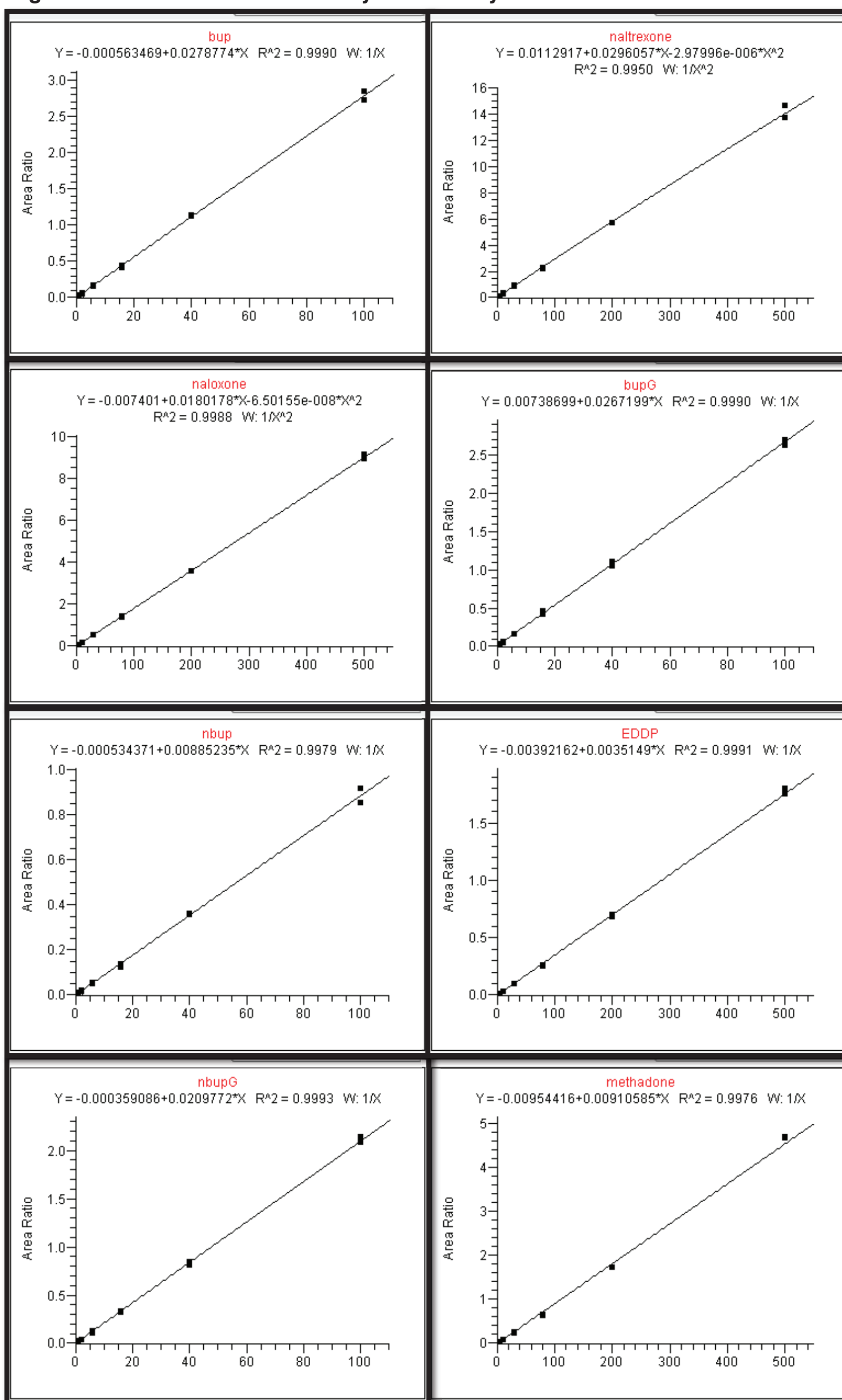

Table 2. Correlation coefficient values for all analytes

Analyte	r^2 day 1	r^2 day 2	r^2 day 3
buprenorphine	0.9949	0.9976	0.9983
norbuprenorphine	0.9985	0.9969	0.9979
buprenorphine glucuronide	0.9974	0.9982	0.9990
norbuprenorphine glucuronide	0.9993	0.9993	0.9993
methadone	0.9974	0.9976	0.9994
EDDP	0.9995	0.9991	0.9986
naloxone	0.9985	0.9942	0.9988
naltrexone	0.9951	0.9924	0.9950

Figure 3. Lower limit of quanititation (LLOQ) for all analytes.

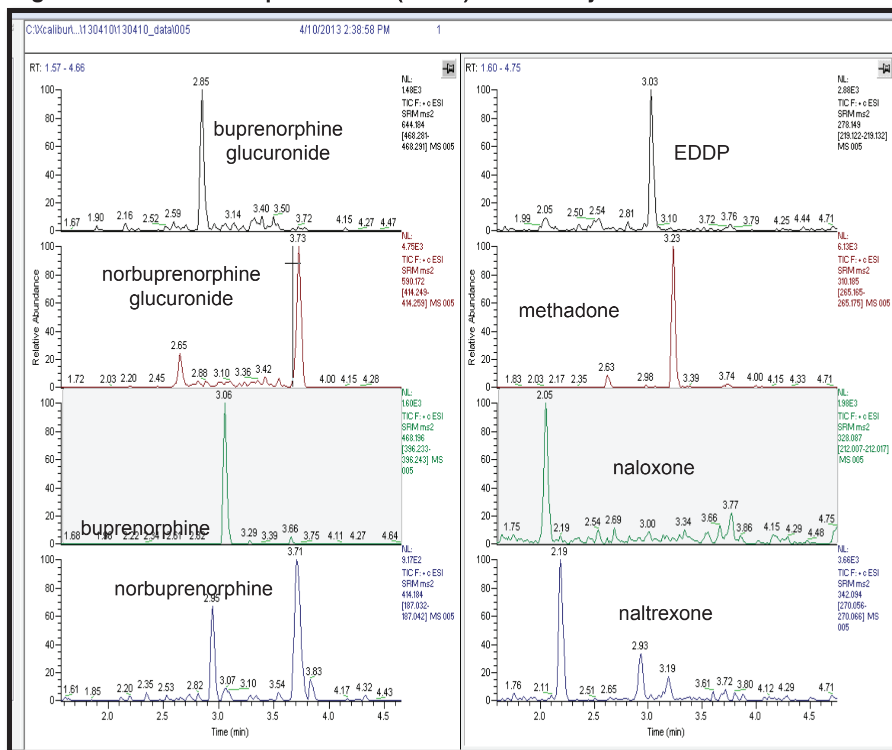

Figure 4 shows the matrix blank that is injected after the highest standard in the calibration curve often referred to as the upper limit of quantitation (ULOQ). This matrix blank (n=2) is used to assess the level of carryover for each analyte. The signal in the matrix blank cannot be greater than 20% of the LLOQ signal. All analytes have zero carryover at the retention time of interest with one exception: methadone has an average carryover of about 4.7%, but this is still well within the allowance of 20% of the LLOQ.

Figure 4. Carryover as shown in the matrix blanks injected after the ULOQ.

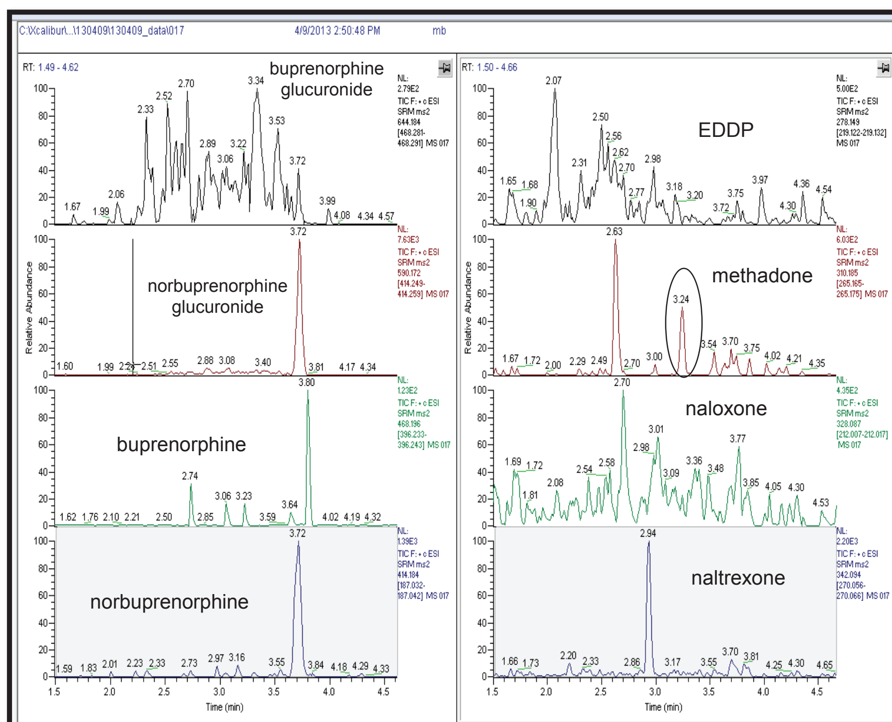

Table 3 shows the resulting quality control data from the interday and intraday accuracy and precision. Three consecutive days of runs were summarized to show the ending RSD percentages. All compounds had RSD values of ≤10% of the expected concentrations showing excellent accuracy and precision. The third column in the table shows the expected QC value with column 4, 5, and 6, showing the QC averages (run in replicates of 5) for each day. Then, in column 7, the overall average is calculated along with the standard deviation (SD) in column 8. Lastly, the %RSD can be seen in column 9.

Table 3. Quality control data summary.

Analyte	(ng/mL)	Expected	Day 1	Day 2	Day 3	Average	SD	%RSD
buprenorphine	Low QC	3.00	3.05	2.81	2.79	3.0	0.1	3.3
	Mid QC	40.0	36.4	38.7	40.0	38.0	1.8	4.7
	High QC	80.0	71.0	79.3	79.8	77.0	4.9	6.4
norbuprenorphine	Low QC	3.00	2.89	3.18	3.27	3.0	0.2	6.7
	Mid QC	40.0	37.4	39.1	37.8	38.0	0.9	2.4
	High QC	80.0	76.5	76.6	77.6	77.0	0.6	0.8
buprenorphine	Low QC	3.00	3.31	2.88	2.86	3.0	0.3	10.0
glucuronide	Mid QC	40.0	36.3	39.4	39.5	38.0	1.8	4.7
	High QC	80.0	74.3	79.8	80.9	78.0	3.5	4.5
norbuprenorphine	Low QC	3.00	2.74	3.18	3.01	3.0	0.2	6.7
glucuronide	Mid QC	40.0	37.8	38.8	38.7	38.0	0.5	1.3
	High QC	80.0	77.1	76.8	79.2	78.0	1.3	1.7
methadone	Low QC	15.0	14.8	15.6	15.1	15.0	0.4	2.7
	Mid QC	200	197	202	191	197	5.6	2.8
	High QC	400	418	412	409	413	4.9	1.2
EDDP	Low QC	15.0	14.8	14.5	14.6	15.0	0.2	1.3
	Mid QC	200	192	200	190	194	5.3	2.7
	High QC	400	398	411	399	403	7.1	1.8
naloxone	Low QC	15.0	14.7	15.5	16.5	16.0	0.9	5.6
	Mid QC	200	207	196	198	200	6.1	3.1
	High QC	400	396	415	387	399	14.6	3.7
naltrexone	Low QC	15.0	14.7	15.4	15.2	15.0	0.4	2.7
	Mid QC	200	201	192	194	195	4.8	2.5
	High QC	400	383	405	382	390	12.9	3.3

Conclusion

- All 8 compounds show excellent verification results using the Prelude SPLC system in combination with the TSQ Vantage MS. With quality control RSD percentages less than 10% and correlation coefficient values of 0.9924 to 0.9995, these verification analyses are proven to be very successful.
- Due to the low volume and low solvent consumption capabilities of the Prelude SPLC system, these compounds were analyzed for research in less time, using less solvent, and with reduced cost to a standard HPLC system
- The design of the Prelude SPLC system allows for efficient online sample clean-up that demonstrates reproducible, reliable data for all analytes, with a total injection time that less than 6 minutes.

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Quantitative Confirmatory Analysis of the NIDA 5 Panel Using Prelude SPLC System and TSQ Quantum Ultra MS

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Overview

Purpose: Develop and validate a simple and efficient quantitative LC-MS/MS method for SAMHSA-compliant confirmatory analysis of 5 panel drug using novel HPLC system.

Methods: Human urine containing the drugs were spiked with internal standards, enzymatically hydrolyzed, and diluted.

Results: The LC-MS/MS method was developed and validated to comply with SAMHSA guidelines.

Introduction

Effective on October 2011, the new SAMHSA/NIDA guidelines allow implementation of LC-MS technique to perform NIDA-5 panel, urine quantitative confirmatory analysis. LC-MS/MS methods are often less complicated than the previously implemented GC-MS/MS methods because they do not require derivatization. The NIDA-5 panel requires 6 separate quantitative methods for analysis of THCA, opiates, amphetamines, cocaine, phencyclidine and 6-MAM to confirm immunomethod positive samples. Here we developed 6 methods using a single sample preparation procedure, analytical column, mobile phase and instrument configuration. The methods are implemented on new Thermo Scientific™ dual channel Prelude ™ SPLC online sample preparation-liquid chromatography system, which allows method execution in parallel with a different method on each channel or the same method on both channels multiplexed to a single mass spectrometer.

Serial MS detection of multiplexed methods improves mass spectrometer utilization time, increases laboratory throughput and reduces analysis cost. The syringe pumps and high-pressure, low-volume gradient mixing used in the Prelude SPLC system provide enhanced LC performance including improved peak shape and resolution, stable retention times and reduced solvent consumption.

Methods

Sample Preparation

The sample prep procedure includes glucuronide hydrolysis followed by dilution. For each sample a 200-µL aliquot of urine was spiked with 10 µL of internal standard solution and 100 µL of β-glucuronidase enzyme in ammonium acetate buffer, pH=5.0. The samples were incubated at 60 °C for 2 hours. A 200-µL aliquot of methanol was added to each sample to stop enzymatic reaction. Samples were cooled down, centrifuged and diluted 20-fold with water, except for THCA, which was diluted 2-fold with water. Then 20 µL of sample was injected onto the LC-MS/MS system.

Liquid Chromatography

Chromatographic separations were performed with the Prelude SPLC system by direct injection onto a Thermo Scientific™ Accucore™ PFP 50x2.1mm, 2.6 µm analytical column. The column was maintained at room temperature. Mobile phases A and B consisted of 10 mM ammonium formate with 0.1% formic acid in water and methanol, respectively. Separate methods were set up to analyze 6-MAM, BE, PCP, and THCA. One method was set up for the combination of amphetamine, methamphetamine, MDA, MDEA and MDMA. A final method was used for the opiates morphine and codeine along with hydromorphone, hydrocodone, oxymorphone and oxycodone. Figure 1 shows the LC method for analyzing the opiates.

Mass Spectrometry

MS/MS analysis was carried out on a Thermo Scientific™ Quantum Ultra™ triple quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. MRM transitions for each compound are listed in Table 1.

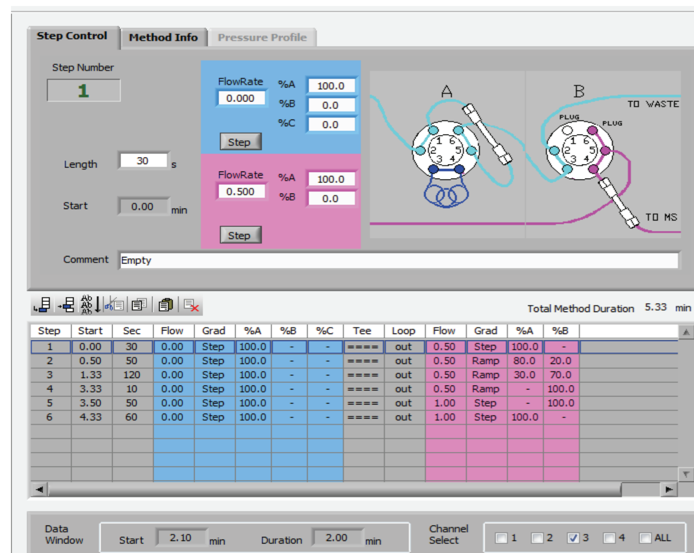
Validation

The calibration standards and quality control (QC) samples were prepared by spiking compounds into blank urine. Samples were processed as described in the Sample Preparation section. Methods were validated in multiplexed mode. Intra- and inter- method precision and accuracy were determined by analyzing a calibration curve along with replicate QCs on three different days. Matrix effects were determined by comparing peak area of samples processed in multiple lots of urine to that of one process in water. Additionally for the opiates, we were able to correlate results obtained with this method to those from a toxicology laboratory validated method.

Data Analysis

Thermo Scientific™ TraceFinder™ software was used for data acquisition and processing. Data were processed with ion ratio confirmation.

FIGURE 1. LC method for separating morphine and codeine.



Results

For each method, performance was within SAMHSA/NIDA guidelines. The quantitation limits (LOQ) for some compounds were lower than required to demonstrate method capability. The linear ranges were 2.5-2000 ng/mL for PCP and THCA; 5-2000 ng/mL for methamphetamine, BE and 6-MAM; 10-2000 ng/mL for morphine, codeine, amphetamine, MDA, and MDMA (Figure 2). The intra-method precision was <13.5%, <3.5%, <14.1%, <6.9%, < 9.6%, <15.9% for PCP, BE, 6-MAM, THCA, opiates and amphetamines respectively. The inter-method precision was <8.9%, <3.6%, <10.9%, <8.8%, <7.0%, <15.3% for PCP, BE, 6-MAM, THCA, opiates and amphetamines respectively. These results are summarized in Table 2. Limited matrix effects were seen and those were largely mediated by deuterated internal standards. The percent recovery for 8 spiked urine donor samples was in range of 80-120% (Table 3). Data collected for opiates with developed methods correlated well with toxicology laboratory data with coefficient of correlation >0.99 (Figure 4). Implementation of the dual channel Prelude SPLC system with syringe pumps improved retention time precision, chromatographic peaks shape and resolution, thus allowing for short, small solvent consumption LC methods while still keeping good data quality.

TABLE 1. List of NIDA 5 compounds MRM transitions, cutoff requirements, LOQ and Linear range

Drug	MRM (Q: Quantifier)	Cutoff (ng/mL)	LOQ (ng/mL)	Linear Range
Amphetamine	136.1-91.3 (Q), 136.2-119.3	250	10	10-5000
Methamphetamine	150.2-91.2 (Q), 150.2-119.2	250	5	5-5000
MDA	180.2-135.2 (Q), 180.2-163.2	250	10	10-5000
MDMA	194.1-163.1 (Q), 194.1-135.1	250	10	10-5000
MDEA	208.1-163.1 (Q), 208.1-135.2	250	10	10-5000
Benzoylecgonine	290.1-168.1 (Q), 290.1-105.1	100	5	5-2000
THCA	354.3-336.3 (Q), 354.3-308.3	15	2.5	2.5-2000
Phencyclidine	244.2 -159.1 (Q), 290.1-105.1	25	2.5	2.5-2000
Morphine	286.11-152.1 (Q), 286.11-165.1	2000	10	10-6000
Codeine	300.2-152.1 (Q), 300.2-165.1	2000	10	10-6000
6-Acetylmorphine	328.1-165.1 (Q), 328.1-211.1	10	5	5-2000

FIGURE 2. Representative calibration curves for BE, THCA, 6-MAM and PCP.

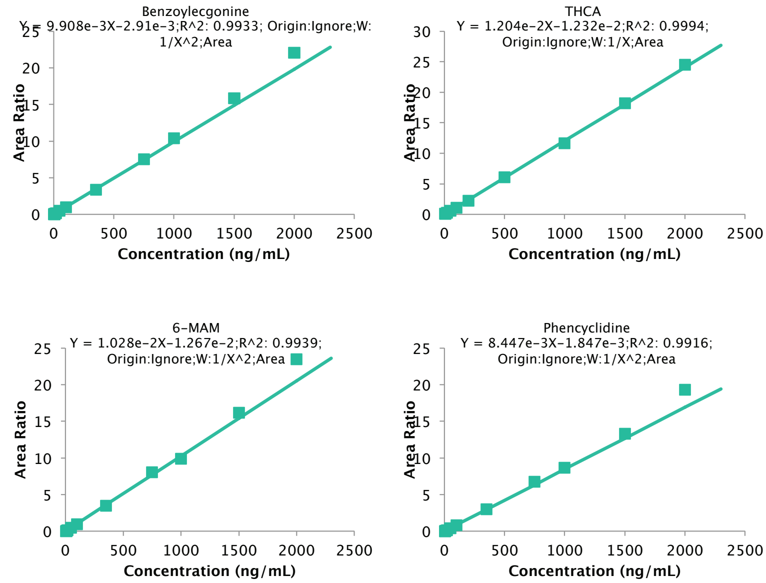


FIGURE 3. Example chromatograms for each method at respective LOQs.

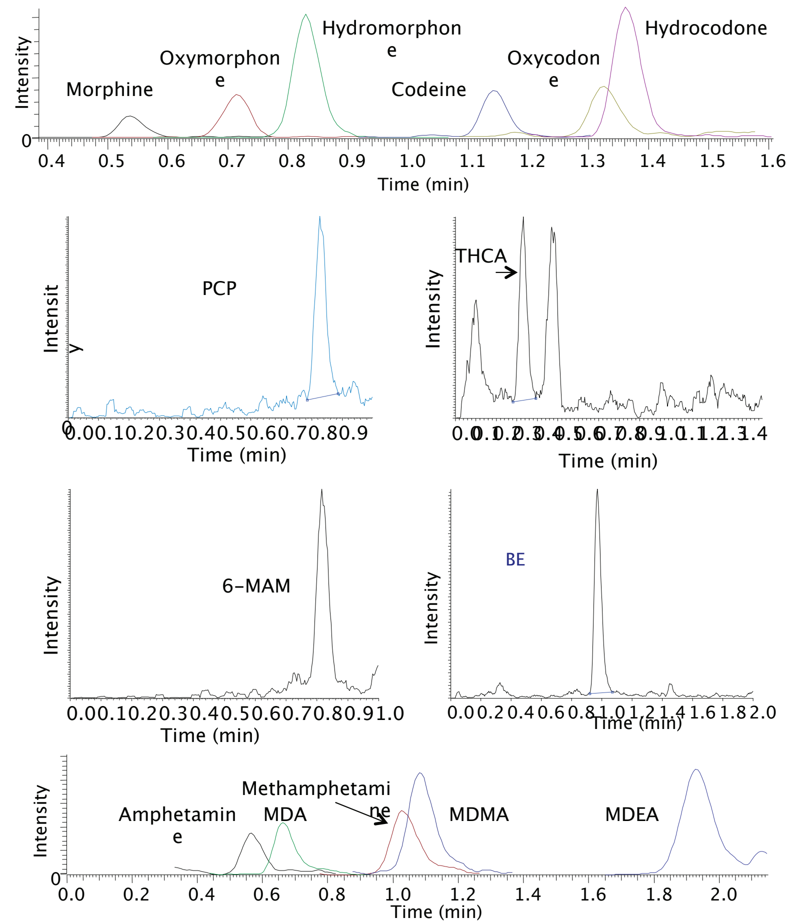


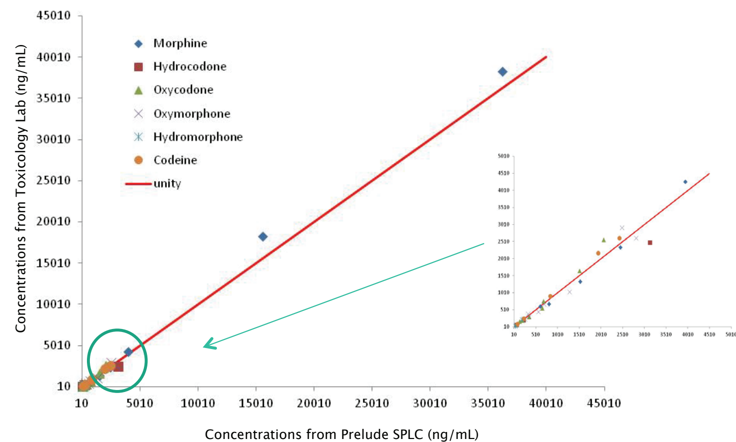
TABLE 2. Intra-method and Inter-method Precision.

Compound	Precision (RSD%)					
	Intra-method			Inter-method		
	LQC	MQC	HQC	LQC	MQC	HQC
Amphetamine	<15.9	<3.68	<2.86	15.33	3.23	2.32
MDEA	<5.33	<3.46	<5.24	3.65	2.88	3.62
MDA	<6.66	<4.15	<11.89	5.84	2.83	2.52
MDMA	<5.52	<4.34	<3.26	4.68	3.31	3.46
Methamphetamine	<5.47	<4.52	<16.63	6.2	4.33	3.79
Benzoylecgonine	<2.21	<2.35	<2.53	1.84	1.8	2.2
Phencyclidine	<6.88	<3.56	<4.33	8.8	3.57	3.63
6-Acetylmorphine	<5.87	<3.39	<4.11	4.69	3.51	3.67
THCA	<7	<2.8	<2.3	8.3	2.5	3.3
Morphine	<8.2	<10.8	<2.2	8.2	4.8	3
Codeine	<7.35	<5.20	<3.68	5.8	3.99	3.77

TABLE 3. Recovery of 11 drugs in 6 different urine lots.

Urine Lot	1	2	3	4	5	6
Amphetamine	100	103	98.3	95.8	101	103
MDEA	94.8	99.9	101	98.3	98.5	94.9
MDA	99.6	107	101	100	102	98.7
MDMA	101	100	97.9	99.3	103	102
Methamphetamine	99.8	101	102	96.1	105	98.5
Benzoylecgonine	106	111	97.6	107	109	106
Phencyclidine	88	84.2	81	83.5	85.6	85.9
6-Acetylmorphine	117	109	104	108	104	105
THCA	95.8	90.2	91.2	93.7	97.8	106
Morphine	96.1	99.8	91	90.7	93.9	92.3
Codeine	102	100	102	103	99.7	104

FIGURE 4. Correlation of data acquired with Prelude-Ultra method compared with data from a toxicology research laboratory validated method.



Conclusion

- An LC-MS/MS method for confirmatory analysis of the 11 drugs in the NIDA 5 panel using the Prelude SPLC and TSQ Quantum Ultra MSwas developed and validated.
- The method has LOQs that satisfy the SAMSHA cutoff requirements for these 11 drugs.
- No matrix interference were observed.
- The method is simple and fast.
- Two-channel multiplexing on Prelude SPLC would allow two different methods multiplexing in two channels and 3 minutes for a sample.

Acknowledgements

We would like to thank Kent Johnson from Pacific Hospital of Long Beach for supplying the comparison samples.

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Quantitative Analysis of THC and Main Metabolites in Whole Blood Using Tandem Mass Spectrometry and Automated Online Sample Preparation

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Overview

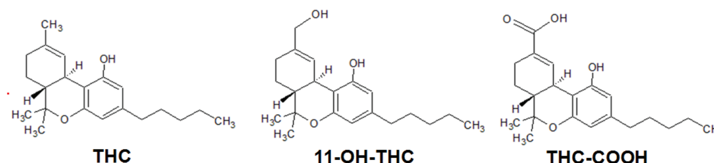
Purpose: Sensitive quantification of THC, 11-OH-THC and THC-COOH from whole blood with Thermo Scientific TurboFlow technology. For confirmation purposes, expected limit of quantification must be close to 0.5 ng/mL.
Methods: Blood samples were treated by protein precipitation followed by an online extraction and analysis by Reverse Phase Liquid Chromatography (RP-LC) coupled to mass spectrometry.

Results: This method was linear from 0.5-100 ng/mL for THC and its metabolites with good repeatability and sensitivity.

Introduction

Cannabis is the most highly used illicit substance around the world, and due to its psychoactive effects, it is of great importance to have analytical procedures for the assessment of the extent of its abuse. The major psychoactive constituent product of cannabis is Δ^9-tetrahydrocannabinol (THC) that is rapidly metabolized mainly in11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-THC) and then in 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), chemical structures are presented on figure 1.

FIGURE 1. Molecular structures of Δ^9-tetrahydrocannabinol (THC) and main metabolites,11-hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC) and 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH).



To have a better understanding of the effects of cannabis abuse, blood analysis is recommended. Nevertheless, THC and 11-OH-THC have short windows of detection in this matrix, and therefore limits of detection for their analysis are often settled to concentrations as low as 0.5 ng/mL.

In recent years, LC-MS has gained ground to GC-MS as a reference method for the analysis and confirmation of drugs of abuse in biological matrices in clinical and forensic toxicology. In the case of cannabinoids, it is particularly interesting to attain high sensitivities without a need for derivatization, but one of the key parameters to achieve sensitivity requirements is the choice of an appropriate sample treatment prior to the LC-MS method.

Thermo Scientific TurboFlow technology is an automated online sample preparation technique that has been coupled to LC-MS/MS for the quantitative analysis of biological samples. Our goal is to develop a method to measure THC and its metabolites by reducing method time while attaining good analytical performances.

Methods

Sample Preparation

A 0.2-mL sample (whole blood) was spiked with internal standards (IS) and then mixed with 0.4 mL of 0.1% formic acid in acetonitrile (v/v) . The mixture was vortexed and stored at 0 °C for 10 min. After a 2 minutes sonication, the mixture was centrifuged at 10,000 rpm for 10 min, and 90 µL of supernatant was injected for LC-MS/MS analysis.

TurboFlow and LC method

The TurboFlow™ method was performed in Focus mode (figure 2) with a Thermo Scientific TurboFlow Cyclone-P column. Analytical separation was carried out on a Thermo Scientific Accucore C18 column (50×2.1 mm, 2.6-μm particle size) . The mobile phases were as follows: loading A : 0.1% formic acid in water; loading C: 0.1% formic acid in acetonitrile; loading D : mixture of isopropanol, acetonitrile, and acetone (40/40/20 v/v/v) ; elutingC : 10mM ammonium formate + 0.1% formic acid in water; elutingD : 0.1% formic acid in methanol. The total LC runtime was 10.4 min (Figure 3).

FIGURE 2. . "Focus Mode Technical" diagram of TurboFlow Technology.

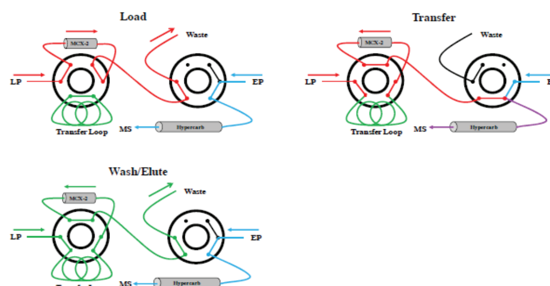


FIGURE 3. TurboFlow and LC method conditions.

Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B	%C	%D
0.00	45	1.50	Step	80.0	-	20.0	-	====	out	0.40	Step	-	-	80.0	20.0
0.75	75	0.10	Step	90.0	-	10.0	-	T	in	0.30	Step	-	-	80.0	20.0
2.00	119	1.50	Step	-	-	100.0	-	====	out	0.40	Ramp	-	-	2.0	98.0
3.98	100	1.00	Step	-	-	-	100.0	====	out	0.40	Step	-	-	2.0	98.0
5.65	15	0.50	Step	-	-	-	100.0	T	out	0.01	Step	-	-	2.0	98.0
5.90	30	1.50	Step	-	-	100.0	-	====	out	0.40	Step	-	-	2.0	98.0
6.40	90	1.50	Step	20.0	-	80.0	-	====	in	0.40	Step	-	-	80.0	20.0
7.90	150	1.00	Step	80.0	-	20.0	-	====	out	0.40	Step	-	-	80.0	20.0

TurboFlow method conditions (Loading Pump) (columns Flow through %D, first group)

LC gradient conditions (Eluting Pump) (columns Flow through %D, second group)

Mass Spectrometry

A Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode for THC and 11-OH-THC and in negative ionization mode for THC-COOH. Data were acquired in the selected reaction monitoring (SRM) mode (Figure 4).

FIGURE 4. MS source parameters and SRM transitions.

Ionization	HESI-II
Spray voltage (V)	3500 (+) 2700 (-)
Vaporizer Temp (°C)	330
Capillary Temp (°C)	270
Sheath gas (AU)	35
Auxilliary gas (AU)	25
Ion sweep gas (AU)	5
Collision gas pressure (mTorr)	25
Q1 (FWMH)	0.4
Q3 (FWMH)	0.7

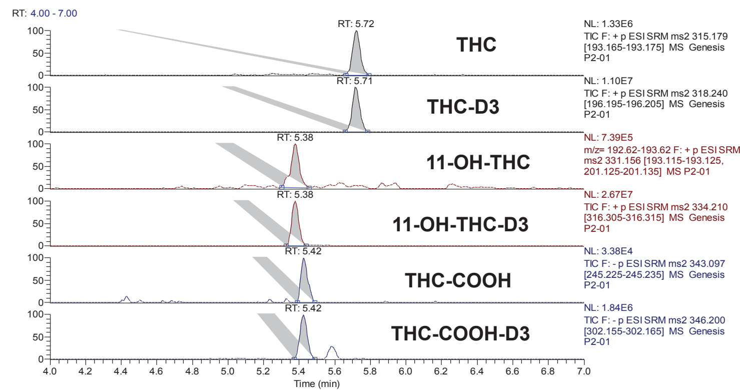
Compound	Parent Ion (m/z)	Product Ion (m/z)	S-Lens	CE	Polarity
THC	315.2	193.2	89	20	+
THC-D3	318.2	196.2	89	23	+
11-OH-THC	331.1	193.1	83	24	+
11-OH-THC-D3	334.2	316.3	83	15	+
THC-COOH	343.1	245.2	118	28	-
THC-COOH-D3	346.2	302.2	119	21	-

Results

Method Development

Different TurboFlow columns (Cyclone, Cyclone P, Fluoro, Phenyl-Hexyl) were evaluated with different loading conditions. Also different separation columns were evaluated (Accucore C18, Hypersil Gold C18, Accucore PFP and Accucore aQ) with different gradients. And finally, transfer optimization was also studied. The final chromatogram is shown in Figure 5.

FIGURE 5. SRM chromatograms of THC, 11-OH-THC and THC-COOH as well as deuterated standards (D3) from a blood sample spiked at 0.5 ng/mL.



Recovery and matrix effects

Precipitation Recovery was obtained by comparing an injection of whole blood spiked with the analytes and then crashed, against whole blood crashed first and then spiked.

On-line extraction Recovery was evaluated by comparing a direct injection of a standard solution to the analytical column against an injection to the TurboFlow column.

Matrix Effects were evaluated by comparing an injection of standard solution to the TurboFlow column against an injection of blood spiked at the same concentration.

Overall recovery was obtained considering both recovery and matrix effects. Results are presented on figure 6.

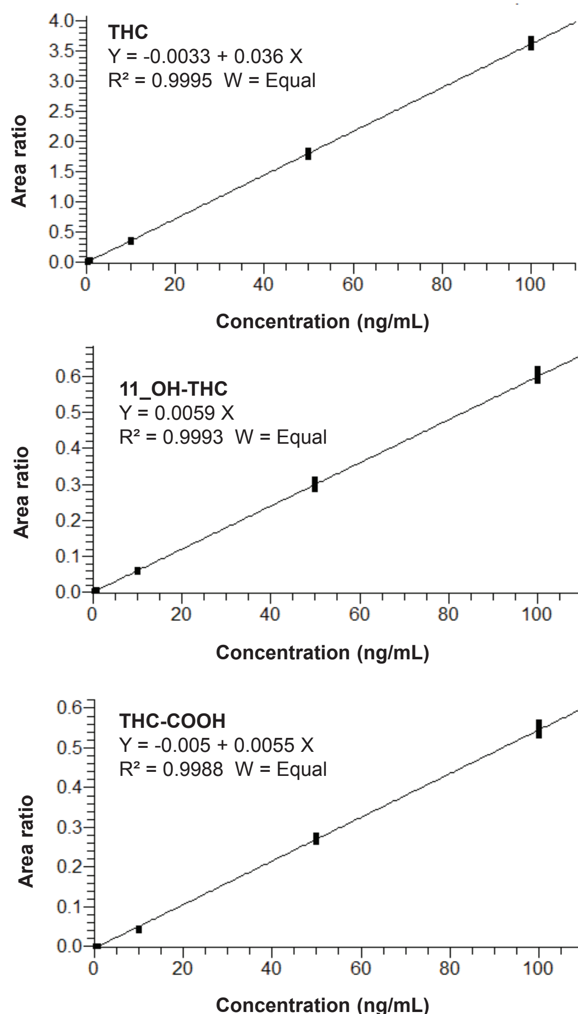
FIGURE 6. Method recovery and matrix effects.

The concentration was 7.5 ng/mL in standard , crashed whole blood and whole blood samples. Injection volume was set to 20µL in all cases and 5 injections were performed in each condition.

Compound	Precipitation recovery	On-line extraction Recovery	Matrix effects	Overall recovery
THC	97%	61%	+ 50%	92%
11-OH-THC	94%	82%	+ 36%	112%
THC-COOH	88%	76%	- 5%	73%

Calibration curves were generated with LCQuan 2.7 SP1 software by injecting whole blood samples spiked with THC, 11-OH-THC and THC-COOH. And crashed before injection Their deuterated (D3) compounds were used as internal standards. With a concentration of 17ng/mL The calibration model was linear with an equal weighting. In these conditions, curves were linear through the calibration range, from 0.5ng/mL to 100ng/mL. The calibration curves are presented in figure 7.

FIGURE 7. Calibration curves for THC, 11-OH-THC and THC-COOH from spiked and crashed whole blood. Calibration ranges goes from 0.5ng/mL to 100ng/mL.



Each calibration point was injected 10 times. The mean calculated concentration, the accuracy (%Diff) and the repeatability (%RSD) for each calibration point are presented in figure 8.

FIGURE 8. Accuracy (%Diff) and repeatability (%RSD) obtained for each calibrator (n=10)

Conc (ng/mL)		0.5	1	10	50	100
THC	Mean	0.49	1.02	10.03	49.5	94.8
	%Diff	-2	+2	+0.3	-1	-5.2
	%RSD	5	3	2	2	2
11-OH-THC	Mean	0.50	1.00	9.97	49.7	94.6
	%Diff	0	0	-0.3	-0.6	-5.4
	%RSD	5	4	3	3	2
THC-COOH	Mean	0.50	1.00	9.95	50.4	94.2
	%Diff	0	0	-0.5	+0.8	-5.8
	%RSD	9	7	2	2	2

Limits of quantification were determined as the lowest concentration for which a 20% RSD is obtained as well as a bias inferior to 20%. The results are presented on figure9.

FIGURE 9. Limits of quantification for THC, OH-THC and THC-COOH in spiked and crashed whole blood samples.

Compound	Concentration (ng/mL)	% RSD (n=10)	Bias (Mean +/- RSD)
THC	0.5	5	0.49 +/- 0.02
11-OH-THC	0.5	5	0.50 +/- 0.03
THC-COOH	0.5	9	0.50 +/- 0.04

The limits of quantification satisfy the requirements for cannabis analysis in whole blood, considering that the limits of detection are expected to be close to 0.5 ng/mL.

Conclusion

- A fast, automated, and analytically sensitive LC-MS/MS method was developed to quantify THC and its metabolites in crashed whole blood.
- The total online extraction and analytical LC runtime was 10.4 minutes. This throughput could be increased by multiplexing this method on a Thermo Scientific Transcend TLX system.
- This method was linear from 0.5 to 100 ng/mL.
- The lower limit of quantitation was at least of 0.5 ng/mL for THC and its metabolites. Good repeatability was obtained for the different calibration levels with %RSD inferior to 10%.
- Correlation between GC-MS and this analytical method is being performed by Analysis – Expertise laboratory.

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AN613: Detection of Stanozolol Glucuronides in Human Sports Drug Testing by Means of High-Resolution, Accurate-Mass Mass Spectrometry

AN496: Screening in Equine Doping Control Analysis with Ultrahigh Resolution and Accurate Mass

AN346: MS/MS as an LC Detector for the Screening of Drugs and Their Metabolites in Race Horse Urine

AN350: Rapid Quantitative and Confirmational Screening for Drugs in Race Horse Urine by ESI-LC-MS/MS and MS^3

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Detection of Stanozolol Glucuronides in Human Sports Drug Testing by Means of High-Resolution, Accurate-Mass Mass Spectrometry

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Key Words

Sports doping, antidoping testing, Q Exactive Focus, long-term metabolite, anabolic agents, 16-oxo-stanozolol, stanozolol glucuronide, epistanozolol

Goal

To demonstrate the utility of direct dilute-and-shoot analysis of glucuronic acid conjugates of stanozolol by means of liquid chromatography and high-resolution, accurate-mass mass spectrometry in sports antidoping testing. To characterize and validate, by means of commercially available 3'-OH-stanozolol glucuronide, "dilute and inject" and confirmation methods, which will allow for the unambiguous identification of stanozolol misuse in routine doping-control samples.

Introduction

The analysis of the anabolic steroid stanozolol (Figure 1a) has proved to be challenging for gas chromatography mass spectrometry (GC-MS) methods due to stanozolol's peculiar physicochemical properties. The uncovering of stanozolol abuse by means of its major urinary metabolite 3'-OH-stanozolol (Figure 1b) as accomplished by Schänzer and Donike[1] initiated investigations into the metabolic fate of this anabolic agent. The molecular features of stanozolol and its metabolites demand sophisticated derivatization and separation steps for GC/MS-based methodologies. Methods based on liquid chromatography with electrospray-ionization tandem mass spectrometry (LC-MS/MS), on the other hand, provide benefits such as lower limits of detection (LODs) and detection windows with expanded metabolite identification. 3'-OH-stanozolol glucuronide (Figure 1c) is the latest metabolite analyzed at 25–50 pg/mL in human urine. In the present study, the use of high-resolution, accurate-mass mass spectrometry for the detection of 3'-OH-stanozolol glucuronide is outlined. Complementary information on N-conjugated glucuronide metabolites of stanozolol and 17-epistanozolol and the use of these in routine doping controls is provided.

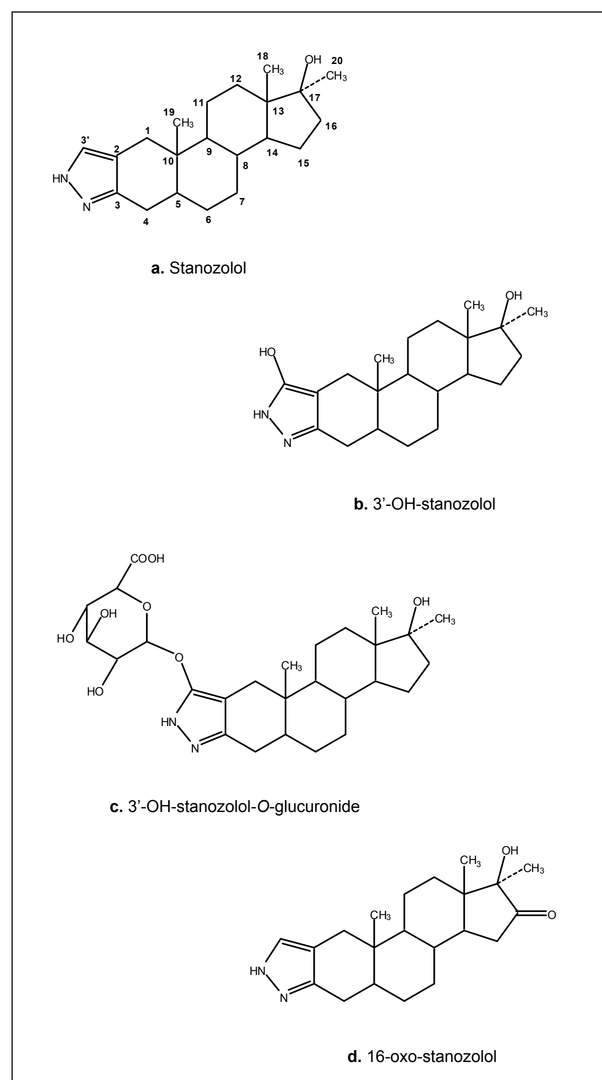

Figure 1. Chemical formulae of stanozolol (a. $C_{21}H_{32}ON_2$, mol wt = 328.2515), 3'-OH-stanozolol (b. $C_{21}H_{32}O_2N_2$, mol wt = 344.2464), 3'-OH-stanozolol-O-glucuronide (c. $C_{27}H_{40}O_8N_2$, mol wt = 520.2785), and 16-oxo-stanozolol (d. $C_{21}H_{30}O_2N_2$, mol wt = 342.2307)

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Experimental

Administration Samples

Two healthy male volunteers (56 and 61 years of age) received a single oral dose of 5 mg of stanozolol (Winstrol®). Urine samples were collected prior to (blank) and up to 28 days post administration of the drug. The urine specimens were stored at -20 °C until preparation and analysis. The study was approved by the local ethical committee and written consent was obtained from both participants.

Sample Preparation

Ninety microliters of urine were enriched with 10 µL of an acetonitrile solution containing the internal standard methyltestosterone (1 µg/mL). The samples were vortexed for 10 s and subjected to LC-MS/MS analysis.

Confirmatory analyses were conducted by applying 1 mL of urine to a solid-phase extraction (SPE) cartridge preconditioned with 2 mL of water and 2 mL of methanol. After the sample had passed through, the resin was washed with 2 mL of water and the analytes eluted with 2 mL of methanol. The organic phase was evaporated to dryness and reconstituted in 100 µL of solvents A (0.1% formic acid) and B (acetonitrile) (1:1, *v/v*) for LC-MS/MS analysis.

LC-MS/MS

The analyses were conducted using a Thermo Scientific™ Accela™ 1250 liquid chromatograph interfaced via a heated electrospray ionization (HESI-II) source to a Thermo Scientific™ Q Exactive™ Focus mass spectrometer. The LC was equipped with a Nucleodur® C18 Pyramid analytical column, 50 x 2 mm, particle size 1.8 µm, (Macherey-Nagel, Düren, Germany) and a corresponding precolumn (4 x 2 mm, particle size 3 µm). The mobile phases 0.1% formic acid (A) and acetonitrile (B) were used to perform a gradient elution at 200 µL/min from 99% of A to 100% of B in 7 min, followed by re-equilibration for 3 min.

The mass spectrometer settings were as follows:

Ionization mode	Positive
Spray voltage	4000 V
Source temperature	300 °C
Full Scan	
Resolution setting	35,000 (FWHM) at *m/z* 200
Mass range	*m/z* 100–1000
Targeted Higher Energy Collisional Dissociation (HCD)	
Preselected ions	*m/z* 505.25 (for stanozolol- and 17-epistanozolol glucuronide) *m/z* 521.25 (for 3'-OH-stanozolol glucuronide)
Resolution setting	35,000 (FWHM) at *m/z* 200
Mass ranges	*m/z* 50–535 and 50–550
Automatic gain control	2×10^5
Maximum IT fill time	200 ms
Isolation window	1.2 Da
Applied collision energy	55 and 72 eV
Collision gas	Nitrogen

Method Characterization

Due to the lack of certified reference material for the newly identified conjugates, the specificity (20 blank urine samples from 10 male and 10 female volunteers), limit of detection (LOD), and ion suppression/enhancement were determined with 3'-OH-stanozolol glucuronide only. In the case of the confirmatory assay, the recovery, linearity, and intra- and interday precision (at 25, 100, and 200 pg/mL), together with the identification capability, were also determined with 3'-OH-stanozolol glucuronide.

Results and Discussion

Stanozolol Metabolites

Administration study urine samples were collected after oral application of 5 mg of stanozolol and subjected to LC-MS/MS with high-resolution, accurate-mass capability in both MS and MS/MS modes. In agreement with earlier initial testing protocols, urine samples were injected into the LC-MS/MS system without further sample preparation, except for the addition of the internal standard methyltestosterone (at 100 ng/mL). Targeted product ion scan experiments [parallel reaction monitoring (PRM)] were performed on precursor ions of various different metabolites, including particularly the glucuronide(s) of stanozolol and its 17-epimer (precursor ion $[M+H]^+$ at *m/z* 505.29) and hydroxylated phase-I-metabolites (precursor ion $[M+H]^+$ at *m/z* 521.29). These yielded a series of signals, which were assigned to stanozolol metabolites by means of accurate masses of the intact protonated molecules and the respective aglycons obtained via collisional activation. A typical post-administration sample (5 days) and a blank urine specimen are shown in Figure 2.

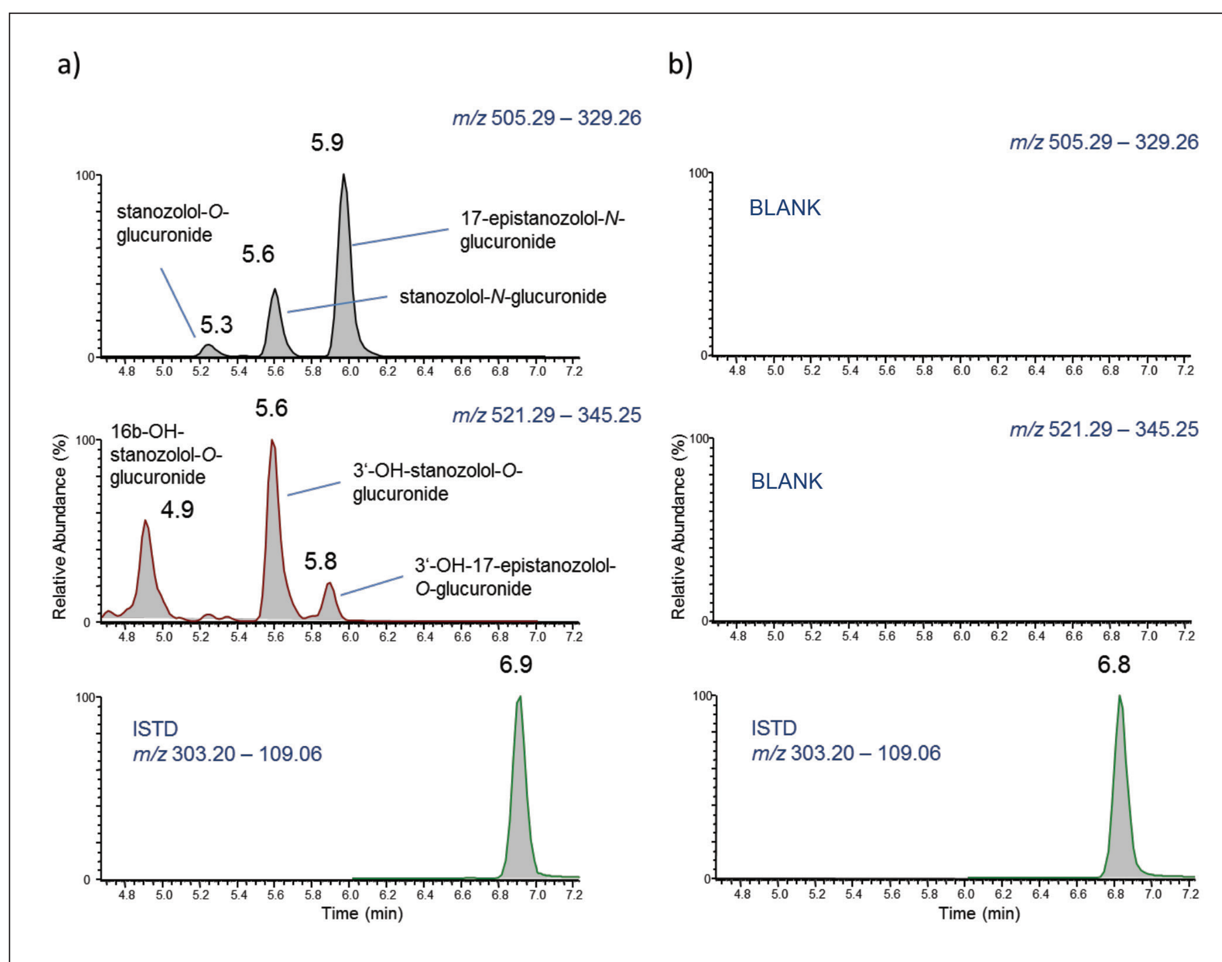

Figure 2. Extracted ion chromatograms of a) post-administration sample (5 days after administration of 5 mg of stanozolol) and b) blank urine sample. Considering accurate masses of precursor and product ions, hydrolysis experiments, and comparison to reference standards, structures were shown as indicated next to each peak.

The MS/MS experiments on *m/z* 505.29 resulted in three distinct signals as depicted in Figure 2a (top). Stanozolol comprises a hydroxyl group at C-17 and was thus expected to produce a glucuronic acid conjugate; however, the origin of the two additional species of identical sum formula was to be clarified and the structure of the 17-O-glucuronidated compound to be verified. Therefore, a urine sample containing predominantly the substances eluting at 5.14 and 5.53 min (3 h post-administration sample) was subjected to enzymatic hydrolysis with β-glucuronidase that reportedly cleaves β-O-glucuronic acid conjugates of steroids.[2] Following hydrolysis, the urine sample was reanalyzed, demonstrating the disappearance of peak 1 (5.14 min), while peak 2 (5.53 min) remained at its initial abundance (Figure 3a, top). Hence it was concluded that peak 1 corresponded to the hydrolysable 17-*O*-glucuronide of stanozolol while peak 2 was attributed to a nonhydrolysable glucuronic acid conjugate bearing the glucuronide moiety at a nitrogen atom of the pyrazole residue. The product ion mass spectra of the analytes are depicted in Figure 3b and did not reveal significant differences that would allow localization of the conjugation site. However, the putative stanozolol-*O*-glucuronide (Figure 3b, top) required more collision energy to dissociate (CE = 45 eV) than the corresponding stanozolol-*N*-glucuronide (Figure 3b, middle) and 17-epistanozolol-*N*-glucuronide (Figure 3b, bottom), which were collisionally activated with 35 eV only. Under increasing CE values (as shown in the insets measured at CE = 65 eV), both *N*- and *O*-conjugated metabolites yielded the diagnostic product ions of stanozolol (e.g. *m/z* 81.0452).

Figure 3. a) Extracted ion chromatograms of a post-administration sample (3 hours after application of 5 mg of stanozolol), indicating the presence of stanozolol-O-glucuronide (at 5.1 min) and stanozolol-N-glucuronide (at 5.5 min), b) product ion mass spectra of the analytes, c) structures

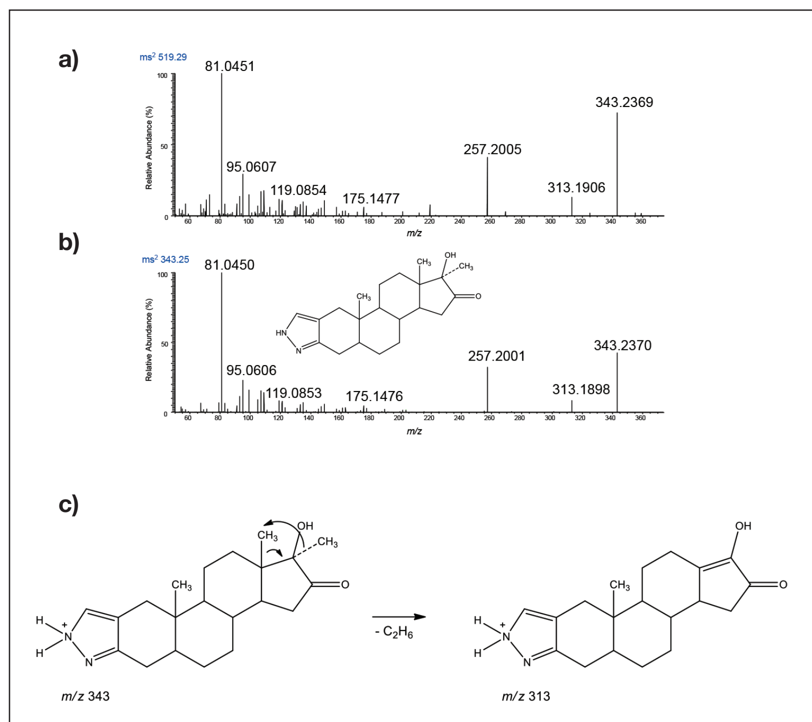

Figure 4. Product ion mass spectra of the protonated molecules $[M+H]^+$ of a) glucuronic acid conjugate of a metabolite observed in administration study urine samples attributed to 16-oxo-stanozolol-glucuronide at *m/z* 519, and b) reference standard of 16-oxo-stanozolol. The elimination of 30 Da resulting from the loss of C_2H_6 is suggested to include the methyl residues at C-15 and C-17 as illustrated under c).

Recent findings of long-term metabolites generated from 17-methyl-17-hydroxy-steroids such as metandienone, oxandrolone, dehydrochloromethyltestosterone, and oxymetholone, fueled the search for analogous metabolites in the case of stanozolol. A common feature of the aforementioned long-term metabolites under ESI-MS/MS conditions is the elimination of formaldehyde (30 Da), which also served as indicator in the present study. In the product ion mass spectrum of the precursor ion $[M+H]^+$ at *m/z* 519, which was attributed to a hydroxylated and glucuronidated analog of 17-hydroxymethyl,17-methyl-18-norstanozolol, the ion of the aglycon was observed at *m/z* 343.2369 with the experimentally determined elemental composition of $C_{21}H_{30}O_2N_2$. In addition, a product ion at *m/z* 313.1899 (-30 mass units, Figure 4a) was present. However, the accurate masses revealed the difference of C_2H_6 rather than CH_2O between *m/z* 343 and 313, demonstrating that the analyzed species was not 17-hydroxymethyl,17-methyl-18-norstanozolol but 16-oxo-stanozolol comprising the same elemental composition as corroborated by the analysis of the respective reference substance (Figure 4b). The peculiar loss of ethane (30 Da) was suggested to originate from the steroidal D-ring including C-18 and C-20 and the introduction of a C-13 – C-17 double bond as shown schematically in Figure 4c. Here, deuterium labeling of either C-18 or C-20 would provide further insights and will be subject of future studies.

Excretion Study Urine Samples

In order to estimate the utility of the newly identified metabolites to prolong and/or improve the detection of stanozolol abuse, the traceability of stanozolol-*O*-glucuronide, stanozolol-*N*-glucuronide, 17-epistanozolol-*N*-glucuronide, 16β-OH-stanozolol-*O*-glucuronide, 4β-OH-stanozolol-*O*-glucuronide, and 3'-OH-stanozolol-*O*-glucuronide by the above mentioned screening method was assessed in administration study urine samples. In Figure 5, the intensities (log scale) of analyte signals were plotted against the time points of urine sampling, demonstrating considerably longer visibility of 17-epistanozolol-*N*-glucuronide, which was detected up to 672 h (28 days) post-administration.

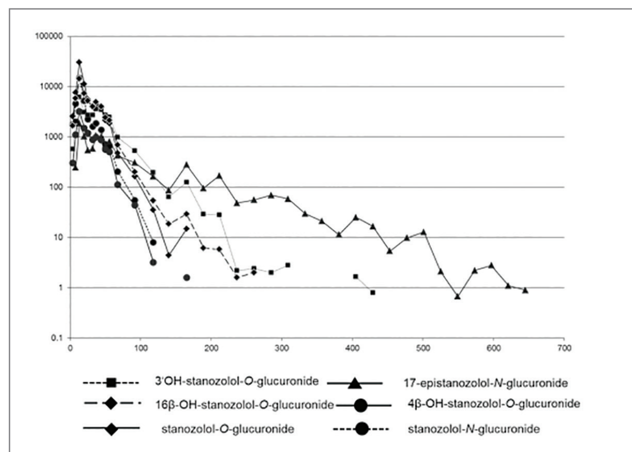

Figure 5. Pharmacokinetics of six metabolites monitored in the administration study urine samples collected after application of 5 mg of stanozolol. The N-glucuronide of 17-epistanozolol was detected up to 28 days.

Methods Characterization / Validation

Before using 3'-OH-stanozolol glucuronide and the metabolites for doping control purposes, the fit-for-purpose of initial testing and confirmation approaches were determined using typical methods. The resulting method characteristics and validated parameters are summarized in Table 1.

Table 1. Method characteristics and validated parameters

Dilute-and-Inject Assay			Confirmation Assay								
								Intraday Precision (n=30)		Interday Precision	
LOD (pg/mL)	Specificity	Ion Suppression	LOD (pg/mL)	Specificity	Ion Suppression	Recovery (%)	Calibration Curve	(n=30+30+30)	CV (%)	Concentration (pg/mL)	CV (%)
20	No Interference (10+10)	3–45%	5	No Interference (10+10)	15–84%	106	25–150 pg/mL	25	15	25	16
							Linear (r^2 = 0.994)	100	9	100	10
								250	7	250	7

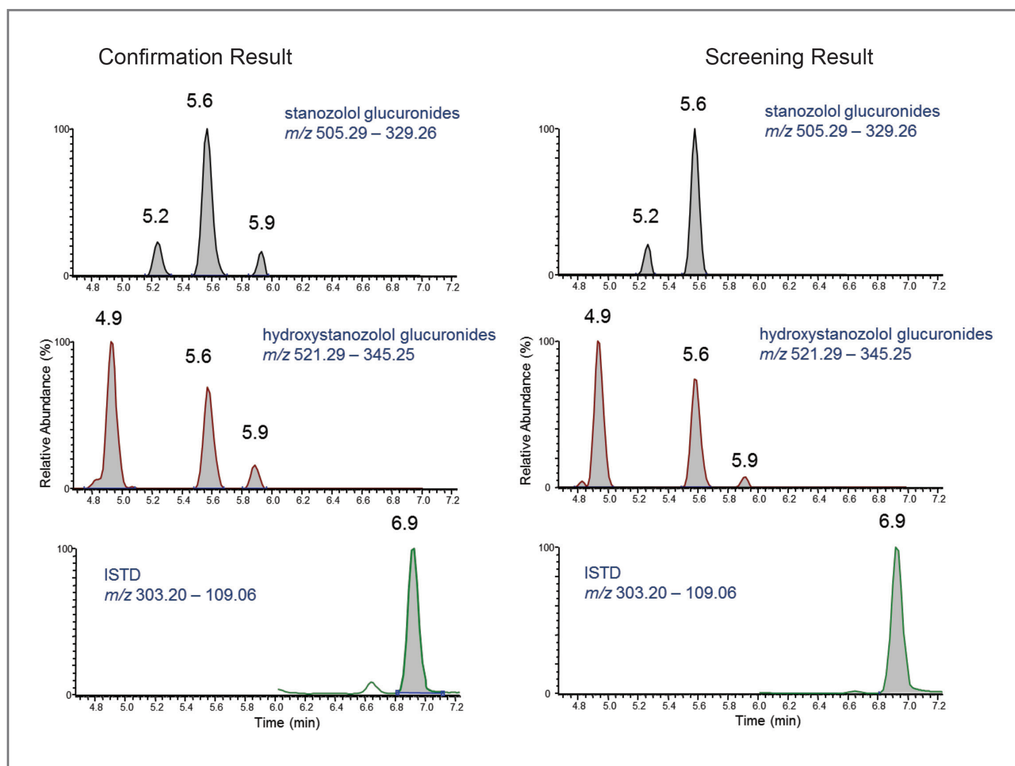

Figure 6. Chromatograms of an authentic doping control routine sample representing an adverse analytical finding for stanozolol in both screening and confirmation assays.

Conclusion

In this study, the utility of direct dilute-and-shoot analysis of glucuronic acid conjugates of stanozolol by means of liquid chromatography and high-resolution, accurate-mass mass spectrometry in sports antidoping testing was assessed and demonstrated. Characterized and validated by means of commercially available 3'-OH-stanozolol glucuronide, dilute-and-inject and confirmation methods were established, which allowed for the unambiguous identification of stanozolol misuse in routine doping control samples. Additionally, new long-term metabolites for the detection of stanozolol abuse were observed in administration study urine samples. These new target analytes, assigned as stanozolol-*N*-glucuronide and 17-epistanozolol-*N*-glucuronide, were characterized by mass spectrometry, and hydrolysis experiments. Both proved particularly useful as target compounds, enabling the determination of drug abuse for up to 28 days post-administration of 5 mg of stanozolol. Since high-resolution, accurate-mass mass spectrometry has been found to be essential for the successful identification of lowest amounts of stanozolol metabolites in human urine, expansion of its use in doping control is encouraged.

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Screening in Equine Doping Control Analysis with Ultrahigh Resolution and Accurate Mass

Yves Moulard, Yves Bonnaire, Laboratoire des Courses Hippiques, Verrières-Le Buisson, France; Bénédicte Duretz, Thermo Fisher Scientific, Les Ulis, France; Dennis Nagtalon, Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- Exactive
- LC/MS
- Orbitrap Technology
- Forensic Toxicology
- ToxID Software

Introduction

Triple quadrupole or tandem mass analyzers have been used most frequently in the accurate identification, confirmation, and quantitation of prohibited compounds in a single analysis. In addition, ion trap and quadrupole time-of-flight mass analyzers have been useful for screening and confirming results. However, these technologies cannot address the main requirements of equine doping control analysis such as:

- Data re-interrogation
- Analyze and monitor a vast number of compounds
- Fast and easy method development, instrument operation, and data interpretation
- Efficient separation of analytes from interferences present in the matrix
- Highly confident identification of compounds

Here we present a screening approach that uses ultrahigh resolution (R = 50,000) and accurate mass in positive and negative mode for the screening of illicit substances in urine matrix using the Thermo Scientific Exactive benchtop mass spectrometer. More than 120 analytes are screened using this method. Confirmation is made using the exact mass of the analytes in positive and negative mode (if available) and the retention time.

Goal

To demonstrate a new approach using ultrahigh resolution (> 50,000) and accurate mass for the screening of illicit substances in a urine matrix using the Exactive™ mass spectrometer, a new high performance benchtop LC/MS instrument equipped with Thermo Scientific Orbitrap technology, for doping control analysis.

Figure 1. Thermo Scientific Exactive high performance benchtop LC/MS system

Experimental

Sample preparation

Solid phase extraction (SPE) was used for sample pretreatment and clean up. The details of the procedure are described below.

- To 5 mL of urine add 25 µL of hydrocortisone d3 at 10 µg/mL
- Add 1 mL of phosphate buffer
- Add 50 µL of β glucuronidase and 50 µL of protease
- Incubate for 1 hour at 55 °C
- Centrifuge at 4,000 rpm for 30 minutes
- Transfer the supernatant to a tube
- Add 5 mL of water
- Condition the C18-HF cartridge with 3 mL of methanol and 3 mL of water
- Load the sample and wash the cartridge with 3 mL of water and 3 mL of hexane
- Elute with 3 mL of a mixture containing dichloromethane and ethanol
- Evaporate to dryness
- Reconstitute with 100 µL of a mixture containing water and acetonitrile (80/20)

Instrumentation Method

HPLC conditions

Chromatographic analyses were performed using Shimadzu binary pumps LC-20ADxr (Champs sur Marne, France). The chromatographic conditions were as follows:

Column: Reversed-phase, silica-based C18 (3.5 µm, 150 x 2.1 mm) column

Flow rate: 0.3 mL/min

Injection volume: 10 µL

Mobile phase: A: Water containing 0.1% formic acid
B: Acetonitrile containing 0.1% formic acid

Gradient:

T(min)	A(%)	B(%)
0.0	80	20
5.0	80	20
20.0	50	50
25.0	0	100
25.2	80	20
30.0	80	20

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Mass Spectrometry conditions

MS analysis was carried out on an Exactive benchtop mass spectrometer with an electrospray ionization (ESI) source (Figure 1). The MS conditions were as follows:

Ion Polarity:	Polarity switching scan dependent experiment
Spray Voltage:	4500 V in positive mode and –3900 V in negative mode
Sheath gas pressure (N_2):	45 (arbitrary units)
Auxiliary gas pressure (N_2):	3 (arbitrary units)
Capillary temperature:	300 °C
Resolution:	50,000 (FWHM)
AGC Target Value	500,000

Results and Discussion

The screening method was set up for the identification and confirmation of more than 100 compounds, including anabolic agents, steroids, anesthetics, anti-inflammatory agents, and diuretics, as listed in Table 1

Acquisition was performed using the full MS scan mode with polarity switching and external calibration. All data were reprocessed using 5 ppm mass accuracy. Figure 2 shows the sensitivity obtained for a urine sample spiked with 4 compounds: dexamethasone, flumethasone, triamcinolone acetonide, and triamcinolone. The injected concentrations were 50 pg/mL for dexamethasone and flumethasone and 1 ng/ml for triamcinolone and triamcinolone acetonide. In the positive mode, the analytes were identified as protonated species and in the negative mode, as formate adducts. As data acquired was in full scan MS mode, re-interrogation of the data file, particularly for non-targeted or unknown compounds or metabolites, is easily made possible.

Thousands of real urine samples have been analyzed using this approach. Figure 3 shows an example of a real sample that has been analyzed using this method.

All data have been processed using Thermo Scientific ToxID software. ToxID™ software for Exactive processes data using the mass accuracy and retention time of the analytes. An example of the automatically generated report can be seen in Figure 4.

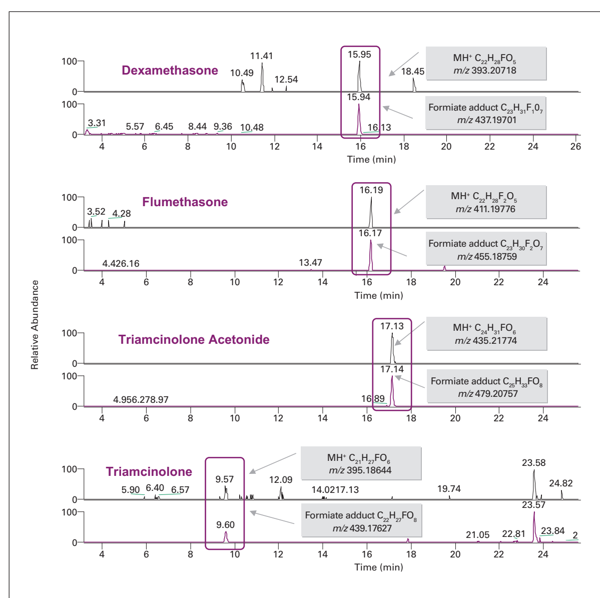

Figure 2: Extracted ion chromatograms for dexamethasone, flumethasone, triamcinolone acetonide, and triamcinolone in the positive and negative modes using 5 ppm mass accuracy

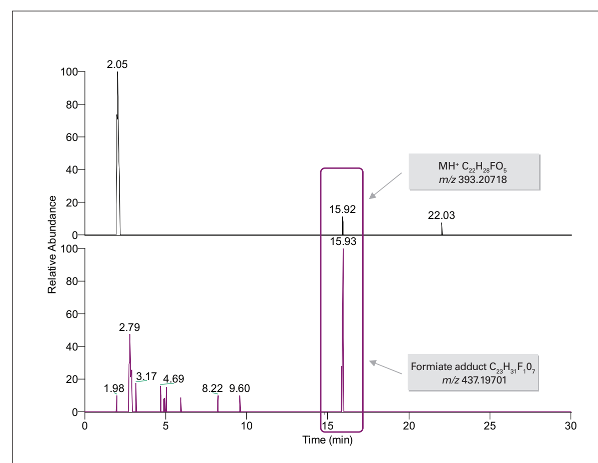

Figure 3: Dexamethasone identified in a real sample in positive and negative mode

Table 1: List of compounds monitored in the screening.

Index	Compounds	Index	Compounds	Index	Compounds
1	20 Beta dihydrocortisol	42	Diazoxide	83	Naftidrofuryl
2	4 Methylamino antypirine	43	Dichlorisone	84	Niketamide
3	5' Hydroxy Omeprazole	44	Diphenydramine	85	Nimesulide
4	Acepromazine	45	Diphylline	86	Nordazepam
5	Acide ethacrynic	46	Etamiphylline	87	Omeprazole
6	Althiazide	47	Etophylline (Etofylline)	88	Oxazepam
7	Ambroxol	48	Fenspiride	89	Oxyphenbutazone
8	Amcinonide	49	Fludrocortisone	90	Paramethasone
9	Amitryptylline	50	Flufenamic acid	91	Pentoxyphylline
10	Antipyrine (phenazone)	51	Flumethasone	92	Petidine (meperidine)
11	Beclomethasone	52	Flunisolid	93	Phenobarbital
12	Bendroflumethiazide	53	Flunixin	94	Phenylbutazone
13	Benzocaine	54	Fluocinolone acetonide	95	Phenytoin
14	Benzoylecgonine	55	Fluocinonide	96	Piroxicam
15	Benzydamine	56	Fluorometholone	97	Prednisolone
16	Betamethasone	57	Fluoroprednisolone	98	Prednisone
17	Budesonide	58	Flurandrenolide	99	Probenicid
18	Buflomedil	59	Fluticasone propionate	100	Procaine
19	Bumetanide	60	Furosemide	101	Prolintane
20	Bupivacaine	61	Guaifenesin	102	Promazine
21	Butorphanol	62	Halcinonide	103	Pyrilamine
22	Caffeine	63	Hydrochlorothiazide	104	Ranitidine
23	Capsaicine	64	Hydroflumethiazide	105	Sildenafil
24	Carbetapentane	65	Hydroxy Lidocaine	106	Sildenafil hydroxy
25	Chlorothiazide	66	Hydroxy Meloxicam	107	Sulindac
26	Chlorpheniramine	67	Hydroxy Piroxicam	108	Tenoxicam
27	Chlorpromazine	68	Hydroxy Tenoxicam	109	Tetracaine
28	Chlorthalidone	69	OH-Triamcinolone Aceto.	110	Tetrahydrogestrinone
29	Cimetidine	70	Imipramine	111	Tetramisole
30	Clenbuterol	71	Indapamide	112	Theobromine
31	Clobetasol	72	Isoflupredone	113	Theophylline
32	Cortisol	73	Ketamine	114	Timolol
33	Cortisol d3	74	Ketoprofen	115	Tixocortol pivalate
34	Cortivazol	75	Ketorolac	116	Tramadol
35	Cyclothiazide	76	Lidocaine	117	Triamcinolone
36	Dantrolene	77	Meloxicam	118	Triamcinolone acetonide
37	Dantrolene hydroxy	78	Mepivacaine	119	Triamcinolone hexacetonide
38	Desonide	79	Meprednisone	120	Trichlormethiazide
39	Desoximethasone	80	Methyl phenidate	121	Tripelennamine
40	Dexamethasone	81	Metocarbamol	122	Xipamide
41	Diazepam	82	Morphine	123	Xylazine

LCH
Summary Report

Raw File Name: C:\Documents and Settings\benedicte.duretz\Mes documents\ClinicalToxicologyForensic\Toxicology
Config File Name: C:\Documents and Settings\benedicte.duretz\Mes documents\ClinicalToxicologyForensic\Toxicology
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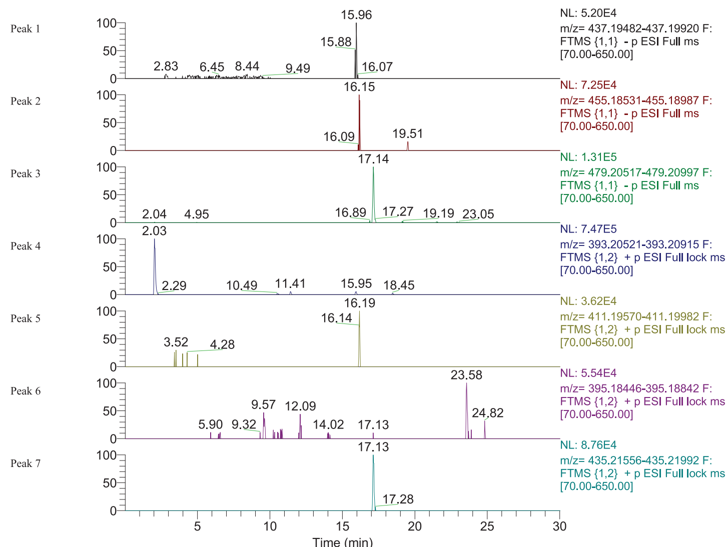
Screening Conditions: Based on accurate mass scans. Exact mass window (ppm): 5, RT window(min): 0.50.



Peak Number	Compound Name	Expected m/z	Detected m/z	Delta (mDa)	Delta (ppm)	Expected RT	Actual RT	Intensity
1	Dexamethasone Neg	437.19701	437.19907	2.1	4.7	16.04	15.96	52002
2	Flumethasone Neg	455.18759	455.18982	2.2	4.9	16.27	16.20	65542
3	Triamcinolone Aceto.	479.20757	479.20956	2.0	4.2	17.22	17.14	131328
4	Dexamethasone Pos	393.20718	393.20673	-0.5	-1.2	16.04	15.95	43018
5	Flumethasone Pos	411.19776	411.19714	-0.6	-1.5	16.28	16.19	36204
6	Triamcinolone Pos	395.18644	395.18570	-0.7	-1.9	9.67	9.57	25986
7	Triamcinolone Aceto. Pos	435.21774	435.21698	-0.8	-1.7	17.21	17.13	87591

Figure 4: ToxID report – short summary style

Conclusion

The Exactive high performance LC/MS demonstrates high resolving power (up to 100,000) and precise mass accuracy for easy, routine analysis and data re-interrogation of urine samples for illicit substances in equine doping control analysis.

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Application Note: 346

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MS/MS as an LC Detector for the Screening of Drugs and Their Metabolites in Race Horse Urine

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Wayne Skinner and Scott Stanley, University of California–Davis

Key Words

- LCQ Advantage MAX™
- Surveyor™
- Data Dependent™
- Drug Screening
- Metabolite ID
- Toxicology

Introduction

Imipramine is a tricyclic antidepressant drug that is not a Drug Enforcement Administration controlled substance but has been classified by the Association of Racing Commissioners International Inc. as a class two drug in horses. Desipramine is a major metabolite of imipramine. These two analytes were analyzed on-line by LC–PDA MS/MS from extracts of horse urine. The urine sample was first treated with β-glucuronidase to hydrolyze glucuronide conjugates of imipramine and desipramine. This was followed by solid phase extraction. The concentration of imipramine and desipramine in the sample was determined by the internal standard method using the peak area ratio and linear regression analysis.

This application note presents a rapid method for quantitation of imipramine and desipramine in horse urine. It illustrates the advantages of MS/MS detection in terms of specificity, sensitivity and unambiguous identification, for the analysis of drugs and their metabolites.

Goal

1) Develop a rapid method to identify and quantitate tricyclic antidepressant imipramine and its major metabolite desipramine in horse urine.
2) Demonstrate the advantages of using MS/MS to identify and confirm the detection of imipramine and its metabolites.
3) Determine presence and structure of minor metabolites using Data Dependent LC-MS/MS analysis.

Experimental Conditions HPLC

LC system: Thermo Scientific Surveyor MS Pump, Surveyor Autosampler and Surveyor PDA Detector

Mobile phase: A: water containing 0.2% formic acid
B: Acetonitrile containing 0.2% formic acid

Column: 50 ! 2.1 mm, 5 µm Thermo Scientific Hypersil™ C18 Column

Injection volume: 1 µL

Flow rate: 200 µL/min

Gradient:

Time (min)	% A	%B
0	98	2
0.2	98	2
8	25	75
9	10	90
10	10	90
10.01	98	2
15	98	2

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Mass Spectrometer

Mass spectrometer: Thermo Scientific LCQ Advantage MAX
Ionization mode: Positive electrospray ionization (ESI)
Capillary temperature: 275 °C
Spray voltage: 4.5 kV
Sheath gas: 30 units
Sweep gas: 8 units

Analyte	MH+	Isolation Width	Collision Energy %	Scan Range	Quantifying MS/MS Product Ions
Imipramine	281.2	1.5	30	75-285	86
Desipramine	267.2	1.5	30	70-290	236
Clomipramine (internal standard)	315.2	4	35	85-320	270

Table 1: MS parameters for imipramine, desipramine, and clomipramine (internal standard)

Standards

Calibration standards were prepared as follows:

Calibration level	Volume of Imipramine and Desipramine working standard solution (µL)	Volume of Clomipramine working standard solution (µL)	Equivalent to Imipramine in the urine (ng/mL)	Equivalent to Clomipramine in the urine (ng/mL)
C1	1:1 Dilution of C2	10	15.6	500
C2	1:1 Dilution of C3	10	31.3	500
C3	1:1 Dilution of C2	10	62.5	500
C4	1:1 Dilution of C2	10	125	500
C5	1:1 Dilution of C2	10	250	500
C6	1:1 Dilution of C2	10	500	500
C7	1:1 Dilution of C2	10	1000	500
C8	1:1 Dilution of C2	10	2000	500
C9	1:1 Dilution of C2	10	4000	500
C10	160	10	8000	500

Imipramine, desipramine and clomipramine working standard solutions were 50 ng/mL

Samples and Internal Standard

Imipramine was administered to the horse and a urine sample drawn after 0, 2, 4, 8 and 24 hours, post dose. One mL of the urine sample was spiked with 10 µL of 50 ng/µL clomipramine internal standard.

Sample Preparation

The calibration standard and urine samples were treated with β-glucuronidase to hydrolyze glucuronide conjugates of desipramine and imipramine, followed by solid phase extraction.

Results and Discussions

LC-UV-MS/MS analysis of imipramine and desipramine

Figures 1 and 2 show the analysis of tricyclic antidepressant imipramine, its major metabolite desipramine, and the internal standard clomipramine by LC with MS/MS and UV detection, respectively. Figure 1 shows base peak and extracted ion chromatograms for the three analytes along with MS and MS/MS spectra. The MS and MS/MS spectra help in unambiguous identification of these analytes and represent the high specificity that can be obtained from such an analysis. Further, the MS/MS spectra can be stored in a library and used for rapid confirmation of the drug and its metabolite. Figure 2 shows total spectra obtained from a PDA detector as well as UV trace at 254 nm and 280 nm. The position of elution of the three compounds had to be determined by sequential injections of individual analytes. As illustrated in Figure 2, the UV spectra for these compounds appear almost identical, making their unambiguous identification difficult.

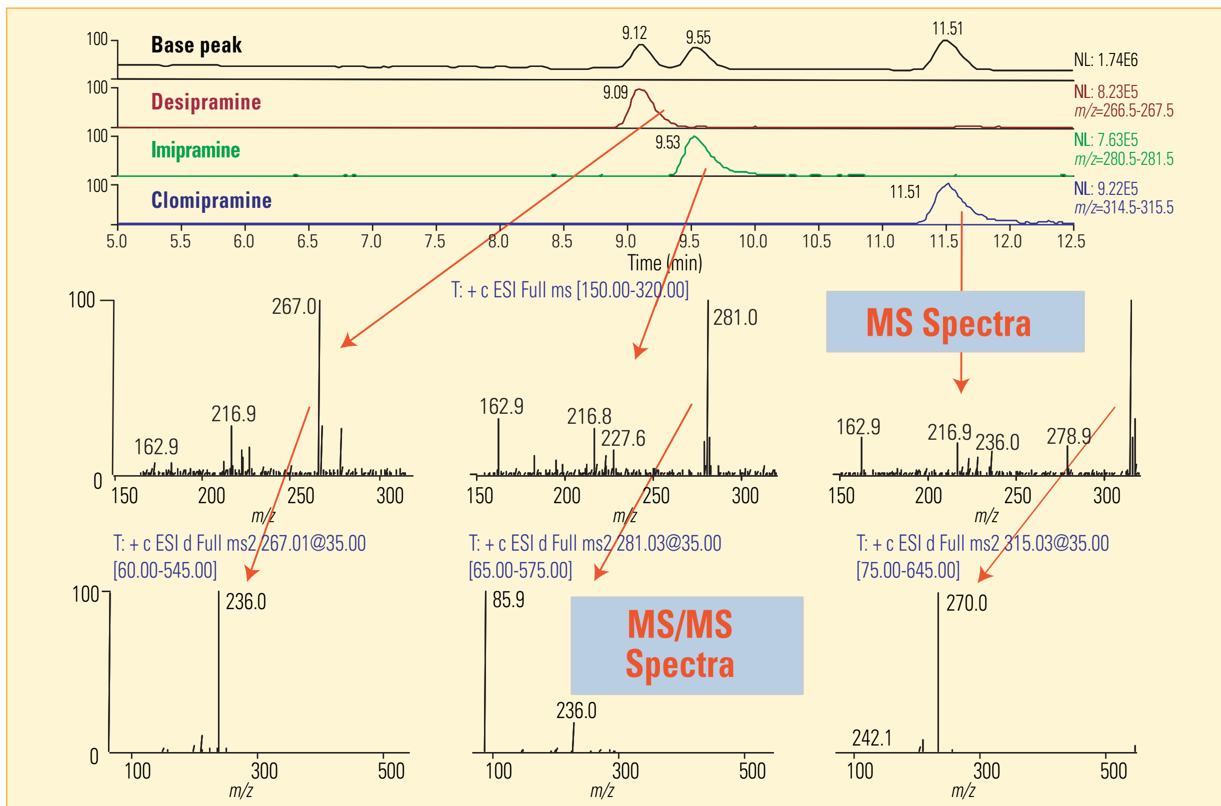


Figure 1: LC-MS/MS analysis of imipramine, desipramine and clomipramine (internal standard)

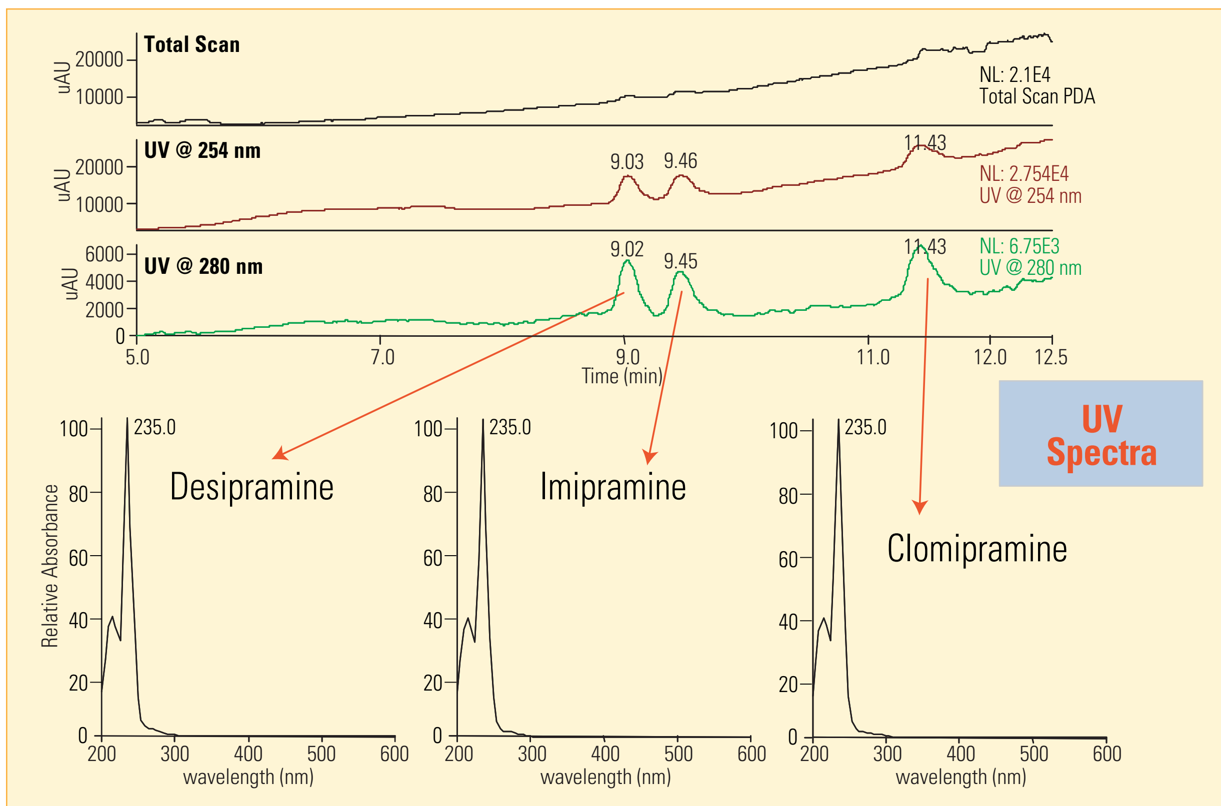


Figure 2: LC-UV analysis of imipramine, desipramine and clomipramine (internal standard)

Figure 3 shows chromatograms obtained for the analysis of imipramine, desipramine and clomipramine (IS) with MS and UV detection at levels of 5 and 0.5 ng on-column. At 0.5 ng on-column, both imipramine and desipramine could be easily identified when MS was used as a detector whereas these analytes were hardly visible in the UV trace. The concentration of clomipramine is the same at both these levels. This illustrates the excellent sensitivity that can be obtained during analysis by LC-MS/MS.

Quantitation of imipramine and desipramine in horse urine

Figures 4 and 5 show calibration curves obtained for imipramine and its major metabolite desipramine in horse urine with clomipramine used as an internal standard. The coefficient of correlation is 0.9896 for calibration curve of imipramine and 0.9836 for the calibration curve of desipramine. The % CV values are less than 7% for the imipramine calibration curve and 15% for the

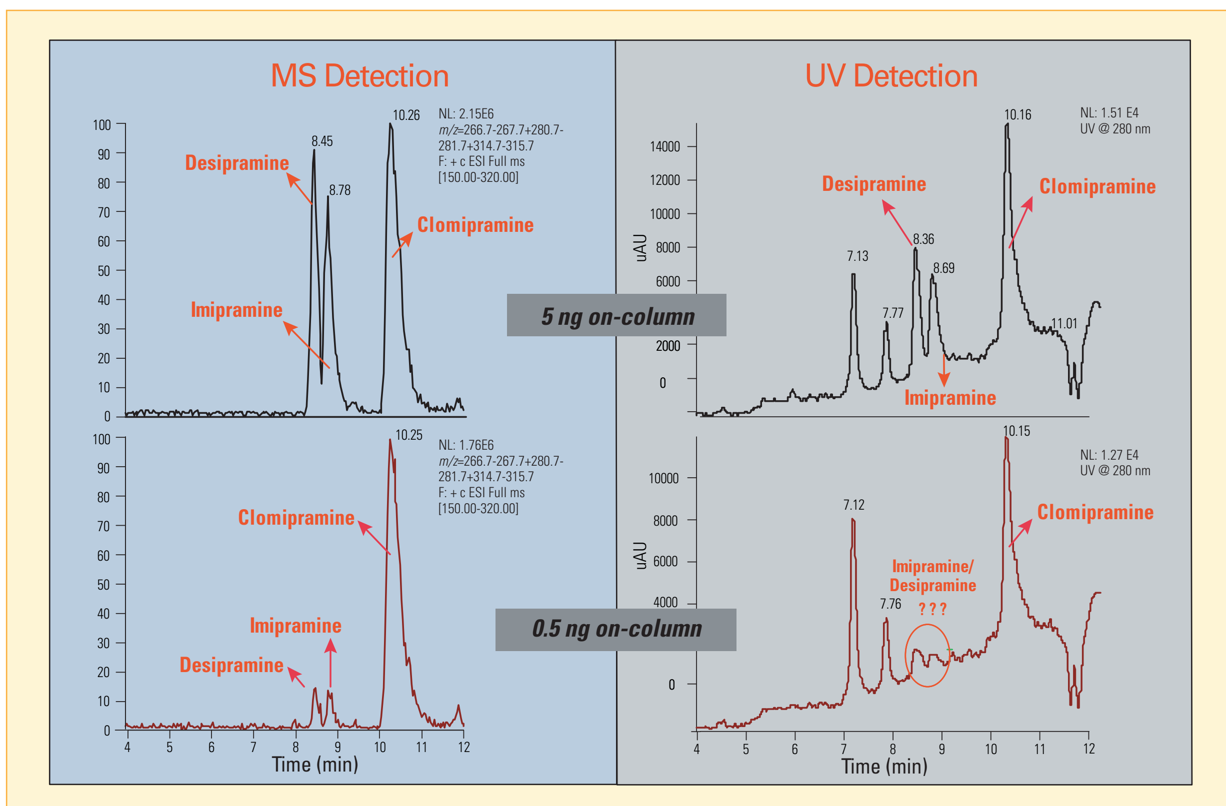
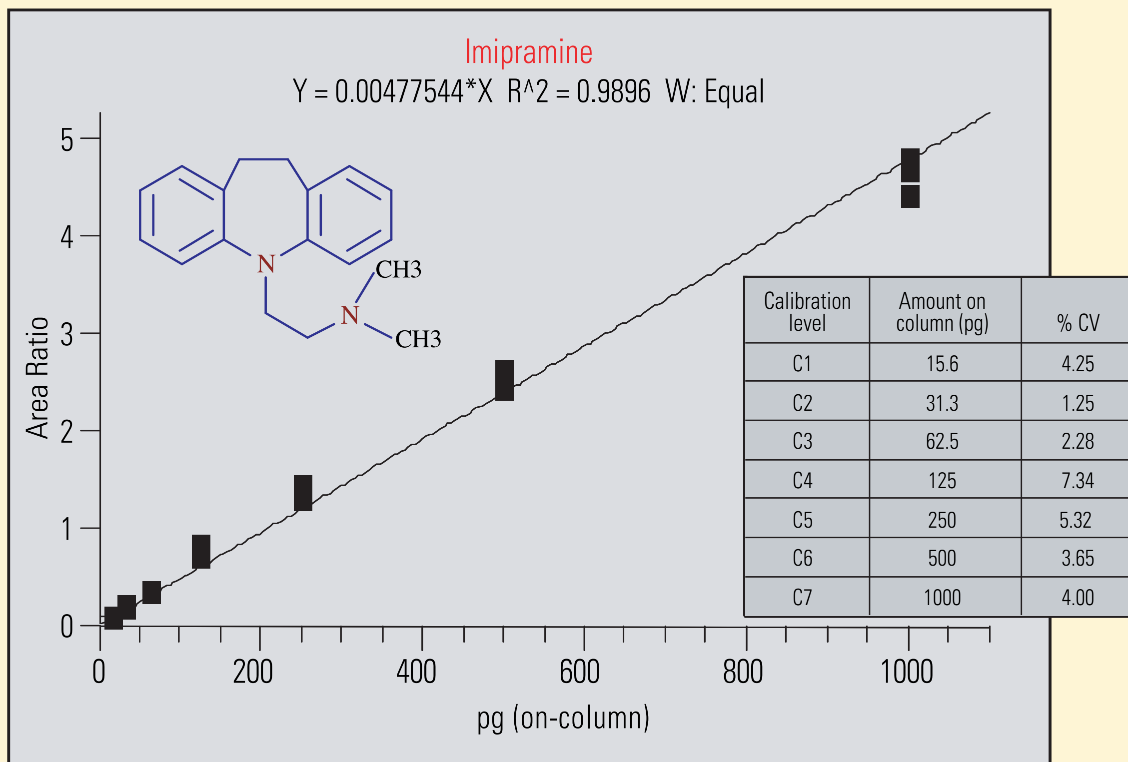
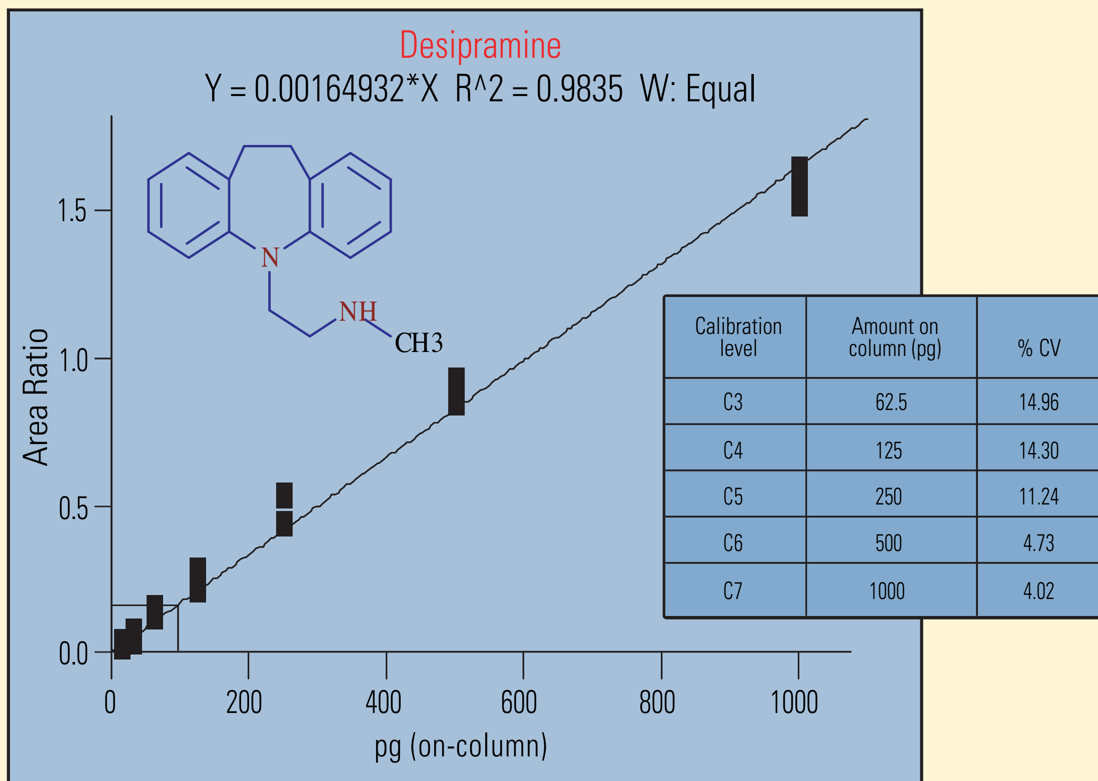


Figure 3: Comparison of MS and UV detection at two different concentrations



Calibration level	Amount on column (pg)	% CV
C1	15.6	4.25
C2	31.3	1.25
C3	62.5	2.28
C4	125	7.34
C5	250	5.32
C6	500	3.65
C7	1000	4.00

Figure 4: Calibration curve for imipramine in horse urine



Calibration level	Amount on column (pg)	% CV
C3	62.5	14.96
C4	125	14.30
C5	250	11.24
C6	500	4.73
C7	1000	4.02

Figure 5: Calibration curve for desipramine in horse urine

desipramine calibration curve. Figure 6 shows analysis of imipramine and desipramine in horse urine sample drawn two hours post-administration of the drug. The amount of imipramine and its major metabolite desipramine was determined using the calibration curves shown in Figures 4 and 5. Table 2 shows the amount of these two analytes as determined in horse urine. For the sample drawn two hours post-administration of the drug, the amount of imipramine and desipramine was determined to be 28 and 1567 ng/mL, respectively. The amount of desipramine determined at this time is above the upper limit of quantitation for the calibration curve shown in Figure 5.

Time (hr)	Imipramine (ng/mL)	Desipramine (ng/mL)
0+	17.56	20.85*
2	28.12	1567.16**
4	4.25*	189.06
8	6.75*	96.56
24	6.11*	13.11*

Table 2: Determination of imipramine and desipramine in horse urine for samples drawn at different times post injection of the drug (*below lower limit of quantitation, **above upper limit of quantitation)

Identification of metabolites of imipramine

A urine sample from the race horse obtained two hours after administration of the drug was also analyzed by Data Dependent LC-MS/MS, with MS/MS on the top two most intense ions to determine the presence of other metabolites. Figure 7 shows the workflow for such an analysis. The extracted ion chromatograms in Figure 8 show the presence of four additional metabolites: desmethyl desipramine, OH desipramine, OH-imipramine, and N-Oxide of imipramine, as well as their MS/MS fragmentation pattern. As indicated by the two peaks in the extracted ion chromatogram for *m/z* 297.2, imipramine is metabolized to two metabolites that have the same *m/z*. In this case, the MS/MS fragmentation pattern enables unambiguous distinction between the two metabolites.

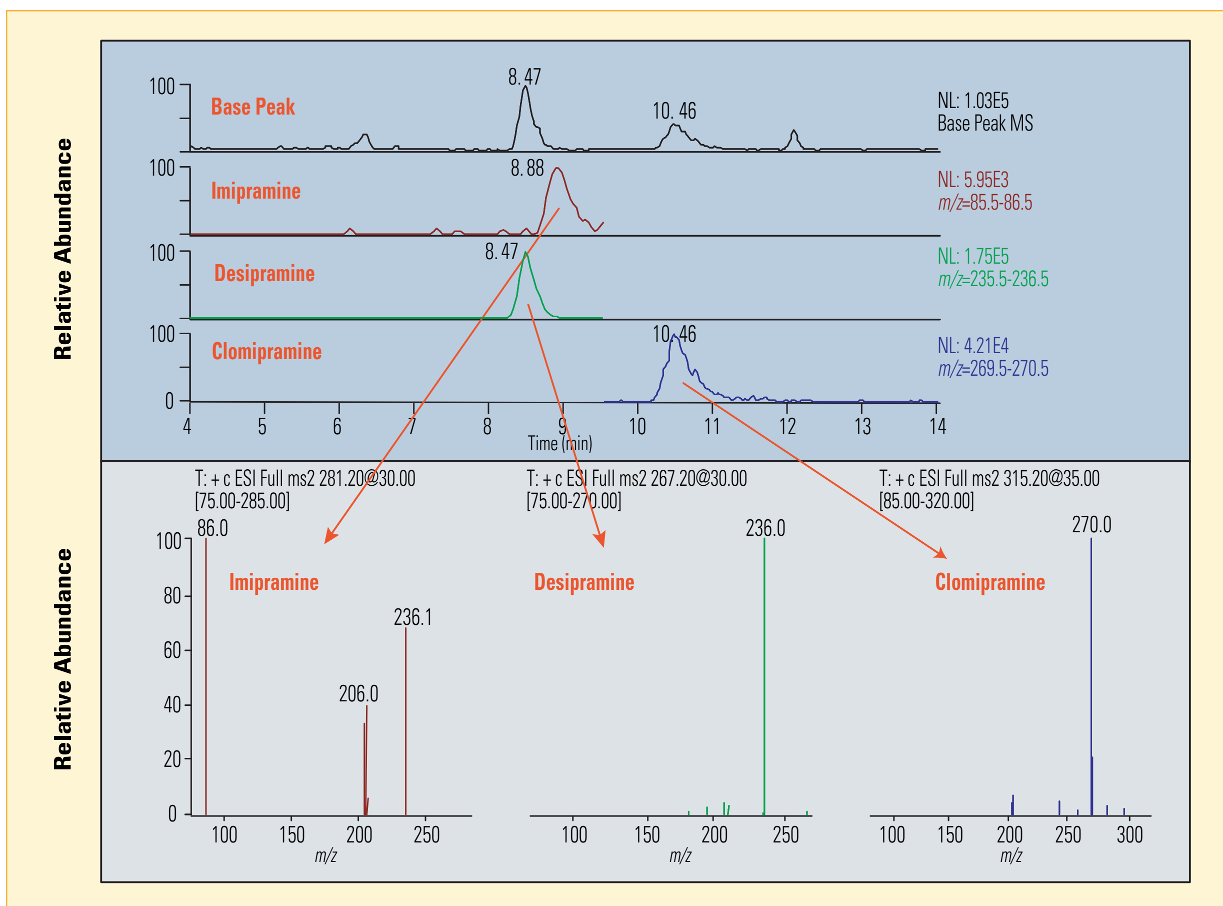


Figure 6: Analysis of imipramine and desipramine in horse urine for sample drawn two hour post-administration of drug

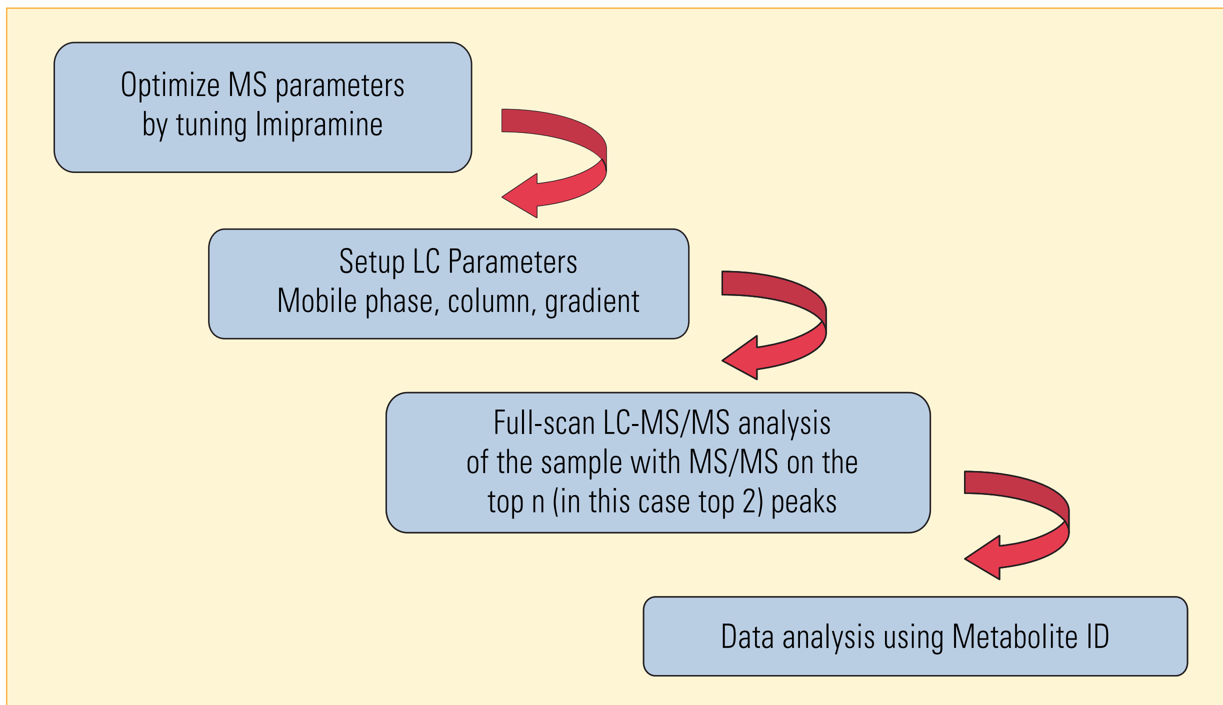


Figure 7: Workflow for the identification of imipramine and its metabolite in horse urine

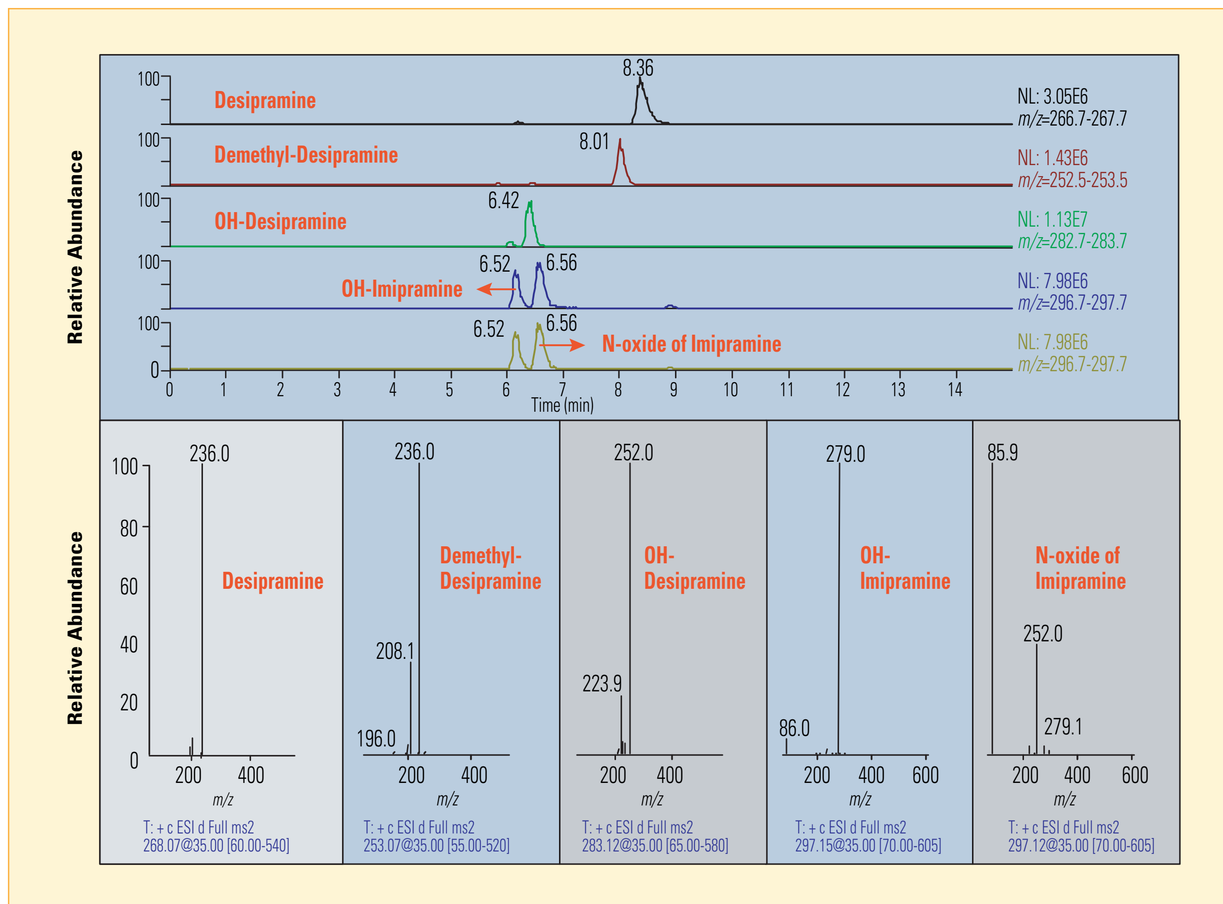


Figure 8: Data Dependent LC-MS/MS analysis of metabolites of imipramine

Conclusions

Full scan MS/MS analysis using a Thermo Scientific LCQ Advantage MAX ion trap mass spectrometer provides the selectivity and sensitivity necessary to support ADME/Tox studies of imipramine in horse urine. Analysis of drugs and their metabolites in complex biological samples using MS/MS detection enables unambiguous identification of these analytes. Data Dependent LC-MS/MS analysis facilitates presence and structural determination of several co-eluting minor metabolites. MS/MS information is invaluable in the identification of metabolites with the same *m/z* (e.g., OH-imipramine and N-oxide of imipramine).

Acknowledgements

The authors wish to thank Marta Kozak for sample analyses and Julian Phillips for presenting this project at the HPLC 2004 conference.

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Application Note: 350

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Rapid Quantitative and Confirmational Screening for Drugs in Race Horse Urine by ESI-LC-MS/MS and MS^3

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Key Words

- LTQ™
- Confirmation
- Drug Screening
- MS^3
- Quantitation

Introduction

Drugs of abuse in the horse racing industry encompass a variety of chemical classes and are typically analyzed from a complex urine matrix. These factors render the rapid and effective diagnostic screening of these drugs at low levels difficult. Traditionally, quantitation has been performed by triple quadrupole mass spectrometry using reaction monitoring (SRM) mode. However, this method does not monitor structurally diagnostic fragmentation. Thus, a second step involving derivatization and GC/MS confirmation was required.

Goal

To develop a simple and fast, yet rugged LC/MS based method capable of simultaneous qualitative and quantitative analysis. We have evaluated the application of the Thermo Scientific LTQ linear ion trap mass spectrometer for providing low levels of detection, good reproducibility, and wide linear dynamic range required for reliable quantitation, with simultaneous structural confirmation using diagnostic full-scan MS/MS or MS^3 mass spectrometry.

Experimental Conditions

Sample Preparation

Standards and Unknowns: Standards of the compounds listed in Table 1 were prepared neat and in urine. Urine standards and unknowns were spiked, dried, and reconstituted with 90% water and 10% acetonitrile with 0.1% acetic acid. Typical Instrument Setup settings are shown in Figure 1.

HPLC

HPLC System: Thermo Scientific Surveyor™ LC system

Column: Thermo Scientific BETASIL™ C18, 3 µm, 100 × 2.1 mm

Flow Rate: 350 µL/min

Injection Volume: 10 µL (full loop)

Mobile Phase: (A) Water with 0.1% acetic acid
(B) Acetonitrile with 0.1% acetic acid

Gradient: 92% A to 90% B.

MS

Mass Spectrometry: Thermo Scientific LTQ linear ion trap mass spectrometer

API Source: Thermo Scientific Ion Max™ source with electrospray ionization (ESI) probe

Ion Transfer Capillary: 220 °C; Sheath Gas: 30 units

Auxiliary Gas: 0 units; Sweep Gas: 20 units

Spray Voltage: 4.5 kV; Isolation Width: 3 amu

Normalized Collision Energy™: 28%

WideBand Activation™: Applied as needed (see Table 1)

Ion Polarity Mode: positive or negative (see Table 1)

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Results

Quantitation

Calibration curves were established using neat standards based on ion intensities from full-scan MS/MS chromatograms. Chromatograms for all the compounds listed in Table 1 were obtained in a single chromatographic run at each concentration. Figure 2 shows reconstructed ion chromatograms (RICs) from the analysis of the 50 pg/µL standards. The MS/MS spectra for all the drugs, with the exception of ketoprofen, are shown in Figure 3.

The MS/MS spectra were generated using a Normalized Collision Energy of 28%. The use of Normalized Collision Energy alleviates the necessity to optimize the collision energy for each compound as is necessary in traditional triple-quadrupole analysis, thus making this method extremely easy to set up and run. Compounds that underwent a non-specific water loss were additionally fragmented using WideBand Activation (see Table 1). This mode of fragmentation results in information-rich spectra enabling structural confirmation without requiring an additional MS^3 transition. The compound ketoprofen undergoes a neutral loss outside of the WideBand Activation window and was selected for an MS/MS to MS^3 comparison study and is discussed later.

Chromatographic and mass spectrometric methods were validated using the neat standards; subsequently the experiments were repeated using standards in horse urine. The RICs from these experiments are shown in Figure 4. Using the RICs, calibration curves were created for each of the compounds either neat (Figure 5) or in urine matrix (Figure 6). The calibration curves were linear over the three orders of magnitude assayed. In addition to demonstrating linearity, the quantitative results shown in Tables 2 and 3 demonstrate excellent reproducibility.

SEGMENT	RT	ID#	COMPOUND	*M/Z*	WB	RIC
1	3.40	416	Theobromine	181.0		137 + 163 + 181
	4.44	417	Theophylline	181.0		124 + 137
	4.56	152	Dyphylline	255.1		181
2	5.58	071	Caffeine	195.1		138
	5.71	089	Chlorothiazide	-293.9		214 + 215
	6.02	107	Cromolyn-Na	469.2	✔✔✔	245
	6.20	198	Hydroclorothiazide	-295.8		205 + 232 + 269
	6.49	311	Pemoline	177.0		106
3	7.20	614	Petoxifyline	279.1		181
4	8.95	117	Dexamethasone	393.1	✔	355 + 337 + 319
	9.60	481	Boldenone	287.1	✔	121 + 135 + 173
	10.16	499	Ketoprofen†	255 (209)		209 (105 + 194)
5	11.28	216	Indomethacin	358.0		139 + 174
	11.33	130	Diclofenac	295.9	✔	215 + 250
	11.93	175	Flufenamic Acid	282.1		264
	12.05	235	Meclofenamic Acid	295.9	✔	242 + 243

† *Ketoprofen was analyzed by both MS/MS and MS^3 for comparison study.*
✔ *WB denotes use of wideband activation during MS/MS fragmentation.*

Table 1: List of target compounds; corresponding RT (retention time), segment (method segment see Figure 1), *m/z* denotes isolation mass and Ion Polarity, WB–WideBand Activation, RIC–masses used in generation of Reconstructed Ion Chromatograms for quantitation.

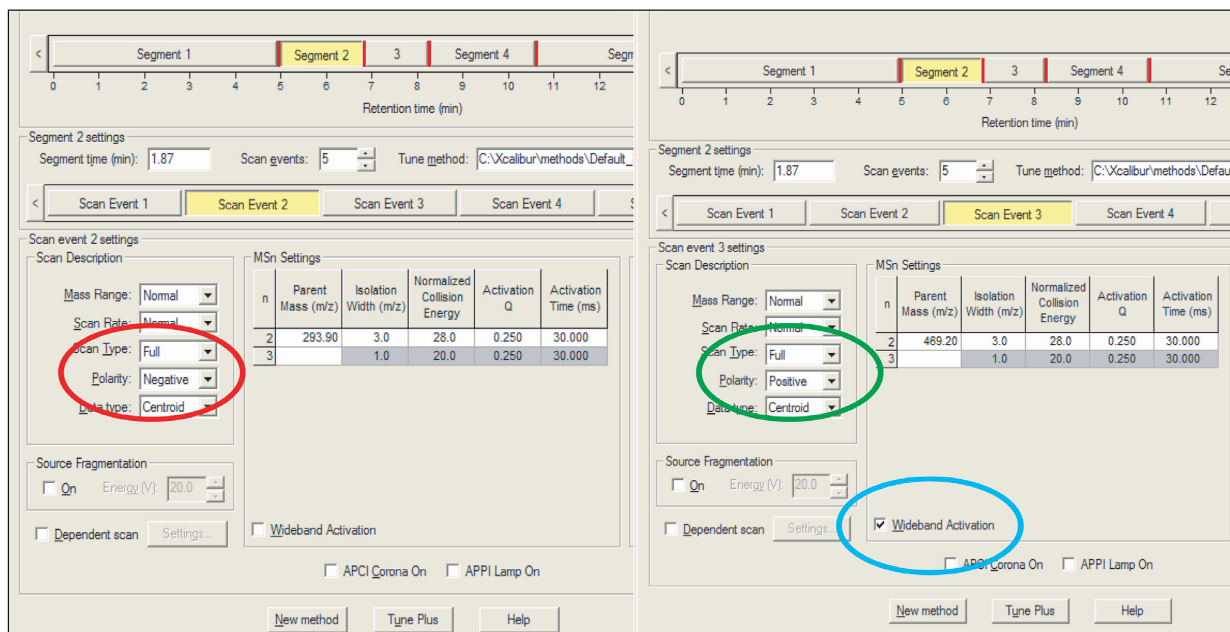


Figure 1: Instrument Setup settings for chlorothiazide (segment 2, scan event 2) and cromolyn-Na (segment 2, scan event 3)

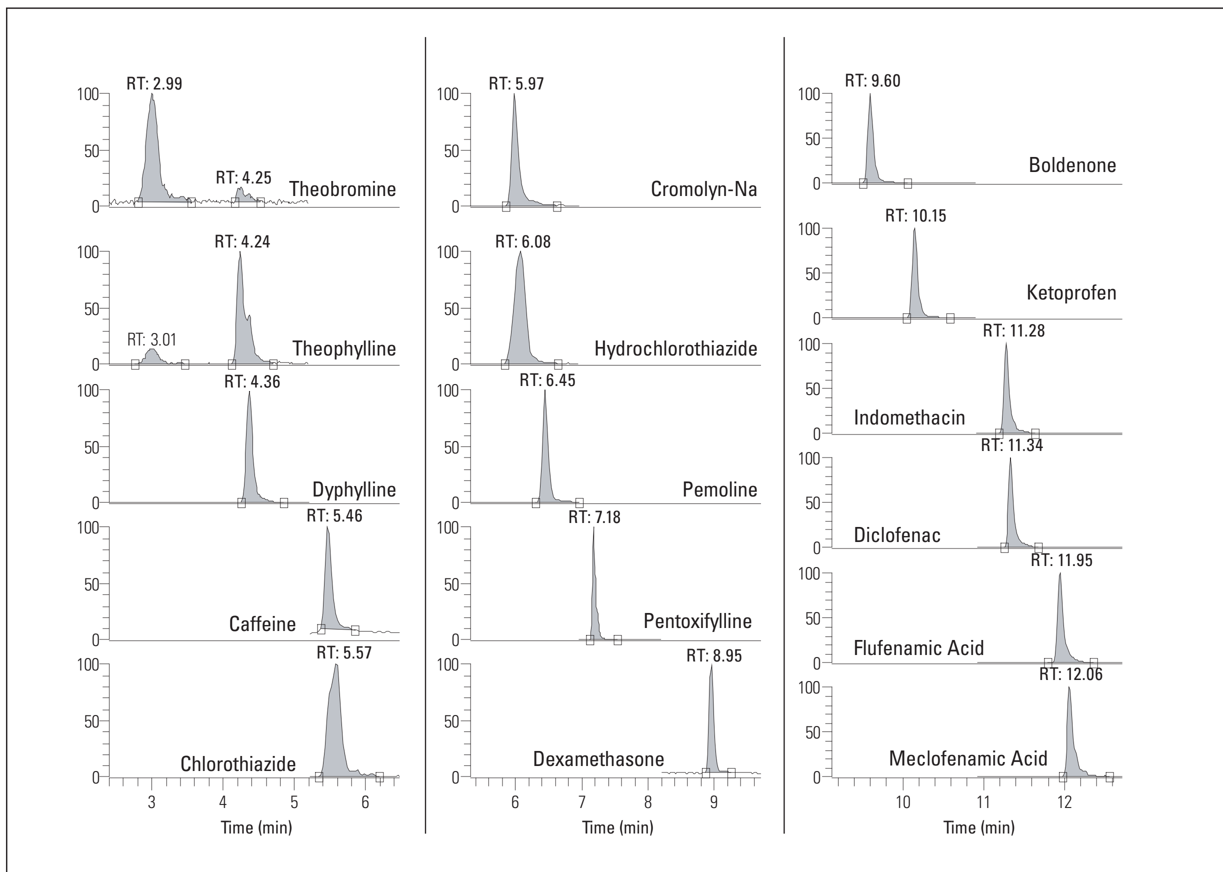


Figure 2: Reconstructed ion chromatograms, using the product ions listed in Table 1 (RIC column), from the analysis of 10 µL of the 50 pg/µL standards in solvent

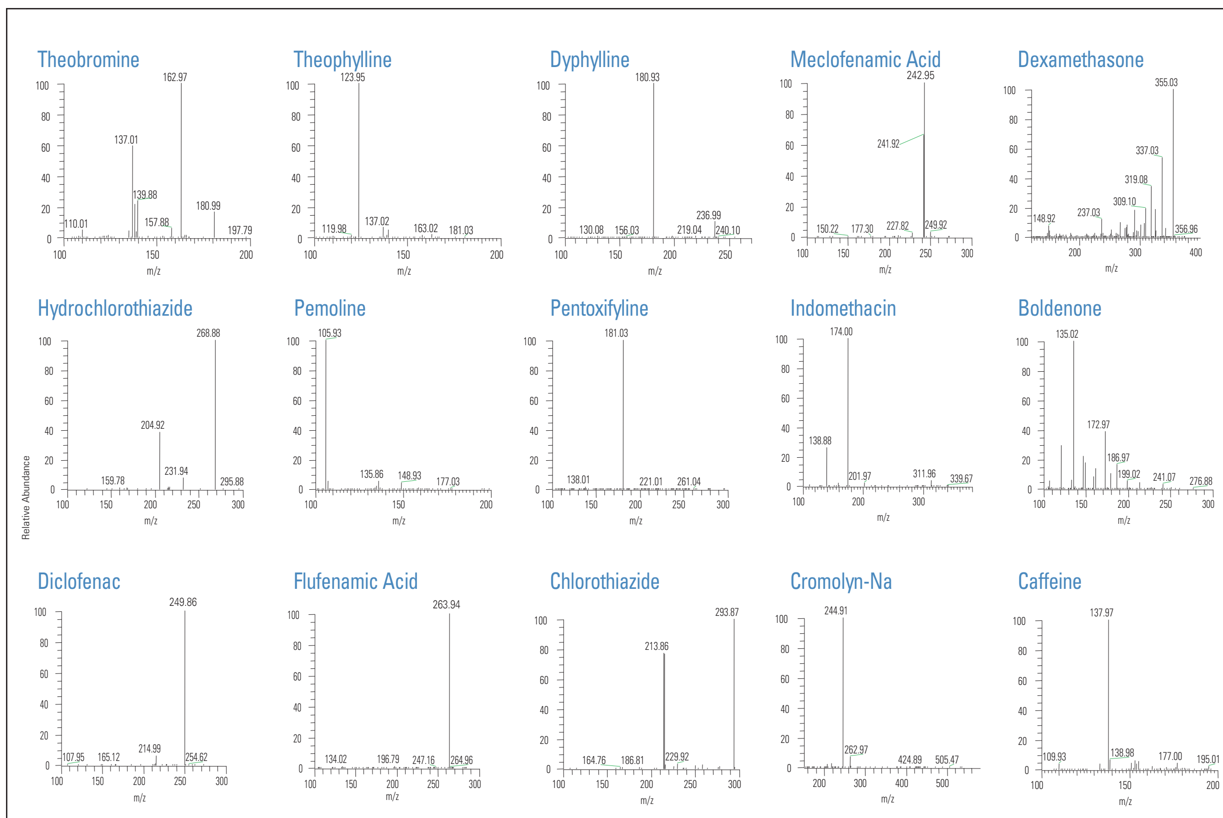


Figure 3: Full-scan MS/MS spectra corresponding to compounds depicted in Figure 2

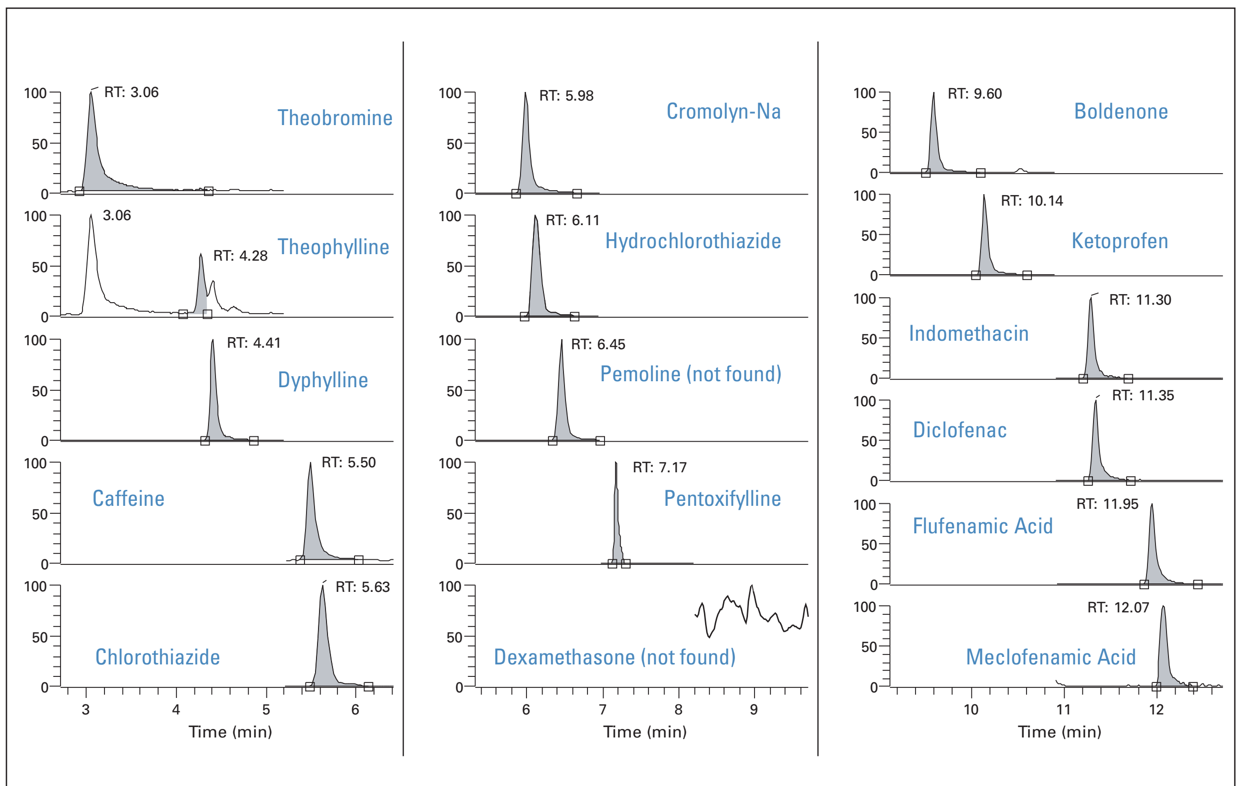


Figure 4: Reconstructed ion chromatograms, using the product ions listed in Table 1 (RIC column), from the analysis of a 10 µL injection of the 50 pg/µL standard in horse urine

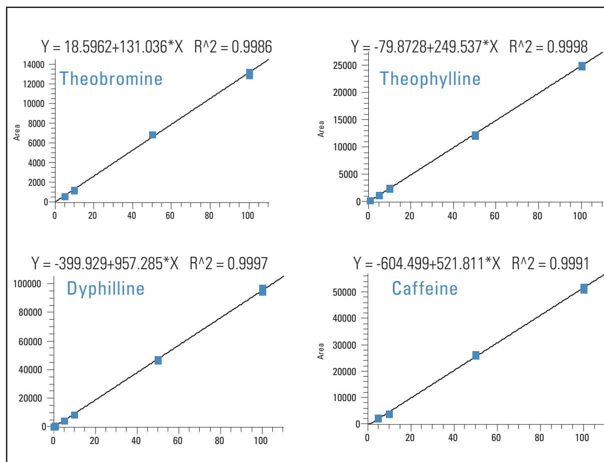


Figure 5: Representative calibration curves from standards prepared in solvent

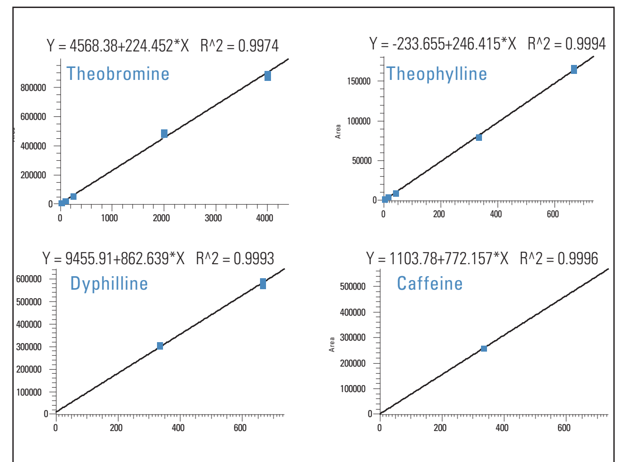


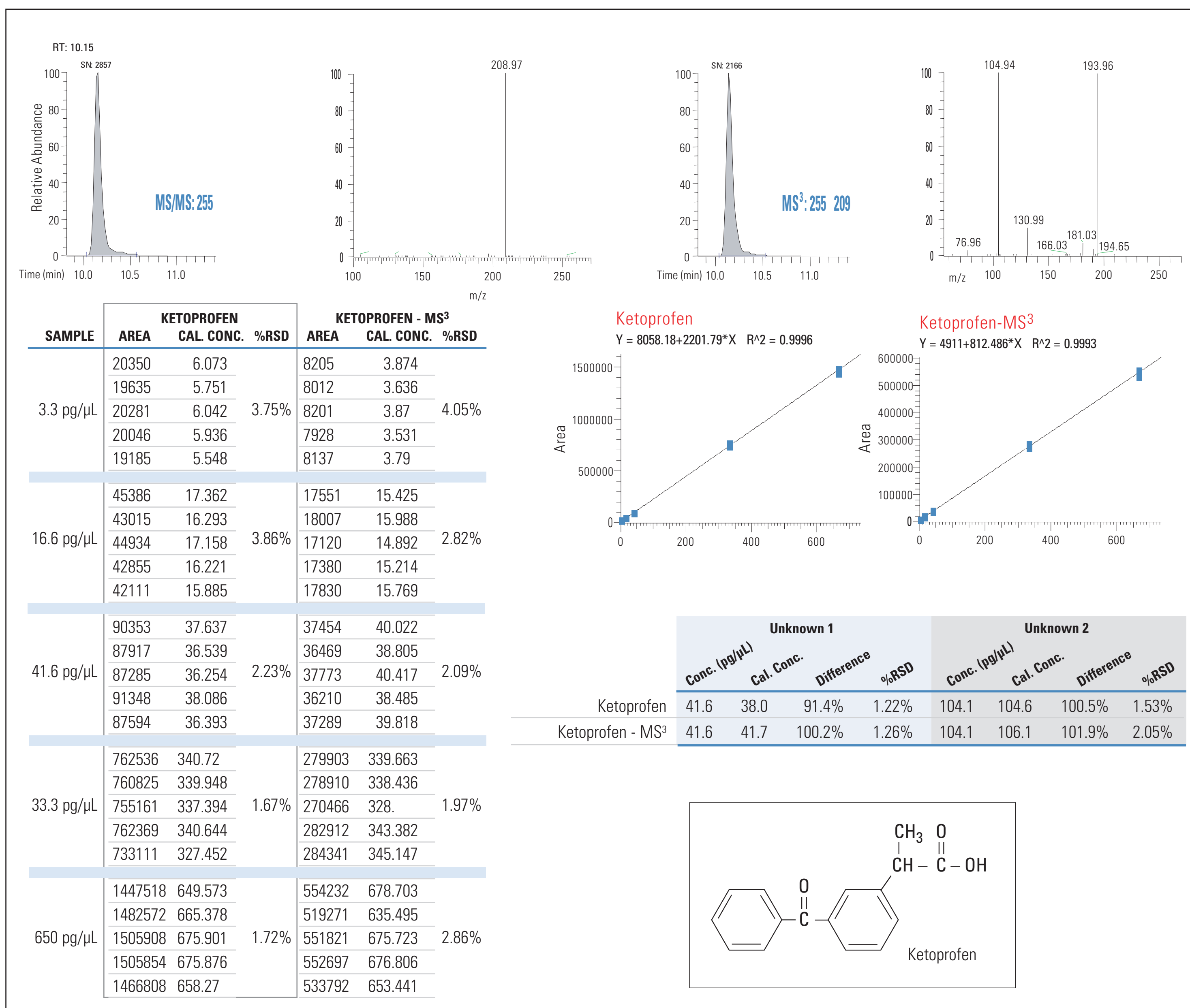
Figure 6: Representative calibration curves from standards prepared in horse urine

The %RSD for three replicate injections is less than 10% for all neat standards at the 1 pg/µL level and higher (see Table 2). The results for standards in urine were also excellent. The %RSD, five replicate injections, for the lowest level assayed in urine was less than 10% for most analytes (see Table 3), and commonly less than 3% for mid- and high-concentration samples. To complete the quantitative study, two QC urine samples were analyzed. The results shown in Table 4 demonstrate a high level of quantitation accuracy, with a deviation of less than 10% for most analytes. In addition, excellent reproducibility was demonstrated with the %RSD being less than 8% for all but two compounds (see Table 4).

Ketoprofen – MS/MS vs. MS^3: Ketoprofen undergoes a neutral loss of a 46 amu fragment in MS/MS mode due to the loss of the carboxyl group (see Ketoprofen structure). This is outside of the mass window for WideBand Activation and thus, an MS^3 experiment was performed to generate additional diagnostic ions without sacrificing sensitivity or reproducibility. To demonstrate this, standards and two urine QC samples were analyzed in both MS/MS and MS^3 mode, with results shown in Figure 7. There is no loss of sensitivity, accuracy, or reproducibility in obtaining this additional information. The %RSD from the MS/MS and MS^3 data are virtually identical. While the sensitivity remains unchanged, the accuracy in the analysis of the unknowns is actually improved in the MS^3 experiments (see Figure 7).

Robustness

To assess the ruggedness of the method, a 166 pg/µL standard in horse urine was assayed over 100 consecutive injections. The results are displayed in Figure 9. The mean and coefficient of variation (%CV) for four compounds: theobromine, caffeine, pentoxyphylline, and ketoprofen were determined to be less than 4% for all four compounds.



SAMPLE	KETOPROFEN AREA	KETOPROFEN CAL. CONC.	KETOPROFEN %RSD	KETOPROFEN - MS3 AREA	KETOPROFEN - MS3 CAL. CONC.	KETOPROFEN - MS3 %RSD
3.3 pg/µL	20350	6.073	3.75%	8205	3.874	4.05%
	19635	5.751		8012	3.636	
	20281	6.042		8201	3.87	
	20046	5.936		7928	3.531	
	19185	5.548		8137	3.79	
16.6 pg/µL	45386	17.362	3.86%	17551	15.425	2.82%
	43015	16.293		18007	15.988	
	44934	17.158		17120	14.892	
	42855	16.221		17380	15.214	
	42111	15.885		17830	15.769	
41.6 pg/µL	90353	37.637	2.23%	37454	40.022	2.09%
	87917	36.539		36469	38.805	
	87285	36.254		37773	40.417	
	91348	38.086		36210	38.485	
	87594	36.393		37289	39.818	
33.3 pg/µL	762536	340.72	1.67%	279903	339.663	1.97%
	760825	339.948		278910	338.436	
	755161	337.394		270466	328.	
	762369	340.644		282912	343.382	
	733111	327.452		284341	345.147	
650 pg/µL	1447518	649.573	1.72%	554232	678.703	2.86%
	1482572	665.378		519271	635.495	
	1505908	675.901		551821	675.723	
	1505854	675.876		552697	676.806	
	1466808	658.27		533792	653.441	

	Unknown 1				Unknown 2			
	Conc. (pg/µL)	Cal. Conc.	Difference	%RSD	Conc. (pg/µL)	Cal. Conc.	Difference	%RSD
Ketoprofen	41.6	38.0	91.4%	1.22%	104.1	104.6	100.5%	1.53%
Ketoprofen - MS^3	41.6	41.7	100.2%	1.26%	104.1	106.1	101.9%	2.05%

Figure 7: Comparison of MS/MS to MS^3 quantitation of Ketoprofen

	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA
	0.1 pg/µL			0.5 pg/µL			
Theobromine							
Theophylline							234
Dyphylline	72	0.49	0.52%	427	1	2.40%	784
Caffeine							
Chlorothiazine				75	0	24.53%	149
Cromolyn-Na	176	0.25	42.33%	456	1	1.28%	917
Hydroclorothiazide							255
Pemoline	102	0.34	18.81%	400	1	11.86%	780
Petoxifyline	793	-0.32	3.79%	4308	0	1.86%	8319
Dexamethasone				479	0	2.45%	1017
Boldenone	219	-0.01	14.44%	1261	0	1.97%	2471
Ketoprofen	315	0.60	7.64%	1191	1	9.43%	2381
Indomethacin				103	1	11.11%	212
Diclofenac	64	0.30	11.55%	281	1	8.84%	475
Flufenamic Acid	310	0.22	13.97%	892	1	2.52%	1780
Meclofenamic Acid	14	0.18	17.51%	61	1	41.26%	113

Table 2: Quantitation results for standards prepared in solvent

	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD
	3.3 pg/µL			16.6 pg/µL			41.6 pg/µL			333.3 pg/µL		
Theophylline (A)	1129	6	10.48%	2540	17	5.53%	5185	38	2.82%	45170	334	2.11%
Dyphylline (A)	9832	5	1.94%	22461	17	2.73%	44917	39	0.98%	322434	334	2.24%
Caffeine (A)	6330	6	6.86%	15401	18	4.94%	30016	37	2.14%	258749	336	1.20%
Chlorothiazine (A)	1798	7	5.99%	3834	17	5.48%	7967	37	0.94%	70426	334	1.24%
Hydroclorothiazide (A)	2487	7	3.07%	5684	18	2.05%	11276	38	2.02%	92748	331	1.39%
Pentoxifylline (A)	122524	-2	-6.01%	296023	16	3.35%	605430	49	2.36%	3152583	332	0.97%
Boldenone (A)	13426	7	2.77%	33942	19	1.55%	58628	35	2.21%	593649	334	1.24%
Ketoprofen (A)	19899	6	3.75%	43660	17	3.86%	88899	37	2.23%	754801	337	1.67%
Ketoprofen – MS^3 (A)	8097	4	4.05%	17578	15	2.82%	37039	40	2.09%	279306	339	1.97%
Indomethacin (A)	1087	2	28.26%	2382	13	6.78%	6442	48	4.05%	35792	332	2.61%
Diclofenac (A)	2577	3	2.37%	5355	13	5.46%	14471	46	4.62%	78597	332	2.94%
Meclofenamic Acid (A)	290	7	10.78%	447	10	22.75%	2130	44	2.92%	6086	122	16.84%
	6.6 pg/µL			33.4 pg/µL			83.3 pg/µL			333.3 pg/µL		
Cromolyn-Na (B)	11298	9	2.70%	30183	27	1.08%	88171	83	0.80%	698392	675	2.40%
Flufenamic Acid (B)	3442	15	3.59%	7763	21	1.84%	55407	88	2.44%	135790	200	13.92%
	20 pg/µL			100 pg/µL			250 pg/µL			2000 pg/µL		
Theobromine (C)	6293	51	1.86%	17288	92	3.23%	51615	222	1.58%	471898	2009	0.77%

Table 3: Quantitation results for standards prepared in horse urine

AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD	AVERAGE AREA	AVERAGE CALC. CONC	%RSD
1.0 pg/µL		5.0 pg/µL			10 pg/µL			50 pg/µL			100 pg/µL		
		625	5	0.47%	1222	9	2.55%	6856	52	0.41%	12993	99	1.43%
1	1.79%	1208	5	2.58%	2408	10	2.52%	12207	49	1.36%	24967	100	0.66%
1	3.10%	4359	5	0.91%	8593	9	0.72%	46667	49	1.47%	95782	100	1.13%
		2330	6	5.03%	3912	9	3.15%	26131	51	0.85%	51308	99	1.07%
1	22.14%	645	4	13.31%	1294	9	4.88%	7711	53	2.66%	14231	98	1.10%
1	5.66%	4672	5	7.11%	8889	9	2.63%	49369	51	3.40%	96148	100	1.12%
1	7.88%	1170	5	11.66%	2663	10	2.83%	13545	50	1.19%	26856	100	2.72%
1	3.65%	3774	5	2.79%	8117	10	1.35%	42321	50	0.62%	84890	100	1.79%
1	1.13%	44808	5	0.56%	93532	10	1.05%	474442	53	2.08%	877603	98	1.77%
1	5.52%	5758	5	1.14%	11952	10	0.47%	60483	51	2.69%	118294	100	1.68%
1	5.08%	12325	5	2.25%	23958	10	1.07%	120812	50	1.33%	238560	100	1.68%
1	7.15%	11906	5	1.22%	22945	10	3.13%	119082	48	1.17%	253903	101	0.46%
1	2.78%	212	1	2.78%	2259	10	2.80%	11565	50	1.77%	23189	100	0.18%
1	8.40%	2382	5	1.20%	4712	10	3.18%	24920	50	1.14%	50161	100	2.06%
1	6.92%	8546	5	0.39%	17468	10	2.60%	90104	50	0.36%	178996	100	1.28%
1	7.84%	641	5	7.97%	1446	10	12.83%	7294	51	1.39%	14337	100	0.21%

AVERAGE AREA	AVERAGE CALC. CONC	%RSD
650 pg/µL		
96129	667	1.31%
575760	667	3.71%
511382	666	1.21%
143677	666	0.54%
186723	668	1.64%
5840616	667	0.95%
1301762	666	1.22%
1481732	665	1.72%
542362	664	2.86%
61378	667	1.74%
122821	668	2.18%
7065	142	10.74%
1350 pg/µL		
88842	84	2.10%
170496	249	10.77%
4000 pg/µL		
825323	3998	1.85%

	QC Sample				QC Sample 2			
	Conc. (pg/µL)	Cal. Conc.	Difference	%RSD	Conc. (pg/µL)	Cal. Conc.	Difference	%RSD
Theobromine (C)	250.0	231.7	92.7%	1.72%	625.0	615.0	98.4%	1.84%
Theophylline (A)	41.6	38.6	92.7%	1.96%	104.1	103.5	99.4%	2.58%
Dyphylline (A)	41.6	41.3	99.3%	2.02%	104.1	115.5	110.9%	3.38%
Caffeine (A)	41.6	42.4	101.9%	3.30%	104.1	109.6	105.3%	3.05%
Chlorothiazine (A)	41.6	43.0	103.3%	2.64%	104.1	114.4	109.9%	1.65%
Cromolyn-Na (B)	83.3	83.9	100.7%	2.10%	210.0	193.9	92.4%	1.77%
Hydroclorothiazide (A)	41.6	41.8	100.5%	2.64%	104.1	113.1	108.6%	2.27%
Pentoxifylline (A)	41.6	44.5	106.9%	2.23%	104.1	126.5	121.5%	1.43%
Boldenone (A)	41.6	38.8	93.4%	1.04%	104.1	102.4	98.4%	2.63%
Ketoprofen (A)	41.6	38.0	91.4%	1.22%	104.1	104.6	100.5%	1.53%
Ketoprofen – MS^3 (A)	41.6	41.7	100.2%	1.26%	104.1	106.1	101.9%	2.05%
Indomethacin (A)	41.6	49.7	119.5%	5.78%	104.1	116.4	111.8%	1.62%
Diclofenac (A)	41.6	48.4	116.3%	5.89%	104.1	124.1	119.2%	7.94%
Flufenamic Acid (B)	83.3	60.7	72.9%	2.21%	210.0	141.6	67.4%	19.10%
Meclofenamic Acid (A)	41.6	33.3	80.1%	6.74%	104.1	89.8	86.3%	21.76%

Table 4: Quantitation results for the analysis of unknown levels of drugs in horse urine

Conclusions

Positive and negative ion detection of co-eluting drugs was accomplished in a single chromatographic run using automated polarity switching. Drugs that underwent a neutral water loss were further fragmented using WideBand Activation to provide a diagnostically rich MS/MS spectrum for structural confirmation. The compound ketoprofen underwent a prominent, non-specific neutral loss of formic acid and was further analyzed using an MS^3 transition. Full-scan MS^n data was reprocessed to quantify all 16 compounds by reconstructed ion chromatograms (RICs), or post-acquisition MRM, and provided results comparable to triple quadrupole SRM quantitation. It is possible to achieve the low % RSD required in quantitation due to the fast cycle time of the Thermo Scientific LTQ. In the case of non-specific neutral molecule losses, MS^3 experiments generated diagnostic spectra for confirmational purposes while providing quantitative results comparable to the MS/MS data. Results of the ruggedness study demonstrate no appreciable loss of sensitivity or reproducibility across 100 replicate urine injections. Thus, using the Thermo Scientific LTQ two-dimensional linear ion trap, we have demonstrated the development of a simple, rapid, and rugged method capable of confirmational screening and simultaneous quantitation of drugs in horse urine using both full-scan LC/MS/MS and MS^3 spectra.

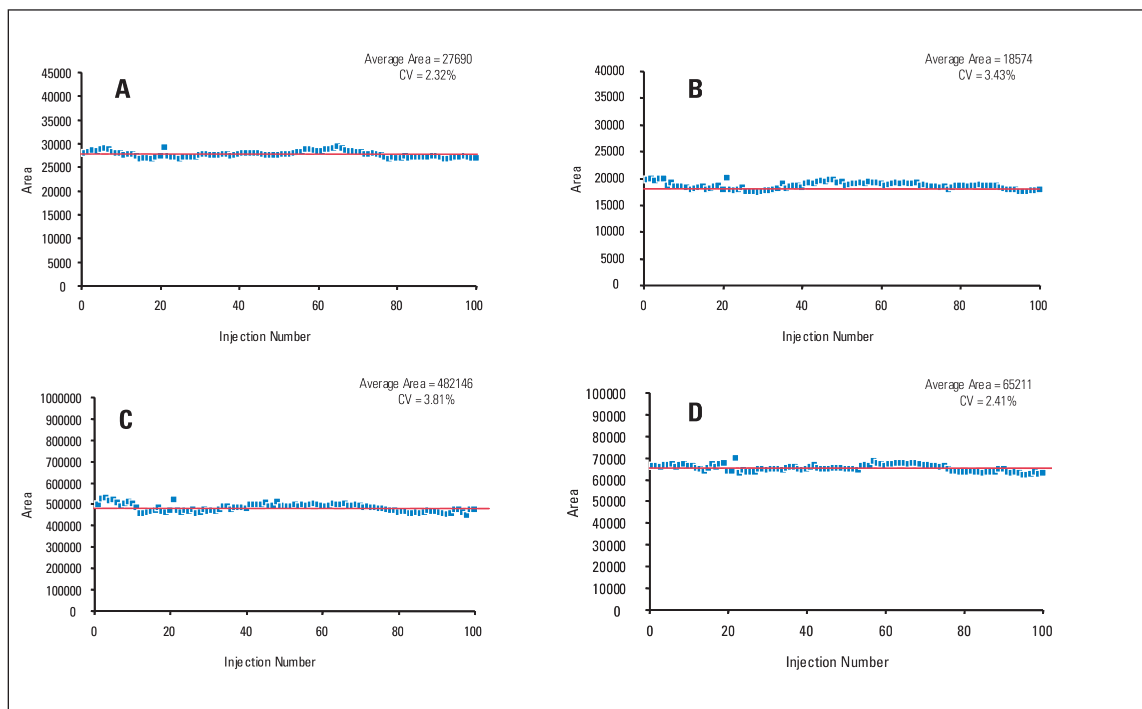


Figure 8: Ruggedness and reproducibility for 100 consecutive injections of a 166 pg/µL standard of theobromine, caffeine, pentoxyphylline, and ketoprofen in urine

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Developing a Method to Protect the Integrity of Racing Using Targeted SRM: Detection and Quantitation of rhEPO/DPO in Horse Plasma

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Key Words

- TSQ Quantum Access™
- HeavyPeptide Labeled Standards
- Proteotypic Peptides
- SRM Method
- Targeted Protein Analysis

Overview

Purpose: To develop a method for the detection and confirmation of rhEPO/DPO in horse plasma using a targeted protein assay and labeled internal standards.

Methods: Combined immunoaffinity separation, enzymatic digestion, and mass spectrometry has been used to confirm the presence of rhEPO in horse plasma[1] The use of an SRM method for targeted protein detection enabled measurements of retention times, ion ratios, and labeled internal standards to confirm and quantify the presence of rhEPO in horse plasma.

Results: Using labeled internal standards, rhEPO was detected, quantified and confirmed in administered horse plasma 72 hours following administration, simulating real world situations.

Introduction

Recombinant human erythropoietin (rhEPO)[2] and Darbepoetin-alpha (DPO)[3] are genetically engineered protein-based drugs used for the treatment of anemia by stimulating red blood cell production. The ability of these agents to stimulate red blood cell production has led to use and abuse by human and equine athletes and, thus, violates the rule of fair competition resulting in their classification as banned substances by the horse racing industry. In addition, continued administration to horses can result in anemia.[3] Despite the negative aspects of rhEPO for horses, a reliable, verifiable, and legally defensible method for identification and confirmation of rhEPO/DPO has been elusive due to the very low concentrations administered. Sample collection is typically acquired only after competition, which could be in excess of 72 hours following administration. Testing of rhEPO/DPO is further confounded by the complexity of the matrices in which the drug is typically found–plasma and urine.

Methods

All experiments were performed using a Thermo Scientific TSQ Quantum Access triple quadrupole mass spectrometer equipped with a Thermo Scientific Surveyor™ MS Pump and MicroAS Autosampler (Thermo Fisher Scientific, San Jose, CA) operated in mSRM mode monitoring six diagnostic peptides that differentiate rhEPO and DPO from equine EPO. (Scheme 1). In addition to the six diagnostic peptides, four stable isotope labeled internal standards for the T_4, T_6, T_{11}, and T_{17} rhEPO proteotypic peptides were used for absolute quantification and additional confirmation of the presence of rhEPO/DPO (Thermo Biopolymers, Thermo Fisher Scientific, Ulm, Germany). Method development was performed using neat rhEPO/DPO protein digests. (Amgen, Inc., Thousand Oaks, CA).

HPLC separations were achieved using a Hypersil Biobasic™ C18 100×0.5 mm column and a binary solvent system consisting of A) 0.1% formic acid and B) MeCN (0.1% formic acid). A gradient profile of 2-40% B in 12 minutes was used at 60 µL/min.

Sample preparation included immunoaffinity separation using rabbit and mouse IgG antibodies linked to magnetic beads. Following separation, the resulting protein was filtered and enzymatically digested with an enzymatic or proteolytic cleavage from which a set of diagnostic peptides representing rhEPO/DPO was chosen as candidate biomarkers for confirmation of the presence of rhEPO/DPO in horse plasma.[1]

Two different sets of samples were prepared and analyzed. The first set was a controlled spiking experiment in which a known quantity of rhEPO was spiked into 1 mL of digested horse plasma to determine detection efficiency. The second sample set was plasma extracted as a function of time following rhEPO administration (iv) of 8000 IU. The time points for extraction ranged from 0 hr to 72 hours. Each of the time point samples was spiked with 10 fmol/µL of the labeled peptide standards.

Results and Discussion

Scheme 1 shows the basis of identification for rhEPO/DPO in equine plasma. The results of enzymatic digestion produced multiple diagnostic markers that can be used to increase the confidence of the presence of the foreign substance in the equine athlete. In addition, the method described enables detection of rhEPO or DPO due to the conserved sequence for each protein over the targeted peptides. Figure 1 shows summed SRM chromatograms for (1A) DPO and (1B) rhEPO using the same SRM transitions. Clearly, the retention times are closely identical for both samples indicating the experimental method is robust for either drug.

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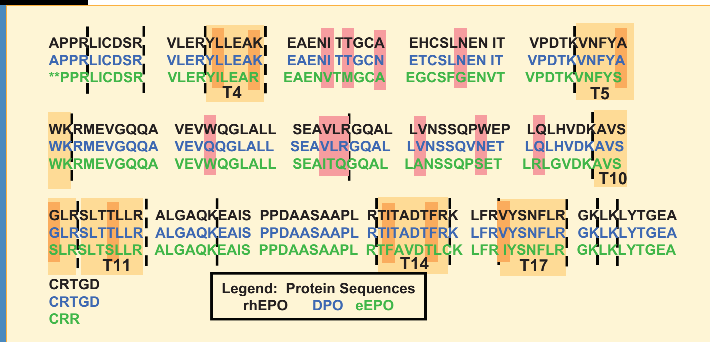
Figure 2 shows the summed SRM chromatograms for the four targeted rhEPO peptides and the labeled analogues. The labeled peptide can be used to confirm the correct elution time as well as the ion ratio provided more than one transition was used to monitor each peptide. A level of 500 amol on column was used to test the detection capabilities of the approach used, which would equate to a concentration of ca. 1.7 ng/mL. Note that the responses of T_4, T_{11}, T_{17} markers were greater than 10000



Scheme 1. Comparison of protein sequences for rhEPO, DPO, and equine EPO. The dashed lines represent sites of enzymatic cleavages and the red boxes highlight non-conserved sequence sites between rhEPO/DPO and equine EPO. The targeted peptides are marked with a gold box.

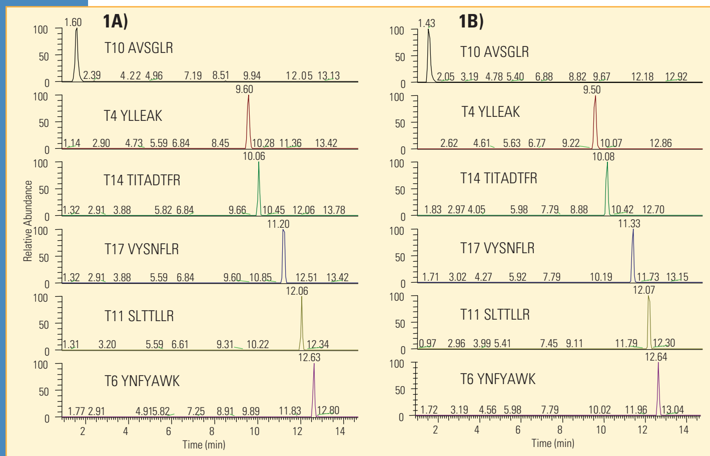


Figure 1: SRM chromatographic traces for each of the targeted peptides for 1A) DPO and 1B) rhEPO enzymatic digest using identical experimental method.

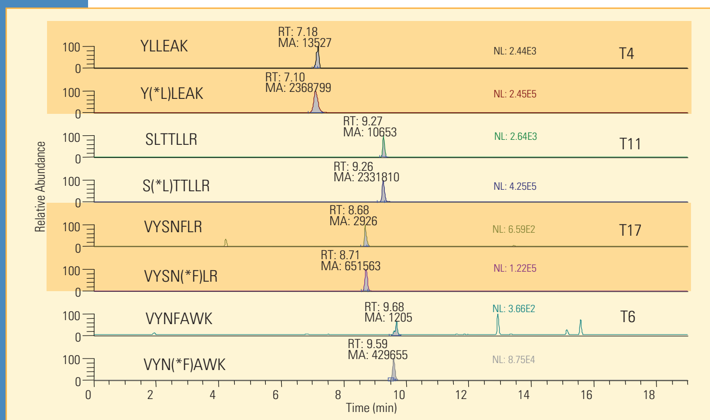


Figure 2: SRM responses for four targeted rhEPO peptides and the corresponding stable isotope labeled peptide. The measured response is for a total of 500 amol on column for the unlabeled rhEPO and 100 fmol for the labeled rhEPO peptides.

counts, indicating lower levels of detection to be about 10x lower (or 0.2 ng/mL) without requiring nanoliter flow rates, which simplifies the experiment and increases the robustness of the method.

In addition to establishing the correct retention times for targeted peptides, the stable-isotope labeled peptides can be used for correct ion ratio determination as an additional means of verification. Figure 3 shows comparative full-scan product ion spectra for the (3A) unlabeled and (3B) labeled T_{11} peptide. Note the y-series detected for each, providing sequencing information and site determination for the stable isotope labeled residue such as the a_2/b_2 fragments as well as the y_6 for the unlabeled peptide. The two product ions used for detecting the T_{11} peptide were the y_4 and y_5 ions. The calculated abundance ratios for the unlabeled and labeled peptides were ca. 25%. The insets to the right of Figure 3 show the measured ion abundance for each SRM transition at 500 amol level. The calculated ratio is within experimental error to be used as an additional means of confirmation for the targeted peptide elution.

Figure 4 shows the quantification curve calculated for the controlled rhEPO spiking of horse plasma. The values show excellent agreement between theoretical and experimentally determined levels based on the integrated peak area ratios between the unlabeled and labeled targeted rhEPO peptides. The %CVs for each was less than 20% at 500 amol level indicating excellent capabilities to quantify the presence of rhEPO in plasma. While a positive confirmation would only require one diagnostic peptide to be present, this method yields four proteotypic peptides that could be used unequivocally to increase the confidence in a positive determination.

The second sample set was used to test the entire workflow. A female horse (500 kg) was administered rhEPO intravenously using 8000 IU (0.08 mg/kg) for four days. Following the injection on the fourth day, blood was withdrawn at 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 24, 48, and 72 hour intervals. Samples for each time point were processed using the method outlined previously, reducing complexity of the resulting protein digest mixture. The protocols of most horse racing commissions require the saliva, urine, and/or blood sample to be taken from the winning horse following completion of a race. The 72 hour time window represents a possible maximum duration between the final doping and racing while maintaining a pharmacological effect following administration of rhEPO/DPO. The 8000 IU dose is also an estimate of the dose required to induce the desired biological effects of increasing oxygen carrying capacity for equine athletes. The proposed protocol must enable a reduction of sample loss through the number of sample purification, filtering, reconstitution, and digestion steps prior to mass spectral analysis. Figure 5 shows the summed SRM chromatograms for the four targeted rhEPO peptides with their stable-isotope labeled internal

standards. Three of the four peptides showed a positive response with little signal attributed to the T_6 peptide at the 72 hour time point. Although the response for the T_4 peptide does not appear to be measurable, closer inspection shows an integrated peak area over 2000 counts observed to have the same retention time as that for the labeled T_4 peptide at 7.27 minutes.

Comparison of chromatographic retention times for the administered rhEPO study with the spiked rhEPO study (Figure 2) showed excellent chromatographic reproducibility, with retention times that shifted less than 6-10 seconds, enabling an additional means of confirmation for the presence of rhEPO in the extracted horse plasma. Based on the integrated peak area ratios for the three detected rhEPO biomarkers, a total of ca. 0.05 ng/mL was present in the horse plasma following a 72-hour delay between rhEPO administration and sample collection. Comparison of LC-MS/MS results with those measured using ELISA show similar levels (0.04 ng/mL–data not presented) indicating excellent agreement between the two methods.

Using a stable-isotope labeled internal standard provides two clear advantages: identification of the correct retention times, as shown above, and determination of the correct ion ratio for the monitored product ions. Figure 3 demonstrates the consistency of the ion ratios measured following CID for both full scan MS/MS detection as well as SRM analysis for the T_{11} labeled and unlabeled rhEPO peptides. The same measurements can be used to confirm the presence of rhEPO at each time point. Figure 6 shows the measured ion abundance for the y_4 and y_5 fragment ions for the unlabeled and labeled T_4 peptides at the time points of 72, 10, and 0.5 hrs following the final rhEPO administration. The measured ion ratios for the unlabeled T_4 peptides were consistently between 20 and 25% while the ratio for the labeled T_4 peptide was consistently between 30 and 35%. The slight increase in the ratio for the labeled peptide was observed for the three other pairs of signature peptides (see Figure 3).

Figure 7 shows the calculated rhEPO concentration in the extracted horse plasma samples for T_4 and T_6 peptides. The levels were calculated using the integrated area ratios between the targeted rhEPO peptide and their corresponding labeled internal standards. The calculated concentration for two targeted peptides agree with those obtained using two different labeled standards to monitor the concentration of rhEPO in the test sample. In addition to mass spectral determination, ELISA was also used to A) predict the presence of rhEPO and B) calculate the level of rhEPO in plasma at each time point. The ELISA results nicely corresponded with those calculated using the targeted SRM approach; in fact, the levels estimated at 48 and 72 hours agreed well (0.06 and 0.04 ng/mL, respectively), increasing the confidence in the calculated concentrations.

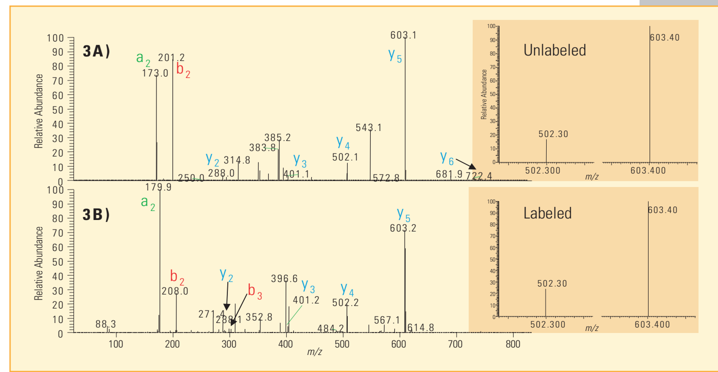


Figure 3: Comparative Quantitation Enhanced Data-Dependent™ MS/MS spectra for 3A) unlabeled and 3B) labeled T_{11} peptides at 50 fmol on column. The inset shows the measured ion intensity for the SRM transitions for the unlabeled and labeled T_{11} peptides at 500 amol on column.

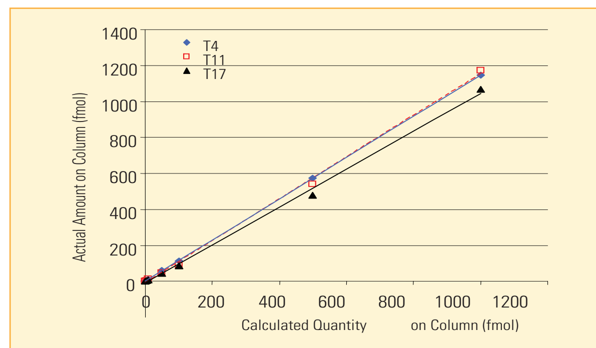


Figure 4: Quantification curve for neat rhEPO analysis. The calculated levels were determined using area ratios of the labeled analogues.

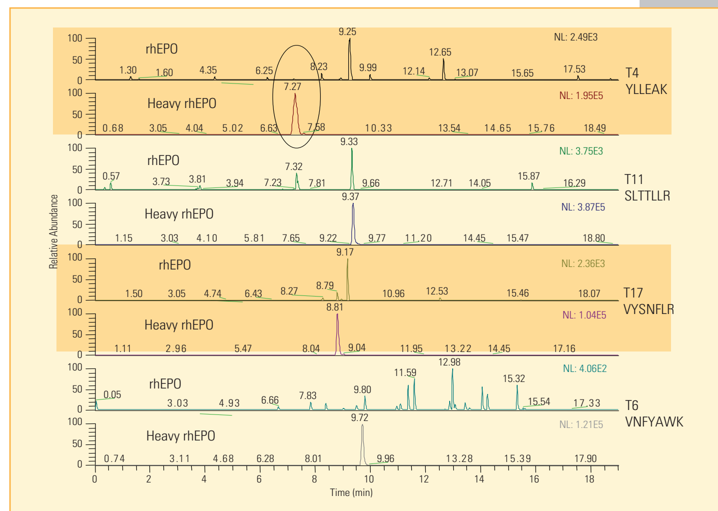


Figure 5: Summed SRM chromatograms for the four targeted rhEPO peptides and their labeled derivatives for the horse plasma extraction sample collected 72 hours following rhEPO administration.

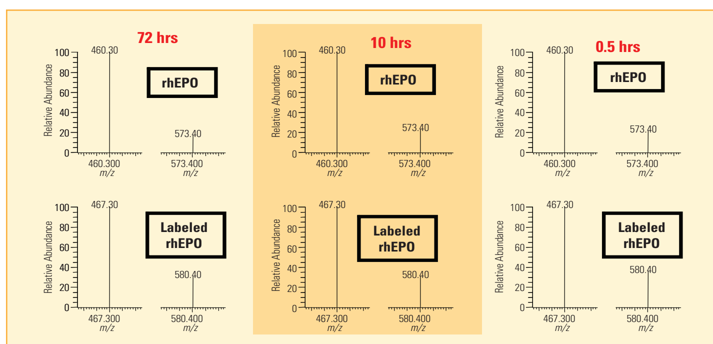


Figure 6: Comparative ion abundance ratios for the T_4 peptide at three different time points for plasma collection. The top row is the measured ion abundance for the unlabeled peptide and the bottom row is the response from the labeled peptide.

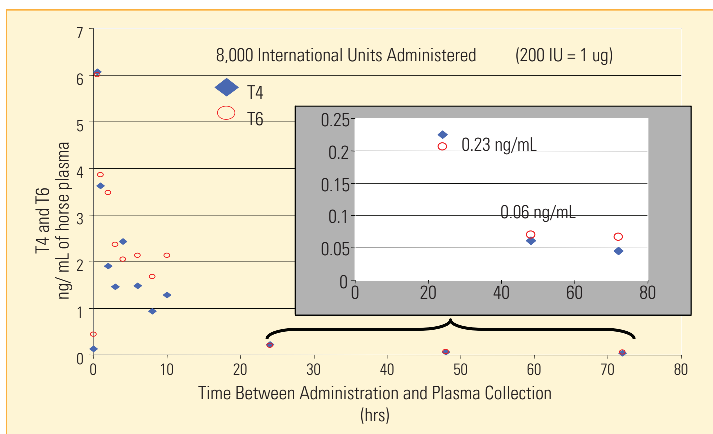


Figure 7: Calculated rhEPO levels in extracted horse plasma as a function of time delay between administration and sample collection. The calculated levels were based on the area ratios of T_4 and T_6 targeted rhEPO peptides and the labeled internal standards.

Conclusions

The approach presented here provides a sensitive and selective method for preparing and analyzing horse plasma for the presence of rhEPO or DPO. The advantages of this method include the ability to use up to six diagnostic peptides to confirm or refute the presence of either illegal protein-based drug.

The use of stable-isotope labeled analogues provides further means of confirming the presence of diagnostic peptides based on chromatographic retention times and ion ratios.

The sensitivity demonstrated enabled detection up to 72 hours following the last administration of rhEPO, increasing the confidence that the described method is useful in the racing industry to maintain a level field of competition.

Of particular interest is the measured sensitivity that was achieved using microspray, increasing the analysis time while simplifying the experimental method and thus, enabling more laboratories the option of employing rhEPO/DPO screening.

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Targeted Quantitation of Insulin and Its Therapeutic Analogs for Research

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Overview

Purpose: To perform simultaneous qualitative and quantitative analyses of endogenous insulin and/or therapeutic analogs at biological levels for research.

Methods: We used a pan-anti insulin antibody in Thermo Scientific™ Mass Spectrometric Immunoassay (MSIA) D.A.R.T.'S™ pipette tips for highly-selective affinity purification of all insulin analogs. Analogs were detected, verified, and quantified using high-resolution, accurate-mass (HRAM) MS and MS/MS data from a Thermo Scientific™ Q Exactive™ mass spectrometer.

Results: We achieved a lower-limit-of-detection (LLOD) of 15 pM in plasma for all variants used with linear regressions of 0.99 or better. Further, we demonstrate inter- and intra-day CV's of < 3% and spike and recovery resulted in recoveries of 96–100%.

Introduction

The measurement of insulin is a paramount metric in clinical research, therapeutic research, forensic, and sports doping applications. Conventional insulin analytical methods are plagued by the inability to differentiate endogenous insulin from exogenous insulin analogs. The use of LC/MS can overcome this shortcoming[1]; however, the LC/MS methods to date lack the analytical sensitivity demanded by the field. Therefore, a highly selective sample interrogation workflow is required to address the complexity of plasma samples and, ultimately, for accurate and sensitive LC/MS detection and quantification. To meet these requirements, a MSIA research workflow was developed for the high-throughput, analytically sensitive quantification of insulin and its analogs from human donor plasma.

Methods

Sample Preparation

For spike and recovery studies, both neat and donor plasma samples containing a mix of insulin and its analogs were prepared. Insulin was added at three different amounts that spanned the dynamic range to the donor plasma. Up to four analogs were prepared in a single sample. For the limit-of-detection and limit-of-quantification studies, 1.5 pM to 960 pM insulin was added to bovine serum albumin in phosphate buffered saline. Additionally, either 0.05 nM of a heavy version of insulin or porcine insulin was added as an internal reference standard to each well of 500 μL plasma.

Samples were then addressed for the first stage in the MSIA workflow. Targeted selection was achieved using insulin MSIA Disposable Automated Research Tip's (D.A.R.T.'S) (Figure 1). The affinity purification step in the MSIA workflow was automated by the Thermo Scientific™ Versette™ automated liquid handler. Following extraction, intact insulin analogs were eluted with 75 μL 70:30 water/acetonitrile with 0.2% formic acid with 15 μg/mL ACTH 1-24. The final concentration was adjusted to 75:25 water/acetonitrile with 0.2% formic acid for LC/MS analysis.

Liquid Chromatography

A Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system was used for all experiments. 100 μL of each sample was separated on a 100 x 1 mm Thermo Scientific™ ProSwift™ column using a linear gradient (10–50% in 10 min) comprised of A) 0.1% formic acid in water and B) 0.1% formic acid in acetonitrile. The column was heated to 50 ºC.

Mass Spectrometry

All data was acquired using a Q Exactive Orbitrap mass spectrometer operated in data-dependent mode with dynamic exclusion enabled. Full scan MS data was acquired with a resolution setting of 70,000 (at *m/z* 200) and using a mass range of 800–2000 Da. A targeted inclusion list was used to trigger MS/MS events and MS/MS was acquired with a resolution setting of 17,500 (at *m/z* 200).

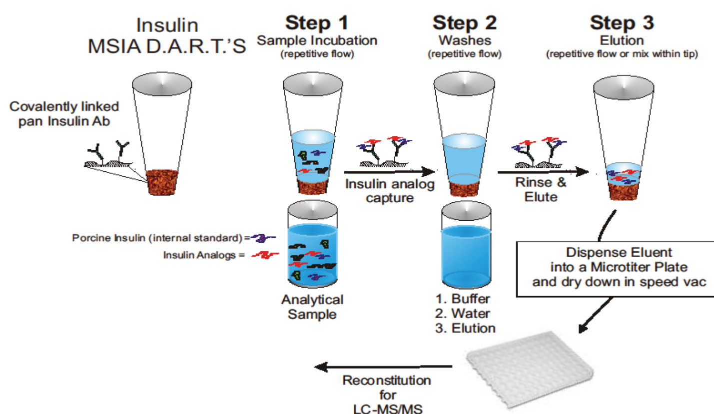
Data Analysis

Thermo Scientific™ Pinpoint™ software version 1.3 was used to analyze all LC/MS data. HRAM measurements were used for qualitative and quantitative measurement of insulin and its analogs.

The three most abundant precursor charge states per analog and the six most abundant isotopes per charge state provided qualitative validation for insulin and its analogs. Qualitative scoring was based on mass error, precursor charge state distribution, isotopic overlap, and corresponding LC elution peak profiles. Product ion data was used for sequence verification.

For quantification, a mass tolerance of ±5 ppm was used for all data extraction. Amounts of each insulin analog were determined by converting area-under-the-curve (AUC) values, normalized to the AUC of the internal reference, which was calculated from standard curve data.

FIGURE 1. Targeted selection using insulin MSIA D.A.R.T.'S.. First, insulin and its analogs are selectively bound. Then, a wash step removes background compounds. Lastly, the insulin and insulin variants are eluted into a new plate, which is ready for LC/MS analysis.



Results

Qualitative Validation of Insulin and Its Analogs

One of the primary limitations of current insulin analytical methods is the inability to distinguish between endogenous and exogenous insulin analogs. The immobilized insulin pan-antibody in the MSIA D.A.R.T.'S recognizes a common epitope region in the β-chain that is conserved across all of the analyzed variants. This allows the capture and detection of all variants from the sample as long as the β-chain epitope region remains conserved. Further, utilizing full scan MS mode in the analysis stage of the MSIA workflow enables simultaneous detection of multiple insulin analogs and the ability to screen for unsuspected insulin analogs post-acquisition.

LC/MS detection using HRAM MS data provided the analytical selectivity to distinguish insulin variants from the background signal using the accurate mass of multiple precursor charge states and isotopes. Figure 2 demonstrates the HRAM data analysis approach. Figure 3 shows simultaneous LC/MS detection of insulin variants. Further, fragmentation patterns from data-dependent MS/MS acquisition can also be used to confirm the identity of insulin variants (data not shown).

FIGURE 2. HRAM MS data analysis in Pinpoint software version 1.3. Extracted ion chromatograms for each targeted insulin variant were created using the isotopic *m/z* values from three precursor charge states. Integrated AUC values from each isotope were then co-added to generate the reported values. Additionally, each insulin variant was qualitatively scored based on 2a) comparative peak profiles (peak start and stop, apex, and tailing factors) as well as 2b) isotopic distribution overlap.

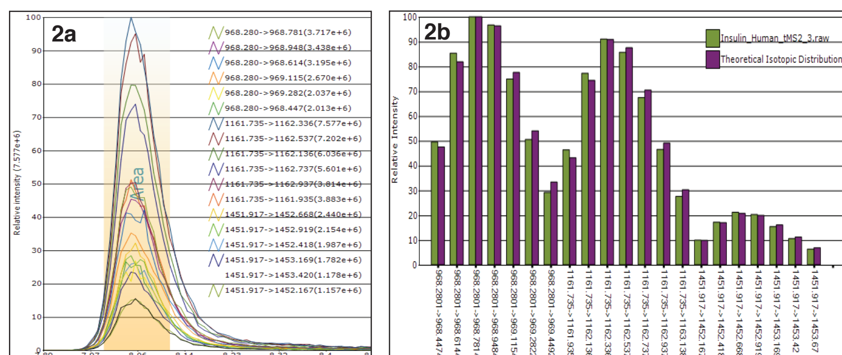
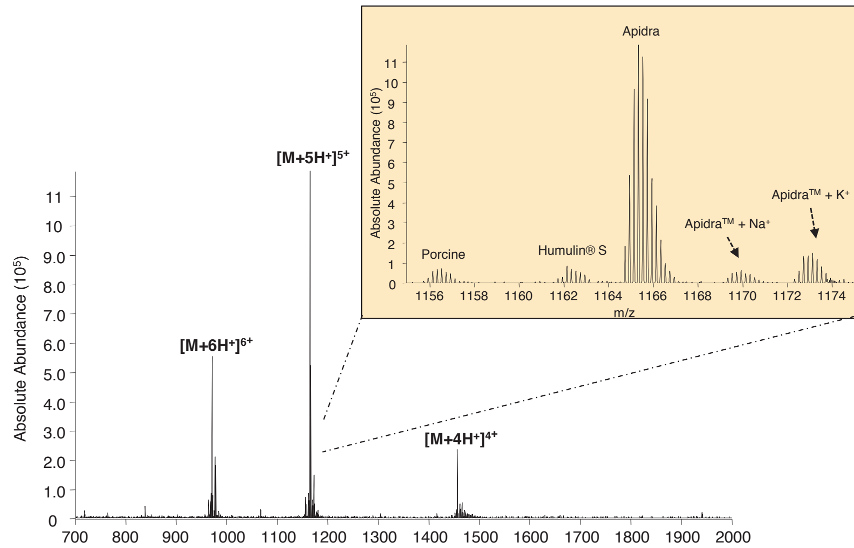


FIGURE 3. Simultaneous LC/MS detection of four insulin variants. Apidra™ (0.48 nM), Humulin® S (0.06 nM), Lantus™ (0.48 nM) , and porcine as the internal standard were processed from the same sample and detected simultaneously. The inset shows an enlargement of the 5+ charge state, and shows all three variants. Lantus elutes 0.5 minutes prior to the three displayed insulin variants.

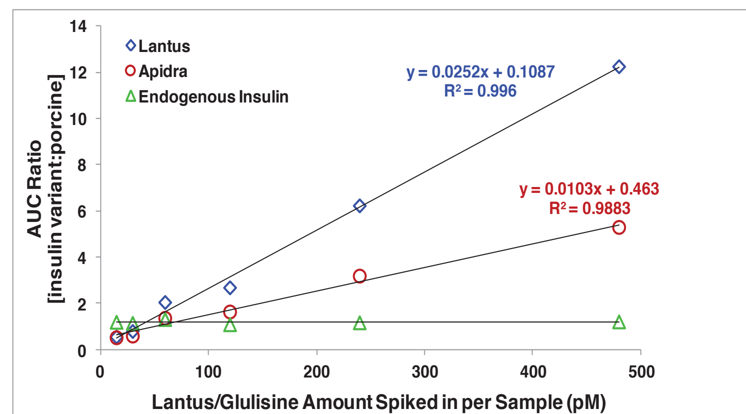


Quantitative Measurement of Insulin and Its Analogs

Additional limitations to high-throughput targeted quantification of insulin and its analogs in research are inefficient sample preparation protocols that result in their lack of analytical sensitivity and robustness. Using the insulin MSIA workflow described above, we achieved an LLOQ and LOD of 15 pM (87 pg/mL) for the intact variants in plasma. Quantification curves for Lantus and Apidra are shown in Figure 4. Tables 1 and 2 display LOQ and LOD.

Further, reproducibility studies demonstrated inter- and intra-day CVs of < 3% (Tables 3 and 4) and spike and recovery resulted in recoveries of 96–100% (Table 5). In addition to the improved sensitivity, the MSIA workflow significantly reduces the background matrix. The reduced complexity affords shorter LC gradients, and, therefore, shorter LC/MS analysis times.

FIGURE 4. Quantification curves for Lantus and Apidra. Lantus and Apidra were spiked into donor plasma at different concentrations. The endogenous insulin from the donor plasma is also plotted. Since the same amount of donor plasma was used for each sample, the level of endogenous insulin remains static. All AUC values were normalized to the porcine AUC response.



Method Characteristics for the MSIA Insulin Research Workflow

The LLOQ for the insulin MSIA research workflow is 15 pM (highlighted in red in Table 1), which was determined as the lowest concentration where we could achieve a %CV of <20% and an accuracy within ±20%.

An LOD of 15 pM (highlighted in red in Table 2) was also achieved for the insulin MSIA workflow. The LLOD was determined as the lowest concentration where the mean total area was greater than four standard deviations of the background signal added to the mean total area for the blank.

TABLE 1. Limit of quantification

STD Conc. (pM)	Mean (5 Curves)	StDev	%CV	Accuracy
0	7.42	1.02		
7.5	10.56	0.95	9.04%	40.80%
15	16.78	1.42	8.46%	11.87%
30	28.96	1.12	3.85%	-3.46%
60	58.41	1.61	2.75%	-2.66%
120	115.93	1.96	1.69%	-3.39%
240	232.65	2.80	1.20%	-3.06%
480	473.25	14.41	3.04%	-1.41%
960	963.31	6.47	0.67%	0.34%

TABLE 2. Limit of detection

STD Conc. (pM)	Mean Total File Area	4 × StDev	Plus 4 × StDev
0	2.37E+05	2.20E+05	4.57E+05
7.5	2.80E+05		
15	4.79E+05		
30	8.93E+05		

TABLE 3. Intra-day repeatability

STD Conc. (pM)	Mean (3 Controls x 5 Curves)	StDevp	%CV	Accuracy
50.00	51.21	1.33	3	2.43%

TABLE 4. Inter-day repeatability

STD Conc. (pM)	Mean (3 Controls x 5 Curves)	StDevp	%CV	Accuracy
50.00	51.07	0.81	2	2.15%

Sample	Spike Conc. (pM)	Exp. Conc. (pM)	Average (pM)	Exp Recovery Conc. (pM)	% Yield
Neat_1	0.00	43.79	44.59		
Neat_2		45.59			
Neat_3		44.38			
Low_1	19.50	65.08	64.11	19.52	100.12%
Low_2		63.65			
Low_3		63.61			
Medium_1	199.50	241.19	237.56	192.97	96.73%
Medium_2		239.80			
Medium_3		231.70			
High_1	919.50	960.91	928.63	884.05	96.14%
High_2		905.35			
High_3		919.64			

Conclusions

- Automated sample extraction is amenable to high-throughput analysis, thus decreasing sample preparation times.
- Insulin MSIA D.A.R.T.'S equivalently extract multiple insulin variants present at different concentrations for simultaneous detection and quantification for research.
- HRAM MS affords qualitative confirmation and quantification of the insulin variants present in one LC/MS run.
- Pinpoint software version 1.3 provides automated data extraction, confirmation, and quantification for all insulin analogs.
- Reduced complexity affords shorter LC gradients, and, therefore, shorter LC/MS analysis times.
- An LLOD < 15 pM and an LLOQ of 15 pM (87 pg/mL) in 0.5 mL of plasma were achieved.
- Intra- and inter-day repeatabilities were < 3%, thus making the insulin MSIA workflow highly reproducible.

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Case Study

“What we liked about the Transcend system is that it offered an opportunity to reduce our sample preparation down to a limited few steps.”

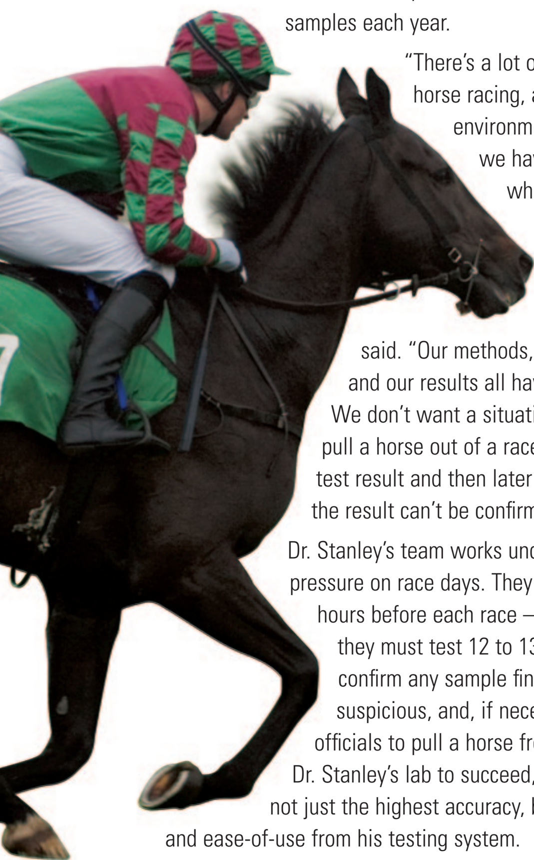
Dr. Shawn Stanley,
Chief Analyst, Singapore Turf Club

Analysis of Equine Doping Using TurboFlow Technology and Multiplexing with LC-MS/MS

Singapore Turf Club Case Study

Introduction

The Singapore Turf Club ran its first race in 1842, for the then-sizable purse of $150. Today hundreds of millions of dollars are won and lost at the track every year. With that kind of money at stake, the fair outcome of a race must be beyond question. As chief analyst at the Singapore Turf Club's testing lab, Dr. Shawn Stanley is tasked with making sure there is never a doubt. Dr. Stanley uses some of the most advanced mass spectrometry technology in the world to test more than 15,000 horse blood and urine samples each year.



"There's a lot of money in horse racing, and the legal environment is such that we have to ensure whatever calls we make from the lab are defensible," Dr. Stanley said. "Our methods, our technology and our results all have to be solid. We don't want a situation where we pull a horse out of a race based on a test result and then later on it turns out the result can't be confirmed."

Dr. Stanley's team works under intense pressure on race days. They have just two hours before each race – during which they must test 12 to 13 samples, confirm any sample finding that is suspicious, and, if necessary, notify officials to pull a horse from a race. For Dr. Stanley's lab to succeed, he requires not just the highest accuracy, but also speed and ease-of-use from his testing system.

The Challenge

Screening for performance-enhancing drugs in horses is an incredibly challenging problem because race officials are usually looking for illegal steroids that have similar molecular structure as naturally occurring steroids in animals. Further complicating the testing process is the fact that horse urine and blood are both complex, dirty matrices – making the separation especially challenging.

Dr. Stanley previously used a mass spectrometry system that required liquid-liquid extraction, but said he needed a system that was both faster and more reliable. Dr. Stanley adopted the Thermo Scientific Transcend TLX-4 system – the only truly independent, parallel, multichannel U-HPLC system. The Transcend™ system, powered by Thermo Scientific TurboFlow technology, provides advanced capability compared to traditional LC separation front-end systems, offering high throughput, online sample extraction, superior data quality and ease-of-use.

Sample Preparation

Sample preparation was the biggest bottleneck in Dr. Stanley's lab and a major reason he became interested in the TLX-4 system.

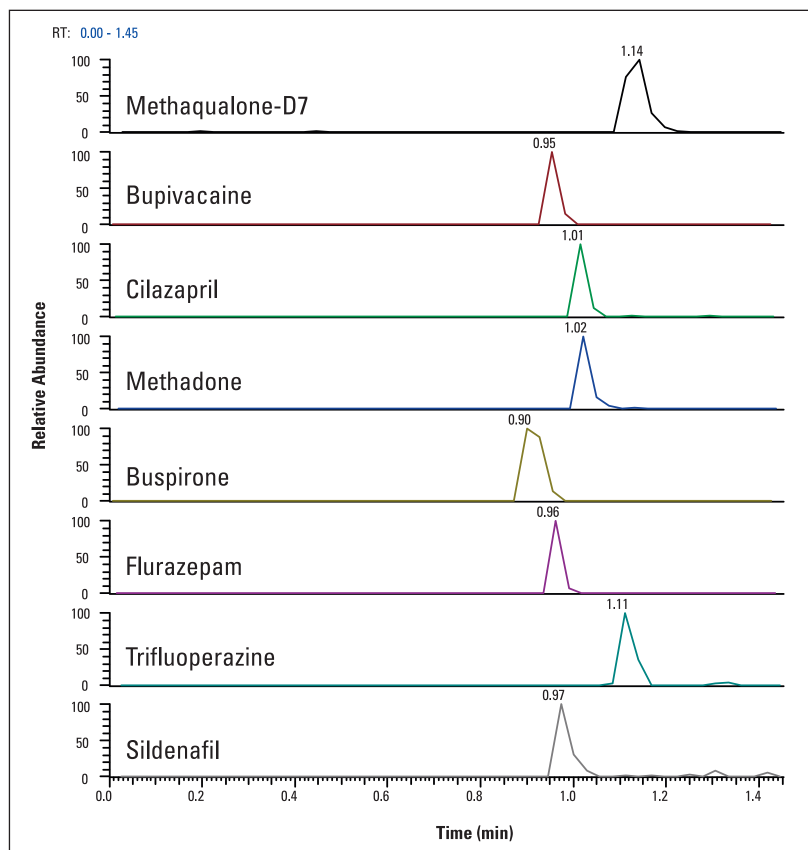
Transcend systems save time because they allow the user to inject an untreated sample, like plasma or urine, directly into the system, eliminating time-consuming sample prep processes such as liquid-liquid extraction, solid-phase extraction and protein precipitation. No preparation is necessary because Transcend uses an innovative TurboFlow™ method to separate analytes from biological fluids prior to MS/MS analysis.

"What we liked about the Transcend system is that it offered an opportunity to reduce our sample preparation down to a limited few steps," Dr. Stanley said. "We didn't have to wait for a sample to dry down; we could even put the sample on directly, although for robustness we do a little sample preparation in the pre-race analysis."

Dr. Stanley estimates the Transcend system shaves 20 minutes off the two hours it used to take him to run a batch of samples – a huge savings on a typical race day consisting of a dozen races or more. The decision to pull a horse or keep it in the race can have huge legal implications for the track. Dr. Stanley said he needs the best data he can get to support his calls.

"Now we have the luxury of spending more time confirming a sample that is flagged. We don't have to make decisions instantly, so there's a lot less pressure," Dr. Stanley said. "Before, we were running so short of time that if there were any hiccups in the second stage of confirmation, it might be too late to call a horse out of the race."

The Transcend TLX-4 system with the Thermo Scientific TSQ Quantum Ultra mass spectrometer provided a 30 second data window for eight antibiotic calibration standards.



Multiplexing

Because of the volume of screening work in Dr. Stanley's lab, multiplexing is a necessity. The multiplexing capability of the Transcend system was a key reason he adopted it.

"Our previous instrument ran four samples at once, so it was always sampling each channel 25 percent of the time, which decreases your sensitivity a lot; you lose more than 50 percent compared to just running a single channel," Dr. Stanley said.

The Transcend system delivers a huge increase in throughput, enabling users to run two or four different methods simultaneously on one mass spectrometer.

These unique capabilities do not come at the cost of data quality. Because the operation of each multiplexed LC system is staggered and parallel, the mass spectrometer is dedicated solely to a single sample stream during the critical elution step, maintaining data quality and sensitivity throughout the process. The TLX-4 system quadruples the throughput of a single channel system, reducing typical mass spectrometer idle time from 75 percent to 4 percent.

Online Operations

With a staff of 19 and several different groups of technicians working on the system, Dr. Stanley identified ease-of-use and the ability to unify all online operations on a single software platform as critical factors in his decision.

All of the Transcend system online operations are controlled by Thermo Scientific Aria software – including multiplexing, pump, valve and autosampler operation.

"From the first day we had the system up and running, we were getting results. Literally, we were using it in eight hours and understood it pretty well," Dr. Stanley said. "The Aria software made it simple to play around with the various parameters, and the graphic interfaces were easy to follow."

Conclusion

The Transcend system reduced sample preparation time and increased mass spectrometry throughput for the Singapore Turf Club. In addition, the system virtually eliminated ion suppression by removing 99 percent of all endogenous phospholipids.

The opportunity to purchase a complete solution that is ready to run out of the box is one reason Singapore Turf Club turned to Thermo Scientific technology. "Other companies said, 'Buy a bit of this, and a bit of this and this, and we'll put it together for you and make it work,'" Dr. Stanley said. "But we can't afford to spend the next two years doing a research project. These are frontline instruments and we needed something with the kind of reliability Thermo Fisher Scientific offers."

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Sports Anti-Doping Peer Reviewed Articles

Tudela, E.; Deventer, K.; Geldof, L.; Van Eenoo, P. Urinary detection of conjugated and unconjugated anabolic steroids by dilute-and-shoot liquid chromatography-high resolution mass spectrometry. *Drug Test. Anal.* [Online early access]. DOI: 10.1002/dta.1650. Published online: April 21, 2014. http://onlinelibrary.wiley.com/doi/10.1002/dta.1650/abstract.

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Hostrup, M.; Kalsen, A.; Auchenberg, M.; Rzeppa, S.; Hemmersbach, P.; Bangsbo, J.; Backer, V. Urine concentrations of oral salbutamol in samples collected after intense exercise in endurance athletes. *Drug Test. Anal.* [Online] **2014,** *6* (6), 528–532. http://onlinelibrary.wiley.com/doi/10.1002/dta.1568/abstract.

Kaabia, Z.; Dervilly-Pinel, G.; Popot, M. A.; Bailly-Chouriberry, L.; Plou, P.; Bonnaire, Y.; Le Bizec, B. Monitoring the endogenous steroid profile disruption in urine and blood upon nandrolone administration: An efficient and innovative strategy to screen for nandrolone abuse in entire male horses. *Drug Test. Anal.* [Online] **2014,** *6* (4), 376–388. http://onlinelibrary.wiley.com/doi/10.1002/dta.1520/abstract.

Sports Anti-Doping Webinars

WB64303: Comprehensive Anti-doping Detection using the Q Exactive Orbitrap Mass Spectrometer for Equine Urine Analysis

Equine anti-doping analysis is a changing and dynamic field of science. The constant introduction of new drugs and biopharmaceuticals present challenges to the integrity of sports. In this webinar Dr. Scott Stanley will discuss how advancements of modern mass spectrometry, specifically, the Thermo Scientific™ Orbitrap™ MS have enabled anti-doping laboratories to significantly expand the drug coverage for both human and equine athletes.

Comprehensive Anti-doping Detection using the Q Exactive Orbitrap Mass Spectrometer for Equine Urine Analysis

WB64009: How to Streamline Sample Preparation for Equine Drug Screening using LC/MS

Learn improved LC-MS solutions for equine drug screening by maximizes sensitivity and selectivity while minimizing sample handling using automated on-line sample cleanup by TurboFlow™ technology coupled ultra high-resolution liquid chromatography (UHPLC). The capability, productivity and reliability of the Thermo Scientific™ Transcend™ II system is evaluated in this presentation. Experiences in developing and validating new drug screening methods, as well as transferring existing methods, will also be discussed.

How to Streamline Sample Preparation for Equine Drug Screening using LC/MS

WB64303: Comprehensive Anti-doping Detection using the Q Exactive Orbitrap Mass Spectrometer for Equine Urine Analysis

Comprehensive Anti-doping Detection using the Q Exactive Orbitrap Mass Spectrometer for Equine Urine Analysis Equine anti-doping analysis is a changing and dynamic field of science. The constant introduction of new drugs and biopharmaceuticals present challenges to the integrity of sports. In this webinar Dr. Scott Stanley will discuss how advancements of modern mass spectrometry, specifically, the Thermo Scientific™ Orbitrap™ MS have enabled anti-doping laboratories to significantly expand the drug coverage for both human and equine athletes.

Comprehensive Anti-doping Detection using the Q Exactive Orbitrap Mass Spectrometer for Equine Urine Analysis

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Translational Research Application Notes

AN619: Automated High-Throughput Data Processing for Targeted Multiplexed Insulin Analog Detection and Quantification

MSIA1004: Selected-Reaction Monitoring-Mass Spectrometric Immunoassay Analysis of Parathyroid Hormone and Related Variants

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Automated High-Throughput Data Processing for Targeted Multiplexed Insulin Analog Detection and Quantification

Scott Peterman[1], Kwasi Antwi[2], Bryan Krastins[1], Eric E. Niederkofler[2], and Mary Lopez[1]
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Key Words

Q Exactive, insulin, insulin variants, Pinpoint, HRAM, high resolution, accurate mass, MSIA, mass spectrometric immunoassay, automation, research

Goal

Present an automated, multiplexed, high-throughput data processing workflow for detection and targeted quantification of insulin and its analogs at concentration ranges of 3.75 to 960 pM in biological matrices.

Introduction

The development of robust biotechnology-based methods to create recombinant peptide hormones with altered peptide sequences has produced hormone variants designed to fit specific needs such as insulin analogs. The development of these variants requires accompanying advances in the analytical technologies used for their detection and quantification in research applications. Routine, global sample preparation, data acquisition, and data processing methods that address expected concentration levels in biological matrices are needed.[1] Traditional global sample preparation and detection methods tend to decrease assay selectivity and sensitivity. Rapid, targeted quantitation of many closely related analytes places significant demands on the software tools used.

To address the analytical requirements of routine detection and quantification of peptide variants in biological matrices, a complete workflow that employs multiplexed Thermo Scientific™ MSIA™ (mass spectrometric immunoassay) technology was created.[2] MSIA technology couples global affinity capture sample preparation with high-resolution, accurate-mass (HRAM) spectrometric detection.[3] The Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap™ mass spectrometer was used to collect full-scan HRAM data.[4] Thermo Scientific™ Pinpoint™ software provided automated qualitative and quantitative HRAM data processing. The complementary research paper published in *Proteomics* provides a detailed description of the MSIA-HRAM method and results for targeted quantification of intact insulin and its analogs in human serum and plasma.[5]

Experimental

Reagents

Insulin analogs Humulin® S (Lilly, 100 IU/mL), Apidra® (Sanofi Aventis, 100 U/mL), Lantus® (Sanofi Aventis, 100 U/mL), NovoRapid® (Novo Nordisk, 100 U/mL), and Hypurin Porcine (CP Pharmaceuticals, 100 U/mL) were provided by Dr. Stephen Morley (Sheffield Hospital, UK). Bovine serum albumin (BSA, Calbiochem) prepared at 50 g/L in phosphate-buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4) served as the biological matrix. Bovine insulin, TWEEN® 20, and phosphate-buffered saline were obtained from Sigma-Aldrich®. Ultra-pure water, trifluoroacetic acid, and ammonium acetate were obtained from American Bioanalytical. ACTH 1-24 was obtained as a carrier peptide from Bachem®. LC-MS grade water, LC-MS grade acetonitrile, and formic acid were Fisher Chemical brand. Thermo Scientific™ MSIA™ D.A.R.T.'S (Disposable Automation Research Tips) were coupled with anti-human insulin antibody.

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Sample Preparation

Two sets of samples were prepared. First, a dilution series of Humulin S, Apidra, Lantus, NovoRapid, and bovine insulin, prepared in the presence of porcine insulin (50 pM) and covering an analytical concentration of 1.5 to 960 pM, were spiked into a phosphate-buffered saline-bovine serum albumin (PBS/BSA) matrix. The second set consisted of Apidra, Lantus, and NovoRapid spiked individually into human plasma at the same concentration range (1.5 to 960 pM). For quantitation curve development, both Apidra and Lantus were spiked into plasma across the same concentration range (1.5 to 960 pM). Porcine insulin was again spiked into each sample at 50 pM as an internal standard.

Mass Spectrometric Immunoassay

The affinity capture of insulin was achieved using insulin-specific MSIA D.A.R.T.'S mounted onto the 96-channel pipetting head of the Thermo Scientific™ Versette™ automated liquid handler. After rinsing the insulin MSIA D.A.R.T.'S with 15 cycles of a single aspiration and dispensing 150 µL 10 mM PBS, the insulin MSIA D.A.R.T.'S were immersed into the samples and 100 aspiration and dispense cycles of 250 µL were performed. The multiple cycles allowed simultaneous affinity enrichment of all of the insulin analogues as well the internal standard. The MSIA D.A.R.T.'S were then rinsed with PBS (15 cycles) from another microplate, followed twice by water (15 cycles) from two additional microplates (150 µL aspirations and dispenses, from 200 µL in each well).

The affinity-captured insulin analogs were eluted to a microplate by aspirating and dispensing 80 µL of 15 µg/mL ACTH 1-24 in 33% acetonitrile/0.4% (v/v) trifluoroacetic acid (TFA) 100 times from a total of 100 µL volume in each well. The eluates were dried down in a Thermo Scientific™ Speed Vac™ concentrator until dry and then resuspended in 100 µL reconstitution buffer 25% acetonitrile/0.2% formic acid (v/v)). The microplate was sealed and vortexed for 30 seconds to ensure proper reconstitution, and then spun-down prior to loading samples onto the LC.

LC/MS Method

Samples were analyzed using a generic LC/MS method. A Thermo Scientific™ UltiMate™ 3000 RSLCnano LC system was used for all LC/MS experiments. To begin, 100 µL of each sample was separated on a 1 x 250 mm Thermo Scientific™ ProSwift™ RP-4H column using a linear gradient (10–50% in 10 minutes) comprised of A) 0.1% formic acid in water and B) 0.1% formic acid in acetonitrile. The column was heated to 50 °C.

All data were acquired using a Q Exactive mass spectrometer operated in data-dependent/dynamic exclusion mode. A resolution setting of 70,000 (FWHM) at *m/z* 200 was used for full-scan MS and 17,500 for MS/MS events. Full-scan MS data were acquired using a mass range of 800–2000 Da. A targeted inclusion list was used to trigger all data-dependent events.

Data Analysis

All data were processed using Pinpoint software (revision 1.4). HRAM MS data extraction was used for quantification. To provide additional levels of qualitative analysis, the three most abundant precursor charge states per insulin variant were used, as well as the six most abundant isotopes per charge state. A mass tolerance of ±7 ppm was used to extract all data. Qualitative scoring was based on mass error, precursor charge state distribution, isotopic overlap, and corresponding LC elution peak profiles measured for each sample. Product ion data were used for sequence verification. The measured area-under-curve (AUC) values for porcine insulin were used as the internal standard for all samples.

Results and Discussion

To assess the workflow, the insulin variants were spiked into two different matrices and processed. The effects of the matrix, competitive binding/extraction of all insulin variants, and automated data extraction, verification, and quantitation were evaluated. The HRAM data acquisition capability of the Q Exactive mass spectrometer enabled downstream automated qualitative and quantitative data processing using Pinpoint software. By acquiring data in a nontargeted manner, post-acquisition methods can be used to process the data for any insulin variant sequence or modification. To increase the qualitative information obtained, multiple target-specific attributes per insulin variant were chosen for analysis by the software.

Qualitative Data Processing Strategy

Figure 1 shows the base peak chromatogram for the human plasma sample spiked with 960 pM of Lantus and Apidra insulin analogs and 50 pM of porcine insulin extracted using MSIA. The data acquisition time range used was 3.5 to 5.5 minutes. The chromatographic trace shows two peaks eluting, with the peak at 4.52 minutes attributed to the Lantus insulin analog and the peak at 4.72 minutes attributed to porcine, human, and Apidra insulin analogs. The inset shows the averaged HRAM mass spectrum around the +5 precursor charge states for the insulin analogs. The observed relative abundance of Apidra to porcine (ca. 6%) was in close agreement with the spiked amounts of 960 to 50 pM, respectively. The observed relative abundance of endogenous human insulin was equivalent to that of porcine. The remaining peaks in the mass spectrum were attributed to adduct formation during ionization. Despite the large difference in the amounts present in the plasma samples, there was little interference observed when detecting all insulin variants. The resolution of the Q Exactive mass spectrometer was more than sufficient to baseline resolve the isotopic profiles for the +5 charge state across the dynamic range.

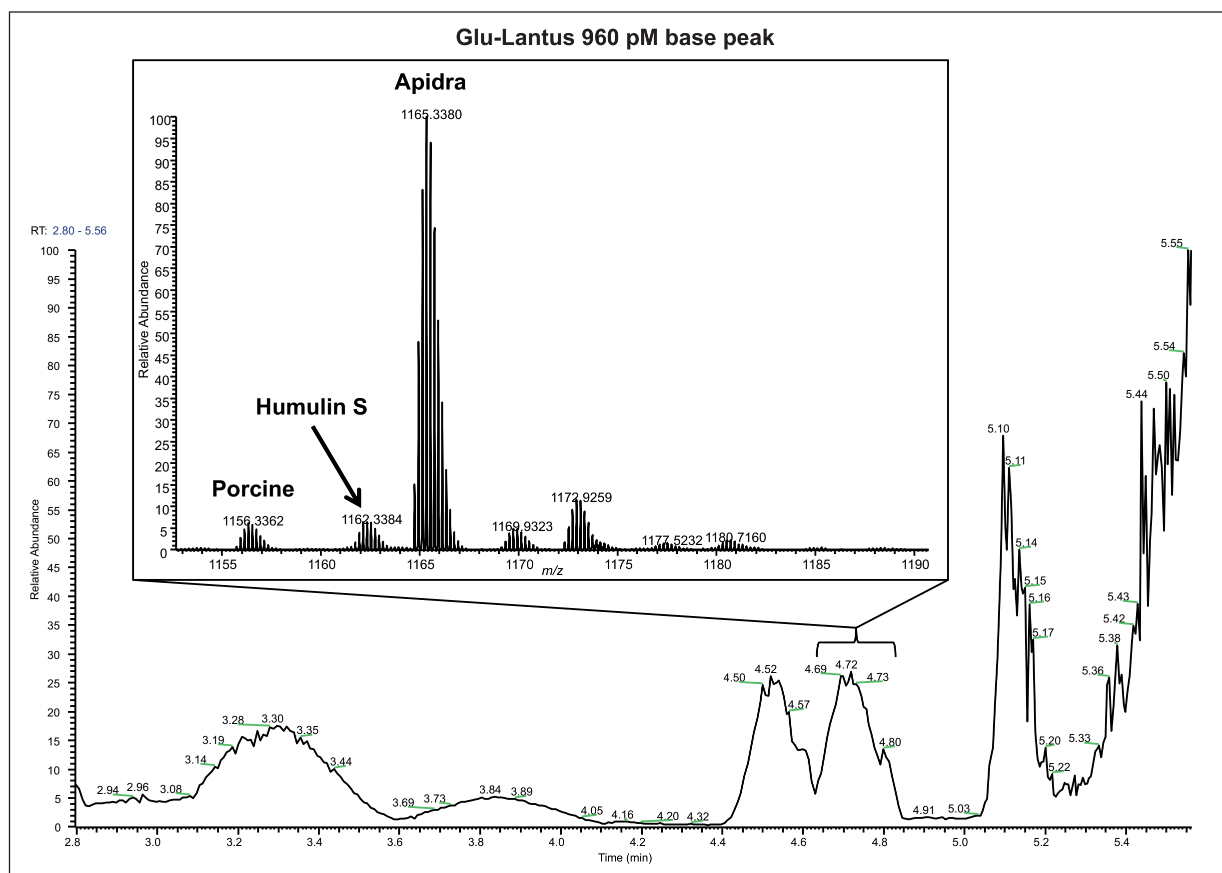

Figure 1. Base peak chromatogram for the MSIA-extracted human plasma sample spiked with 960 pM of both Apidra and Lantus insulin variants and 50 pM of porcine insulin (internal standard). The inset shows the summed mass range covering three of the four insulin variants.

Figure 2a shows the initial data extract using multiple isotopes per charge state. Pinpoint software determined the isotopic distribution and *m/z* list and created the theoretical profile based on user-defined sequence and possible modifications. Each isotopic *m/z* value was used to create an extracted ion chromatogram (XIC) with a ±7 ppm window, providing first-level qualitative analysis. The resulting XICs were overlaid to determine the retention time (Figure 2b) and AUCs were calculated for all isotopes. The overlaid peak profiles enabled scoring the LC component based on common peak shapes (peak state/end, apex, and symmetry) for the collection of isotopes of one or more precursor charge states. The color-coding capability of Pinpoint software facilitated data review.

The AUC values for each isotope were calculated and then used to determine background interference. Figure 2c shows the Pinpoint software-generated bar chart used to evaluate the isotopic distribution profile of human insulin.

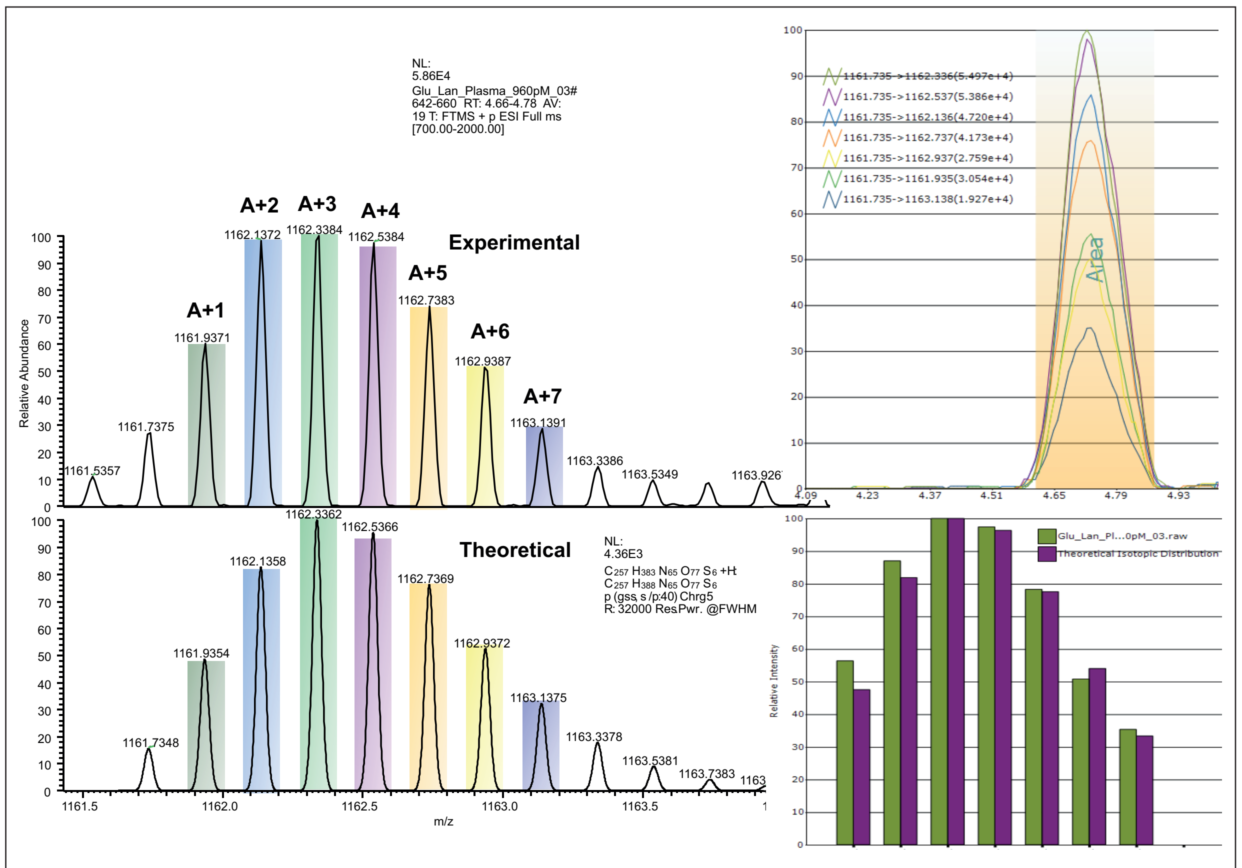

Figure 2. Data processing using Pinpoint software. Figure 2a shows the targeted data extraction based on isotopic *m/z* values for the seven most abundant isotopes, and a ±7 ppm extraction tolerance based on the theoretical isotopic distribution. Figure 2b shows the overlaid XICs for each of the targeted isotopes. The AUC values for each isotope were used to evaluate the scoring shown in Figure 2c, where the relative AUC values for the collective isotopic distribution were compared to the theoretical value.

LC-MS methods, particularly those employing HRAM detection, provide significant advantages over enzyme-linked immunosorbent assay (ELISA), capillary electrophoresis (CE), and ultraviolet (UV) methods because the selectivity of MS allows detection of co-eluting analogs. Three co-eluting insulin analogs shown in Figure 1 were easily separated based on the accurate *m/z* values of each precursor charge state and corresponding isotopes. Comparative analyses for the three insulin variants (porcine, human, and Apidra), including XICs and the total isotopic distribution, are shown in Figure 3. Pinpoint software automatically calculated the dot product correlation coefficient for the charge states that was used to evaluate isotopic distribution overlap and filter results. Here the dot product scores for each charge state and analog were greater than 0.9, an excellent match.

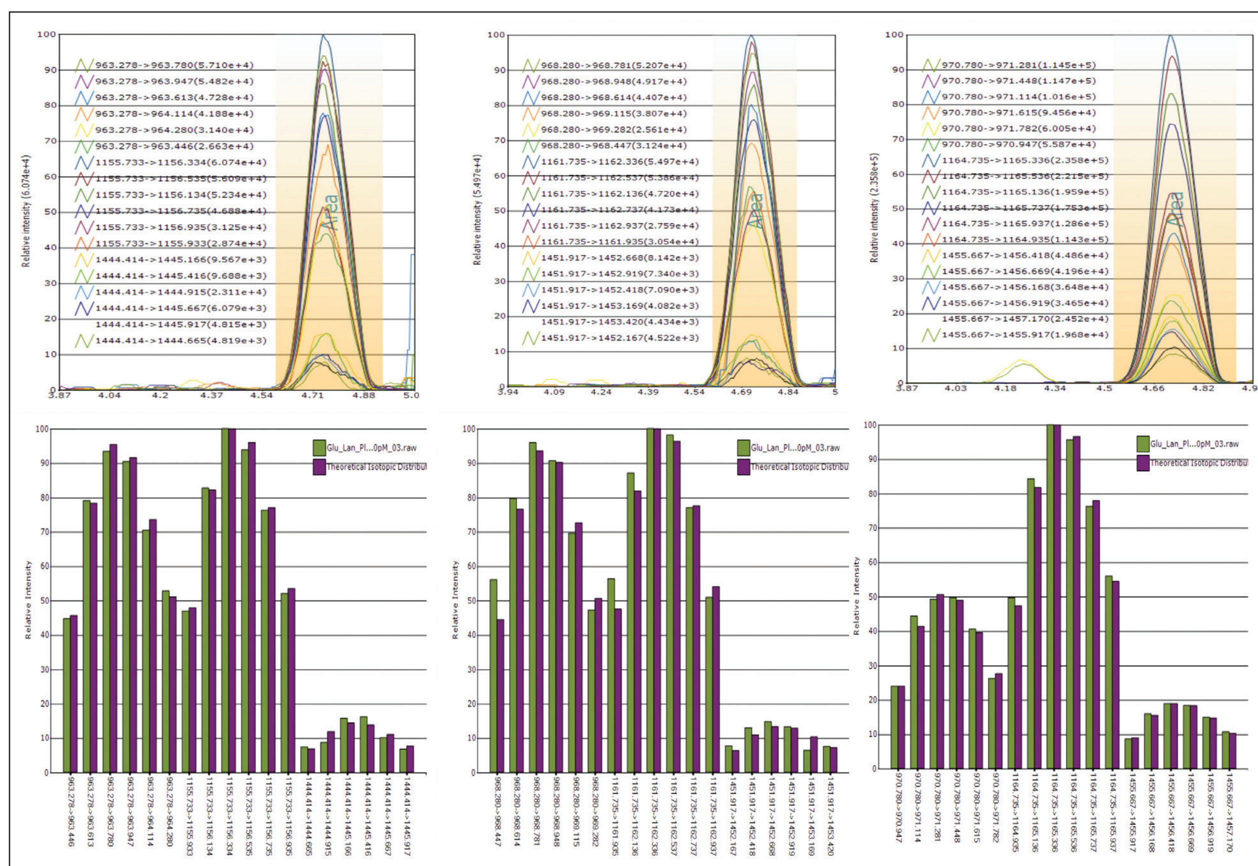

Figure 3. Comparative analysis of the three co-eluting insulin variants. Overlaid XICs for the six isotopes and three precursor charge states per insulin variant for (3a) porcine, (3b) human, and (3c) Apidra. The isotopic distribution analysis for the collection of precursor charge states and Pinpoint software calculated dot product correlation coefficients per charge state are shown.

The data analysis method provided an additional dimension of qualitative scoring beyond the isotopic distribution of a single charge state: the relative distribution of precursor charge states. For example, the measured abundances of the +5 and +6 charge states of porcine and human insulin were nearly equivalent, while the measured abundance of the +5 charge state of Apidra was twice that of the +6 charge state. The +4 precursor for all three showed relatively poor response and would likely be excluded from the final quantitation method.

Quantitative Data Processing Strategy

Robust data processing and reporting incorporate both qualitative and quantitative strategies. After data were collected using the HRAM global acquisition method, a Pinpoint software Main Workbook for the targeted insulin sequences was created, the processing parameters set, and the RAW files loaded for automated data processing. This was performed in three steps. Figure 4 shows the list of insulin variant sequences imported from a FASTA file containing all of the insulin variant sequences. A FASTA file is a text-based format for representing either nucleotide sequences or peptide sequences, in which nucleotides or amino acids are represented using single-letter codes.

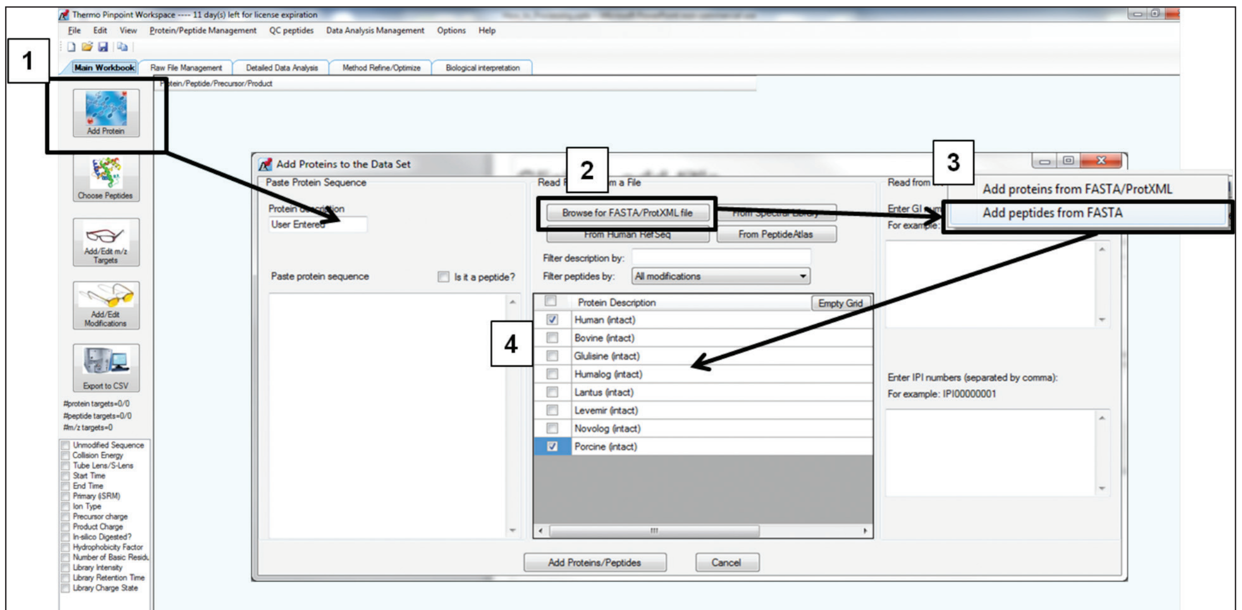

Figure 4. The targeted insulin variant list was created by opening the “Add Protein” window (1), selecting means of importing variant sequences (2), defining each entry as a peptide, and then selecting analytes to be included (4).

Modifications were then added to the sequences. Because insulin is comprised of multiple disulfide bonds linking the alpha and beta chain, mass shifts were added to determine the correct chemical formula (Figure 5).

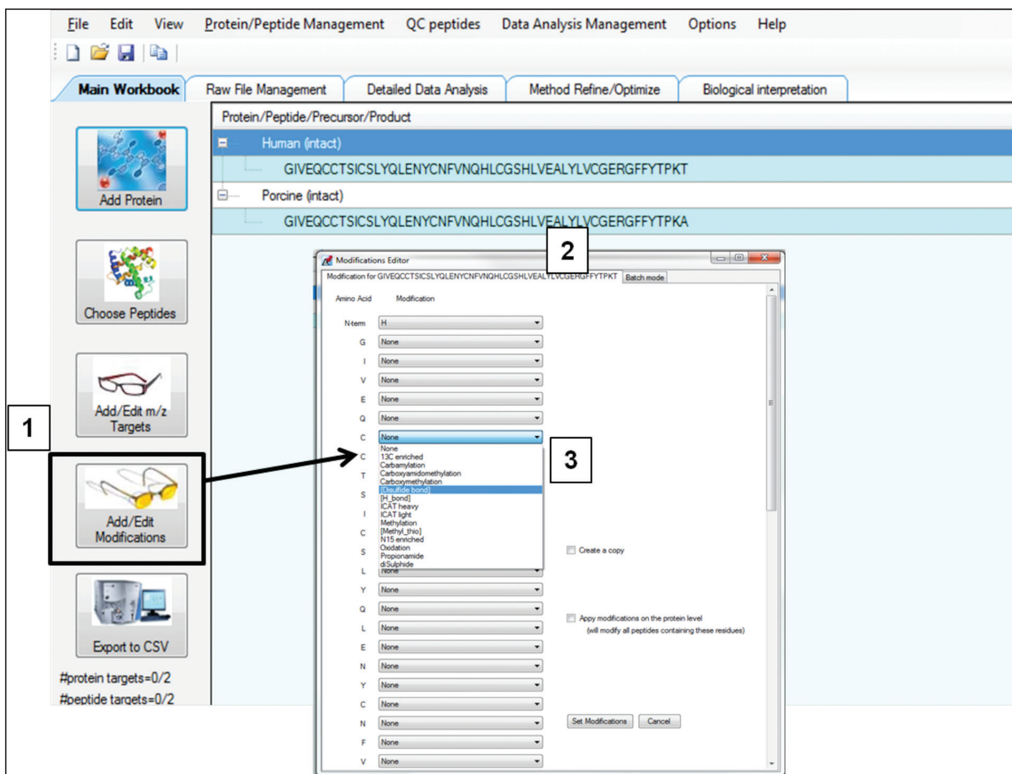

Figure 5. Mass shifts can be applied globally or locally by choosing Add/Edit Modifications (1). which opens the working window (2). Here the specific amino acid residue to which the modification was applied was selected and desired modification chosen (3).

For each insulin variant, three different disulfide bonds were selected and assigned to three different Cys residues as well as the addition of H_2O to account for two chains. Since the targeted quantitation was performed on the MS signal, the specific Cys residues were not important.

After the sequences had the correct modifications (chemical formulas), the *m/z* values were assigned (Figure 6). Determination of precursor charge states and isotopes linked the *m/z* values used for targeted data extraction to the appropriate insulin variant sequence.

Figure 6. Method to assign *m/z* values to each targeted insulin variant. “Add/Edit *m/z* Targets” (1) was selected to display sequence-specific information for the highlighted sequence. The Batch Mode Tab was used to apply the settings globally. To determine the *m/z* for a specific sequence, the precursor charge state was selected (2), and the isotopes checked (3).

For larger targets, at least six isotopes per charge state were selected to increase the qualitative information used for quantitation. In addition, multiple charge states were incorporated into the automated data extraction.

After the Main Workbook was created, the RAW files were batch processed as shown in Figure 7.

Figure 7. RAW data for processing was imported by clicking on the top bar (1) and selecting RAW files. The extraction parameters—including precursor mass tolerance (2) and retention time window for data extraction (3)—were set. After the extraction values were selected and data processing had begun, the group names (4) used for data organization were assigned.

For quantitative experiments, the expected values per group were entered (Figure 8).

After all values were set, automated data processing was completed in 30 minutes. Pinpoint software consolidated qualitative and quantitative results in an interactive display that facilitated review and customized reporting (Figure 9). The top left table displays the AUC values for the levels of the spiked analytes (e.g. isotopes, precursor charge states, and the summed values). The method of reporting AUC values enabled display of specific values for each level, which could be expanded or reduced as desired. Each entry was scored based on the calculated dot product for isotopic distribution, making it easy to determine which rows failed the filter.

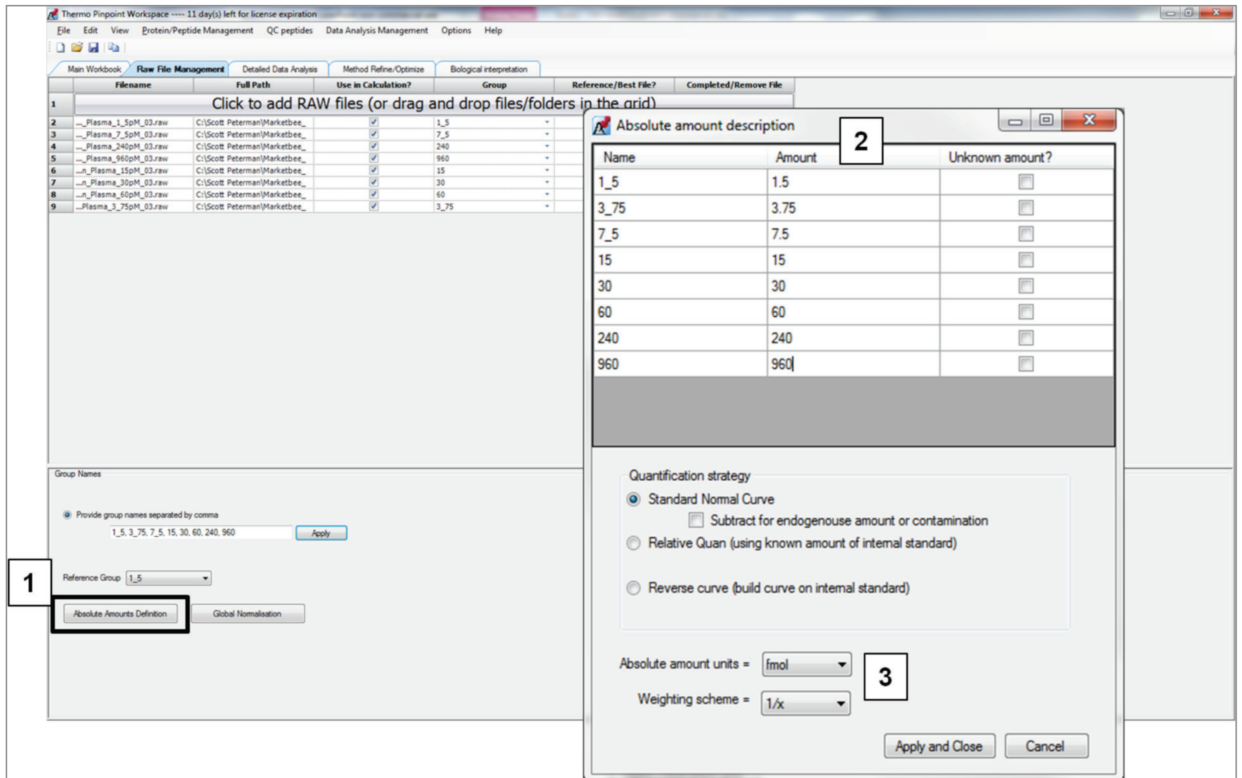

Figure 8. Quantitative parameters were defined by clicking on “Absolute Amounts Determination” (1) to open the dialog box containing user-defined group names (2). Values were assigned or defined as unknown. Finally the units to display and the weighting scheme to apply to the calibration curve (3) were selected.

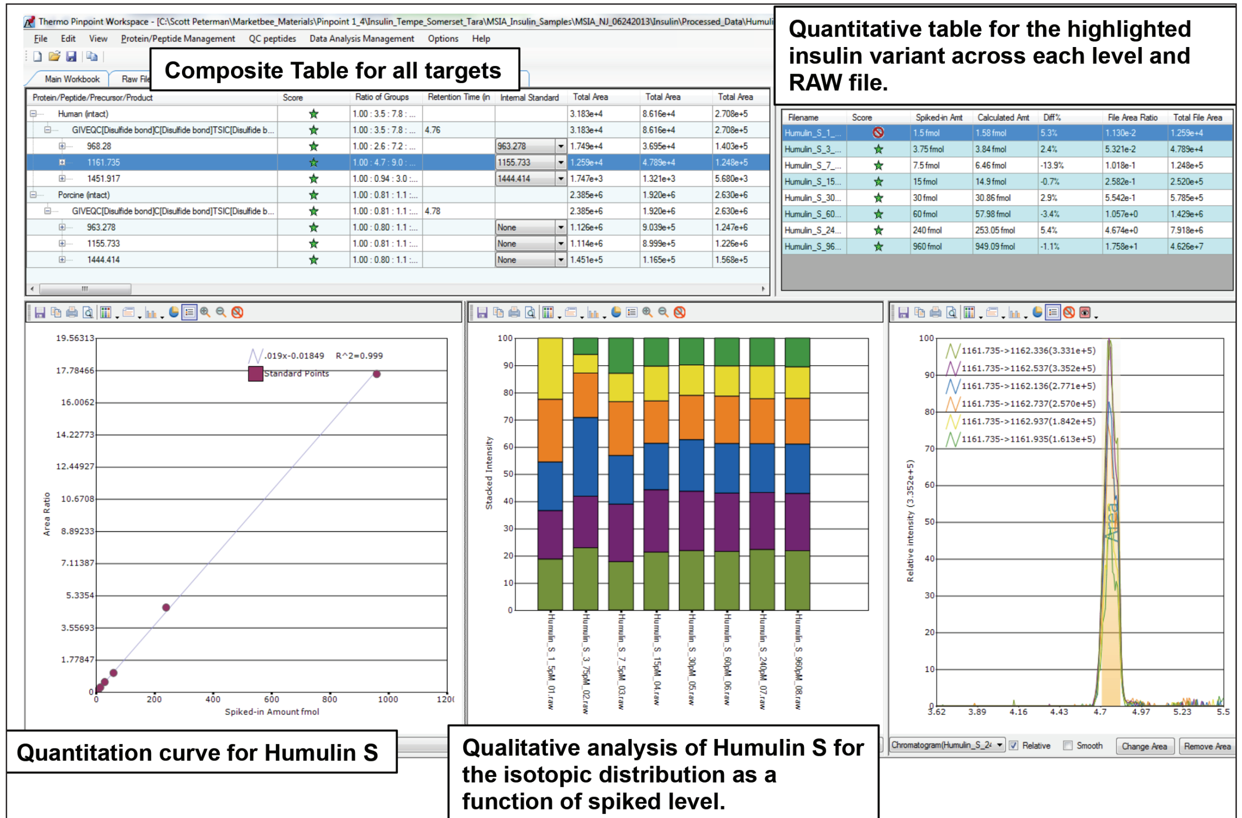

Figure 9. Automated data processing using Pinpoint software. The Detailed Data Analysis tab provided the composite quantification results for Humulin S from 1.5 to 960 pM spiked into PBS/BSA matrix.

Quantitation curves were generated based on user-defined levels. To best match the relative AUC values, each charge state was used to normalize the corresponding charge state for human insulin. This provided a significant benefit as relative abundance values are generally not equivalent, and simply summing and normalizing AUC response per target will bias towards the most abundant charge state. Normalizing at each charge state and showing the summed response for each individual charge state, takes advantage of HRAM data to increase the discriminatory power of the method.

As shown in Figure 9, the top right table shows the cumulative quantitation results for the row highlighted and contains all of the values used to evaluate the quantitation, including the %RSD and calculated amounts. Specific values can be added or subtracted. When a row in the table is selected (highlighted in blue), the accompanying results are graphically displayed in the bottom half of the window. The bottom left of Figure 9 shows the normalized quantitation curve for human insulin. The curve is weighted by 1/x and the equation is displayed. The bar chart (bottom middle) displays the relative AUC values for the six isotopes across the spiked levels. As reported in the upper right table, only the lowest spiked amount (1.5 pM) could not be used to identify all six isotopes. The graph on the lower right displays the XIC data for any single RAW file selected and can be changed by clicking on any point in the quantitation curve or bar chart.

The cumulative results from individual quantitative curves for the first sample set were overlaid to demonstrate that the workflow was global in its ability to quantify the different insulin variants (Figure 10). Each curve, when normalized to porcine insulin, had a linear regression of 0.98 or greater for all precursor charge states, isotopes, and reported summed AUC values. The difference in slopes was attributed to the relative ionization efficiency and antibody binding coefficients of the different insulin analogs. The workbook used to process the insulin quantitation curves spiked into PBS/BSA was also able to process the insulin quantitation curves spiked into human plasma.

Figure 11 summarizes the qualitative aspects of the quantitation as a function of the levels spiked and the confidence in the isotopic overlap between experimental and theoretical values. The response at each level represents over 30 measurements where all charge states across each quantitation curve were considered. As expected, the lower levels resulted in a lower average dot product correlation coefficient (0.58 for all insulin variants spiked at 1.5 pM). However with six isotopes considered, the coefficient was discriminatory and accurately defined potential background interference that would disrupt the true isotopic distribution in the four most abundant isotopes. Even at very low analyte levels, the quality of the quantitation is quite high. All other levels generated average dot product correlation coefficients greater than 0.8. The average dot product coefficients for porcine measurements were consistently above 0.97 for the 50 pM level.

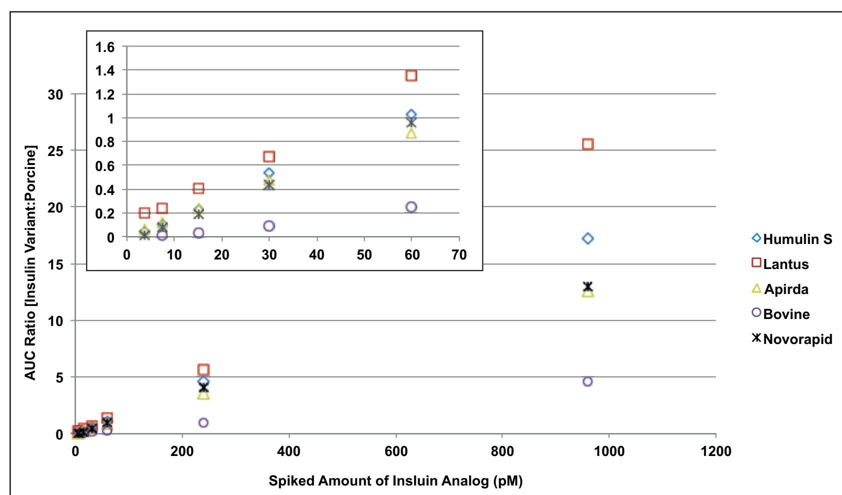

Figure 10. Comparative quantitation curves for all insulin analogs spiked into PBS/BSA matrix. The AUC values were normalized against the measured AUC value for porcine.

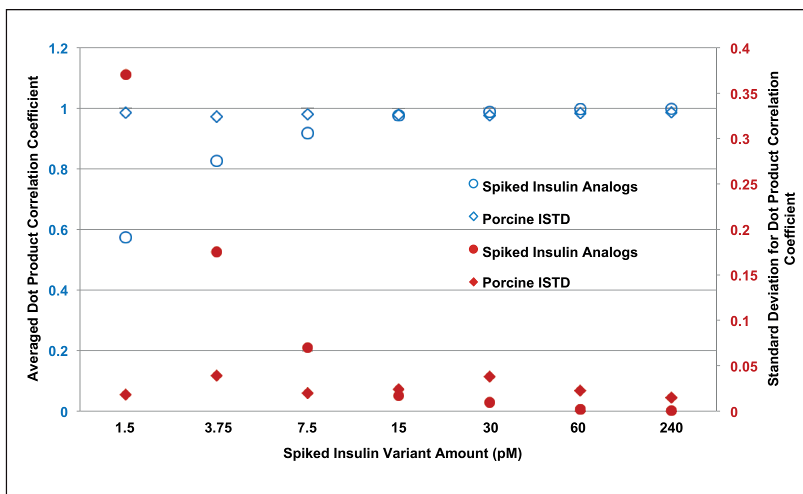

Figure 11. Dot product correlation coefficients for each insulin analog spiked into both matrices, and for porcine insulin. The standard deviation was also plotted per spiked level.

In combination with the average dot product coefficient, the standard deviation for the measurements demonstrated the workflow's effectiveness. The deviation in the isotopic distribution showed that each insulin variant was routinely detected with four or more isotopes. Predicted relative abundance values were maintained at the 1.5 pM level.

Figure 12 shows the RSD values for all insulin analogs as a function of amounts spiked in the PBS/BSA and human plasma matrix. The greatest spread in measured %RSD was at the 1.5 pM level where three of the 11 measurements exceeded 20% spread. Only one measurement at the 3.75 and 30 pM level exceeded 20% spread in %RSD. The errors were attributed to using only one measurement per level, per quantitation curve. The overall groupings per level were well within acceptable error. It should also be noted that the lowest four levels used in this study were 50–100 times lower than the previously published lower limits of quantitation.

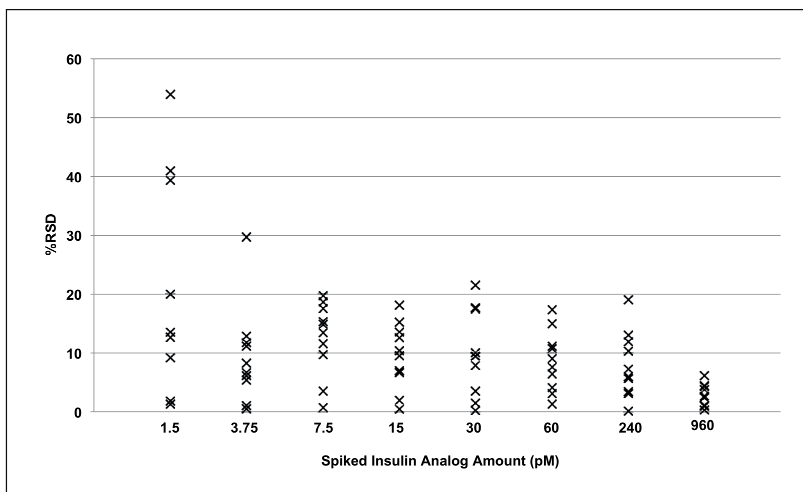

Figure 12. Spread in %RSD values for all insulin analogs across the spiked levels. The %RSD values are based on the individual quantitation curves.

Conclusion

An automated, high-throughput data processing workflow for detection and targeted quantification of insulin and its analogs at low levels in biological matrices was presented for use in research applications.

Full-scan MS data could be analyzed due to the low noise and selectivity of the MSIA extraction technology. Co-eluting insulin analogs were easily separated and identified based on the accurate *m/z* values of each precursor charge state and corresponding isotopes. Detection and quantification ranges were 1.5 to 960 pM in a plasma matrix. Even at low analyte levels, the quality of the quantitation was high. Linear regression values for the method were all better than 0.98 using a 1/x-weighting scheme. The average dot product coefficients for the porcine insulin ISTD were consistently above 0.97 at the 50 pM level. Though the lowest four levels used in the study were 50–100 times lower than previously published, %RSD values were acceptably low.

The HRAM data acquisition capabilities of the Q Exactive mass spectrometer enabled streamlined qualitative and quantitative data processing and reporting using Pinpoint software. This approach enabled quantification of HRAM MS data using the precursor charge state distribution as well as the isotopic distribution for evaluation of potential background interference. By acquiring data in a nontargeted manner, post-acquisition methods can be used to process the data later for any insulin variant sequence or modification. Data processing was performed in 30 minutes using a single Pinpoint software workbook containing all of the targeted insulin variants. To achieve robust quantitation, the Pinpoint software data processing method used the precursor charge state distribution and isotopic distribution analysis for evaluation of potential background interference. Pinpoint software capabilities, such as color-coding capability and the Main Workbook, which consolidates qualitative and quantitative tables and graphs in an interactive display, facilitated data review.

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Application Note MSIA1004

Selected-Reaction Monitoring–Mass Spectrometric Immunoassay Analysis of Parathyroid Hormone and Related Variants

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Key Words
LTQ Orbitrap XL, TSQ Vantage, Pinpoint Software, parathyroid hormone, PTH, biomarkers, MSIA

Goal

To develop a highly sensitive and selective selected-reaction monitoring–mass spectrometric immunoassay analysis (SRM-MSIA)-based method for the concurrent detection and quantification of full-length parathyroid hormone (PTH) [amino acid (aa)1–84] and two N-terminal variants [aa7–84 and aa34–84] for clinical research use.

Introduction

Parathyroid hormone is produced in the parathyroid glands through the two-step conversion of prepro-PTH (115 amino acids) to pro-PTH (90 amino acids) to the 84 amino acid peptide (PTH1–84). Conventional PTH measurements typically rely on two-antibody recognition systems coupled to a variety of detection modalities.[1] The most specific modalities are able to differentiate between different truncated forms of PTH and are referred to as second- and third-generation PTH assays.[2] The key to the application of these later-generation assays is the ability to selectively detect and quantify various PTH forms. In particular, two variants are the subject of increased research investigation: full-length PTH1–84 and PTH missing the 6 N-terminal amino acids (PTH7–84). Because of the inability of existing tests to detect microheterogeneity,[3] these variants were historically considered as a single PTH value (by the first-generation assays). The classification of each variant as its own molecular entity, and the analysis of each independently, suggest an antagonistic relationship between the two different forms in regard to calcium homeostasis.[4] In fact, there is mounting research showing that the ratio between PTH1–84 and PTH7–84 could have future clinical relevance for distinguishing between hyper-parathyroid bone turnover and adynamic bone disease.[5-7]

The ratio of PTH1–84 to PTH7–84 is an example of the potential utility of the microheterogeneity within the PTH protein. Another PTH variant, PTH1–34, has been identified as exhibiting biochemical activity comparable to the full-length protein. There are indications that the microheterogeneity of PTH has yet to be fully characterized, challenging researchers' efforts to determine the utility and/or confounding effects on present-day methods. Accurate examination of known PTH variants and the simultaneous evaluation of other possible variants requires a degree of analytical freedom that universally escapes conventional methods. This work describes mass spectrometric immunoassays that, although specifically designed for the detection of PTH1–84 and PTH7–84, also facilitate the simultaneous discovery and evaluation of further microheterogeneity in PTH.

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Experimental

Approach

In addition to the well-characterized truncated PTH variants, PTH1–84 and PTH7–84, four other molecular versions have been reported in the literature as present in human biofluids (primarily plasma or serum). Aligning these fragments to the sequence of PTH1–84 produced a variant map revealing forms stemming predominantly from N-terminal truncations (Figure 1). A conserved region (among several variants) was evident between residues 48 and 84. This region was suitable for immunoaffinity targeting to capture ragged N-terminal variants (for example, PTH1–84 and PTH7–84). Postcapture digestion of retained PTH (and variants) created the basis for SRM-MSIA,[8-11] for which surrogate peptides representative of the different PTH variants were selected for analysis.

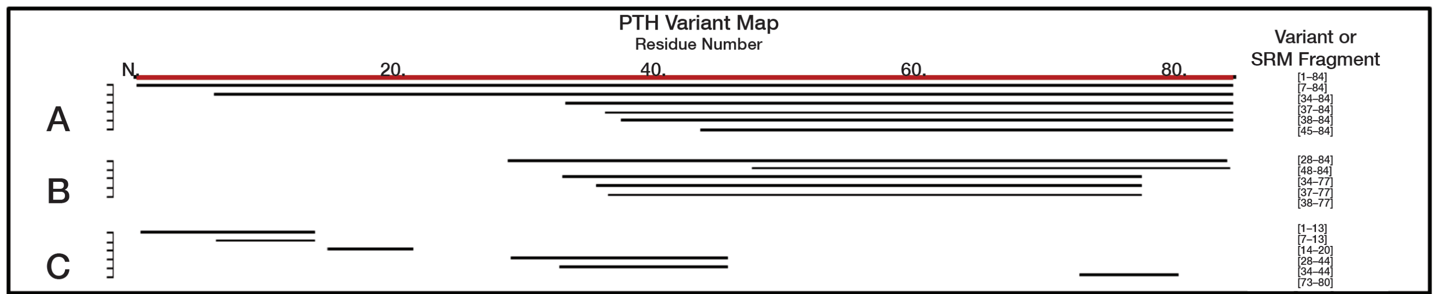


Figure 1. PTH variant map. (A) N-terminally truncated PTH variants identified previously.[7, 12] (B) Variants added to map by top-down MS analysis. (C) Conserved and truncated tryptic fragments chosen for SRM-MSIA.

Reagents

Goat polyclonal anti-PTH39–84 antibody was purchased from Immutopics International. Recombinant human PTH (rhPTH) was obtained from Bachem. Premade 0.01 M HEPES-buffered saline with 3 mM EDTA and 0.05% (vol/vol) surfactant P20 (HBS-EP) was purchased from Biacore. Thermo Scientific™ Pierce™ premixed 2-[morpholino]ethanesulfonic acid–buffered saline powder packets and Thermo Scientific synthetic heavy-labeled peptides were used. High purity solvents from Fisher Chemical brand were used.

Samples

A total of 24 plasma samples were used in the research study: 12 from individuals with previously diagnosed severe renal impairment or end-stage renal disease (ten males and two females; mean age 66.7 years) and 12 from healthy individuals (ten males and two females; mean age 65 years). Among the individuals with renal failure, three were Hispanic, two were Asian, two were African American, and six were Caucasian. The ethnicity information for the healthy sample donors was not available.

Calibration Curves Samples

Samples for creation of calibration curves were prepared from pooled human plasma by step-wise, 2-fold serial dilution of an initial sample containing rhPTH at a concentration of 1000 ng/L (eight steps, range 1000–7.8 ng/L). Samples were frozen at -80 °C until use.

Sample Preparation and Immunocapture

Purification and concentration of the PTH was accomplished by immunoaffinity capture. Extraction of PTH from plasma was carried out with proprietary Thermo Scientific™ Mass Spectrometric Immunoassay (MSIA™) pipette tips derivatized with the PTH antibodies via 1,1 -carbonyldiimidazole chemistry.[13-17] After extraction, PTH was digested, separated by liquid chromatography, and analyzed by high-resolution MS/MS on an ion trap-Orbitrap™ hybrid mass spectrometer and by SRM on a triple quadrupole mass spectrometer as described below.

Sample Elution and Trypsin Digestion

Bound proteins were eluted from the tips into a 96 well plate by pipetting 100 µL of 30% acetonitrile/0.5% formic acid up and down for a total of 15 cycles. Samples were lyophilized to dryness and then resuspended in 30 µL of 30% n-propanol/100 mmol/L ammonium bicarbonate, pH 8.0, diluted with 100 µL of 25 M acetic acid containing 100 ng of trypsin. Samples were allowed to digest for 4 hours at 37 °C. After digestion, samples were lyophilized and resuspended in 30 µL of 3% (vol/vol) acetonitrile/0.2% (vol/vol) formic acid/glucagon/PTH heavy peptides.

High-Resolution LC-MS/MS

High-resolution LC-MS/MS analysis was carried out using a Thermo Scientific™ EASY-nLC™ system and Thermo Scientific™ LTQ Orbitrap XL™ hybrid ion trap-Orbitrap mass spectrometer. Samples in 5% (vol/vol) acetonitrile/0.1% (vol/vol) formic acid were injected into a Thermo Scientific™ Hypersil GOLD™ aQ fused-silica capillary column (75 µm x 25 cm, 5 µm particle size) in a 250 µL/min gradient of 5% acetonitrile/0.1% formic acid to 30% acetonitrile/0.1% formic acid over the course of 180 minutes. The total run time was 240 minutes and the flow rate was 285 nL/min. The LTQ Orbitrap XL MS was operated at 60,000 resolution (FWHM at *m/z* 400) for a full scan for data-dependent Top 5 MS/MS experiments (CID or HCD). The top 5 signals were selected with monoisotopic precursor selection enabled, and +1 and unassigned charge states rejected. Analyses were carried out in the ion trap or the Orbitrap analyzer. The experiments were performed using collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD) fragmentation modes.

SRM Methods

SRM methods were developed on a Thermo Scientific™ TSQ Vantage™ triple stage quadrupole mass spectrometer with a Thermo Scientific™ Accela™ pump, a CTC PAL® autosampler (Leap Technologies), and a Thermo Scientific™ Ion Max™ source equipped with a high-flow metal needle. A mass window of 0.7 full width at half maximum (FWHM, unit resolution) was used in the SRM assays because the immunoenriched samples had a very high signal-to-noise ratios. Narrower windows were necessary when the matrix background was significant and caused interferences that reduced signal-to-noise in the SRM channels. Reversed-phase separations were carried out on a Hypersil GOLD column (1 mm x 100 mm, 1.9 µm particle size) with a flow rate of 160 µL/min. Solvent A was 0.2% formic acid in LC-MS-grade water, and solvent B was 0.2% formic acid in Fisher Scientific™ Optima™-grade acetonitrile.

Software

Thermo Scientific™ Pinpoint™ software was used for targeted protein quantification, automating the prediction of candidate peptides and the choice of multiple fragment ions for SRM assay design. Pinpoint software was also used for peptide identity confirmation and quantitative data processing. The intact PTH sequence was imported into the software and digested with trypsin *in silico*. Then, transitions for each peptide were predicted and tested with recombinant PTH digest to determine those peptides and transitions delivering optimal signal. After several iterations, a subset of six peptides with multiple transitions was chosen.

Further tests were conducted with this optimized method. After the target peptides were identified, heavy arginine or lysine versions were synthesized to be used as internal quantitative standards. Target peptides were subsequently identified and quantified by coeluting light- and heavy-labeled transitions in the chromatographic separation. Time alignment and relative quantification of the transitions were performed with Pinpoint software. All samples were assayed in triplicate.

Results and Discussion

Top-Down Analysis and Discovery of Novel Variants

The approach described herein coupled targeting a common region of PTH by use of a polyclonal antibody (raised to the C-terminal end of the protein) with subsequent detection by use of SRM MS. Numerous PTH variants were simultaneously extracted with a single, high-affinity polyclonal antibody, and the selection of the epitope was directed by the target of interest (i.e., intact and N-terminal variants). The primary goal was to differentiate between intact PTH1–84 and N-terminal variant PTH7–84 while simultaneously identifying any additional N-terminal heterogeneity throughout the molecule. The results of these top-down experiments allowed the development of an initial standard profile for PTH. Clearly, this profile is not finite, and may be expanded to include additional variants found through literature search and/or complementary full-length studies. However, this standard profile provided an initial determination of target sequences for developing specific SRM assays.

Selection of Transitions for SRM

During LC-MS/MS analysis, multiple charge states and fragmentation ions were generated from each fragment, resulting in upwards of 1000 different precursor/product transitions possible for PTH digested with trypsin. Empirical investigation of each transition was not efficient. Therefore, a workflow incorporating predictive algorithms with iterative optimization was used to predict the optimal transitions for routine monitoring of tryptic fragments (Figure 2). The strategy facilitated the translation of peptide intensity and fragmentation behavior empirically obtained by high-resolution LC-MS/MS analyses to triple quadrupole SRM assays. Inherent to the success of the workflow was the similarity of peptide ion fragmentation behavior in these ion trap and triple quadrupole instruments.[12] Empirical data from such LC-MS/MS experiments were used in conjunction with computational methods (*in silico* tryptic digestions and prediction of SRM transitions) to enhance the design of effective SRM methods for selected PTH peptides.

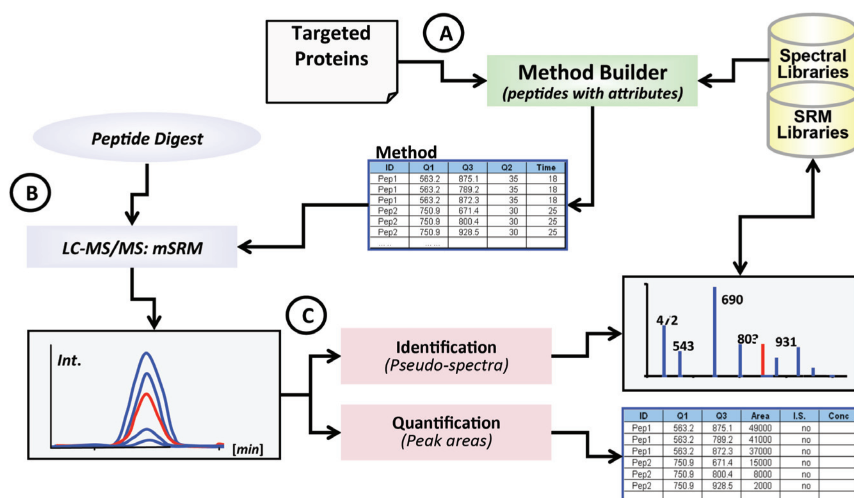


Figure 2. Pinpoint workflow for development of multiplexed SRM assays. [Q = quadrupole; mSRM = multiple SRM; Int. = intensity; I.S. = internal standard; Conc = concentration. Time measurements are in minutes (min).]

The initial list of transitions was queried empirically to produce an LC-MS/MS profile based on four tryptic peptides that collectively spanned >50% (45 of 84 amino acids) of the full PTH sequence. SVSEIQLMHNLGK [amino acid (aa)1–13] was monitored to represent PTH species with an intact N-terminus, such as PTH1–84. Other tryptic peptides, HLNSMER (aa14 –20), DQVHNFVALGAPLAPR (aa28–44), and ADVNVLTK (aa73–80) were included for monitoring across the PTH sequence. In addition, transitions for two truncated tryptic peptides, LMHNLGK (aa7–13) and FVALGAPLAPR (aa34–44), were added to the profile to monitor for truncated variants PTH7–84 and PTH34–84, respectively. In total, 32 SRM transitions tuned to these six peptides were used to monitor intact and variant forms of PTH (Figure 1).

Generation of Standard Curves and Limits of Detection and Quantification

rhPTH was spiked into stock human blood plasma to create calibration curves for all target tryptic peptides through serial dilution. As illustrated in Figure 3 for peptides LQDVHNFVALGAPLAPR (aa28–44) and SVSEIQLMHNLGK (aa1–13), SRM transitions for the four wild-type tryptic fragments exhibited linear responses (R^2 = 0.90–0.99) relative to rhPTH concentration, with limits of detection for intact PTH of 8 ng/L and limits of quantification for these peptides calculated at 31 and 16 ng/L, respectively. Standard error of analysis for all triplicate measurements in the curves ranged from 3% to 12% for all peptides, with <5% chromatographic drift between replicates. In addition, all experimental peptide measurements were calculated relative to heavy-labeled internal standards. CVs of integrated areas under the curve for 54 separate measurements (for each heavy peptide) ranged from 5% to 9%. Monitoring of variant SRM transitions showed no inflections relative to rhPTH concentration, owing to the absence of truncated variants in the stock rhPTH.

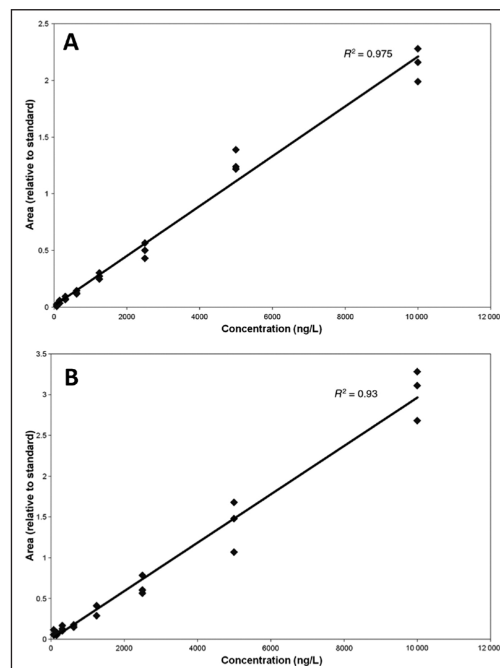


Figure 3. SRM calibration curves for PTH peptides.
(A) Peptide LQDVHNFVALGAPLAPR aa28–44.
(B) Peptide SVSEIQLMHNLGK aa1–13.

Evaluation of Research Study Samples

Initial SRM data were acquired from replicate plasma samples. The light and heavy peptides coeluted precisely in all samples. Further SRM experiments were carried out on the cohort of renal failure (n = 12) and normal (n = 12) samples. The most prominent PTH variant in the renal failure samples was PTH34–84. To quantify this observation with SRM, all samples were interrogated to determine the expression ratios of renal failure to normal for the various target peptides, including FVALGAPLAPR (aa34–84), which should be specific to the 34–84 variant. Chromatographic data from single renal-failure samples for peptides FVALGAPLAPR (aa34–44) and SVSEIQLMHNLGK (aa1–13) are shown in Figure 4. The peak integration area and individual coeluting fragment transitions for each peptide are illustrated. Similar chromatograms were obtained for peptides LQDVHNFVALGAPLAPR (aa28–44), HLNSMER (aa14–20), and ADVNVLTK (aa73–80) (data not shown). The sample variances and expression ratios of renal-failure samples to normal samples for each peptide are shown in Figure 5. The expression ratios for the peptides ranged from 4.4 for FVALGAPLAPR (aa34–44) to 12.3 for SVSEIQLMHNLGK (aa1–13). Notable quantities of peptide LMHNLGK (aa 7–13) were not detected in these samples. Sample variances illustrated in the scatter plots in Figure 5 demonstrate that the renal failure and normal samples groups were clearly segregated by the five target peptides.

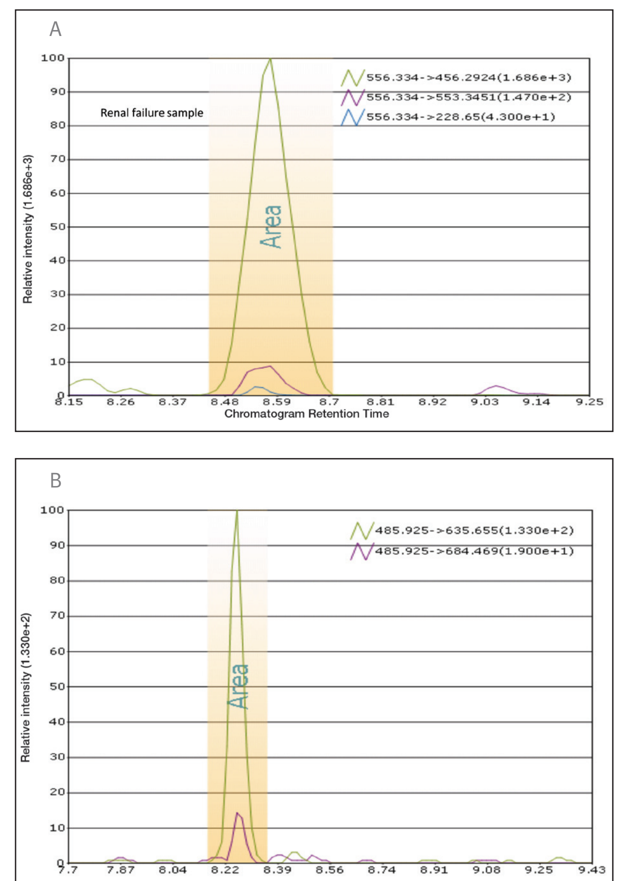
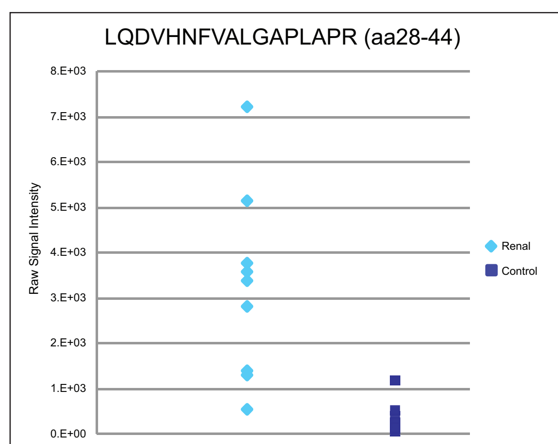
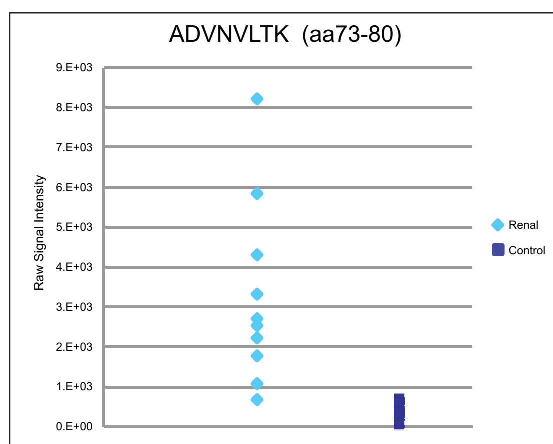
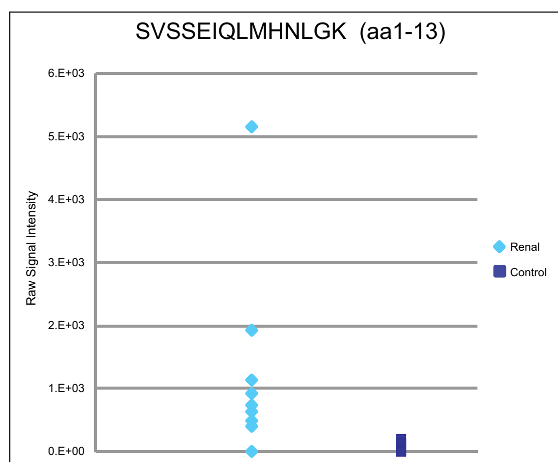
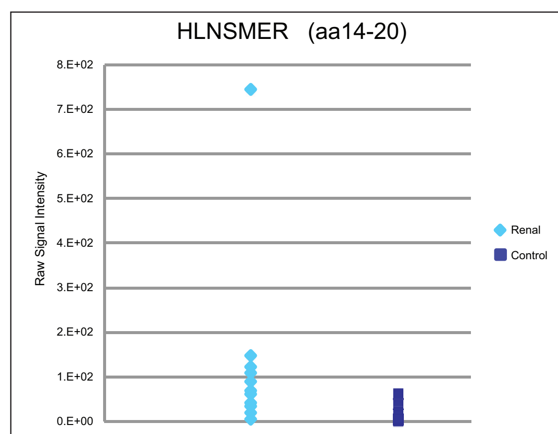


Figure 4. Pinpoint software SRM data from samples of normal and renal failure patients. Chromatographic data illustrate peak integration area and individual fragment transitions for peptides from single renal failure samples. (A) Semitryptic peptide FVALGAPLAPR (aa34–44), specific to the 34–84 variant (see Figure 1). (B) Tryptic peptide SVSEIQLMHNLGK (aa1–13).









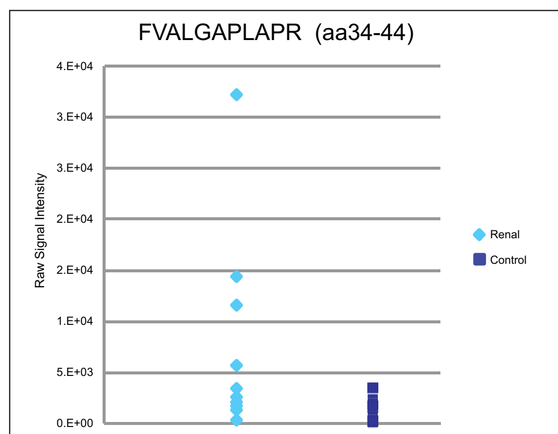


Figure 5. SRM quantitative ratios and sample variances of PTH peptides in samples from renal failure patients (Renal) and healthy controls. Ratios refer to the average value of the renal cohort divided by the average value of the healthy control cohort.

Conclusion

An SRM-MSIA-based analysis method was developed capable of simultaneously monitoring full-length PTH and truncated variants with analytical metrics suitable for clinical research use. Using a workflow incorporating postcapture tryptic digestion, surrogate peptides representative of PTH1–84 and PTH7–84 were generated and then monitored using SRM. In addition, tryptic fragments spanning other regions of PTH were incorporated into the analysis. Relative ion signals for these species confirmed that the clinical research method was functional and created the basis for a standard PTH profile. This standard profile was expanded to include a peptide representative of a novel variant, PTH34–84, clipped at the N-terminus. In total, 32 SRM transitions were analyzed in a multiplexed method to monitor nonvariant PTH sequences with >50% sequence coverage, as well as the two truncated variants. Peptides exhibited linear responses (R^2 = 0.90–0.99) relative to the limit of detection for an intact recombinant human PTH concentration of 8 ng/L. Limits of quantification were 16–31 ng/L, depending on the peptide. Standard error of analysis for all triplicate measurements was 3%–12% for all peptides, with <5% chromatographic drift between replicates. The CVs of integrated areas under the curve for 54 separate measurements of heavy peptides were 5%–9%.

Pinpoint software was used to develop and implement "intelligent SRM" data acquisition strategies, increasing instrument efficiency by avoiding the need to monitor all of the specified transitions at all times. Use of these techniques may be particularly advantageous for clinical research laboratories in methods where a large number of PTH variants are monitored, or where the analyzed sample contains a complex mixture of PTH-derived peptides and components produced by digestion of compounds in the sample matrix.

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Targeted Kinase Inhibitor Profiling Using a Hybrid Quadrupole-Orbitrap Mass Spectrometer

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Key Words
Q Exactive, targeted peptide quantification, msxSIM, kinome profiling by MS, desthiobiotin nucleotide probes

Goal
To identify and quantify kinase inhibition by staurosporine using kinase active sites probes in combination with targeted, multiplexed SIM (msxSIM).

Introduction
Protein kinases are key enzymes involved in a wide array of complex cellular functions and pathways. Misregulation or mutation of protein kinases underlies numerous disease states, including tumorigenesis, making them ideal candidates for drug development. However, identifying specific kinase inhibitors is challenging due to the high degree of homology among subfamily members of the 500+ human kinases. In addition, overlapping kinase substrate specificity and crosstalk between cellular signaling pathways can confound attempts to identify kinase inhibitor targets *in vivo*.

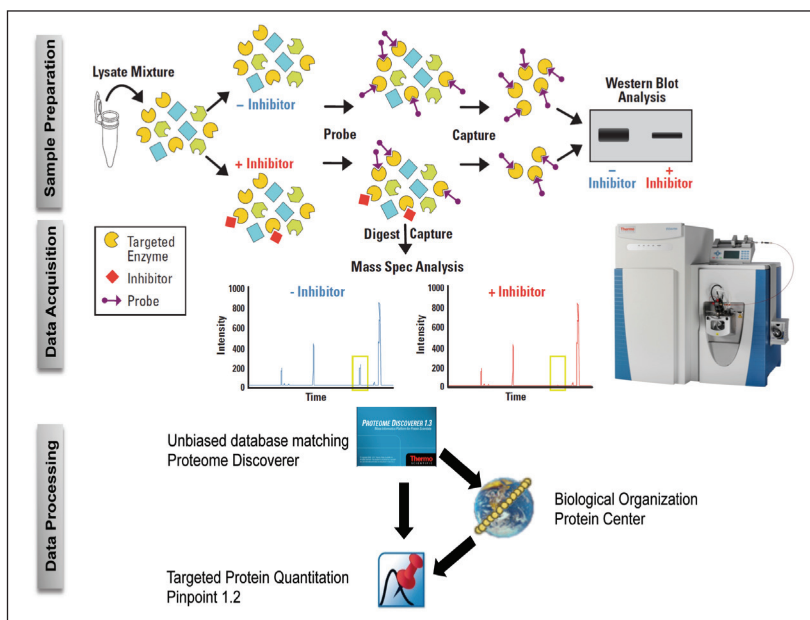

Figure 1. Integrated workflow of sample preparation/enrichment, data acquisition and data processing for global kinase identification and drug inhibition profiling

An emerging technology for identifying kinase inhibitor targets is based on chemical proteomic profiling of kinase inhibitor specificity and binding affinity. This technology combines mass spectrometry (MS)-based identification and quantitation with small molecule probe binding and enrichment to determine kinase active site occupancy during inhibition. One of these methods uses novel biotinylated ATP and/or ADP probes that irreversibly react with conserved lysine residues of kinase ATP binding sites.[1,2] Selective enrichment of active-site peptides from labeled kinase digests dramatically reduces background matrix and increases signal for MS analysis of low-abundance kinase peptides. Using this method, more than 400 different protein and lipid kinases from various mammalian tissues and cell lines have been identified and functionally assayed using targeted acquisition on an ion trap mass spectrometer.[1] The assays are available commercially from ActivX Biosciences as their KiNativ™ kinase profiling services.

Our approach incorporates desthiobiotin-ATP and -ADP probes with a hybrid quadrupole-Orbitrap™ mass spectrometer into an integrated workflow for global kinase identification and inhibition analysis (Figure 1). This workflow leverages the unique capabilities of the mass spectrometer for acquisition of MS and MS/MS spectra in unbiased or targeted modes with Orbitrap ion detection for high resolution and mass accuracy. A multiplexed data acquisition method was also employed to maximize instrument duty cycle and quantification of low-abundance kinase peptides through selected ion monitoring (SIM) on a nano-LC timescale. Overall, this approach resulted in significant improvements to kinase active-site peptide detection and relative quantitation for kinase inhibitor profiling.

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Experimental

Sample Preparation

K562 leukemia cells (ATCC) were grown in RPMI media supplemented with 10% FBS. Cell lysates (1 mg) were desalted using Thermo Scientific™ 7K Zeba™ Spin Desalting Columns and labeled with 5 µM of Thermo Scientific ActivX™ Desthiobiotin-ATP or -ADP probes for 10 minutes as described previously.[3] For inhibitor profiling, cell lysates were pretreated with 0, 0.01, 0.3, 1, 3, and 10 µM of staurosporine before addition of the desthiobiotin nucleotide probes. Labeled proteins were reduced, alkylated, desalted, and digested with trypsin. Desthiobiotin-labeled peptides were captured using Thermo Scientific High-Capacity Streptavidin Agarose Resin for two hours, washed and eluted using 50% acetonitrile/0.4% TFA for MS analysis.

LC-MS

All separations were performed using a Thermo Scientific EASY-nLC™ nano-HPLC system and a binary solvent system comprised of A) water containing 0.1% formic acid and B) acetonitrile containing 0.1% formic acid. A 150 x 0.075 mm capillary column packed with Magic™ C18 packing material was used with a 0.57% per minute gradient (5%–45%) flowing at 300 nL/min at room temperature. The samples were analyzed with a Thermo Scientific Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer in either data-dependent or targeted fashion. Details of the acquisition methods are summarized in Tables 1 and 2.

Table 1. Mass spectrometer parameter settings used for discovery experiments

Parameter	Setting
Source	Nano-ESI
Capillary temperature (˚C)	250
S-lens RF voltage	50%
Source voltage (kV)	2
Full-MS parameters	
Mass range (*m/z*)	380–1700
Resolution settings (FWHM at *m/z* 200)	140,000
AGC Target	1×10^6
Max injection time (ms)	250
Dynamic Exclusion™ duration (s)	70
Top n MS/MS	10
MS/MS parameters HCD	
Resolution settings (FWHM at *m/z* 200)	35,000
AGC Target	2×10^5
Max injection time (ms)	250
Isolation width (Da)	2
Intensity threshold	8×10^2 (0.1% underfill)
Collision energy (NCE)	27
Charge state screening	Enabled
Charge state rejection	On: 1+ and unassigned rejected
Peptide match	On
Exclude isotopes	On
Lock mass enabled	No
Lowest *m/z* acquired	10

Table 2. Mass spectrometer parameter settings used for targeted experiments

	Full	msxSIM
AGC Target Value	1×10^6	2×10^5
Max Injection Time (ms)	250	250
Resolution (FWHM at *m/z* 200)	140,000	140,000
Isolation Window (Da)	500–1300	4
# Multiplexed Precursors	-	4

Data Analysis

Thermo Scientific Proteome Discoverer™ software version 1.3 was used to search MS/MS spectra against the International Protein Index (IPI) human database using both SEQUEST® and Mascot® search engines. Carbamidomethyl (57.02 Da) was used for cysteine residue static modification. Desthiobiotin (196.12 Da) modification and oxidation were used for lysine and methionine residues, respectively. Database search results were imported into Thermo Scientific Pinpoint™ software version 1.2 for high-resolution, accurate-mass (HR/AM) MS-level quantitation. Data extraction was based on the four most-abundant isotopes per targeted peptide. The area under the curve (AUC) values were summed for the total AUC values reported. The relative AUC values for each of the isotopes were compared against the theoretical isotopic distribution for further confirmation and evaluation of potential background interference. The half-maximal inhibitory concentration (IC_{50}) values were determined by plotting AUC values for each peptide versus inhibitor concentration to generate a dose response curve of inhibitor binding (K_d) as described previously.[2]

Results & Discussion

Building a Kinase Active-Site Peptide Library

While protein kinase sequences and active sites are readily known from protein databases, it is still challenging to build a method for detection, verification, and quantification of kinase peptides. Our method focused on identifying and quantifying kinase active-site peptides since a large majority of these peptides are unique for their respective kinase. In addition, these peptides provide direct insight into kinase active-site inhibition for kinases that have multiple kinase domains.

To build a list of kinase active-site peptides, untreated cell lysate samples were labeled with the ActivX Desthiobiotin-ATP or -ADP probes for active-site peptide enrichment. An initial, unbiased Top10 data acquisition method (Table 1) was used to generate spectral libraries for database searching using Proteome Discoverer software. These search results were used to determine peptide sequences, desthiobiotin modification sites, and protein kinase family members. In addition, this experiment provided key data required for subsequent targeted acquisition methods including peptide retention times, precursor and product ion charge states, and HCD product ion distribution.

Peptide sequences were validated using Thermo Scientific ProteinCenter™ software for protein functional annotation. Figure 2 shows the proteins identified from peptides labeled with either desthiobiotin-ATP or -ADP probes. Both probes show high specificity for labeling known ATP binding proteins (77% and 83%, respectively) with protein kinases representing 13% of the total proteins identified using either probe. Although both probes enriched similar numbers of kinases with a modest degree of overlap, there was preferential binding of each probe for specific kinases as previously reported.[1]

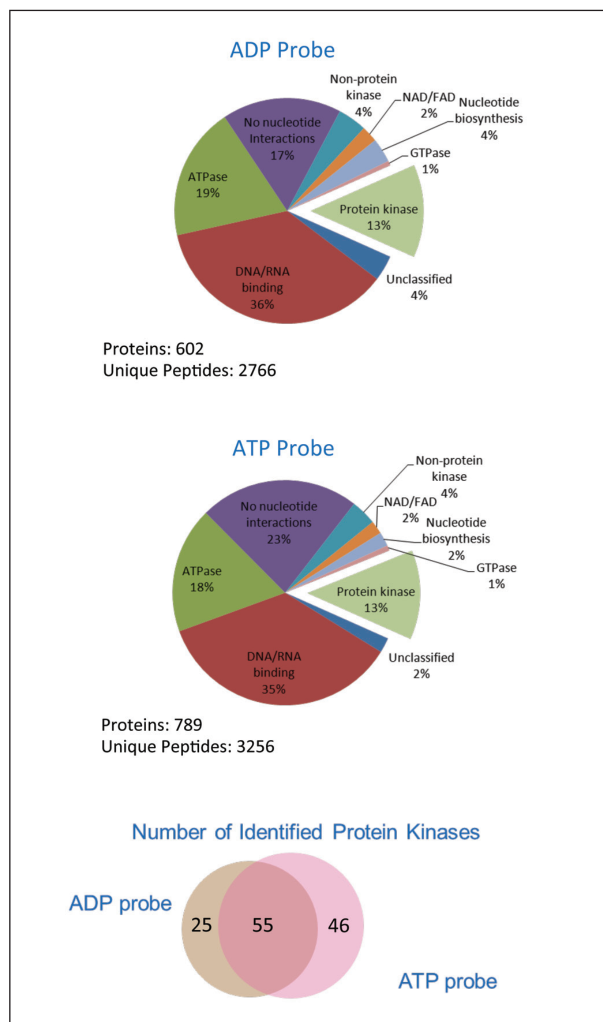

Figure 2. Proteins identified after desthiobiotin-ATP and -ADP probe labeling and enrichment categorized by protein function using Protein Center software. The Venn diagram shows the distribution of the resulting protein kinases identified using each probe.

The high resolution (up to 140,000) and mass accuracy of Orbitrap detection, as well as the ability to use the C-trap to collect/enrich the concentration of ions, facilitated kinase active-site peptide identification at the MS and MS/MS levels. Figure 3A shows an HR/AM MS spectrum at the retention time of the targeted active-site peptide **DIKAGNILLTEPGQVK** from Tao1/3. The complexity of the spectrum is typical even after active-site peptide enrichment and contains numerous highly charged peptides. The speed of the Q Exactive instrument for serial HR/AM MS and MS/MS acquisition, coupled with the C-trap's enrichment of the ion concentration in the Orbitrap mass analyzer, enabled the unbiased selection and sequencing of the +3 precursor despite it being the 14th most abundant peptide. Figure 3B shows the experimentally measured isotopic distribution and the corresponding theoretical isotopic distribution for the **DIKAGNILLTEPGQVK** +3 precursor charge state. Each isotope had less than 5 ppm mass error and less than 15% error compared to the theoretical isotope intensity distribution.

Figure 3C shows the HCD MS/MS spectra database search result matching 25 b- and y-type fragment ions. The average mass error for the fragment ions was less than 2 ppm across an order of magnitude range of measured product ion intensities. The ability to maintain a constant mass error across the entire mass spectrum is a particular advantage of Orbitrap detection, as it greatly increases peptide identification confidence. Ultimately, 126 kinase active-site peptides identified using Proteome Discoverer software were imported into Pinpoint software to evaluate relative abundance and generate an inclusion list for scheduled targeted acquisition (Figure 3D).

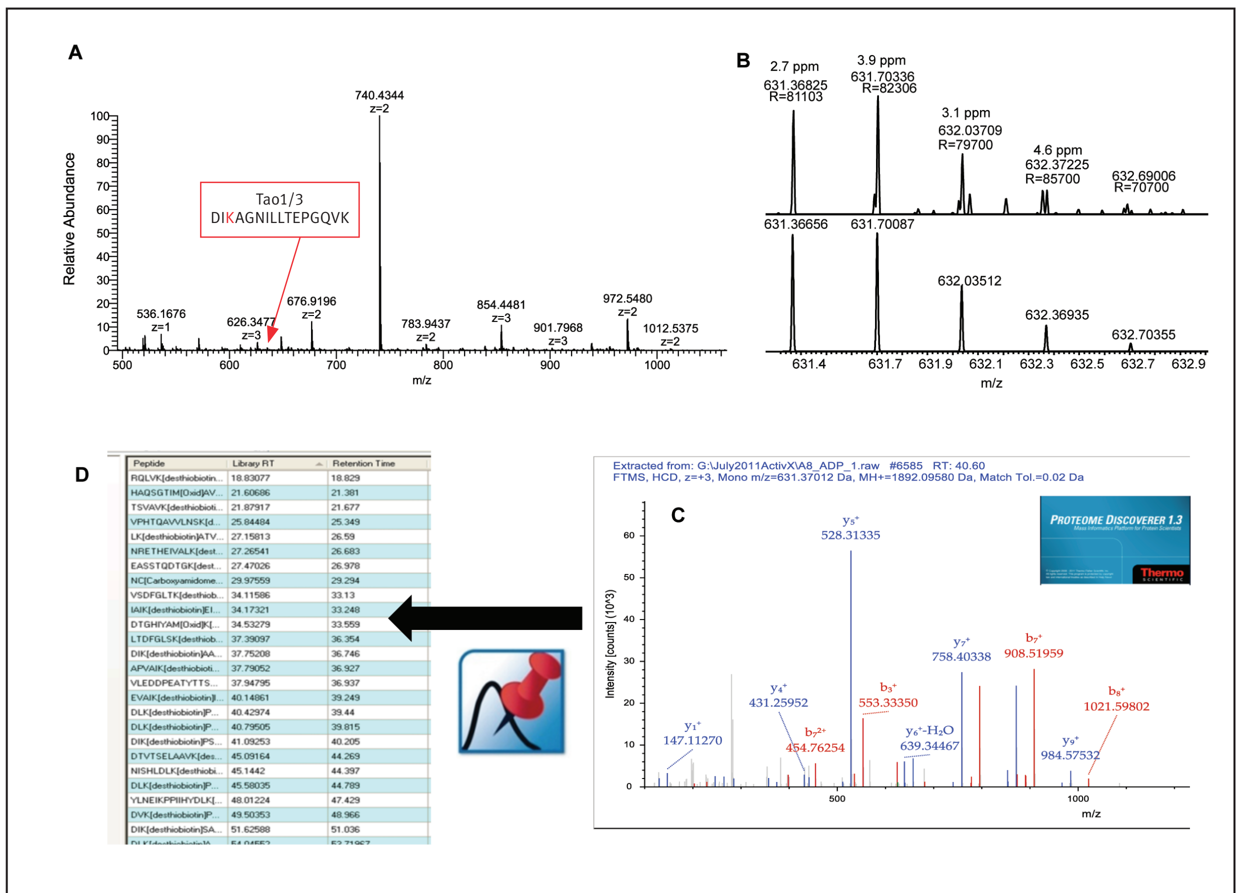

Figure 3. Processing strategy for building kinase active-site peptides spectral libraries and target lists. Example active-site peptide **DIKAGNILLTEPGQVK** from Tao1/3 kinase (A, B) was identified using Proteome Discoverer software from the HCD product ion spectrum (C) with Pinpoint software used to automate target-list method building (D).

Targeted Active-Site Peptide MS Method Development and Acquisition

Pinpoint analysis of kinase active-site peptide relative abundance in the enriched samples revealed a large dynamic range of peptide signals. More abundant active-site peptides were quantified using HR/AM full-scan MS and not included in the targeted experiment. Lower-abundance peptides were selected for a multiplexed SIM (msxSIM) acquisition to improve peptide ion signal intensity. A major advantage of the Q Exactive mass spectrometer is simultaneous C-trap filling during Orbitrap detection (Figure 4A and B).[4] This greatly reduces cycle times as selected ions can be trapped and detected in parallel. When targeted msxSIM events are scheduled, this benefit can be enhanced further by the trapping of multiple target ions in parallel. During Orbitrap detection, the C-trap is sequentially filled by ions defined by narrow mass ranges. Our experiment limited the multiplexing to a maximum of 4 mass windows but software capabilities enable up to 10 mass ranges to be collected and analyzed simultaneously. Cycling between full-scan MS and msxSIM is used to maintain target selectivity.

This approach of switching between full-scan MS and msxSIM was applied for targeted analysis of the ULK3 kinase active-site peptide **NISHLDLKPQNILLSSLEKPHLK** (Figure 4C). A narrow retention time region is displayed in centroid mode for which a total of 1.2 seconds was used to acquire data in both full-scan and SIM modes. The extracted ion chromatogram (XIC) plot from the full-scan mode shows a large number of peaks despite filtering using a mass tolerance of ±5 ppm. The highlighted region covers the expected elution time for the targeted active-site peptide, which was only 3% of the base peak intensity. The XIC profile for the SIM event shows that the scheduled, targeted data acquisition method clearly measures the peak of interest without interference.

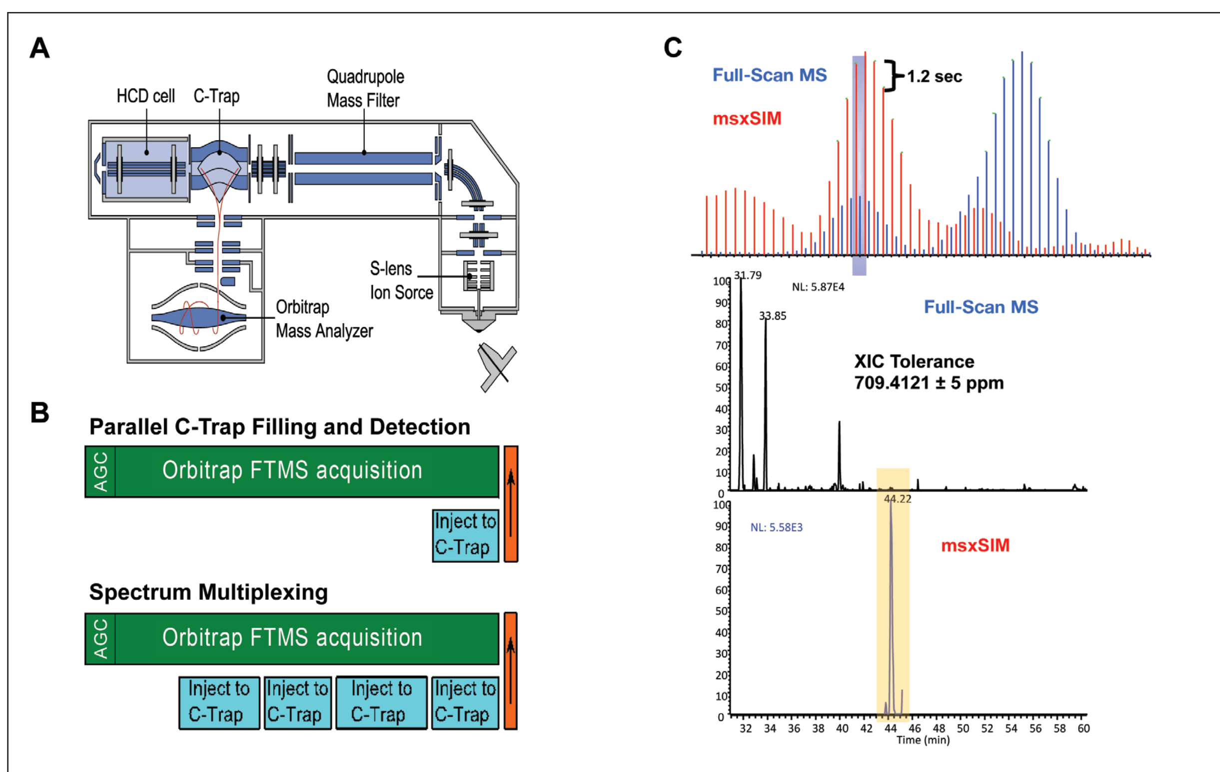

Figure 4. The Q Exactive mass spectrometer (A) is capable of multiple C-trap filling during Orbitrap detection (B) and producing a multiplexed spectrum (C).

Multiplexing the SIM event facilitated selective data acquisition for two additional active-site peptides that co-eluted in the same retention time window as the ULK3 active-site peptide (Figure 5A). Despite a large number of background ions in the full-scan MS scan, three target *m/z* values are easily acquired in the msxSIM window. Quadrupole mass filtering around the targeted *m/z* values enables greater accumulation of the target peptide precursor ions. Figure 5B shows the narrow mass region centered at the **NISHLDLKPQNILLSSLEKPHLK** +4 precursor *m/z* value for the full-scan MS, SIM, and theoretical isotopic distribution. Both full MS and SIM scans show a co-isolated matrix ion in the +3 charge state. However, the high resolution of the Orbitrap mass analyzer facilitates separation of the background signal from the targeted peptide even though the mass difference between the matrix ion and the A+2 target isotope is only 0.05 Da (76 ppm) at 20% relative intensity. The SIM event also provided greater fine structure compared to full-scan MS with increased signals for target peptide isotopes. Each peptide was confirmed in a separate targeted MS^2 experiment. Excellent retention-time correlation was observed between targeted msxSIM and targeted MS^2 experiments (data not shown).

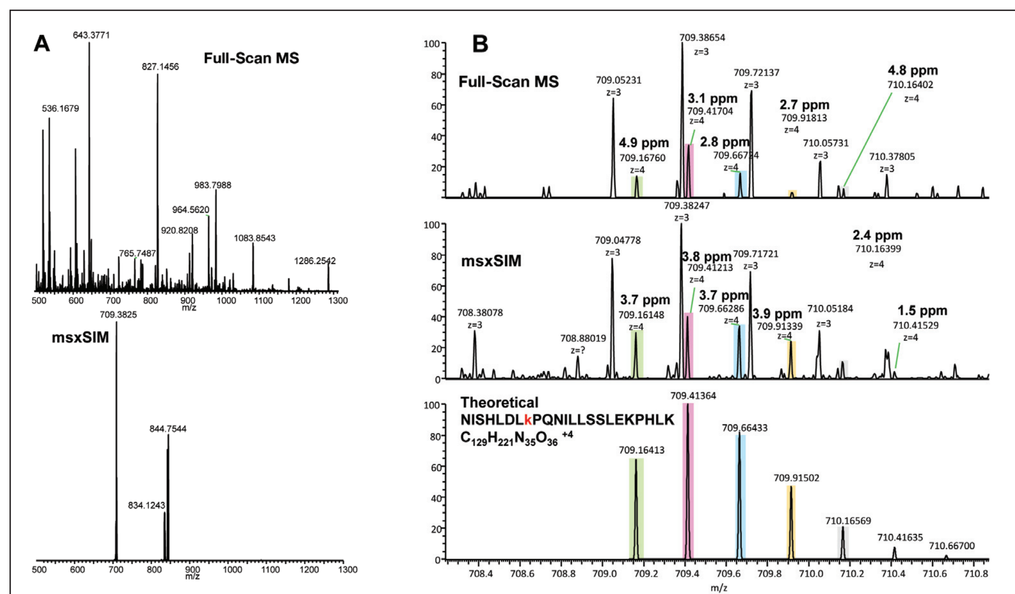

Figure 5. Comparative HR/AM mass spectra for the targeted active-site peptide **NISHLDLKPQNILLSSLEKPHLK** between full-scan MS and msxSIM at a determined retention time (A). Zoom in shows the +4 precursor charge state compared to the theoretical precursor isotopic distribution used for verification (B).

Post-acquisition data verification, integration, and relative quantitation using HR/AM MS data (full-scan and msxSIM data) was performed using Pinpoint software. Figure 6A shows the individual isotopic XICs (displayed in centroid mode) and the measured AUC values. A total of 1.8 seconds was needed to cycle from the msxSIM event to full-scan MS acquisition and back to msxSIM detection resulting in 12 scans acquired across the elution peak. User-defined sequence and modifications are used to calculate the theoretical *m/z* value for the four most abundant isotopes per charge state. An XIC extraction tolerance of ±5 ppm was used for full MS and msxSIM scans. Finally, an XIC is performed for each isotope independently, grouped, and then overlaid to identify and confirm target peptides versus retention time across all attributed precursor charge states and corresponding isotopes (Figure 6B).

High resolution and mass accuracy are also used to maintain target ion selectively for both full-scan MS and msxSIM MS analysis. Pinpoint software can determine potential background interference per isotopic XIC. Figure 6C shows the isotopic distribution overlap between experimental and theoretical values for the +4 and +5 precursor charge states of the **NISHLDLKPQNILLSSLEKPHLK** peptide. The dot-product correlation coefficients for the charge states are 0.95 and 0.98, respectively. The consistent peak shape and relative isotopic distribution increase confidence in the measured signal being attributed to the target peptide and not to the matrix background. Figure 6D shows the consolidated histogram for the +4 charge state for all biological conditions and technical replicates. The calculated correlation coefficient was greater than 0.95 for all files, indicating the externally calibrated mass accuracy was maintained for the duration of the sample analysis.

Kinase Inhibitor Profiling and IC_{50} Determination

Using the combined full-scan MS and targeted msxSIM methods described, relative abundance of kinase expression can be readily assessed for multiple samples or treatment conditions. Kinase inhibitor profiling is a powerful application that can be assessed by measuring changes in kinase active-site peptide relative abundance before and after drug treatment. Inhibitor binding affinities can also be determined by titrating inhibitor concentrations to determine IC_{50} values from dose-response curves. Targeted msxSIM acquisition greatly aids this type of analysis as inhibited kinases peptide signals will be decreased as inhibitor concentrations increase.

Figure 7 shows the relative quantitation of three co-eluting active-site peptides measured using the msxSIM method from samples treated with increasing amounts staurosporine, a broad-specificity kinase inhibitor. Figure 7A shows the overlaid XIC plots for each targeted peptide per sample with two technical replicates. The GCK kinase (**DTVTSELLAAVKIVK**) and ULK3 kinase (**NISHLDLKPQNILLSSLEKPHLK**) both show significant inhibition at < 0.1 µM staurosporine while PKR kinase (**DLKPSNIFLVDTK**) shows modest inhibition to staurosporine treatment. Relative peptide signal intensity was used to plot dose-response curves and calculate IC_{50} values for each kinase (Figure 7B). For staurosporine kinase inhibition profiling, comprehensive data is also available from several alternative assays and is very similar to values obtained in the present study.[5]

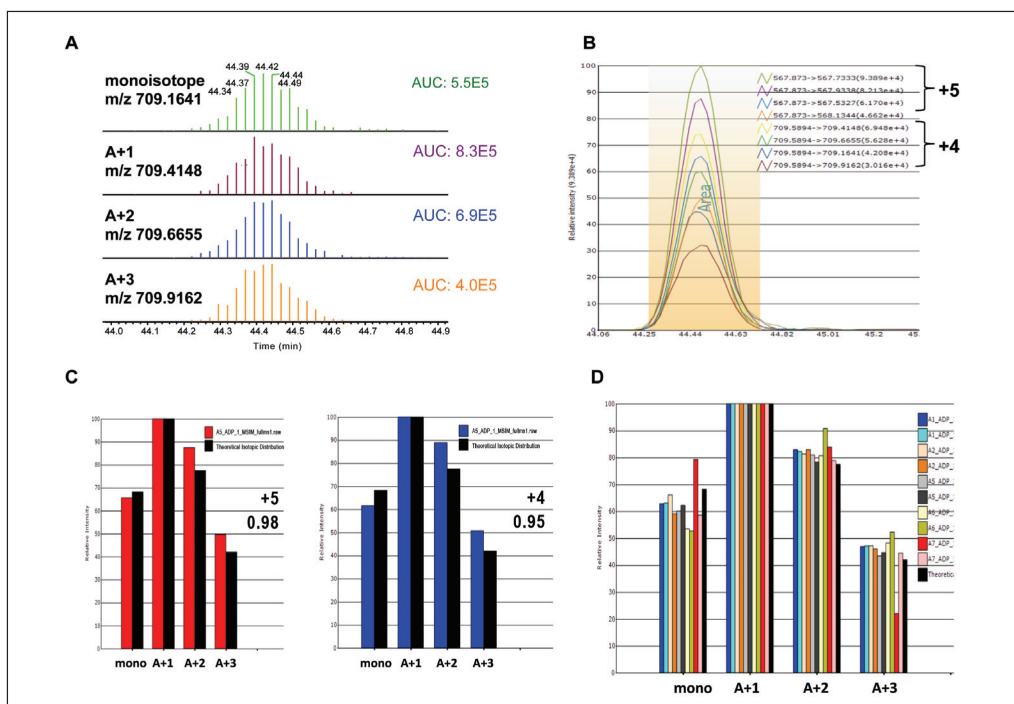

Figure 6. Data processing strategy using Pinpoint software for HR/AM MS and msxSIM data where (A) shows the individual isotopic XIC plots in centroid display mode, (B) shows an overlaid XIC plot for retention-time determination, (C) shows the isotopic distribution overlap between experimental and theoretical profiles for each charge state, and (D) shows the resulting isotopic distribution for all resulting RAW files acquired in the study.

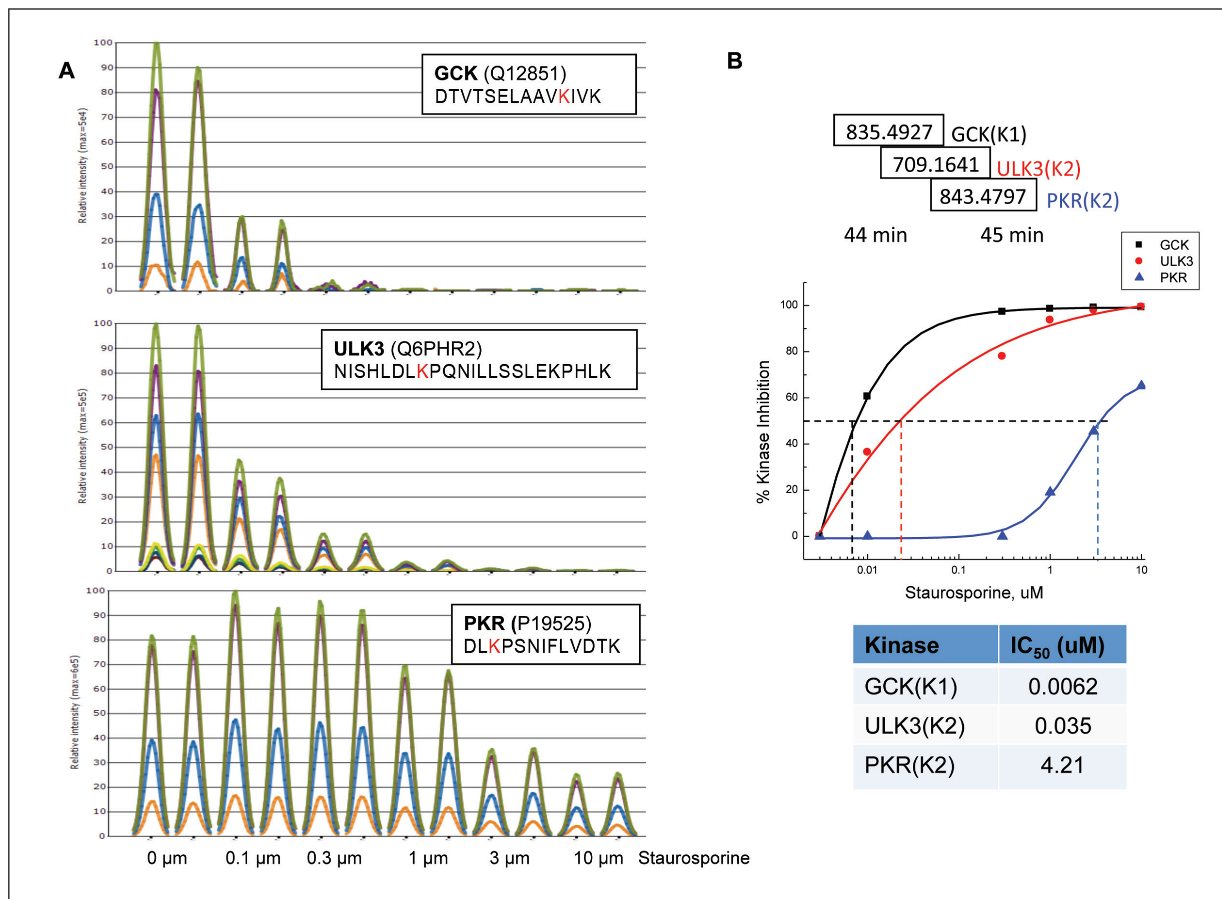

Figure 7. Targeted msxSIM analysis for low-level co-eluting active-site peptides. Comparative overlaid XIC plot at each titrated staurosporine level (A). Dose-response curves of the measured peptide signal inhibition as a function of titrated staurosporine concentration with calculated IC_{50} values for each active-site peptide (B).

Table 3 lists all kinases quantified in this study after staurosporine treatment. By comparing relative intensity of untreated to staurosporine-treated (10 μM) samples, kinases were grouped by their percent inhibition. This analysis clearly shows which kinases bind to staurosporine and can be used to identify kinase subfamilies that share common inhibition profiles.

Kinase	% Inhibition	Kinase	% Inhibition	Kinase	% Inhibition	Kinase	% Inhibition
ABL1/2	75%	EphB4	88.00%	MARK2	100.00%	PLK1	0.00%
ACK	100.00%	Erk1/2	0.00%	**MARK3**	100.00%	PKN2	100.00%
AMPKa1/2	100.00%	FAK1	100.00%	MASTL	0.00%	PRPK	0.00%
ATR	50.00%	**FER**	100.00%	MET	0.00%	PRP4	40.00%
ATM	0.00%	**GCK**	100.00%	**MLKL**	0.00%	**PRKDC**	0.00%
ARAF	0.00%	GCN2	75.00%	MLK3	100.00%	PRKCI	0.00%
BAZ1B	100.00%	JAK1	100.00%	**MST3**	93.00%	ROCK1/2	100.00%
BRAF	0.00%	IKKa	0.00%	MST4	100.00%	RSK1	95.00%
CaMK1a	75%	IKKb	0.00%	**NDR1/2**	95.00%	**RSK1/2/3**	100.00%
CaMK2g	100.00%	ILK	10.00%	NEK1	20.00%	**RSK2(1)**	100.00%
CAMKK2	100.00%	IRE1	90.00%	NEK3	0.00%	RSK2(2)	0.00%
CASK	100.00%	**IRAK4**	100.00%	NEK4	0.00%	**SLK**	100.00%
CDC2	50.00%	KHS1/2	100.00%	NEK7	0.00%	SMG1	20.00%
CDK5	100.00%	LATS1	100.00%	NEK8	0.00%	SRPK1/2	50.00%
CDK6	65.00%	LKB1	100.00%	NEK9	40.00%	STLK6	0.00%
CDK7	100.00%	LOK	100.00%	OSR1	30.00%	SRC	100.00%
CDK8/11	100.00%	LYN	100.00%	p70S6Kb	0.00%	TAO1/3	100.00%
CDK9	0.00%	MAP2K1/2	90.00%	p70S6K	15.00%	TAO2	100.00%
CDK10	100.00%	**MAP2K3**	100.00%	PAN3	0.00%	**TBK1**	100.00%
CDK12	0.00%	**MAP2K4**	95.00%	PDK1	85.00%	TEC	100.00%
CDK13	0.00%	**MAP2K6**	100.00%	PHKg2	100.00%	TP53RK	100.00%
CHK1	100.00%	MAP3K1	80.00%	PKCi	80.00%	**TLK1/2**	100.00%
CHK2	97.00%	MAP3K2	50.00%	PITSLRE	0.00%	**ULK3**	100.00%
CSK	85.00%	MAP3K4	0.00%	PKD2	100.00%	Wnk1/2/4	0.00%
EF2K	0.00%	**MAP3K5**	100.00%	PKD3	100.00%	YES	100.00%
EGFR	30.00%	MARK1/2	100.00%	**PKR**	30.00%	ZAK	0.00%
EphA2	88.00%						

% Inhibition > 90% | 60%-89% | 30%-59% | <30%

Table 3. Identified kinases (active-site peptides) and corresponding relative quantitation determined using HR/AM MS data after inhibitor treatment. Kinase peptides measured using targeted multiplexed SIM are shown in bold.

Conclusion

Thermo Scientific ActivX Desthiobiotin-ATP and -ADP probe technology combined with Q Exactive LC-MS analysis creates a powerful workflow enabling global kinase identification and drug-inhibition profiling. These novel active-site probes specifically captured ATPase subfamily members and improved kinase MS detection for inhibitor selectivity profiling and binding affinity assessment. Unique data acquisition methods incorporating HR/AM full-scan MS and multiplexed SIM events were used to build spectral libraries and target kinase active-site peptides. Multiplexed SIM events were also used to maximize cycle times for co-eluting active-site peptides which increased signals 7-fold over background. This increased dynamic range enabled low-level peptide quantitation necessary for IC_{50} determination. Integrated software further facilitated automated method building and data processing to reduce analysis time. This workflow was successfully used to identify and quantify inhibition of over 126 kinases in a 60 minute gradient using the same instrument platform.

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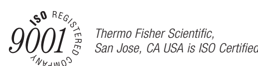

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Translational Research Poster Notes

Improving Throughput for Highly Multiplexed Targeted Quantification Methods Using Novel API-Remote Instrument Control and State-Model Data Acquisition Schemes

PN64078: Targeted Quantitation of Insulin and Its Therapeutic Analogs for Research

PN64139: Enrichment of EGFR/PI3K/AKT/PTEN Proteins for Research Using Immunoprecipitation and with Mass Spectrometry-Based Analysis

PN64149: Real-Time Qualitative and Quantitative Global Proteomics Profiling Using a Hybrid Data Acquisition Scheme

PN64144: Characterizing Qualitative and Quantitative Global Changes in the Aging Heart using pSMART, a Novel Acquisition Method

PN64039: Targeted Multiplexed Protein Quantitation Using Serial Immunoaffinity Extraction Coupled to LC-MS

MSACL 14 PN: Improving Throughput for Targeted Quantification Methods by Intelligent Acquisition

MSACL 14 PN: Spectrum Library Retention Time Prediction Based on Endogenous Peptide Standards

HUPO 2013 PN: Comprehensive Peptide Searching Workflow to Maximize Protein Identifications

ASMS 2013 PN: High-Resolution, Accurate-Mass (HR/AM) and Intelligent Acquisition-Enabled Global Discovery and Quantification of Histones, Histone PTMS, and Histone Modification Enzymes in Mesenchymal Stem Cells

A Phospho-Peptide Spectrum Library for Improved Targeted Assays

ASMS 2013 PN: Improving Label-Free Quantification of Plasma and Serum Proteins Using a High-Resolution Hybrid Orbitrap Mass Spectrometer

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Improving Throughput for Highly Multiplexed Targeted Quantification Methods Using Novel API-Remote Instrument Control and State-Model Data Acquisition Schemes

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Overview

Automated remote multiplexed targeted protein quantification utilizing real-time qual/quan processing for increased quantitative accuracy over large dynamic ranges.

Introduction

Targeted quantification has become a very popular technique to verify putative biomarker candidates in large clinical cohorts of samples. These candidates are usually generated following a biomarker discovery experiment or derived from a biological hypothesis, for example, a pathway or biophysical interaction. These lists are usually large, containing upwards of 100–1000 proteins spanning several orders of magnitude dynamic concentration range. This presents analytical challenges for conventional SRM assays both in terms of method development and throughput. We propose using high-resolution, accurate-mass (HR/AM) mass spectrometry (MS) and MS/MS schemes in conjunction with validated spectral libraries for automated method building, data acquisition, verification, and quantification in real-time using novel acquisition schemes.

Methods

K562 colon carcinoma cells were grown in heavy and light media, collected and mixed at different ratios to cover a 20-fold dynamic range. All samples were digested and analyzed on a quadrupole Thermo Scientific™ Orbitrap™ mass spectrometer equipped with a nanospray ion source. Data was acquired in two steps to simulate traditional workflows. Initial experiments employed unbiased data-dependent MS/MS acquisition resulting in peptide/protein identification as well as building of a spectral library. The spectral library contains relative retention time, precursor charge state distribution, and product ion distributions, creating a unique verification/quantification scheme. A highly multiplexed, targeted protein list was created from the spectral library and used for automated data acquisition and processing real time to facilitate changes to the acquisition scheme.

The scheme in Figure 1 describes the methodology in more detail. The first step is to characterize the LCMS parameters using the PRTC kit. The next step is to build a list of proteins that we are interested in. This will typically come from a pathway study or a discovery experiment. The next is to build a spectral library for this list of proteins. This can be built via predictive algorithm or empirical observations. This turns into a spectral library lookup table. The look-up table includes the precursor *m/z* values for the defined charge state as well as the expected retention time window, which are used to initiate product ion spectral acquisition based on the presence of multiple precursor isotopes during the expected elution window. Once the signal for multiple precursor isotopes surpasses the user-defined intensity threshold, a higher-energy collision dissociation (HCD) spectrum is acquired and immediately compared against the spectral library generating a dot-product correlation coefficient to determine spectral overlap and to check if the targeted peptide has been detected previously. If the calculated correlation coefficient surpasses the user-defined acceptance value, HCD product ion spectra will continue to be acquired across the elution profile. This is shown in Figure 2.

FIGURE 1. Strategy for large-scale targeted quantification based on high IQ data acquisition scheme

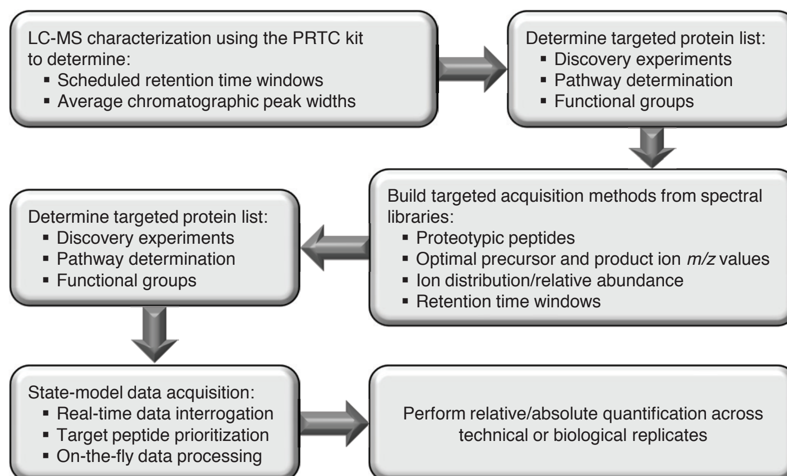
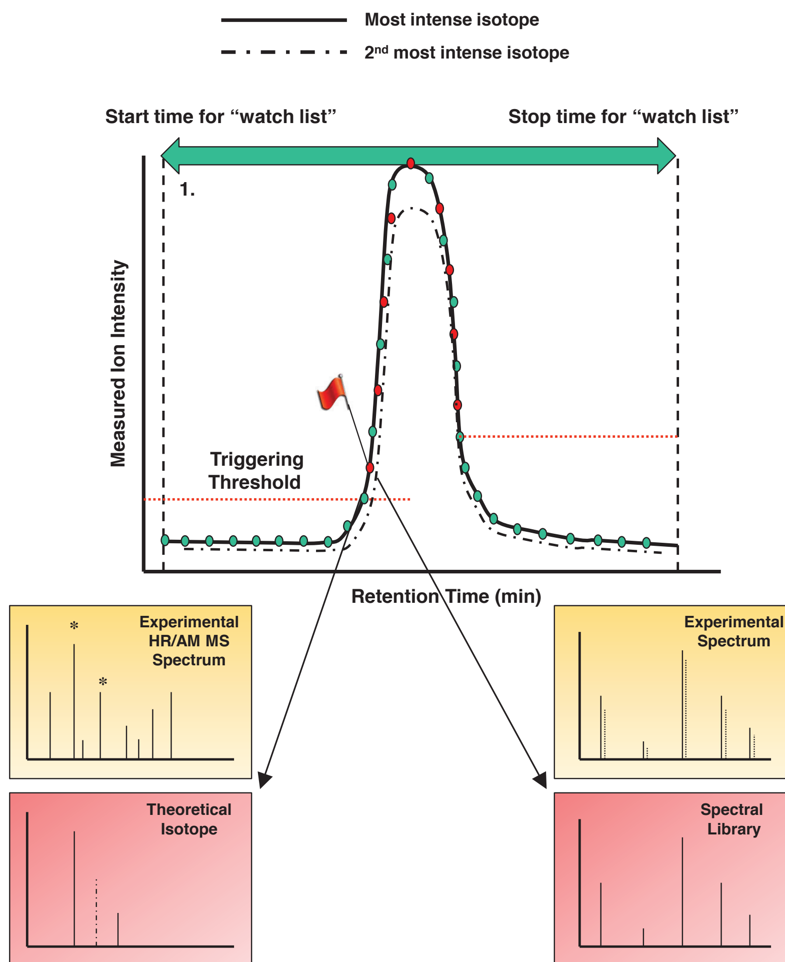


FIGURE 2. Pictorial representation of high IQ data acquisition schemes for targeted peptide quantification using a targeted scanning window, target elution identification, and real-time product ion spectral acquisition. Both precursor and production spectral matching is performed to increase the selectivity of data acquisition.



Results

Highly multiplexed targeted protein quantification requires significant steps of method refinement prior to implementation. While the determination of proteins is relatively straightforward based on biology, the selection of peptides as surrogate biomarkers and corresponding *m/z* values (precursor and product ions) used to uniquely identify and quantitate the peptide targets becomes challenging. Generally, retention times and acquisition windows must be determined to maximize instrument cycle time to achieve robust quantification. To expedite complex experimental method development, we have created a unique spectral library procedure based on an analytically rigorous discovery data acquisition scheme. The local spectral library contains both LC and MS information that can be readily enlisted to build robust methods requiring few refinement steps.

To first test our methods, a protein mix was spiked in equine plasma (containing PTRC kit). Spectral library was first built on the neat protein mixture. Experiments performed on the quadrupole Orbitrap mass spectrometer facilitate unique product ion collection and detection schemes to not only increase data acquisition, but perform state-model data acquisition, increasing the ability for quantification. Figure 3 shows the result of the data acquisition scheme, with MS/MS events acquired for the various peptides, showing the benefit of increased efficiency of triggering events. Figure 4 shows the distribution of the retention of the various peptides, and as expected, most peptides elute in the middle of the gradient. Figure 5 shows the CV distribution for the peptides over four acquisitions (by summing the area of top eight product ions).

FIGURE 3. Result from high IQ data acquisition scheme for a small list of peptides. The graphs show the MS/MS events for the various peptides, and the effective gain in duty cycle.

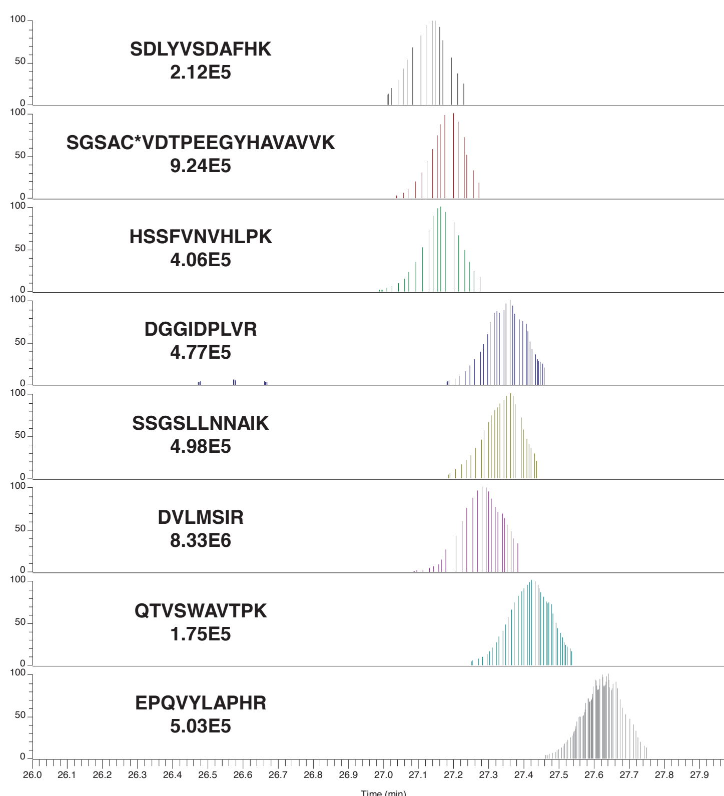


FIGURE 4. The number of targeted peptides in each retention time window

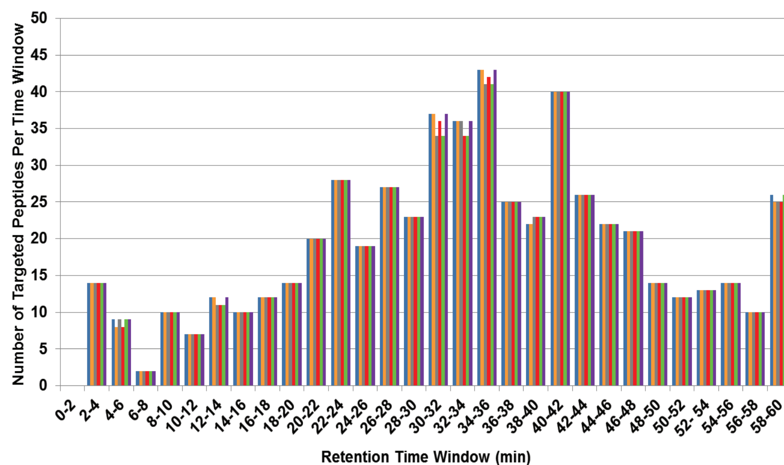
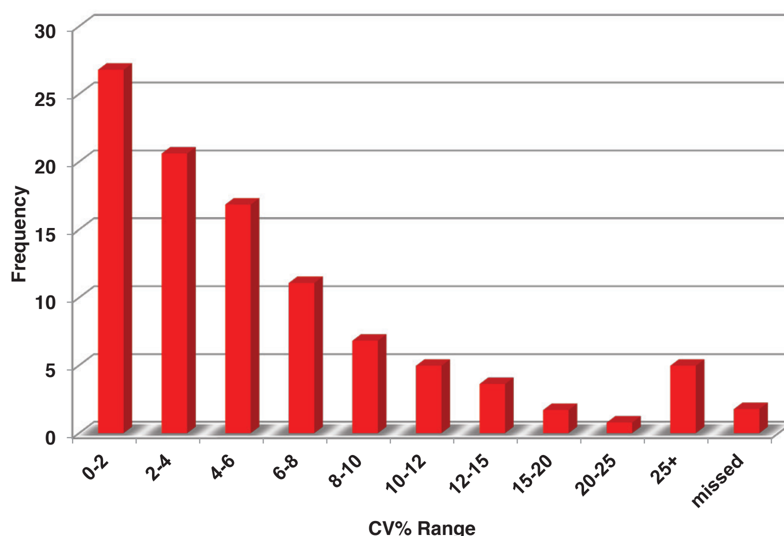


FIGURE 5. CV distribution for the initial peptide list

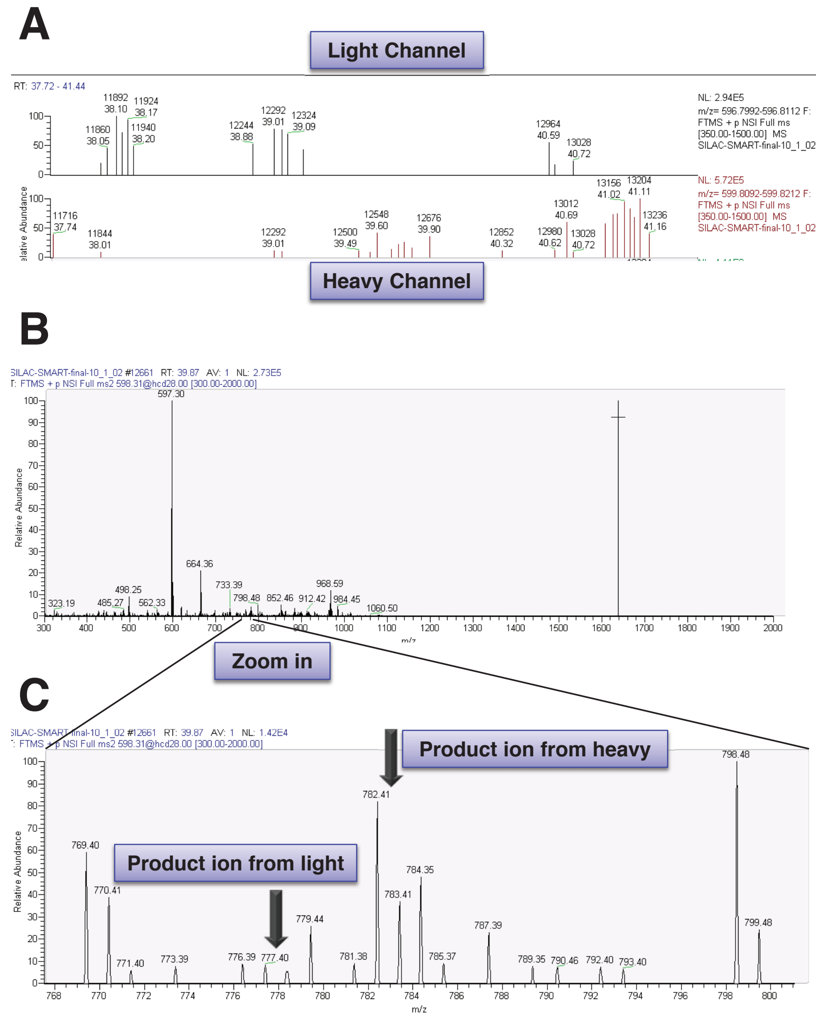


K562 Cell Line

2,100 proteins were selected from the K562 cell line and imported into the new algorithm. The algorithm utilizes the spectral library information to select unique peptides and create precursor and product ion information used to perform real-time qualitative and quantitative analysis. In total, 3,800 peptides were chosen and 20-fold range digest was created.

Figure 6 shows an example where the ratio of 1:10 could not be calculated using the full scan MS1 (panel A), but could be calculated in tandem MS/MS scan (panel B, and zoom-in, panel C).

FIGURE 6. The benefit of MS/MS scan (with higher S/N) compared to full scan. Ratio of 1:10 could not be calculated in full scan (panel A), but it could be calculated in tandem MS/MS scan (panel C).



Conclusion

The developments here resulted in the successful qualitative/quantitative analysis for over 3,000 peptides representing over 2,000 proteins in this complex leukemia cell digest. Successful quantification was determined for proteins spiked at over a 20-fold range and the ability to change instrument acquisition parameters for increased sensitivity.

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Targeted Quantitation of Insulin and Its Therapeutic Analogs for Research

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Overview

Purpose: To perform simultaneous qualitative and quantitative analyses of endogenous insulin and/or therapeutic analogs at biological levels for research.

Methods: We used a pan-anti insulin antibody in Thermo Scientific™ Mass Spectrometric Immunoassay (MSIA) D.A.R.T.'S™ pipette tips for highly-selective affinity purification of all insulin analogs. Analogs were detected, verified, and quantified using high-resolution, accurate-mass (HRAM) MS and MS/MS data from a Thermo Scientific™ Q Exactive™ mass spectrometer.

Results: We achieved a lower-limit-of-detection (LLOD) of 15 pM in plasma for all variants used with linear regressions of 0.99 or better. Further, we demonstrate inter- and intra-day CV's of < 3% and spike and recovery resulted in recoveries of 96–100%.

Introduction

The measurement of insulin is a paramount metric in clinical research, therapeutic research, forensic, and sports doping applications. Conventional insulin analytical methods are plagued by the inability to differentiate endogenous insulin from exogenous insulin analogs. The use of LC/MS can overcome this shortcoming[1]; however, the LC/MS methods to date lack the analytical sensitivity demanded by the field. Therefore, a highly selective sample interrogation workflow is required to address the complexity of plasma samples and, ultimately, for accurate and sensitive LC/MS detection and quantification. To meet these requirements, a MSIA research workflow was developed for the high-throughput, analytically sensitive quantification of insulin and its analogs from human donor plasma.

Methods

Sample Preparation

For spike and recovery studies, both neat and donor plasma samples containing a mix of insulin and its analogs were prepared. Insulin was added at three different amounts that spanned the dynamic range to the donor plasma. Up to four analogs were prepared in a single sample. For the limit-of-detection and limit-of-quantification studies, 1.5 pM to 960 pM insulin was added to bovine serum albumin in phosphate buffered saline. Additionally, either 0.05 nM of a heavy version of insulin or porcine insulin was added as an internal reference standard to each well of 500 μL plasma.

Samples were then addressed for the first stage in the MSIA workflow. Targeted selection was achieved using insulin MSIA Disposable Automated Research Tip's (D.A.R.T.'S) (Figure 1). The affinity purification step in the MSIA workflow was automated by the Thermo Scientific™ Versette™ automated liquid handler. Following extraction, intact insulin analogs were eluted with 75 μL 70:30 water/acetonitrile with 0.2% formic acid with 15 μg/mL ACTH 1-24. The final concentration was adjusted to 75:25 water/acetonitrile with 0.2% formic acid for LC/MS analysis.

Liquid Chromatography

A Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system was used for all experiments. 100 μL of each sample was separated on a 100 x 1 mm Thermo Scientific™ ProSwift™ column using a linear gradient (10–50% in 10 min) comprised of A) 0.1% formic acid in water and B) 0.1% formic acid in acetonitrile. The column was heated to 50 ºC.

Mass Spectrometry

All data was acquired using a Q Exactive Orbitrap mass spectrometer operated in data-dependent mode with dynamic exclusion enabled. Full scan MS data was acquired with a resolution setting of 70,000 (at *m/z* 200) and using a mass range of 800–2000 Da. A targeted inclusion list was used to trigger MS/MS events and MS/MS was acquired with a resolution setting of 17,500 (at *m/z* 200).

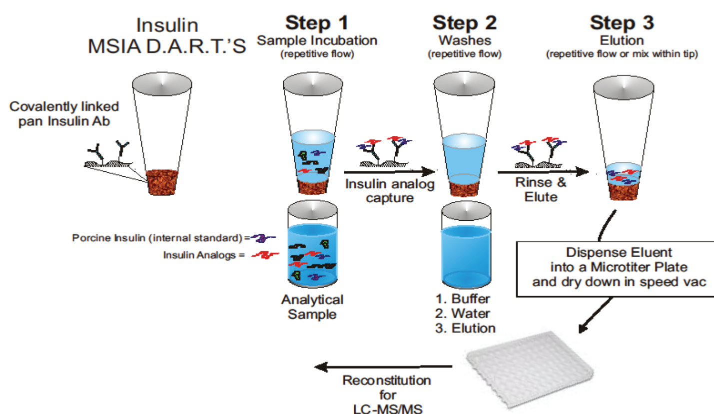
Data Analysis

Thermo Scientific™ Pinpoint™ software version 1.3 was used to analyze all LC/MS data. HRAM measurements were used for qualitative and quantitative measurement of insulin and its analogs.

The three most abundant precursor charge states per analog and the six most abundant isotopes per charge state provided qualitative validation for insulin and its analogs. Qualitative scoring was based on mass error, precursor charge state distribution, isotopic overlap, and corresponding LC elution peak profiles. Product ion data was used for sequence verification.

For quantification, a mass tolerance of ±5 ppm was used for all data extraction. Amounts of each insulin analog were determined by converting area-under-the-curve (AUC) values, normalized to the AUC of the internal reference, which was calculated from standard curve data.

FIGURE 1. Targeted selection using insulin MSIA D.A.R.T.'S.. First, insulin and its analogs are selectively bound. Then, a wash step removes background compounds. Lastly, the insulin and insulin variants are eluted into a new plate, which is ready for LC/MS analysis.



Results

Qualitative Validation of Insulin and Its Analogs

One of the primary limitations of current insulin analytical methods is the inability to distinguish between endogenous and exogenous insulin analogs. The immobilized insulin pan-antibody in the MSIA D.A.R.T.'S recognizes a common epitope region in the β-chain that is conserved across all of the analyzed variants. This allows the capture and detection of all variants from the sample as long as the β-chain epitope region remains conserved. Further, utilizing full scan MS mode in the analysis stage of the MSIA workflow enables simultaneous detection of multiple insulin analogs and the ability to screen for unsuspected insulin analogs post-acquisition.

LC/MS detection using HRAM MS data provided the analytical selectivity to distinguish insulin variants from the background signal using the accurate mass of multiple precursor charge states and isotopes. Figure 2 demonstrates the HRAM data analysis approach. Figure 3 shows simultaneous LC/MS detection of insulin variants. Further, fragmentation patterns from data-dependent MS/MS acquisition can also be used to confirm the identity of insulin variants (data not shown).

FIGURE 2. HRAM MS data analysis in Pinpoint software version 1.3. Extracted ion chromatograms for each targeted insulin variant were created using the isotopic *m/z* values from three precursor charge states. Integrated AUC values from each isotope were then co-added to generate the reported values. Additionally, each insulin variant was qualitatively scored based on 2a) comparative peak profiles (peak start and stop, apex, and tailing factors) as well as 2b) isotopic distribution overlap.

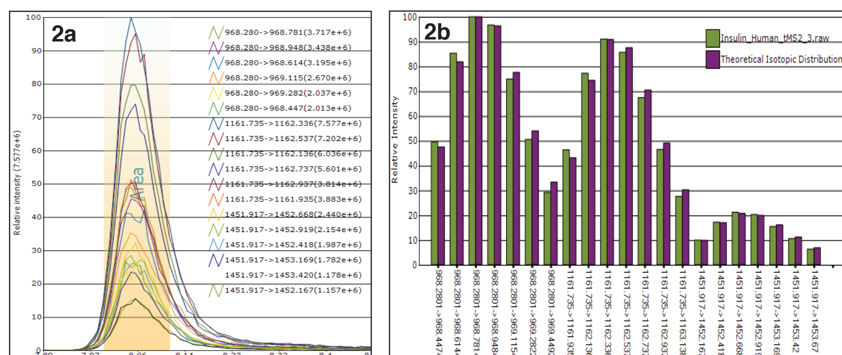
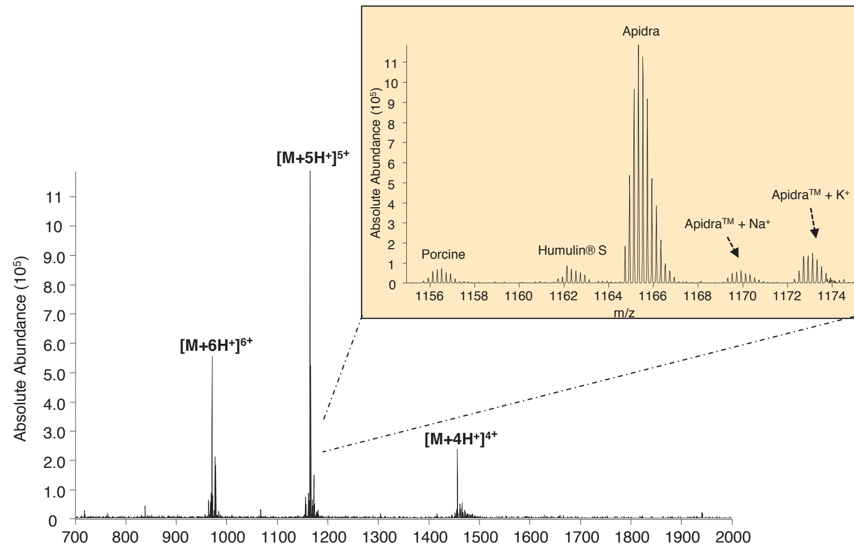


FIGURE 3. Simultaneous LC/MS detection of four insulin variants. Apidra™ (0.48 nM), Humulin® S (0.06 nM), Lantus™ (0.48 nM) , and porcine as the internal standard were processed from the same sample and detected simultaneously. The inset shows an enlargement of the 5+ charge state, and shows all three variants. Lantus elutes 0.5 minutes prior to the three displayed insulin variants.

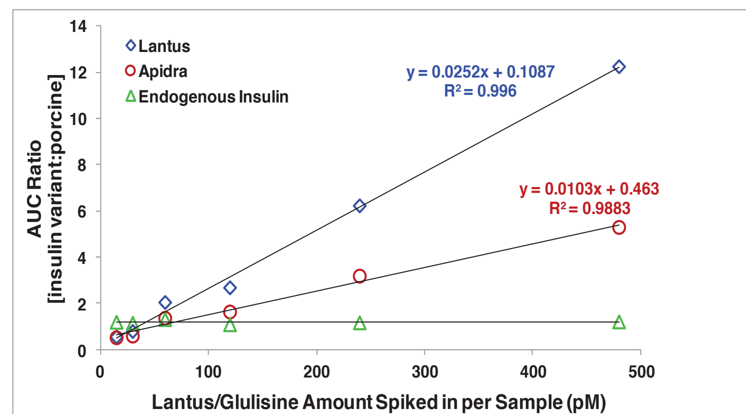


Quantitative Measurement of Insulin and Its Analogs

Additional limitations to high-throughput targeted quantification of insulin and its analogs in research are inefficient sample preparation protocols that result in their lack of analytical sensitivity and robustness. Using the insulin MSIA workflow described above, we achieved an LLOQ and LOD of 15 pM (87 pg/mL) for the intact variants in plasma. Quantification curves for Lantus and Apidra are shown in Figure 4. Tables 1 and 2 display LOQ and LOD.

Further, reproducibility studies demonstrated inter- and intra-day CVs of < 3% (Tables 3 and 4) and spike and recovery resulted in recoveries of 96–100% (Table 5). In addition to the improved sensitivity, the MSIA workflow significantly reduces the background matrix. The reduced complexity affords shorter LC gradients, and, therefore, shorter LC/MS analysis times.

FIGURE 4. Quantification curves for Lantus and Apidra. Lantus and Apidra were spiked into donor plasma at different concentrations. The endogenous insulin from the donor plasma is also plotted. Since the same amount of donor plasma was used for each sample, the level of endogenous insulin remains static. All AUC values were normalized to the porcine AUC response.



Method Characteristics for the MSIA Insulin Research Workflow

The LLOQ for the insulin MSIA research workflow is 15 pM (highlighted in red in Table 1), which was determined as the lowest concentration where we could achieve a %CV of <20% and an accuracy within ±20%.

An LOD of 15 pM (highlighted in red in Table 2) was also achieved for the insulin MSIA workflow. The LLOD was determined as the lowest concentration where the mean total area was greater than four standard deviations of the background signal added to the mean total area for the blank.

TABLE 1. Limit of quantification

STD Conc. (pM)	Mean (5 Curves)	StDev	%CV	Accuracy
0	7.42	1.02		
7.5	10.56	0.95	9.04%	40.80%
15	16.78	1.42	8.46%	11.87%
30	28.96	1.12	3.85%	-3.46%
60	58.41	1.61	2.75%	-2.66%
120	115.93	1.96	1.69%	-3.39%
240	232.65	2.80	1.20%	-3.06%
480	473.25	14.41	3.04%	-1.41%
960	963.31	6.47	0.67%	0.34%

TABLE 2. Limit of detection

STD Conc. (pM)	Mean Total File Area	4 × StDev	Plus 4 × StDev
0	2.37E+05	2.20E+05	4.57E+05
7.5	2.80E+05		
15	4.79E+05		
30	8.93E+05		

TABLE 3. Intra-day repeatability

STD Conc. (pM)	Mean (3 Controls x 5 Curves)	StDevp	%CV	Accuracy
50.00	51.21	1.33	3	2.43%

TABLE 4. Inter-day repeatability

STD Conc. (pM)	Mean (3 Controls x 5 Curves)	StDevp	%CV	Accuracy
50.00	51.07	0.81	2	2.15%

Sample	Spike Conc. (pM)	Exp. Conc. (pM)	Average (pM)	Exp Recovery Conc. (pM)	% Yield
Neat_1	0.00	43.79	44.59		
Neat_2		45.59			
Neat_3		44.38			
Low_1	19.50	65.08	64.11	19.52	100.12%
Low_2		63.65			
Low_3		63.61			
Medium_1	199.50	241.19	237.56	192.97	96.73%
Medium_2		239.80			
Medium_3		231.70			
High_1	919.50	960.91	928.63	884.05	96.14%
High_2		905.35			
High_3		919.64			

Conclusions

- Automated sample extraction is amenable to high-throughput analysis, thus decreasing sample preparation times.
- Insulin MSIA D.A.R.T.'S equivalently extract multiple insulin variants present at different concentrations for simultaneous detection and quantification for research.
- HRAM MS affords qualitative confirmation and quantification of the insulin variants present in one LC/MS run.
- Pinpoint software version 1.3 provides automated data extraction, confirmation, and quantification for all insulin analogs.
- Reduced complexity affords shorter LC gradients, and, therefore, shorter LC/MS analysis times.
- An LLOD < 15 pM and an LLOQ of 15 pM (87 pg/mL) in 0.5 mL of plasma were achieved.
- Intra- and inter-day repeatabilities were < 3%, thus making the insulin MSIA workflow highly reproducible.

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Enrichment of EGFR/PI3K/AKT/PTEN Proteins for Research using Immunoprecipitation and with Mass Spectrometry-based Analysis

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Overview

Purpose: Identification and quantification of EGFR/PI3K/AKT/PTEN proteins for research using an optimized immunoprecipitation to mass spectrometry (IP-MS) workflow.

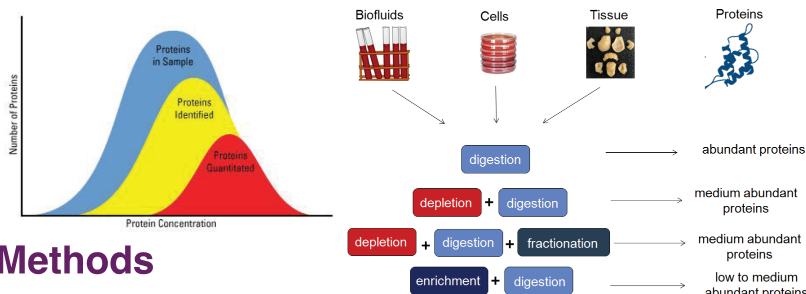
Methods: We evaluated immunoprecipitation with directly coupled antibodies or biotinylated antibodies with immobilized streptavidin resin. EGFR, PI3K, AKT isoforms and PTEN were enriched from two cell lysates using an optimized IP to MS workflow. A multiplex, targeted selected reaction monitoring (SRM)-based MS research method was developed to measure the limit of quantitation (LLOQ) of EGFR, AKT2, AKT1, PTEN, PIK3CA and PIK3R1 tryptic peptides. Multiple targets (EGFR, AKT isoforms, PTEN) were immunoprecipitated simultaneously and quantified by targeted SRM assay.

Results: Immunoprecipitation using magnetic beads resulted in overall higher yield of target protein and less non-specific binding than agarose beads for MS research applications. Enrichment of EGFR, AKT2, AKT1 and PTEN from two cell lysates enabled MS detection and quantitation. Enrichment of as low as 7ng recombinant EGFR in human plasma matrix allowed absolute quantitation by LC-SRM. Multiplexed target immunoprecipitation resulted in simultaneous identification and quantitation by MS.

Introduction

A major bottleneck in the verification of protein biomarkers in clinical research is the lack of methods/reagents to quantify medium to low levels of proteins of interest in human samples. Immunoprecipitation (IP) and mass spectrometry (MS) are complementary techniques that permit sensitive and selective characterization and quantitation of low abundance protein analytes in cell lines, tissue, and biofluids. IP provides both enrichment and high selectivity while the MS provides high selectivity, sensitivity, and multiplexing across a range of analyte concentrations in different matrices. The quantitative evaluation of protein expression and PTM status of EGFR-PI3K-AKT signaling pathway proteins enables the precise characterization of the disease.

FIGURE 1. Enrichment is necessary for medium to low abundant proteins.



Methods

Sample Preparation

EGFR from A431 lysate was immunoprecipitated by direct IP methods (Hydrazide activated polyacrylamide bead, Aldehyde activated agarose bead, NHS-Ester activated magnetic bead, and Epoxy activated magnetic bead) and indirect IP methods (Streptavidin coated polyacrylamide bead and Thermo Scientific™ Pierce™ Streptavidin magnetic bead). IP eluted samples were evaluated by western blot and in-solution trypsin digestion followed by MS analysis. IP conditions were optimized for enrichment of medium to low abundant targets (EGFR, AKT isoforms, PTEN and PIK3CA) for MS applications. Multiplex IP was performed to enrich EGFR, AKT isoforms and PTEN targets simultaneously from HEK293 lysate with biotinylated antibodies and with Pierce Streptavidin coated magnetic beads.

Liquid Chromatography and Mass Spectrometry

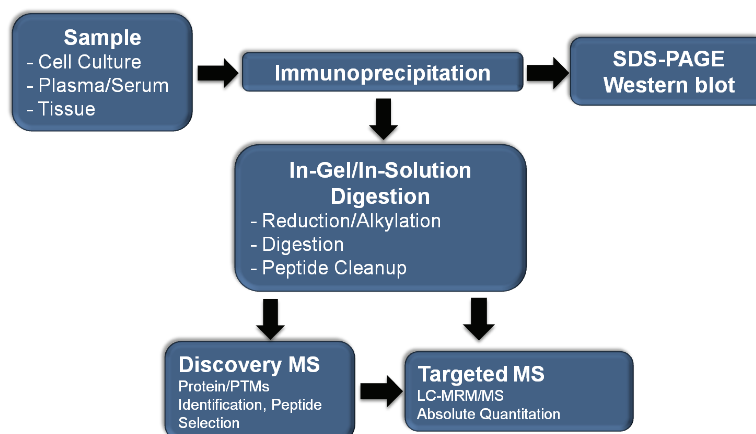
IP eluates were reconstituted in 6M Urea, 50mM Tris-HCl pH 8 followed by reduction, alkylation and trypsin digestion overnight. Prior to MS analysis, tryptic digest samples were desalted using the Thermo Scientific™ Pierce™ C18 Spin Tips. For discovery MS, the samples were analyzed by LC-MS/MS using a nanoLC system at 300 nL/min over a 45 min gradient and Thermo Scientific™ Orbitrap XL ™ mass spectrometer (DDA, Top 6, CID). For targeted MS, the samples were analyzed by LC-SRM/MS with the Thermo Scientific ™ TSQ Vantage ™ mass spectrometer and Thermo Scientific™ Easy nanoLC II system.

Data Analysis

Discovery MS data were analyzed with Thermo Scientific™ Proteome Discoverer™ 1.4 and Scaffold 4.0 software to assess percent sequence coverage, spectral counts and PTMs. For targeted LC-SRM/MS data analysis, Thermo Scientific ™ Pinpoint™ and Skyline software were used to measure limit of quantitation (LOQ) and target analyte concentration.

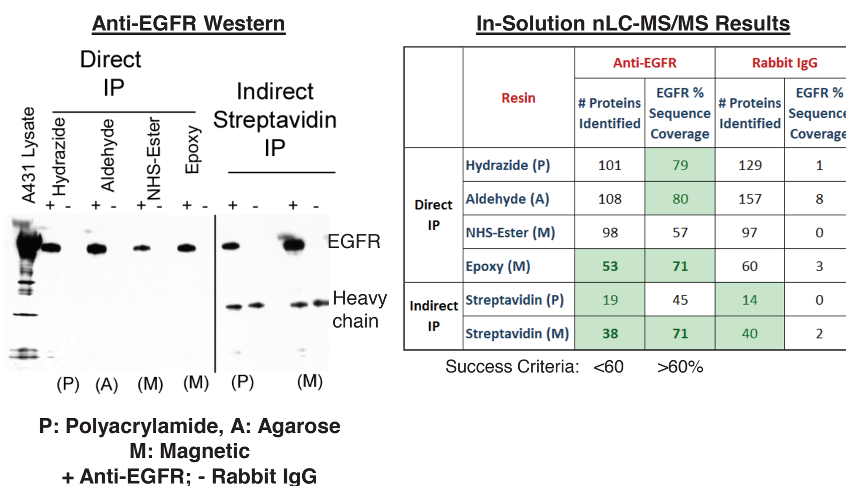
Results

FIGURE 2. Experimental workflow for IP-MS research method development.



Protein targets are immune-enriched from matrix and analyzed by silver stain or Western blot after gel electrophoresis. IP samples are also digested with trypsin and analyzed by nLC-MS/MS to identify candidate quantitative peptides. Heavy isotope-labeled, quantitative peptide standards are then used in targeted SRM or MRM research methods for absolute quantitation.

FIGURE 3. Evaluation of EGFR immunocapture efficiency and selectivity.



	Resin	Anti-EGFR		Rabbit IgG	
		# Proteins Identified	EGFR % Sequence Coverage	# Proteins Identified	EGFR % Sequence Coverage
Direct IP	Hydrazide (P)	101	79	129	1
	Aldehyde (A)	108	80	157	8
	NHS-Ester (M)	98	57	97	0
	Epoxy (M)	53	71	60	3
Indirect IP	Streptavidin (P)	19	45	14	0
	Streptavidin (M)	38	71	40	2

Success Criteria: <60 >60%

P: Polyacrylamide, A: Agarose
M: Magnetic
+ Anti-EGFR; - Rabbit IgG

EGFR immunoprecipitation was used to evaluate directly coupled antibody or biotinylated antibody with immobilized streptavidin resin. A) Capture efficiency was determined by Western blot. B) EGFR sequence coverage and background proteins were determined by LC-MS/MS after elution and trypsin digestion. IP using magnetic beads resulted in fewer background proteins identified and higher EGFR sequence coverage.

Benefits of Magnetic Beads for IP-MS

- Lower background: Minimal non-specific binding
- High signal to noise: Easy and efficient washing, less void volume reduces the chance of losing sample
- Easy handling: Easy separation of resin
- Time and effort: Less washing and faster incubation (60 minutes start to finish)
- Better reproducibility: Product and handling consistency
- Ab savings: All binding on outer surface
- Automation: Improves throughput and reproducibility

FIGURE 4. Identification of multiple phosphorylation sites for EGFR peptides.

A EGFR

82 unique peptides, 130 unique spectra, 143 total spectra, 861/1210 amino acids (71% coverage)

693-Phosphothreonine by PKD/PRKD1

1166-Phosphoserine

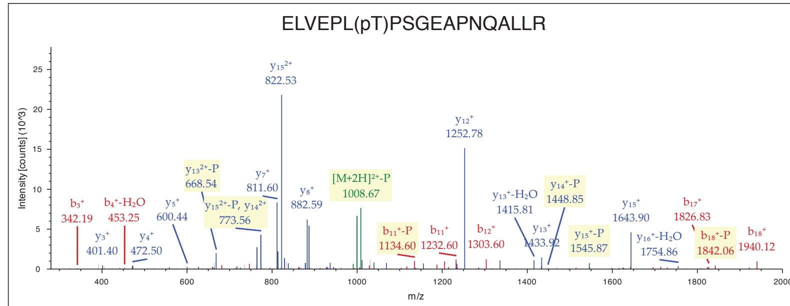
991-Phosphoserine

AKT2

31 unique peptides, 38 unique spectra, 47 total spectra, 395/481 amino acids (82% coverage)

451-Phosphothreonine

B



A) IP-MS allowed simultaneous analysis of multiple phosphorylation sites for EGFR and AKT2 peptides. B) MS/MS spectra of ELVEPL(pT)PSGEAPNQALLR peptide showing phosphothreonine residue at T693 of EGFR.

Enrichment of medium to low abundant targets using Thermo Scientific Pierce Streptavidin Coated Magnetic Beads

	Target	A431		HEK293	
		Anti-target Ab	Negative Control	Anti-target Ab	Negative Control
% Sequence Coverage	EGFR	65%	0%	16%	0%
	AKT1	36%	2%	68%	6%
	AKT2	50%	0%	82%	0%
	AKT3	8%	0%	62%	0%
	PTEN	16%	0%	36%	0%
	PIK3CA	0%	0%	0%	0%

EGFR-AKT pathway targets were immunoprecipitated from two cell lines with biotinylated antibodies, captured with Pierce Streptavidin coated magnetic beads, washed, eluted, digested in-solution, and analyzed by LC-MS/MS to assess sequence coverage and identify isoform-specific peptides.

Immunoprecipitation to targeted MS research application (nLC-SRM/MS)

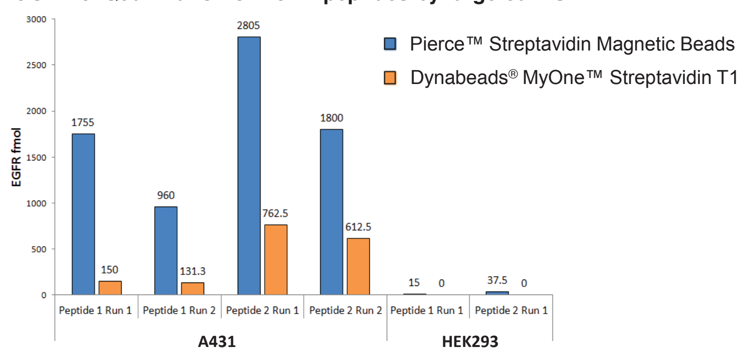
After enrichment by IP, SRM assays enabled the quantitation of EGFR, AKT2, AKT1, PTEN proteins in the low fmol range.

FIGURE 5. Detection and quantitation limits of EGFR, AKT2, AKT1, PTEN, PIK3CA and PIK3R1 peptides.

Target	Peptide No.	LOD (fmol)	LLOQ (fmol)	ULOQ (fmol)	Linearity (R^2)
EGFR	Peptide 1	0.2	3.9	1000	0.9977
	Peptide 2	0.2	3.9	1000	0.9997
AKT2	Peptide 1	0.2	3.9	1000	0.9998
	Peptide 2	3.9	15.6	1000	0.9599
AKT1	Peptide 1	3.9	15.6	1000	0.9541
PTEN	Peptide 1	0.2	3.9	1000	0.9999
	Peptide 2	0.2	3.9	1000	0.9997
PIK3R1	Peptide 1	0.2	3.9	1000	0.9997
	Peptide 2	0.2	3.9	1000	0.9999
PIK3CA	Peptide 1	0.2	3.9	1000	0.9981

All six targets were monitored with linear quantification. EGFR, AKT2, PTEN, PIK3CA and PIK3R1 peptides were quantified from 3.9 fmol to 1000 fmol.

FIGURE 6. Quantitation of EGFR peptides by targeted MS.



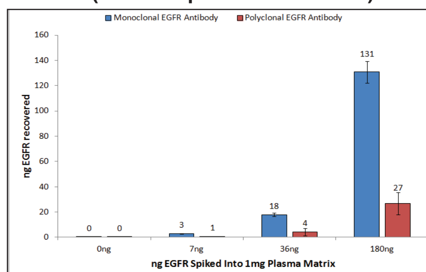
Enrichment of EGFR from two cell lysate allowed for quantitation of two unique EGFR peptides by targeted MS. Better recovery was observed with Pierce Streptavidin (SA) Magnetic beads compared to Dynabeads MyOne Streptavidin T1 beads.

FIGURE 7. Recovery of recombinant EGFR (rEGFR) from plasma matrix.

nLC-MS/MS
(EGFR % Sequence Coverage)

rEGFR	Mono Ab	Poly Ab
0 ng	0%	0%
7 ng	3%	2%
36 ng	20%	12%
180 ng	42%	26%

nLC-SRM/MS
(EGFR Peptide Quantitation)



rEGFR spiked into 1mg plasma is detected and quantitated at >7ng (52 fmol). Monoclonal antibody recovered more rEGFR.

FIGURE 8. Multiplex immunoprecipitation to MS research applications.

nLC-MS/MS

Targets/ HEK293 lysate	% Sequence Coverage
EGFR	17%
AKT2	23%
AKT1	16%
PTEN	11%

nLC-SRM/MS

Targets/ HEK293 lysate	Concentration (fmol)	Concentration (ng)
EGFR	46	6.2
AKT2	96	5.4
AKT1	>ULOQ	>ULOQ
PTEN	89	4.2

EGFR, AKT isoforms and PTEN were enriched simultaneously from HEK293 lysate with biotinylated antibodies, captured with Pierce Streptavidin coated magnetic beads. All four targets were identified and quantified by MS.

FIGURE 9. Summary of EGFR-AKT pathway targets identified and quantified in two cell lines without and with enrichment.

Target	Cell line	Detected by Orbitrap		Quantified by SRM	
		Neat	Enriched-IP	Neat	Enriched-IP
EGFR	A431	+	+	+	+
	HEK293	-	+	-	+
AKT1	A431	-	+	-	+
	HEK293	-	+	-	+
AKT2	A431	-	+	-	+
	HEK293	-	+	-	+
AKT3	A431	-	+	N/A	N/A
	HEK293	-	+	N/A	N/A
Grp94	A431	+	+	N/A	N/A
	HEK293	+	+	N/A	N/A
PIK3CA	A431	-	-	N/A	N/A
	HEK293	-	-	N/A	N/A
PIK3R1	A431	-	-	N/A	N/A
	HEK293	-	-	N/A	N/A
PTEN	A431	-	+	-	+
	HEK293	-	+	-	+

Conclusion

- Immunoprecipitation using magnetic beads for MS research applications resulted in a higher yield of target protein and less non-specific binding than using directly immobilized antibody.
- Enrichment of EGFR, AKT isoforms, and PTEN in A431 and HEK293 lysates enabled detection by discovery MS and quantitation by targeted MS.
- Immunoprecipitation of EGFR and AKT2 resulted in simultaneous analysis of multiple isoforms and phosphorylation sites.
- EGFR, AKT1, AKT2 and PTEN were quantified in the low nanogram range by nLC-SRM/MS in two cell lysates.
- Enrichment of as low as 7ng recombinant EGFR in plasma matrix and 10ng of recombinant PIK3CA/PIK3R1 in cell lysate (data not shown) enabled absolute quantitation by targeted SRM-MS.
- Mulitplex IP to MS allowed simultaneous detection and quantification of EGFR, AKT2, AKT1 and PTEN targets.
- Future work will focus on optimization of IP conditions to enrich lower abundant targets (<1ng total protein).

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Real-Time Qualitative and Quantitative Global Proteomics Profiling Using a Hybrid Data Acquisition Scheme

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Overview

Purpose: Demonstrate a more comprehensive data acquisition scheme for performing global protein/peptide qualitative and quantitative analysis. Evaluate data acquisition performance by comparing to previously published methods.

Methods: Data acquisition strategy based on HR/AM MS used for quantitative analysis with looped narrow precursor DIA events for sequence confirmation. Utilize consolidated spectral library information facilitating real-time data analysis to create a targeted peptide list used for analysis across all technical replicates.

Results: The pSMART acquisition method resulted in more peptides identified and quantified than the standard DIA method with significantly fewer decoy matches resulting in greater quantitation in much less time.

Introduction

The trend in proteomics is to perform global qualitative and quantitative sample analysis without targeted precursor inclusion lists or from MS-driven MS/MS acquisition. The goal is to sample the greatest number of peptides across the gradient enabling global determinations across biological samples which can then determine subsequent targeted, high-throughput experiments. Data independent acquisition (DIA) strategies have become common due to opportunity to archive and interrogate data using any user-defined protein/peptide lists. To increase the sampling and characterization capabilities for global qual/quan experiments, we utilize high resolution/accurate mass (HR/AM) MS and narrow, asymmetric DIA windows as opposed to only DIA data. The decoupling of data sets used for quantitative and qualitative analysis increases quality of both sets. Success is predicated on leveraging high resolution (>70,000 per precursor target) and high mass accuracy to increase selectivity for robust MS quantitation. In addition, the acquisition strategy enables product ion data quality to be significantly increased because only one high quality product ion spectrum is needed for verification. The narrow precursor isolation using high ion accumulation times and higher resolution settings have shown greater sensitivity and selectivity compared to standard DIA data on the same samples.

Methods

Sample Preparation

All experiments were performed using a donor sample of human plasma collected under IRB approved protocols and stored in an EDTA stabilized tube (Becton Dickinson, Franklin Lakes, NJ). A stock solution of human plasma was prepared without depletion using standard trypsin digestion protocols following reduction and alkylation. The concentration of the final stock solution was estimated to be 4 mg/μL, divided into aliquots of 100 μL of 100 ng/μL and frozen until used. Before MS analysis, the sample was spiked with Peptide Retention Time Calibration (PRTC) peptides (Thermo Fisher Scientific, Rockford, IL) to a final concentration of 20 fmol on column. A total of 1 μL was injected on column per experiment.

Liquid Chromatography

All chromatographic separation was performed using a Thermo Scientific™ EASY-nLCII™ LC system with a binary solvent system of (A) 0.2% formic acid in water and(B) 0.2% formic acid in acetonitrile. Samples were loaded onto a 120 x 0.15 mm trapping column packed with 5 μm PS-dvb particles (Polymer Labs) and the analytical separation was performed using a 500 x 0.1 mm column packed with C18 Aq (Bischoff). The samples were eluted from the column with a linear gradient from 5 to 45% B in 180 minutes prior to ramping to 90% B for column regeneration.

Mass Spectrometry

Thermo Scientific ™ Q Exactive™ mass spectrometer was used for all experiments. Two different experiments were performed, standard DIA and peptide-based. Staggered MS and M/MS acquisition Across Retention Time (pSMART) for data analysis. Standard DIA acquisition was performed using 25 Da precursor isolation covering m/z 400-1200 in 32 scan events, 100 msec max ion fill times, 1e6 AGC settings, and 35,000 resolution (@m/z 200). The pSMART acquisition settings for MS was 5e6 AGC setting and 140,0000 resolution (@m/z 200) and DIA events were independently acquired using 5 Da precursor isolation for a precursor range of m/z 400-800, 10 Da for m/z 800-1000, and 20 Da for m/z 1000-1200. Each narrow DIA was acquired using 150 msec max ion fill times, 1e6 AGC settings, and 35,000 resolution (@m/z 200). A custom acquisition script was used to perform real-time data analysis and recording from a global peptide lists.

Data Analysis

Crystal spectral libraries were used to create a comprehensive list of plasma peptides identified and verified based on DDA data acquired at BRIMS over the course of two years and contains 10,288 peptides. The list of peptides contains the sequence (with and without modifications), relative retention times, precursor charge states, product ion m/z values and average product ion distribution. A custom script was used to perform real-time spectral matching for both standard DIA and pSMART data to spectral library information resulting in a final list of identified peptides per injection. Real-time identification was based on retention time overlap, precursor/product ion mass errors, and cosine similarity scoring between experimental product ion distribution and spectral library information. Mass tolerance values were set to 10 ppm for all pSMART data and CS scores of 0.6 or better. Standard DIA data was processed using two different mass tolerance values, 10 and 20 ppm and a CS score threshold of 0.7. Further scoring for standard DIA evaluated the consecutive spectral matches based on mass accuracy and CS scores. The final list of identified peptides was exported to the Pinpoint™ software for quantitation and variance analysis across all technical replicates.

In addition to forward matching analysis, a decoy database was created and used for subsequent data analysis. The same spectral library was used to create two different decoy databases with the first decoy database created by switching precursors and product ions. The two peptide entries used to switch must have similar retention times but precursor m/z value differences in excess of 50 Da. The second decoy database extended the first by further shuffling the relative abundance values per fragment ion. Decoy hits were scored using the same acceptance criteria as that for the forward search.

Results

To determine performance of the pSMART acquisition strategy, experimental analysis of non-depleted human plasma digest was compared to standard DIA data. The evaluation metrics was confident matching as described above. In addition to spectral matching, %CVs were used to determine reproducibility and quantitative capabilities per method.

FIGURE 1. Schematic representation of pSMART data acquisition strategy consisting of HR/AM MS spectral acquisition used for quantitative peptide analysis (red lines) and looped narrow asymmetrical DIA acquisition for qualitative peptide confirmation (black dashes). (1A) User defined loop count dictates MS acquisition cycle time while precursor m/z range, individual DIA precursor isolation range, and max ion fill times dictate total DIA acquisition cycle time. The real-time data processing scheme is displayed in Fig. 1B showing the predicted retention time window read in from the Crystal Spectral Library, precursor XIC, and the single narrow DIA window acquired under the precursor XIC trace.

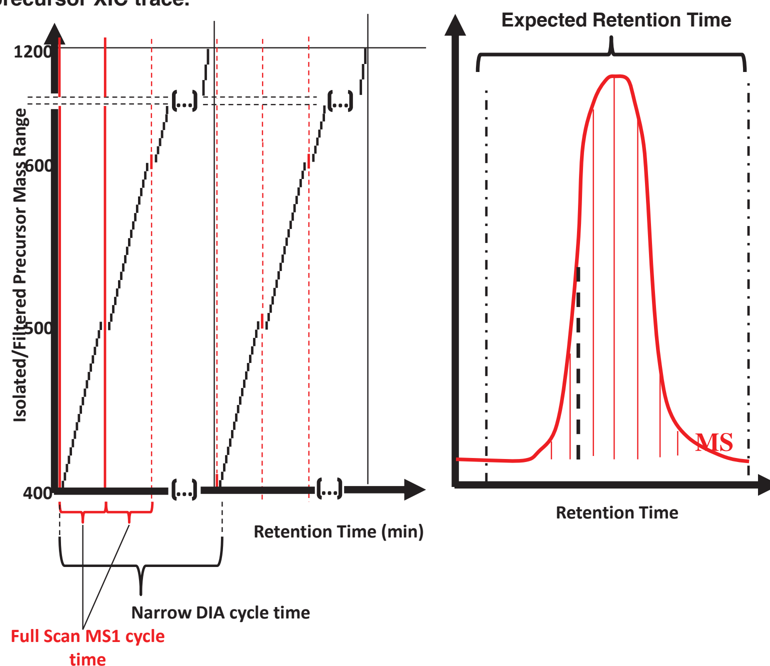


FIGURE 2. Comparative targeted peptide analysis using standard DIA and pSMART methods for the plasma peptide ISASAEELR. (A) Shows the product ion XIC results from the standard DIA dataset compared to (B) which shows the precursor ion XIC results used to determine elution profile, qualitative analysis, and quantitation. The dashed line shows the RT point for matched DIA spectrum for the targeted peptide. Fig. C shows comparative product ion distribution analysis for DIA and pSMART as well as the product ion distribution read in from Crystal. Successful matching is based on CS scores in excess of 0.7 as compared to library product ion distribution. CS scores for DIA is 0.87 compared to 0.81 for pSMART. CS scores for DIA are based on relative product ion AUC values whereas the single narrow DIA spectrum is used to calculate the CS score.

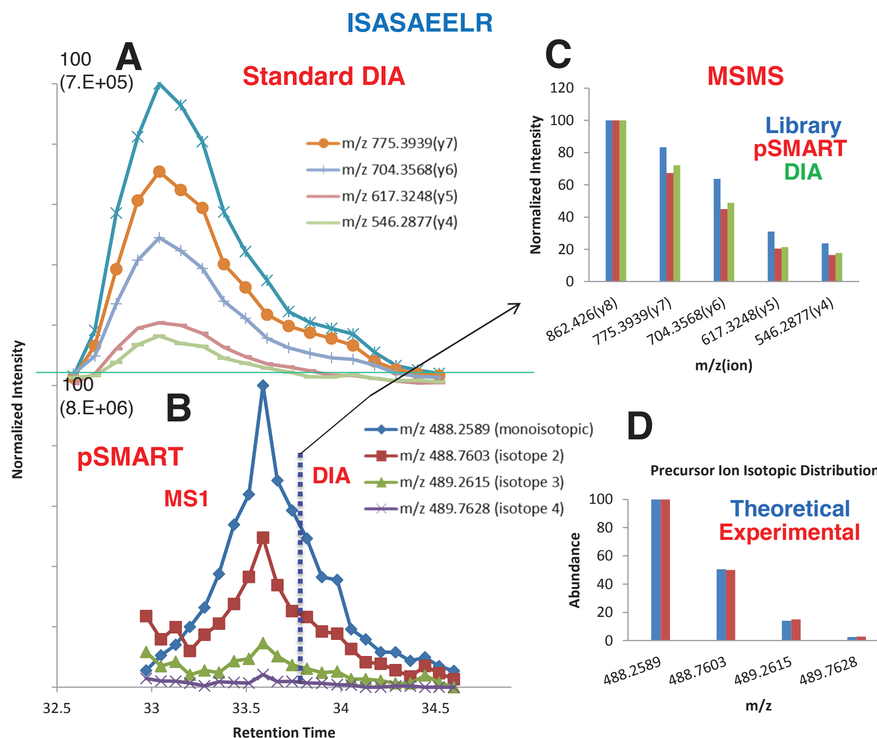


TABLE 1. List of comparative peptide hits using different data processing strategies. Each column lists the forward and decoy hit rates per data set and acceptance criteria used for processing. Mass tolerance criteria used for pSMART data (MS and DIA) was 10 ppm. Decoy libraries used to search experimental data are described above.

Peptide IDStrategy	Standard DIA 20ppm 6 data point across peak	Standard DIA 20ppm 4 data point across peak	Standard DIA 10ppM 6 data point across peak	Standard DIA 10ppm 4 data point across peak	pSMART (10ppm)
Library Hits	2159	3034	1645	2244	2525
Decoy library #1 Hits	983	1790	503	897	287
Decoy library #2 Hits	515	996	182	363	149
Decoy Hit Rate (#1)	46%	59%	31%	40%	11%
Decoy Hit Rate (#2)	24%	33%	11%	16%	6%

Three technical replicates were acquired per data acquisition scheme. The real-time data analysis generates a list of peptide sequences per injection that can be compared across each replicate and data acquisition scheme to determine reproducibility. The peptide numbers listed in Table 1 sums the total number of peptides identified across all technical replicates. Of primary interest is the reproducibility of peptides identified across each injection. Of the 2525 peptides identified across all pSMART, 2285 were identified in all three replicates and ca. another 75 peptides identified in 2/3 injections. In the standard DIA identified a total 2159 peptides from the all replicates but only 1599 peptides were verified across all three technical replicates and ca. another 180 peptides identified in 2/3 replicates.

Of further interest was determining the overlap of identified peptides across all methods as well as those peptides uniquely identified per method. Each list of peptides per method were compared and evaluated displayed in Figure 3.

FIGURE 3. Venn diagram comparing identified peptides per data acquisition used to sample the plasma digest. Each peptide had to be identified in all three technical replicates to be considered.

A secondary set of PRM experiments were performed to target the unique peptides identified per acquisition scheme. A targeted inclusion list of 536 peptides (standard DIA), 656 from pSMART, and 724 peptides identified by all three experiments as a control. The results from each PRM analysis showed over 95% of the 724 peptides verified across all three methods were confirmed using PRM, over 85% of the peptides were confirmed for the pSMART method, and less than 15% of the unique peptides identified by standard DIA were confirmed by the PRM experiment. An example of the comparative data analysis strategy is presented in Figure 4.

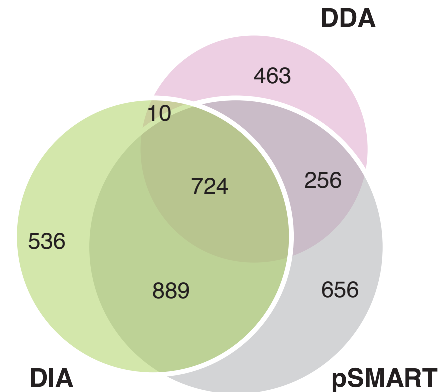


FIGURE 4. Comparative mass spectral analysis of the plasma peptide SLAELGGHLDQQVEEFR that was uniquely identified using pSMART method. A) shows the overlaid precursor isotopic XICs for the +3 charge state and the inset shows the isotopic distribution overlap. The red dashed line indicates the matched narrow DIA window. B) shows the overlaid product ion XIC trace from the PRM experiment. The inset table list the product ions, ion type, and library distribution. C) shows the overlaid product ion XICs from the standard DIA experiment. The inset shows the comparative product ion distribution from each data set as compared to the spectral library entry.

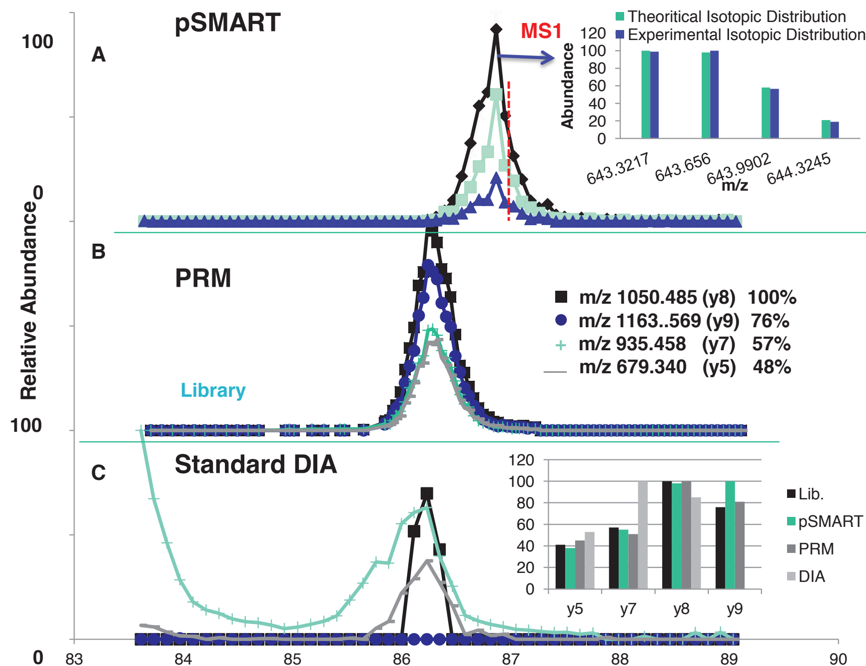
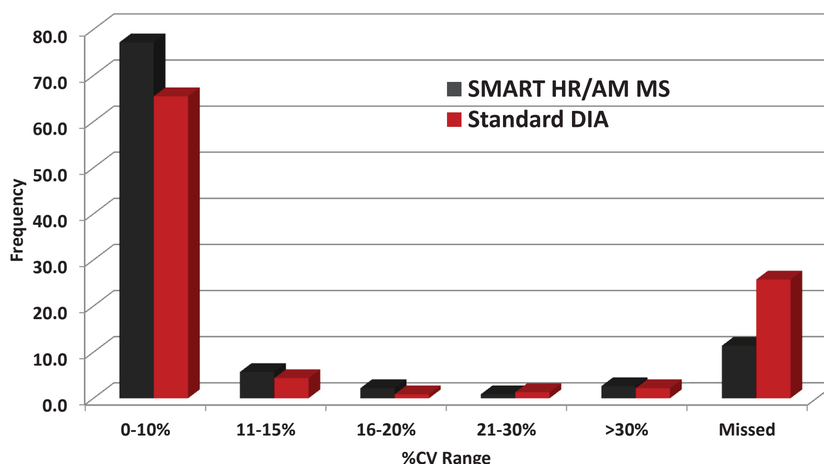


Figure 5. Comparative reproducibility analysis of area values per data acquisition method. The coefficient of variance was determined across the three technical replicates. Area values for the pSMART data was determined from precursor isotopic XICs compared to standard DIA experiments relying on product ion XICs.



Conclusion

The pSMART acquisition method for global qualitative and quantitative analysis was developed to increase performance of sample profiling for proteins and peptides that could be used as putative biomarkers. The pSMART performance analysis resulted in better data as compared to standard DIA methods based on:

- Greater number of plasma peptides routinely identified
- Significantly reduced number of decoy hits using multiple means of matching
- Incorporation of Crystal spectral libraries facilitated real-time data analysis to reduce post-acquisition processing time
- pSMART method is easily adaptable for complex or simple samples as well as different chromatographic peak shapes

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Characterizing Qualitative and Quantitative Global Changes in the Aging Heart Using pSMART, a Novel Acquisition Method

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Overview

Purpose: We have completed a label-free quantitative global profiling and targeted analysis of the cardiac proteome in aging mice using a novel data acquisition method, pSMART.

Methods: The pSMART method was used to acquire qualitative and quantitative data using one HR/AM MS and a series of narrow DIA mass windows. Initial characterization experiments using unbiased DDA facilitated the building of a detailed spectral library which was used for qualitative scoring for the DIA data.

Results: Our pSMART strategy resulted in 30% more peptide identifications per run than a standard DDA run. Additionally, using pSMART, we are able to confirm MS1 quantitation at low abundance levels with MS/MS for each peptide. This novel acquisition enabled quantitation of previously identified peptides as well as novel putative targets of aging. By identifying and quantifying more targets, we were able to better characterize the dynamic proteomic changes of cardio-dysfunction in aging mice.

Introduction

The cardiovascular system undergoes significant changes as it ages. Aging is a complex event that eventually leads to loss of function and abilities over time and has been shown to cause multi-level changes in the heart, from the genomic-transcriptomic level to the cellular-tissue level. These pathological changes in the heart can be followed at the proteomic level.

In this study we introduce a complete workflow that enables label-free quantification of age-related proteomic changes in murine heart tissue.

Methods

Sample Preparation

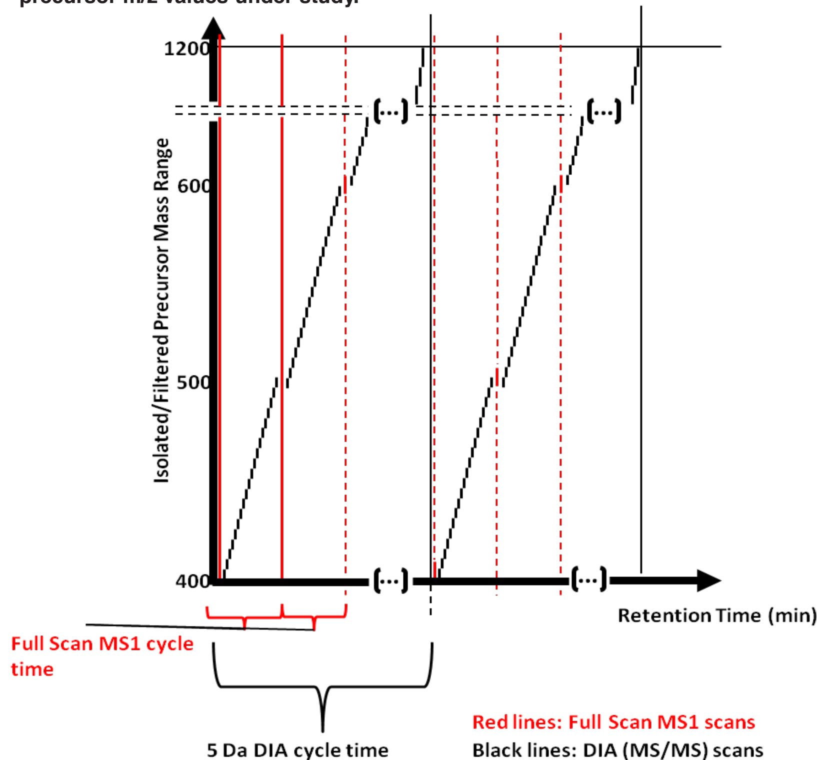
Heart tissue was isolated and homogenized from young (2 months old) and old (2 years old) C57BL/6 mice. Each heart was normalized by weight, homogenized in 8M GuHCl, 250 mM Tris, pH 8.5, using a FastPrep 120. The supernatant was then reduced, alkylated and digested with sequencing grade Thermo Scientific™ Pierce™ trypsin kit overnight. A portion of each of the digested samples was then separated by high pH reverse-phase fractionation into 16 equal fractions. These fractions were used to create the spectral library. Each sample was spiked with Peptide Retention Time Calibration Mixture (PRTC) (Thermo Fisher Scientific Pierce) prior to mass spectrometry analysis.

Mass Spectrometry and Data Analysis

All samples were analyzed on a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer equipped with a Thermo Scientific™ Nanospray Flex™ Ion Source. Data were acquired in two steps to simulate traditional workflows. Initial experiments employed unbiased data-dependent MS/MS acquisition (DDA) for each of the fractionated samples to build the spectral library. Each non-fractionated heart sample was also run with unbiased DDA as well as with our novel pSMART data acquisition method (Figure 1). Each non-fractionated heart sample was run in triplicate.

Thermo Scientific™ Proteome Discoverer™ version 1.4 and Thermo Scientific™ Pinpoint™ version 1.4 software packages were used to analyze both the qualitative and quantitative data. The spectral library resulting from initial fractionated sample runs was used to create reference information to perform confirmation on all peptide targets.

FIGURE1. pSMART data acquisition approach with high resolution accurate mass (HR/AM) MS and 5 Da DIA acquisition. This novel acquisition method consists of two independent loops governed by the loop count for 5 Da DIA acquisition in between each full scan HR/AM MS spectrum. By decoupling quantitative (fullscan MS) and qualitative (5 Da DIA) the method leverages the most sensitive global quantitative method and the user-defined 5 Da DIA acquisition cycle times assure at least one specific DIA window over all precursor m/z values under study.



Results

FIGURE2. Biomarker discovery workflow using pSMART. Our workflow is a two-step process consisting of discovery and global differential analysis. The initial, unbiased characterization using DDA acquisition and sequencing is used to create the spectral library (Crystal) specific for the mouse heart tissue. Global quantitation is performed using pSMART and all data are processed in Pinpoint. The list of peptides and corresponding retention time, precursor and product ion information are read into Pinpoint from Crystal for automated data processing.

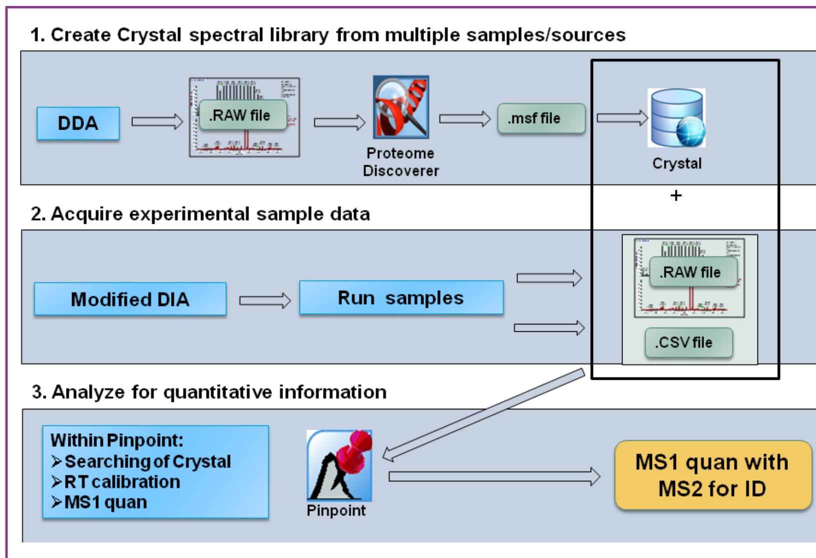
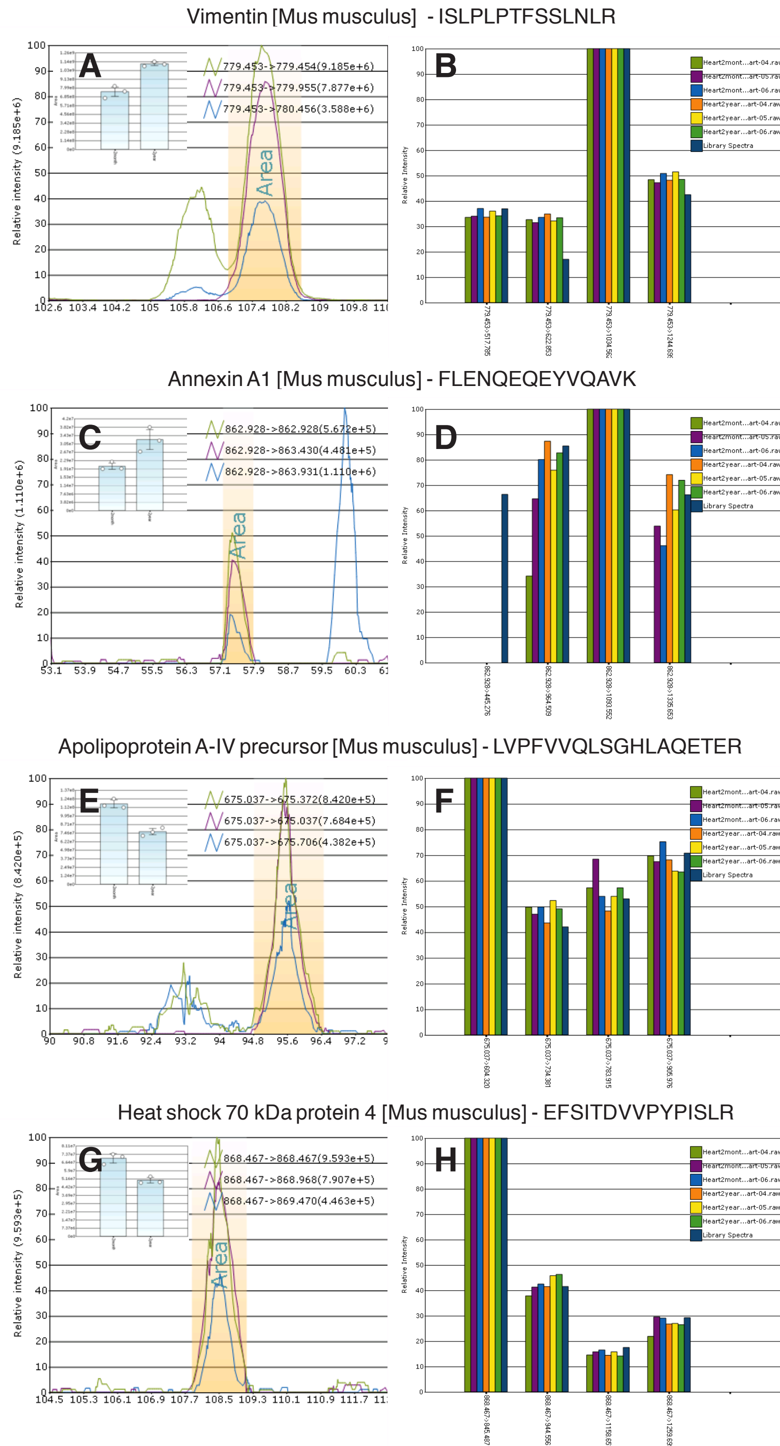


FIGURE3. Quantitative differences in cardiac peptides discovered with pSMART. Relative abundance changes in peptides with age, measured using the MS1 peak areas (DDA)(A,C,E,G) with confirmation of peptide ID using MS/MS composite spectra (DIA) (B,D,F,H). Insets, show the relative areas for young and old in the triplicate data.

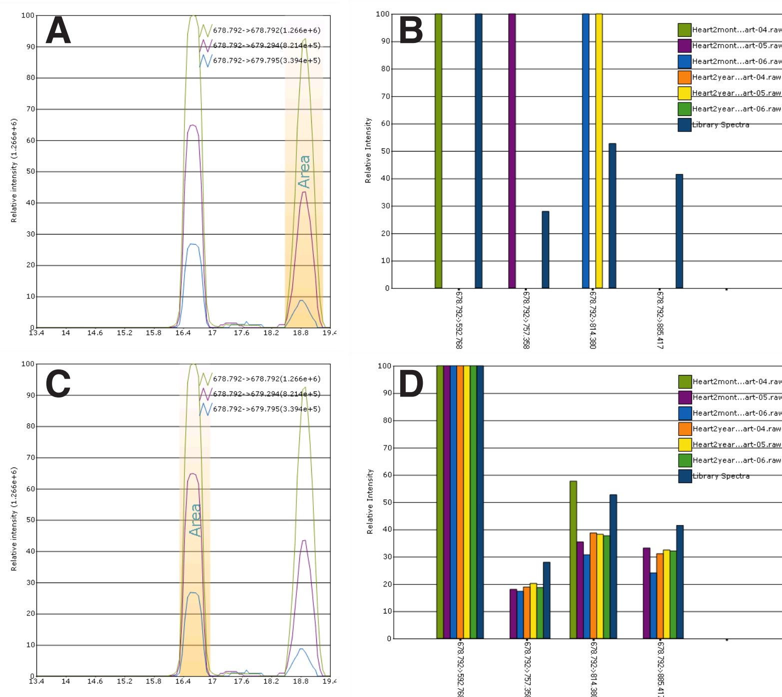


This workflow has several advantages *vis a vis* DDA or DIA alone.

- Narrower acquisition windows result in greater sensitivity and selectivity, since the precursor isolation window is inversely proportional to qualitative and quantitative performance.
- Increased selectivity results in lower FDR.
- Using a hybrid of DDA and DIA allows for confirmation of MS1 quantification due to the many MS/MS fragmentation spectra that are acquired within the narrow windows.
- This workflow is only possible with the increased speed and higher resolution provided by the Fusion MS and other High Resolution Accurate Mass (HR/AM) Orbitrap instruments.

The benefits of the pSMART method listed above enable robust complex sample characterization compared to DDA and standard DIA methods. Decoupling quantitative from qualitative data acquisition leverages the high-resolution capabilities and high charge densities of the Orbitrap mass spectrometer. The combination of data acquisition methods facilitates confident targeted peptide determination and quantification. Figure 4 shows an example of two peaks that are chromatographically resolved but have similar precursor m/z values and isotopic distribution profiles. The HR/AM MS coupled to a series of smaller DIA mass window acquisitions allows for increased confidence in the MS1 quantification. Spectral matching of the specific 5 Da DIA window containing the precursor collected under each peak, clearly identifies the retention time at 16.4 minutes *versus* 18.8 minutes, based on the presence and correct product ion distribution of the spectral library entry,

FIGURE 4. Advantage of pSMART. Using a hybrid of DDA and DIA allows for confirmation of MS1 quantification. A and C are the MS1 peaks for two different peptides. B and D are the composite MS/MS spectra for the top 4 transition ions for the highlighted peaks (A and C, respectively). Given that these two peaks are relatively close in a 3 hour gradient run, it would be difficult to confidently choose the correct MS1peak for label-free quantitation without added MS/MS data provided by pSMART. The highlighted peak in C is the correct precursor for peptide GDP[Hydroxyl]GEAGPQGDQGR, confirmed in D.



The primary challenge to global protein characterization is attributed to data processing. Reliance on spectral libraries not only helps to determine the peptide list used for post-acquisition data processing, but also provides robust scoring metrics to significantly decrease manual peak integration. Basic DIA events using 25 Da precursor isolation windows result in greater numbers of nonsymmetrical, overlaid product ion XIC traces that introduce errors in automated peak picking routines. By decoupling the HR/AM MS and 5 Da DIA data, each acquisition event can leverage high-resolution in both MS and DIA spectra to reduce target ion signal from background. This decreases the need for manual post-acquisition processing. In addition, by modeling sequencing strategies around DDA events (*e.g.* narrow precursor isolation and only one correctly matched product ion spectrum needed for confident sequencing) the selectivity and sensitivity of each 5 Da DIA window can be increased with longer maximum ion fill times and higher resolution settings than previously reported for basic DIA experiments. This combination increases the robustness of automated data processing and provides larger lists of differentially abundant peptides that can be globally evaluated with ROC analyses for classification value. The results of the ROC analysis can provide a starting point for translation to targeted, high-throughput methods.

Conclusion

- We have created a high quality murine heart tissue spectral library with over 5900 protein/19,900 peptide targets at FDR 1%.
- Using the pSMART workflow, we can quantify at MS1 level with high confidence in label-free discovery experiments using MS/MS spectra for confirmation of each quantified precursor ion area.
- The pSMART workflow coupled to HR/AM MS instrumentation provides increased protein assignments with lower FDR compared to standard DDA and DIA methods[1-3].

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Targeted Multiplexed Protein Quantitation Using Serial Immunoaffinity Extraction Coupled to LC-MS

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Bryan Krastins, David Sarracino, and Mary F. Lopez
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Overview

Purpose: Evaluate the reproducibility of serial protein extraction from one sample on an individual or multiple protein extraction.

Methods: Perform HR/AM MS analysis of MSIA D.A.R.T serial extractions from a single sample using one Ab for a single protein or multiple Abs (one per D.A.R.T tip).[1] The relative quantitation on peptides was used to determine depletion and disruption effects from serial dilution.

Results: Pooling of serial extractions increased targeted peptide response ca. 5-10x without significantly increasing background interference. The increased abundance resulting from pooling extractions facilitated greater protein sequence coverage and detection of N- and O-linked glycopeptides and corresponding glycoforms.

Introduction

Targeted protein quantitation using LC-MS provides significant advantages to determining protein sequence variations, truncations, and/or PTMs. To extend the sensitivity, immunoaffinity extraction based on antibody (Ab) capture has been implemented. The benefits of Ab capture for LC-MS studies increases the targeted protein amounts while significantly reducing background matrix effects facilitating faster LC gradients and greater sequence coverage. Performing Ab extraction using Mass Spectrometry ImmunoAffinity (MSIA) capture does not disrupt the sample. This facilitates serial extractions of low sample volumes yet facilitating multiplexed experiments.

Methods

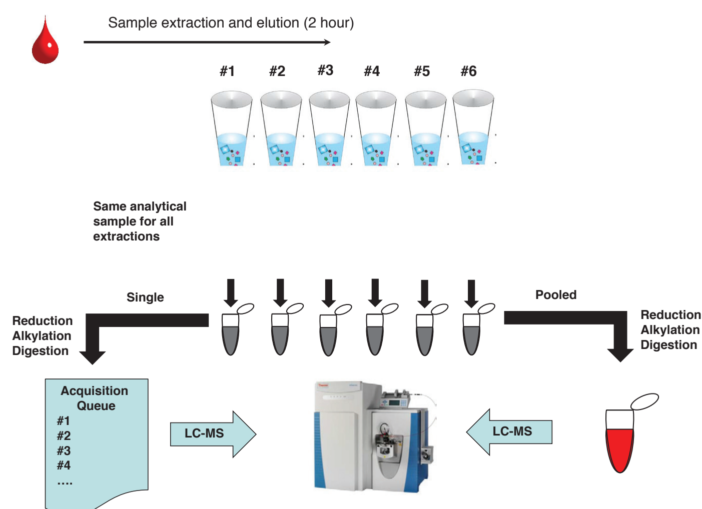
Sample Preparation

A common stock plasma sample was used for all studies. A total of 1 μg of digest was prepared and analyze. A 200 μL aliquot was used for each sample. A set of Thermo Fisher Scientific™ MSIA™ D.A.R.T. tips were used with each tip covalently bound to a specific Ab used to extract a set of targeted proteins. Targeted protein extraction was performed by serial extraction where a new tip was inserted into the same sample well and aspirated prior to collection, reduction, alkylation, and digestion. LC-MS analysis was performed on each individual extraction or pooled for multiplexing.

The first set of experiments used two different samples for the analysis of serial extraction. A total of six extractions were performed with the extracted protein separately collected from each tip, reduced, alkylated, and digested with trypsin and analyzed individually. The second where each of the six extractions were pooled prior to reduction, alkylation, and digestion followed by LC-MS analysis. (Figure 1) The targeted proteins for the first set of experiments was serotransferrin and zinc-α-2-glycoprotein.

A second set of samples were prepared following the same approach targeting four different proteins: serotransferrin, zinc-α-2-glycoprotein, α-1-antitrypsin, and lactotransferrin. Four different MSIA D.A.R.T. tips were used with each set covalently bound to the specific Ab. The same set of samples were used for individual extraction (one tip per sample) followed by reduction, alkylation, and digestion then LC-MS. The second set of samples had each vial processed through serial extraction by different MSIA tip. For example, one sample was first extracted by anti-serotransferrin, then anti-lactotransferrin etc. and the extractions were pooled prior to reduction, alkylation, and digested and ultimately LC-MS.

FIGURE 1. Strategy for testing the effects of serial dilution on sample disruption. Two sample preparation types were individual extraction and analysis vs. pooling samples.



Liquid Chromatography (or more generically Separations)

LC separation was performed using a Thermo Fisher Scientific™ Hypersil Gold™ 100 x 1 mm column with 1.9 μm particle size and a binary solvent system comprised of A) 0.1% formic acid and B) 0.1% formic acid in MeCN. A linear gradient of 5-32% B was performed over 15 minutes prior to column washing and re-equilibration.

Mass Spectrometry

All experiments were performed on a Thermo Scientific™ Q Exactive™ mass spectrometer operated in data dependent/dynamic exclusion mode using a Top 10 acquisition scheme. Full scan MS spectra were acquired using a resolution setting of 70k and all HCD product ion spectra were acquired using 15k.

Data Analysis

Initial unbiased data searching was performed using Thermo Scientific™ Proteome Discoverer™ 1.4 to Thermo Scientific™ Pinpoint 1.4 to search N- and O-linked glycopeptides through the Screening Tool. To total list of peptides (modified and unmodified) were used to performed relative quantitation across all samples. Qualitative assessment was based on retention time overlap with spectral library values, accurate mass (10 ppm tolerance), and isotopic distribution. Negative controls were analyzed by evaluating precursor ion intensities for sample RAW files not expected to contain the targeted protein.

Results

Figure 2. Comparative coverage maps for serotransferrin from DDA experiments of (2a) digested plasma and 2(b) pooled extracted digested. Figure 2c shows the pooled extracted serotransferrin digest coverage map as determined from Proteome Discoverer. The open sequence sites are attributed to either short peptides (3-5 amino acid residues poorly mapped by database searching) and sites of glycosylation.

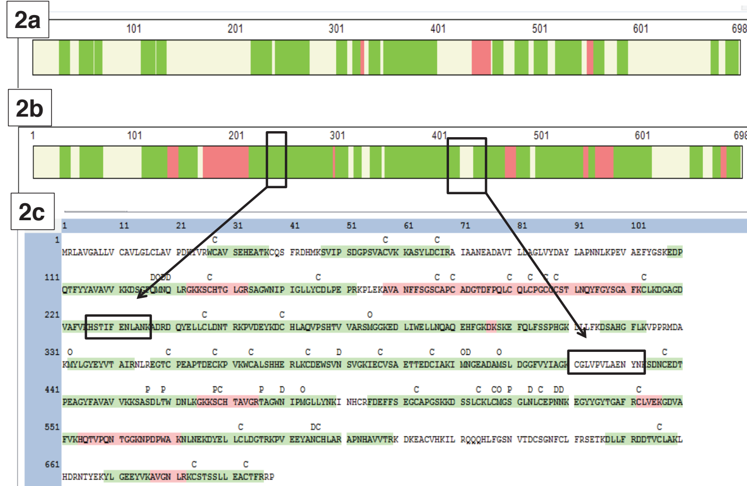


FIGURE 3. Comparative analysis of peptide quantitation as a result of extraction step vs. pooled response for an unlabeled peptide vs. An N-linked glycopeptide. The response is consistent across each peptide as a function of order extracted. The two peptides are boxed in Fig. 2c above.

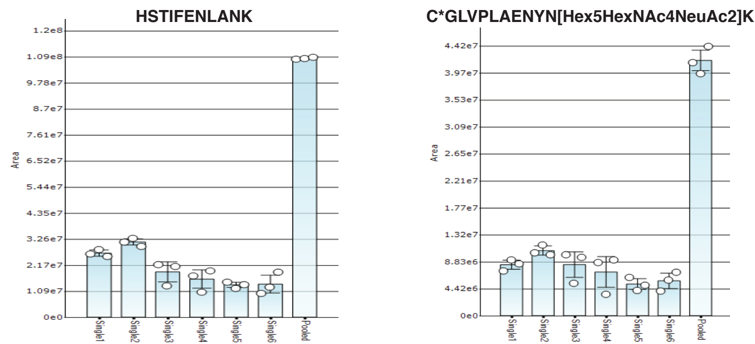


FIGURE 4. Comparative AUC response for all targeted serotransferrin peptides to evaluate the AUC ratio per peptide from the second MSIA extraction across all subsequent MSIA extractions.

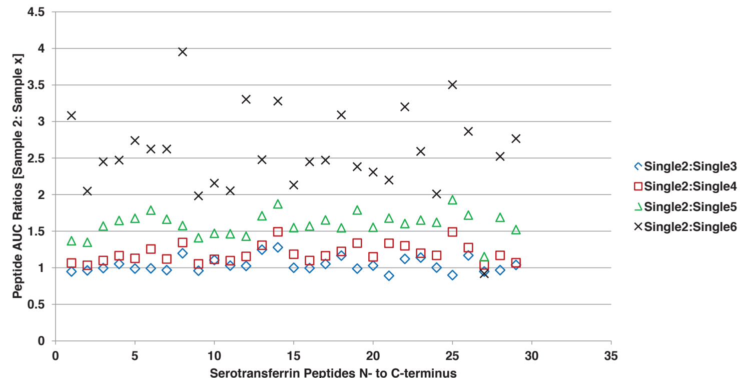


FIGURE 5. Reproducibility of glycan distribution based on comparative AUC ratios for the serotransferrin peptide C*GLVPLAENYN*K.S as a function of MSIA extraction.

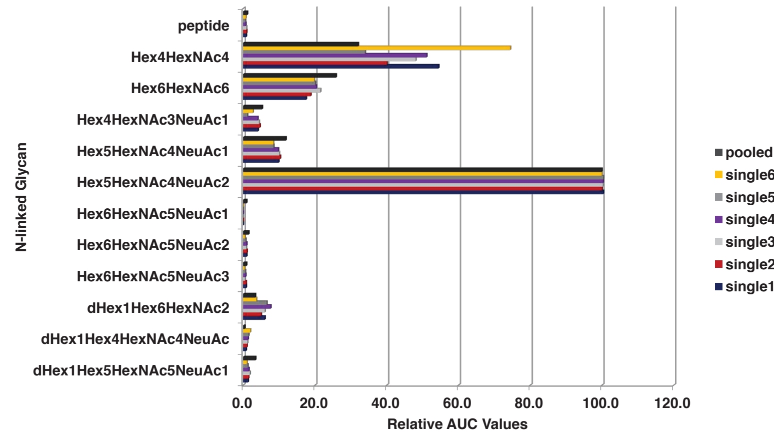
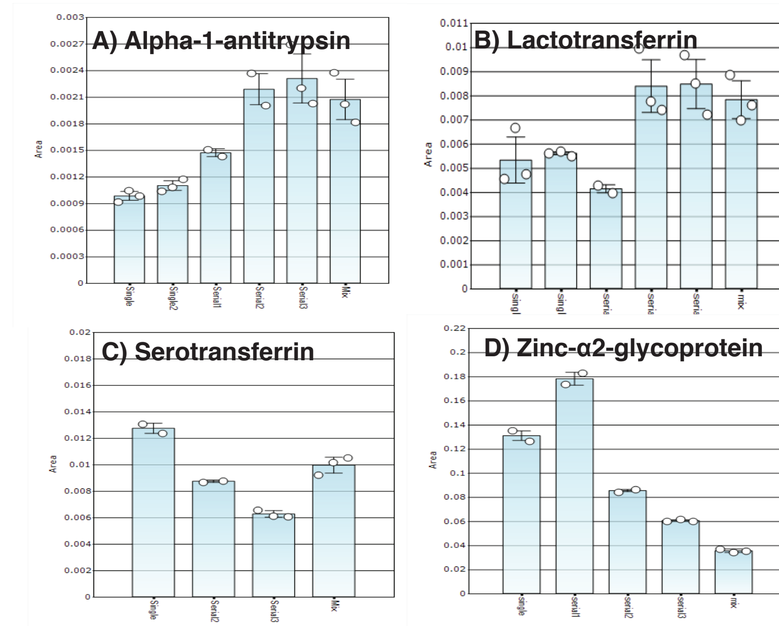


FIGURE 6. Comparative targeted protein response across the different sample preparation and LC-MS strategies. The histograms report the normalized protein response for single MSIA extraction per plasma sample (labeled as "Single") vs. serial MSIA extraction of the same plasma sample (labeled as "Serial"). The numbers behind each sample description represents the degree of post-extraction multiplexing prior to LC-MS analysis. For example, Figure 4A shows Single2 and Serial2 where the alpha-1-antitrypsin extraction was mixed with one other targeted protein extraction prior to LC-MS analysis.



Conclusion

MSIA D.A.R.T. extraction increases options for developing targeted protein quantitation and transition into routine, analysis by:

- Non-disruptive sample extraction process facilitates serial extraction using different MSIA D. A.R.T. tips.
- Serial extraction facilitates efficient multiplexing strategies on low sample volume.
- Serial extraction using same Ab (pooling) increases target protein characterization, including N- and O-linked glycopeptide/glycoform determinations.
- Offline immunoaffinity extraction increases throughput by reducing matrix, enriching signal, and facilitating multiplexed analysis of pooled samples.

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Improving Throughput for Targeted Quantification Methods by Intelligent Acquisition

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Overview

Automated remote multiplexed targeted protein quantification utilizing real-time qual/quan processing for increased quantitative accuracy over large dynamic ranges.

Introduction

Targeted quantification has become a very popular technique to verify putative biomarker candidates in large clinical cohorts of samples. These candidates are usually generated following a biomarker discovery experiment or derived from a biological hypothesis, for example, a pathway or biophysical interaction. These lists are usually large, containing upwards of 100–1000 proteins spanning several orders of magnitude dynamic concentration range. This presents analytical challenges for conventional SRM assays both in terms of method development and throughput. We propose using high-resolution, accurate-mass (HRAM) mass spectrometry (MS) and MS/MS schemes in conjunction with validated spectral libraries for automated method building, data acquisition, verification, and quantification in real-time using novel acquisition schemes.

Methods

K562 colon carcinoma cells were grown in heavy and light media, collected and mixed at different ratios to cover a 20-fold dynamic range. All samples were digested and analyzed on a Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer equipped with a nanospray ion source. Data was acquired in two steps to simulate traditional workflows. Initial experiments employed unbiased data-dependent MS/MS acquisition resulting in peptide/protein identification as well as building of a spectral library. The spectral library contains relative retention time, precursor charge state distribution, and product ion distributions, creating a unique verification/quantification scheme. A highly multiplexed, targeted protein list was created from the spectral library and used for automated data acquisition and processing real time to facilitate changes to the acquisition scheme.

The scheme in Figure 1 describes the methodology in more detail. The first step is to characterize the LCMS parameters using the Thermo Scientific™ Pierce™ PRTC Mixture Kit. The next step is to build a list of proteins that we are interested in. This will typically come from a pathway study or a discovery experiment. The next is to build a spectral library for this list of proteins. This can be built via predictive algorithm or empirical observations. This turns into a spectral library lookup table. The look-up table includes the precursor *m/z* values for the defined charge state as well as the expected retention time window, which are used to initiate product ion spectral acquisition based on the presence of multiple precursor isotopes during the expected elution window. Once the signal for multiple precursor isotopes surpasses the user-defined intensity threshold, a higher-energy collision dissociation (HCD) spectrum is acquired and immediately compared against the spectral library generating a dot-product correlation coefficient to determine spectral overlap and to check if the targeted peptide has been detected previously. If the calculated correlation coefficient surpasses the user-defined acceptance value, HCD product ion spectra will continue to be acquired across the elution profile. This is shown in Figure 2.

FIGURE 1. Strategy for large-scale targeted quantification based on high IQ data acquisition scheme

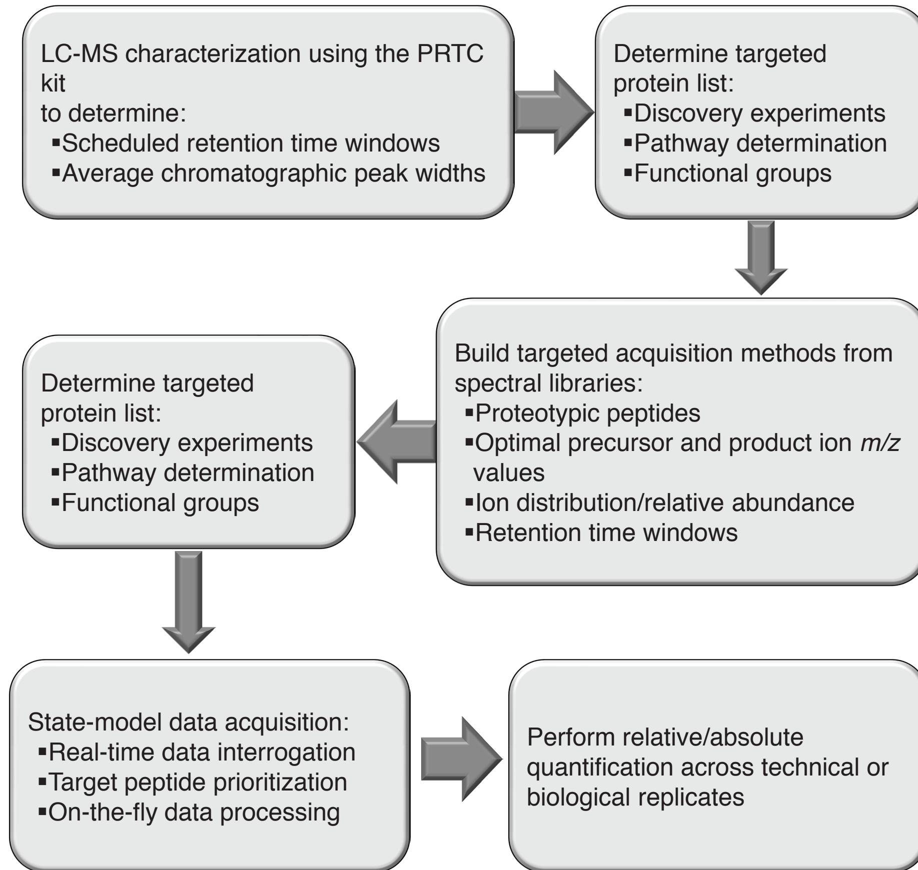
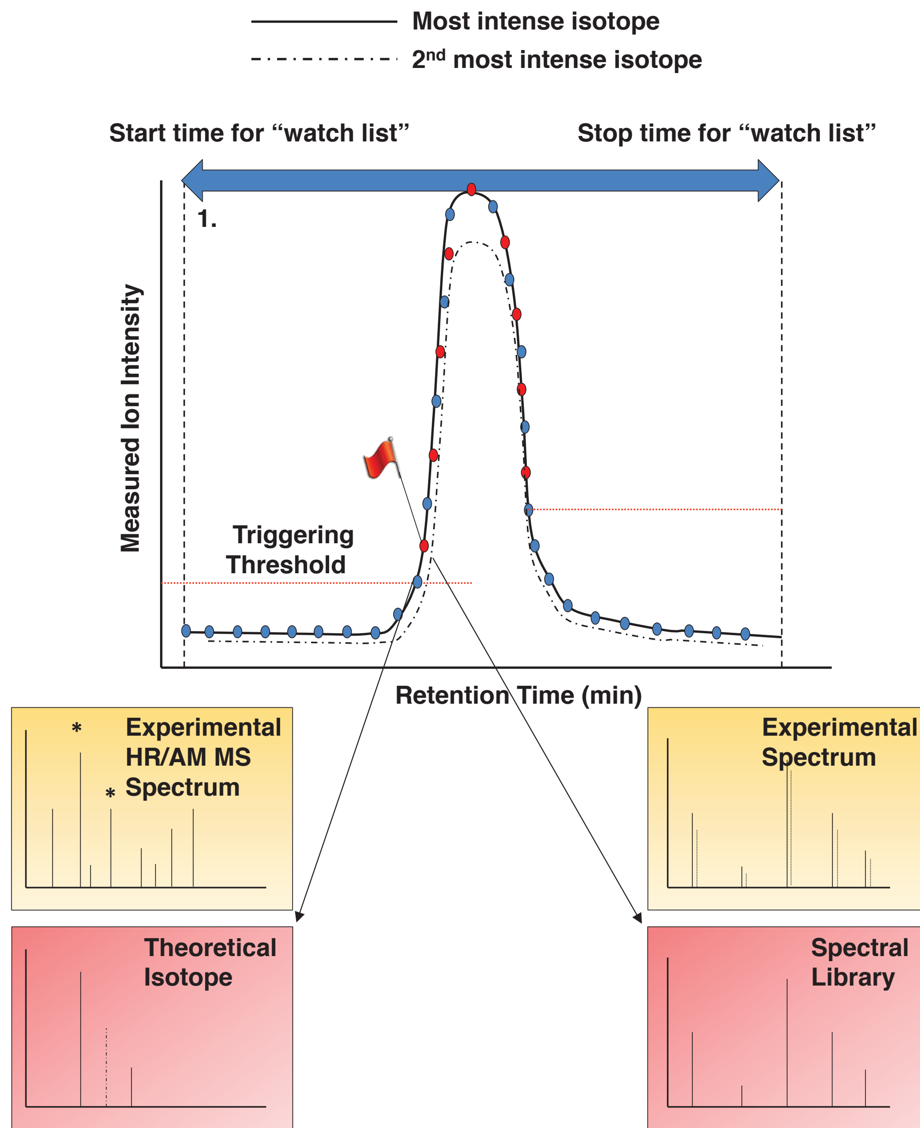


FIGURE 2. Pictorial representation of high IQ data acquisition schemes for targeted peptide quantification using a targeted scanning window, target elution identification, and real-time product ion spectral acquisition. Both precursor and production spectral matching is performed to increase the analytical selectivity of data acquisition.



Results

Highly multiplexed targeted protein quantification requires significant steps of method refinement prior to implementation. While the determination of proteins is relatively straightforward based on biology, the selection of peptides as surrogate biomarkers and corresponding *m/z* values (precursor and product ions) used to uniquely identify and quantitate the peptide targets becomes challenging. Generally, retention times and acquisition windows must be determined to maximize instrument cycle time to achieve robust quantification. To expedite complex experimental method development, we have created a unique spectral library procedure based on an analytically rigorous discovery data acquisition scheme. The local spectral library contains both LC and MS information that can be readily enlisted to build robust methods requiring few refinement steps.

To first test our methods, a protein mix was spiked in equine plasma (containing PTRC kit). Spectral library was first built on the neat protein mixture. Experiments performed on the quadrupole Orbitrap mass spectrometer facilitate unique product ion collection and detection schemes to not only increase data acquisition, but perform state-model data acquisition, increasing the ability for quantification. Figure 3 shows the CV distribution for the peptides over four acquisitions (by summing the area of top eight product ions).

K562 Cell Line

2,100 proteins were selected from the K562 cell line and imported into the new algorithm. The algorithm utilizes the spectral library information to select unique peptides and create precursor and product ion information used to perform real-time qualitative and quantitative analysis. In total, 3,800 peptides were chosen and 20-fold range digest was created.

Figure 4 shows an example where the ratio of 1:10 could not be calculated using the full-scan MS1 (panel A), but could be calculated in tandem MS/MS scan (panel B, and zoom-in, panel C).

FIGURE 3. CV distribution for the initial peptide list.

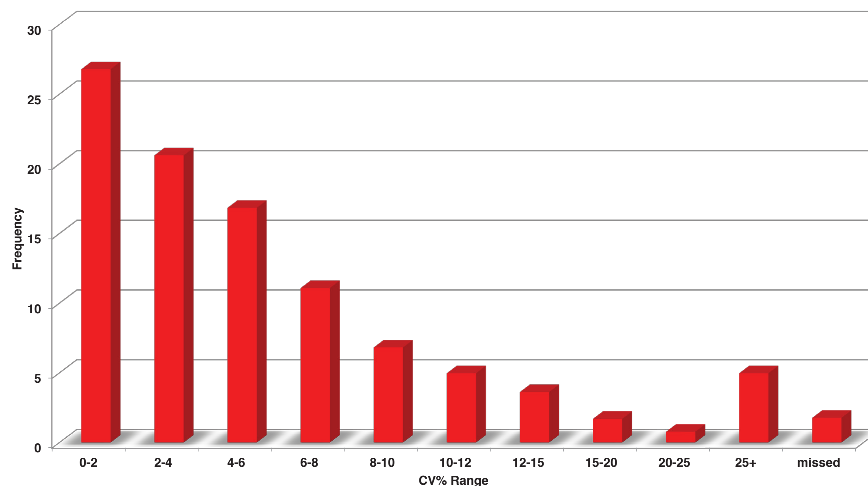
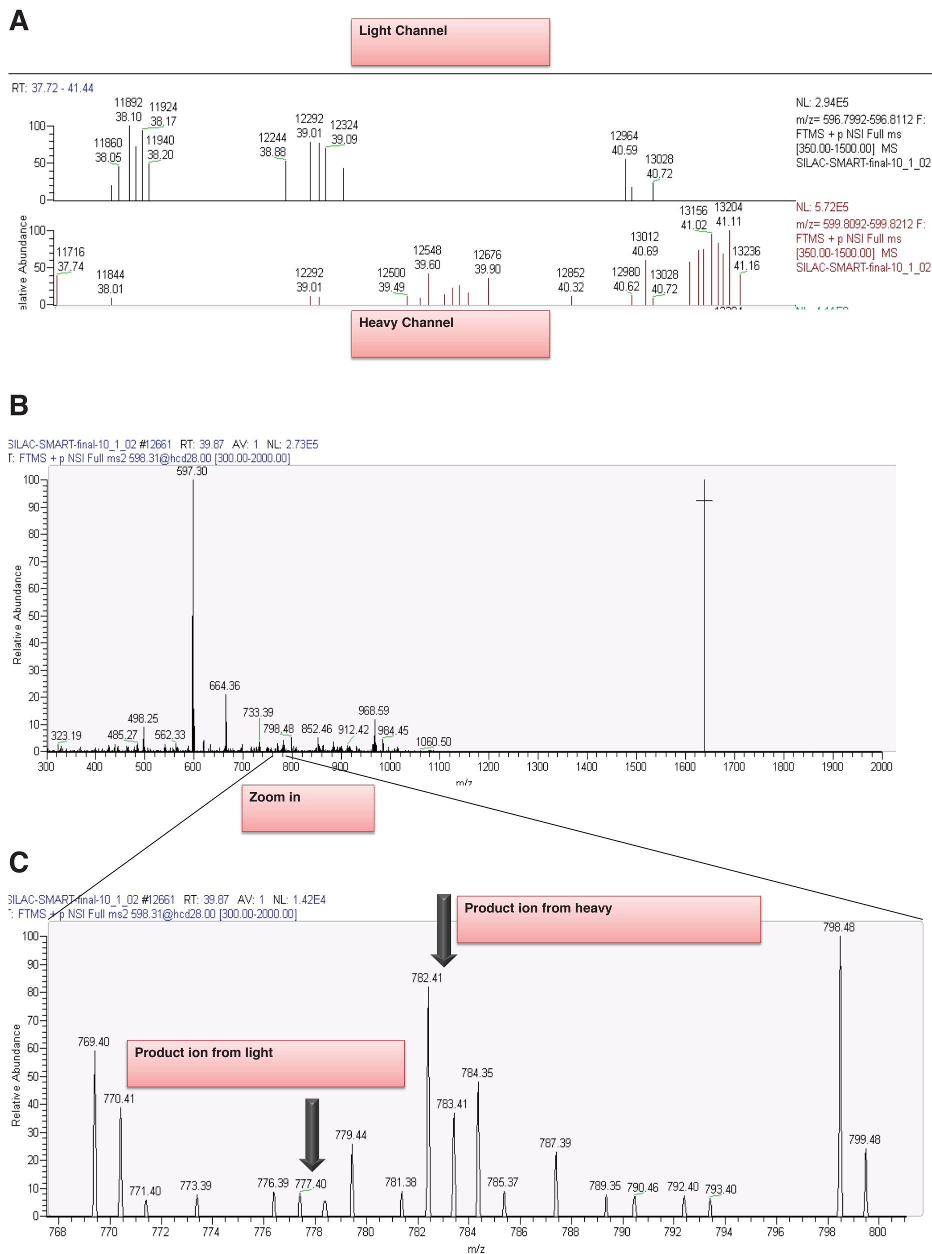


FIGURE 4. The benefit of MS/MS scan (with higher S/N) compared to full scan. Ratio of 1:10 could not be calculated in full scan (panel A), but it could be calculated in tandem MS/MS scan (panel C).



Conclusion

The developments here resulted in the successful qualitative/quantitative analysis for over 3,000 peptides representing over 2,000 proteins in this complex leukemia cell digest. Successful quantification was determined for proteins spiked at over a 20-fold range and the ability to change instrument acquisition parameters for increased analytical sensitivity.

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Spectrum Library Retention Time Prediction Based on Endogenous Peptide Standards

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Overview

Purpose: Accurately estimate peptide retention based on spectrum library data utilizing commonly observed peptides in place of synthetic standards.

Methods: We consolidate many months' worth of LC-MS/MS data into a library of MS/MS spectra. Our automated analysis selects endogenous peptides to act as standards which are used to predict retention times of any peptide in the library.

Results: Seventeen peptides were identified as appropriate endogenous standards. Relative retention time information stored in the library allowed us to predict the retention times of 1750 peptides more accurately than predictions based on hydrophobicity.

Introduction

Spectrum libraries are an invaluable starting point for developing targeted assays (e.g. SRM, PRM) because they provide information about fragmentation patterns and retention times. When library data are collected under a variety of LC conditions, the use of synthetic peptide standards can greatly improve the ability to accurately predict retention time in new experiments. Unfortunately, any samples not including those peptide standards cannot be used in the predictions. We present a method for selecting peptides endogenous to a sample to act as standards and demonstrate their use for predicting retention times of other peptides including those with chemical modifications, which indicate portability to both unmodified and post-translationally modified peptides.

Methods

Sample Preparation

Activity-based protein profiling (ABPP) was performed on various human lung cancer cells and five pairs of tumor and adjacent control human tissue samples. Thermo Scientific™ Pierce™ ActivX ™ desthiobiotin ATP probes were used to interact with ATP utilizing enzymes and lysine close to the active sites were labeled with desthiobiotin.

Liquid Chromatography and Mass Spectrometry

Trypsin-digested samples were run on one of three gradients (2 hr on HPLC, 2 hr on UPLC, 4 hr on UPLC). The validation experiment used a 4 hr gradient on UPLC. Spectra were acquired on a Thermo Scientific™ LTQ Orbitrap™ MS using data-dependent acquisition.

Data Analysis

Peptide identification was done in Thermo Scientific™ Proteome Discoverer™ (PD) software. The spectrum library was built using the Crystal node for PD version 1.4. A custom script was written to analyze the library entries and find appropriate endogenous peptides to use as standards.

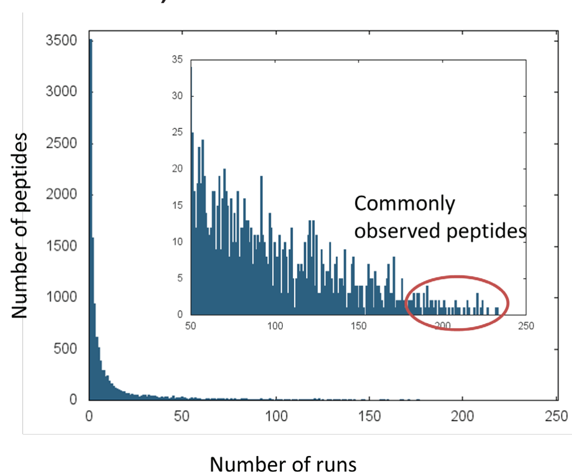
Results

Peptide Frequency in the Spectrum Library

Assembly of the Crystal spectrum library collected the retention time information into one resource. The library contained 220,542 spectra from 250 LC-MS runs including 9,109 peptide sequences (12,063 total with modified forms). As these samples did not contain a synthetic peptide standard, we first sought appropriate endogenous peptides.

The best candidates for peptides to act as retention time landmarks are those most commonly seen from run to run. We looked at the frequency of peptides in the 250 runs used to build the library. No peptides were observed in every run, the most commonly seen peptide having 233 appearances. (Figure 1) We selected the 50 most commonly seen peptides which were seen in no fewer than 185 runs.

FIGURE 1. Frequency of peptide observation. The library collected spectra from 250 DDA runs. Peptides were observed with varying frequency, between 1 and 233 runs. We focused on the 50 most frequently seen peptides (circled in inset).



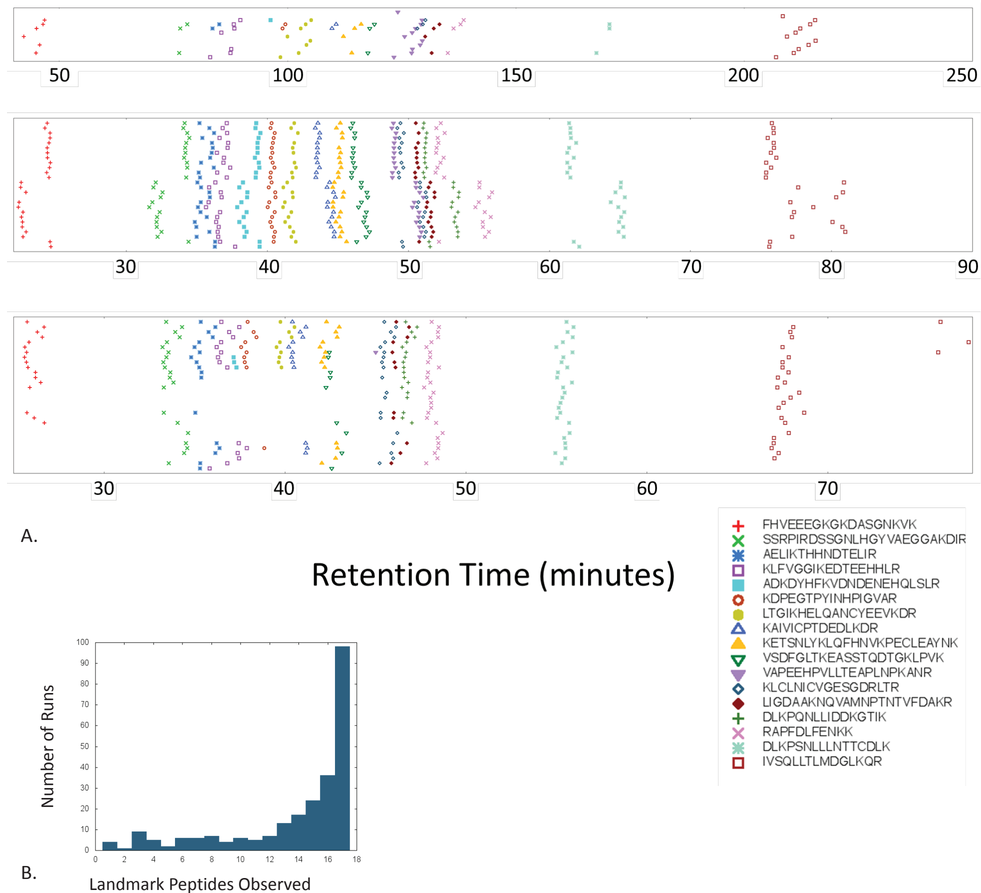
Endogenous Peptides for Retention Time Landmarks

Starting with the 50 most commonly seen peptides, we winnowed down the list to find a set of peptides that both covered the entire elution profile and consistently eluted in the same order relative to each other. An in-house script automated the process. First we record the relative order of the 50 peptides in all 250 runs, for each pair of peptides A and B, keeping track of how often A came before B. Next we use a greedy algorithm to select a consistent set.

- Start the set with one peptide.
- For each remaining peptide, try adding it to the set in the appropriate order.
- If it cannot be placed unambiguously relative to the existing peptides in the set, eliminate this peptide.

We found seventeen that eluted in a consistent order. They are plotted at their observed retention times in several library runs in Figure 2.

FIGURE 2. A. Retention times of landmark peptides in library data. The observed retention times of the seventeen peptides selected to act as landmarks were plotted for 68 runs in the library. Runs from each of three gradients are plotted together. The rank order of the peptides is the same in all runs, but the absolute times differ even for runs with the same gradient. Peptides are distributed across the entire gradient, with a higher density in the early-to-middle times. B. Histogram of number of landmark peptides in each run. Not every peptide was observed in every run, but there are enough in most cases to cover the whole gradient.



Use Relative Retention Times to Estimate RT on New Gradient

The Crystal library computes a relative retention time for each peptide stored in the library as a distance between the two nearest landmark peptides. (Figure 3a) These are used to estimate the retention times on a new gradient (Figure 3b). First, the RT of the landmarks must be measured on the new gradients. Then the relative RTs can be projected on to this new gradient and the average time is taken as the estimate.

We estimated the times of 1750 peptides on a 4 hour gradient. In addition, we compare our estimates to estimates based on peptide hydrophobicity (Krohkin, 2009). The accuracy of the estimate is measured as the difference between the estimated and observed times. Figure 4 plots the accuracy of the two estimation methods as well as accuracy of the library predictions as a function of the observed time. Library predictions are much closer than the hydrophobicity predictions to the observed retention times with most falling with in +/- 10 minutes of the observed time. Predictions are not consistently earlier or later than observed, but there is a slight trend for the prediction to be too early at the beginning of the run and too late at the end of the run. This may be due to having fewer landmarks at the ends of the run.

FIGURE 3. A. Observed retention times of target peptides are stored as the distance between the two nearest landmark peptides. B. Retention time predictions are made by projecting the relative times on to the known times of the landmarks on a new gradient.

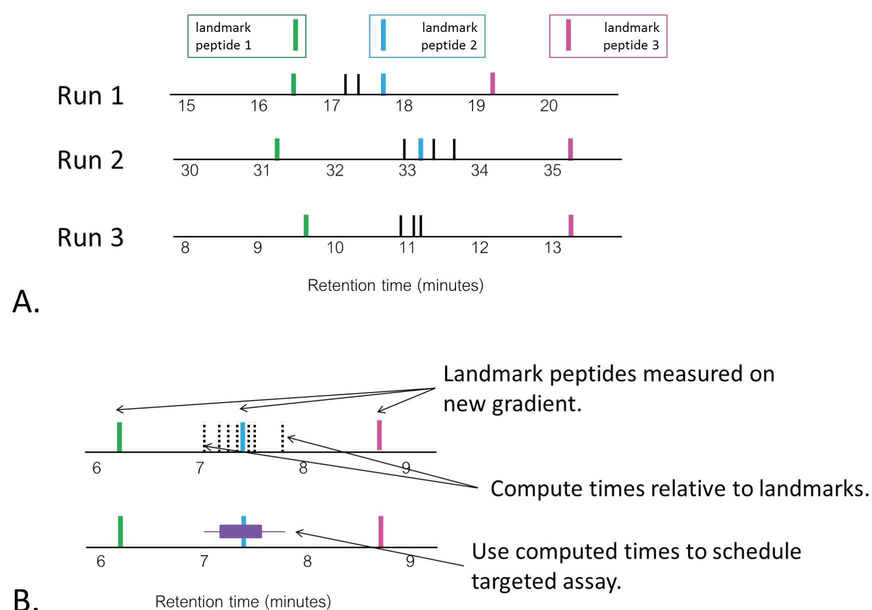
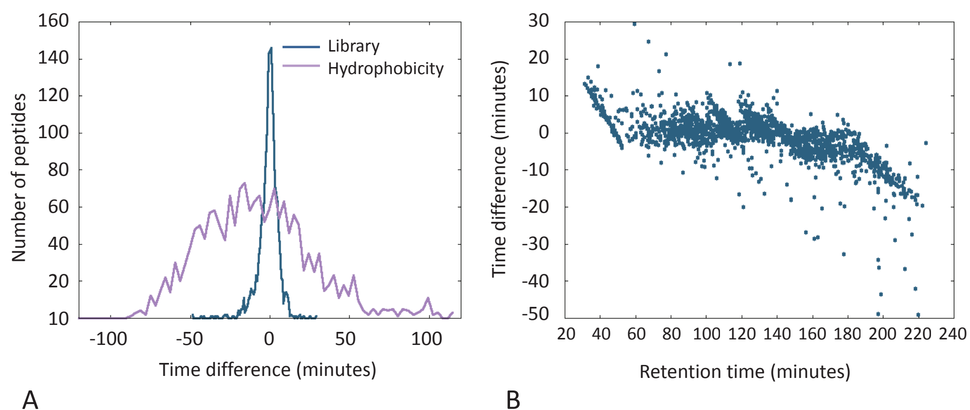


FIGURE 4. Comparison of estimated and observed retention times of 1750 peptides. A. Histogram of predicted minus observed retention times for both prediction methods. B. Library-predicted minus observed retention time vs. the observed retention time.



Conclusion

Endogenous peptides can successfully act as retention time landmarks and accurately estimate RT in new gradients.

- Spectrum libraries capture valuable retention time information.
- Our algorithm finds endogenous peptides with consistent elution behavior to act as standards.
- We can accurately predict the retention time of any library peptide by estimating it relative to the standard peptides. Therefore, comparisons can more easily be made across datasets with accurate mass and retention time measurements (AMT). This capability also enables method transfer to scheduled LC-MRM.
- Library-based estimated retention times are closer to the observed times than predictions made based on hydrophobicity.

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Acknowledgments

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Comprehensive Peptide Searching Workflow to Maximize Protein Identifications

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Overview

Purpose: Development of a comprehensive protein identification workflow to maximize high-confidence peptide/protein identifications including post-translational modifications (PTM) compared to a traditional database search strategy.

Methods: Use of a combination of multiple search engines (e.g., SEQUEST®, Sequest HT, Mascot and MS Amanda) where combinations of PTMs were judiciously chosen for each node based on uniprotKB relative PTM abundances from high quality, manually curated, proteome-wide data[1].

Results: Tremendous enhancement in the high-confidence, Percolator-validated peptide and protein identifications compared to a standard protein identification workflow.

Introduction

Protein identification and characterization by mass spectrometry has become an established method in biological research in recent years. The number of protein identifications from complex biological samples depends on many factors, ranging from data acquisition strategy to MS/MS data searching methods. Unfortunately, only a fraction of spectra generated by the acquisition have confident peptide matches for any complex biological sample. There are several factors that are being overlooked by many users in the conventional data searching strategy, including the appropriate combination of PTMs, coding SNPs[2], isoforms of proteins, and iterative searching strategies that can potentially help to identify unmatched spectra. We developed a comprehensive MS/MS searching workflow in Thermo Scientific™ Proteome Discoverer™ software to maximize high-confidence peptide/protein identifications. The effect of various search strategy factors on peptide identifications were explored. We implemented a process that includes analysis of protein isoforms, missed cleavage sites, semi-tryptic digestion and most importantly, appropriate combination of PTMs in each search node. The workflows were tested on plasma and urine samples analyzed on a Thermo Scientific™ Orbitrap™ hybrid mass spectrometer. The comprehensive workflow was found to make more high-confidence peptide/protein IDs and identify multiple PTMs and partially cleaved peptides in a single run.

Methods

Comprehensive Workflow Development

We developed a comprehensive MS/MS searching workflow in Proteome Discoverer software using a combination of multiple search engines (Figure 1) in an iterative fashion to maximize protein/peptide identifications by considering the most frequently found PTMs1, artefacts (Table 1) and partially cleaved peptides. The combination of PTMs were judiciously chosen based on relative abundances (UniProtKB) of each PTM found experimentally and putatively as described in, from high-quality, manually curated, proteome-wide data1. The workflows were tested on plasma and urine samples analyzed on a hybrid Orbitrap mass spectrometer.

Sample Preparation

In order to evaluate the performance of the comprehensive workflow we took four human samples from two different sources (a) urine and (b) plasma (three samples). Human urine and plasma samples were collected with full consent and approval. The samples were subjected to reduction and alkylation followed by digestion with trypsin.

Liquid Chromatography and Mass Spectrometry

The digested samples were separated with a 5-45% acetonitrile gradient in 0.1% formic acid using a C18 nano-LC column. The urine sample (sample no. 1) and a plasma sample (sample no. 2) were run for 140 minutes and 90 minutes, respectively and the data were acquired with a Thermo Scientific™ LTQ Orbitrap Velos™ MS with Top 11 and Top 10 data-dependent MS/MS respectively, using CID fragmentation. Another two plasma samples (sample nos. 3 and 4) were run for 240 minutes and the data were acquired with the Thermo Scientific™ Q Exactive™ benchtop mass spectrometer, with Top 15 data-dependent MS/MS using HCD fragmentation.

Data Analysis

The acquired data was searched with Proteome Discoverer 1.4 against Uniprot human complete proteome database using the comprehensive workflow (Figure 1, Table1) and compared with the SEQUEST workflow with standard modifications (oxidation at methionine as dynamic modification and alkylation as static modification) coupled with percolator validation (Standard Search).

FIGURE 1. Structure of the comprehensive workflow

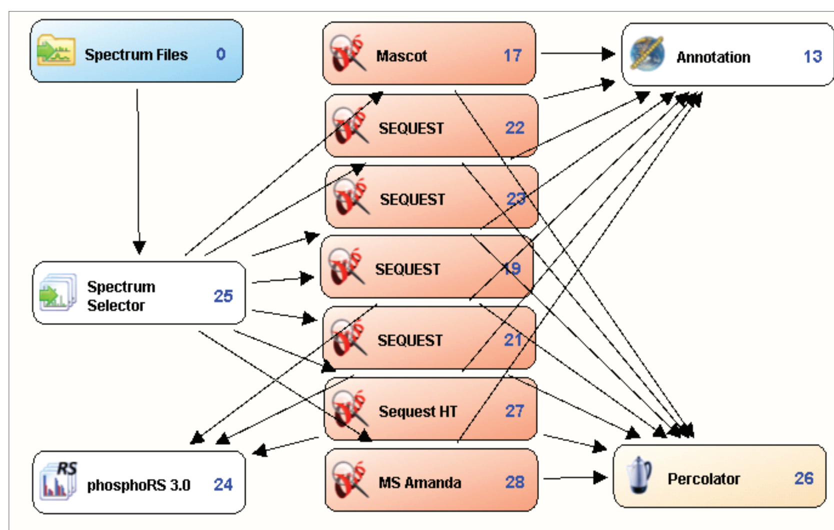

TABLE 1. Parameters and modifications used in comprehensive search workflow

Search Engine	Precursor Mass Tolerance	Fragment Mass Tolerance (Q Exactive MS/LTQ Orbitrap Velos MS)	Missed Cleavage	Enzyme	Static Modification	Dynamic Modification
Mascot	5 ppm	0.02 Da / 0.4 Da	2	Semi Trypsin	Carboxymethyl (C)	Oxidation (M); Acetyl (K); Methyl (K)
SEQUEST	5 ppm	0.02 Da / 0.4 Da	3	Trypsin (Full)	Carboxymethyl (C)	Oxidation (M); ADP-Ribosyl (N,R); Myristoyl (K); Deamidation (N,Q); Phospho (S)
SEQUEST	5 ppm	0.02 Da / 0.4 Da	3	Trypsin (Full)	Carboxymethyl (C)	Oxidation (M); Dioxidation (M); Trimethyl (K,R); Phospho (S,T)
SEQUEST	5 ppm	0.02 Da / 0.4 Da	3	Trypsin (Full)	Carboxymethyl (C)	Oxidation (M); Carbamyl (K,R); Deamidated (N,Q); Amidation (Any C-Terminus)
SEQUEST	5 ppm	0.02 Da / 0.4 Da	3	Trypsin (Full)	Carboxymethyl (C)	Oxidation (M); Methyl (K,R); Dimethyl (K,R); Trimethyl (K,R); Acetyl (K)
Sequest HT	5 ppm	0.02 Da / 0.4 Da	3	Trypsin (Full)	Carboxymethyl (C)	Oxidation (M); Phospho (S,T,Y); Deamidated (N,Q);
MS Amenda	5 ppm	0.02 Da / 0.4 Da	3	Trypsin (Full)	Carboxymethyl (C)	Oxidation (M); Acetyl (K)

Results

We compared the results from our comprehensive searching strategy with a standard search strategy. We found that on average, the number of high-confidence peptide identifications (FDR≤0.01) increased approximately 2-fold with our comprehensive workflow compared to standard searches, whereas the increment in the number of medium confidence peptide identifications (FDR≤0.05) was more than two times compared to standard search (Figure 2).

The comprehensive workflow was found to increase the number of high-confidence protein identifications (FDR≤0.01) by 90% and the high-confidence protein groups by 75% with respect to the standard search condition. Moreover, the comprehensive workflow increases the high-confidence group proteins (with at least two high-confidence peptides for every protein in the group) by 23% (Figure 3).

The comprehensive workflow identified several high-confidence peptides with multiple PTMs which reveals the importance of particular combinations of PTMs in a search node (Table 2).

FIGURE 2. Comprehensive workflow increases number of peptide identifications (sample 1 = urine, sample 2-4 = plasma)

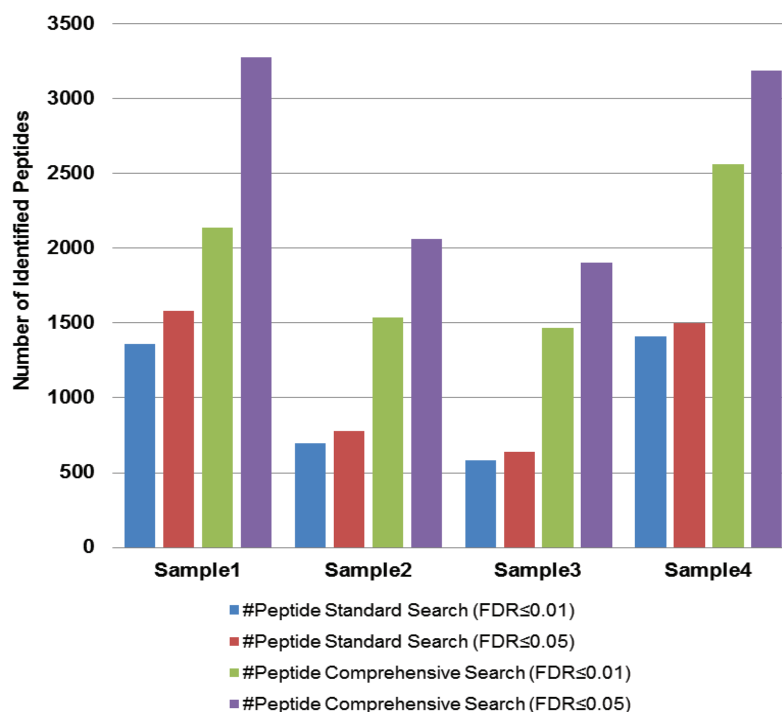

FIGURE 3. The comprehensive workflow increases the number of identified protein groups with at least two peptide hits per protein.

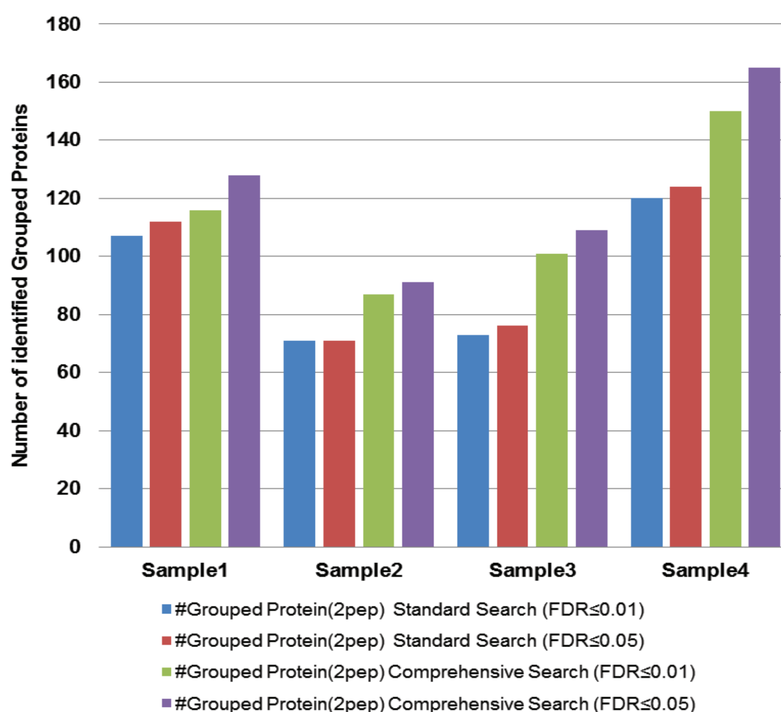

TABLE 2. Examples of peptides containing multiple PTMs from the comprehensive search strategy

Sequence	Modification	q-Value
CCKHPEAKRMPCAEDYLSVVLNQLC VLHEK	C1(Carboxymethyl); C2(Carboxymethyl); K3(Myristoyl); M10(Oxidation); C12(Carboxymethyl); C25(Carboxymethyl)	≤0.001
CYAKVFDEFKPLVEEPQNLIK	C1(Carboxymethyl); K4(Methyl); K10(Acetyl); K21(Methyl)	≤0.001
DKDEAEQAVSR	K2(Acetyl); R11(Trimethyl)	0.008
LVRPEVDVMCTAFHDNEETFLKK	R3(Dimethyl); M9(Oxidation); C10(Carboxymethyl); K22(Acetyl); K23(Acetyl)	0.004
INNEDNSQFK	N3(ADP-Ribosyl); K10(Myristoyl)	0.01
RMPCAEDYLSVVLNQLCVLHEK	R1(Trimethyl); M2(Dioxidation); C4(Carboxymethyl); C17(Carboxymethyl)	≤0.001
SEPKWEVVEPLK	K4(Trimethyl); K12(Dimethyl)	0.004
TCVADESAENCDK	C2(Carboxymethyl); C11(Carboxymethyl); K13(Dimethyl)	≤0.001
YYFNCNNWLSKVEGDRQWCR	C5(Carboxymethyl); K11(Methyl); R16(Trimethyl); C19(Carboxymethyl); R20(Methyl)	0.006

We further investigate the number of matched and unmatched spectra in the data sets comparing the standard search and our comprehensive search strategy. We found that the percentage of matched spectra improves significantly when using the comprehensive search workflow (Table 3).

Table 3. Comparative table for matched spectra

File	Total Spectra	Matched Spectra Standard Search (FDR≤0.05)	Matched Spectra Comprehensive Search (FDR≤0.05)	Matched Spectra Standard Search (FDR≤0.01)	Matched Spectra Comprehensive Search (FDR≤0.01)
Sample1	27215	28.0%	46.7%	26.2%	41.1%
Sample2	14005	15.4%	44.2%	14.4%	39.6%
Sample3	43036	5.1%	13.6%	4.9%	12.1%
Sample4	44450	9.5%	22.3%	9.0%	20.3%

Moreover, the comprehensive search workflow increased sequence coverage of proteins significantly, giving rich information about proteins including PTMs (Table 4).

Table 4. Comprehensive search increases protein coverage

Example	Protein	Sequence Coverage Standard Search (FDR≤0.01)	Sequence Coverage Comprehensive Search (FDR≤0.01)
1	A1AT	28.47%	57.42%
2	ALBU	70.94%	78.00%
3	A2MG	35.35%	53.12%
4	AACT	35.7%	42.55%
5	APOB	14.66%	23.12%
6	CERU	22.44%	37.28%
7	HEMO	38.96%	49.13%
8	TRFE	40.11%	61.17%
9	TTHY	54.42%	62.59%
10	VTDB	31.65%	50.21%

Conclusion

- A comprehensive workflow strategy identified almost twice as many high-confidence peptides compared to the standard search strategy.
- The comprehensive workflow helped increase the number of high-confidence protein identifications and high-confidence protein group identifications by approximately 90% and 75%, respectively, compared to the standard search approach.
- The comprehensive workflow identifies more high-confidence peptides with multiple PTMs.
- The percentage of matched spectra improves significantly when using the comprehensive search workflow in Proteome Discoverer software.

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High-Resolution, Accurate-Mass (HR/AM) and Intelligent Acquisition-Enabled Global Discovery and Quantification of Histones, Histone PTMS, and Histone Modification Enzymes in Mesenchymal Stem Cells

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Overview

Purpose: Development of a real-time, intelligent acquisition strategy for HR/AM global targeted quantification of histones, histone PTMs, and histone modification enzymes

Methods: Mesenchymal stem cells were derived from human adipose tissue and treated with bleomycin. Samples were analyzed on a Thermo Scientific™ Q Exactive™ mass spectrometer using an intelligent acquisition method.

Results: The final assay performed qual/quan studies on 36 proteins and 154 histone and histone-related peptides and modified analogs. The combined approach enabled quantification of previously identified modified peptides as well as novel targets across different samples and were correlated to somatic or stem cell aging (replicative or genotoxic stress-induced senescence).

Introduction

Chromatin is viewed as an operational interface for almost all known nuclear processes. Nucleosomal packaging and histone modifications dictate the different degrees of primary chromatin compaction achieved by additional chromatin structural proteins. For example, euchromatic chromatin fibers contain six nucleosomes per 11 nm; however, heterochromatin consists of 12–15 nucleosomes per 11 nm. A dynamic balance between these two radically different chromatin compaction states is at the very core of the high-level nuclear chromatin organization (nuclear architecture), and is vital for maintaining cell-type identity over time. Functional differences between the cells in an organism are defined by epigenetic factors and epigenetic programs, which are critical for the preservation of functional integrity of the cellular phenotypes (1). However, as a mediator of the external signals, chromatin is anything but static. Nucleosome unwrapping and disassembly events, which must occur during DNA replication, transcription, and DNA repair, can directly influence the state of chromatin compaction. Several lines of evidence obtained in Drosophila, yeast, and plants indicate that chromatin undergoes disassembly during the onset of DNA double-strand breaks (DSB) and repair at the DSB sites. It is still an open question how to quantify the chromatin changes. One of the accepted ways to do such is through cellular fractionation. Based on the salt and detergent concentrations in buffer solutions, three cellular fractions can be obtained: cytoplasmic, nuclear soluble (proteins that are loosely bound to chromatin), and a chromatin fraction (containing tightly bound proteins, or enzymes engaged in chromatin modifications). Tracing histones or histone-modifying activities (HMT, HATs, and HDACs) dynamics in these fractions during biological events permits the assessment of overall chromatin dynamics and full characterization of the cellular stage (2).

In this study, we set out to quantify the changes in the chromatin composition in primary human stem cells upon acute DNA damage and during drug-evoked senescence. This assessment allowed insights into the “access, repair, restore” model of chromatin dynamics upon DNA damage repair and a better understanding of whether or not misregulation of this axis is one of the critical factors of cellular senescence. In our model system, we compared two conditions: 1) Acute DNA damage (2 hrs treatment with bleomycin) and 2) DNA damage-induced cellular senescence (cellular recovery after 5 days of post-bleomycin treatment). Both conditions were compared to the normally proliferating (self-renewing) cells.

Using a novel application of intelligent acquisition and HR/AM MS, we have developed workflows for quantitative global profiling and targeted analysis of histones, histone post-translational modifications (PTMs), and histone modification enzymes.

Methods

Samples

Mesenchymal stem cells were derived from human adipose tissue and treated with bleomycin as follows: nuclear, cytoplasmic, and chromatin fraction samples were isolated from proliferating (bleomycin –) cells as well as cells under conditions of acute DNA damage (bleomycin 2 hrs) and drug-induced senescence (bleomycin 2 hrs followed by a 5-day recovery).

MS Data Acquisition and Analysis

Samples were analyzed on a Q Exactive mass spectrometer. Initial discovery experiments were performed to generate a list of peptides/protein IDs and corresponding spectral libraries. A subset of peptides was selected for targeted quantification across the different samples. Heavy labeled analogs were used for qual/quan data processing. Qualitative and quantitative data analysis was performed using Thermo Scientific™ Pinpoint™ software.

Results

Intelligent Data Acquisition

Initial discovery experiments were performed to help drive targeted quantitative experiments (Figures 1, 2). The discovery experiments were performed in an unbiased data-dependent acquisition across different biological samples to determine proteins and peptides as well as the initial set of PTMs. From the initial discovery results, histones and histone modification proteins were chosen and further targeted discovery experiments were performed to increase the probability of modified peptide identification based on theoretical *m/z* inclusion lists. The combined results from the discovery experiments were used to build a local spectral library consisting of precursor and product ion *m/z* values and relative abundance distribution as well as relative retention time values. A set of peptides from the discovery data was selected based on known and novel PTMs. The spectral library information for the targeted peptides was used to create a targeted inclusion list and reference information to perform qual/quan determination in real time. The real-time feedback facilitated optimization of instrument parameters to maximize instrument duty cycle and detection capabilities resulting in significantly increasing the number of peptides quantified per experiment. The final assay performed qual/quan studies on 36 proteins and 154 histone and histone-related peptides and modified analogs (Table 1). The combined approach enabled quantification of previously identified modified peptides as well as novel targets across different samples and were correlated to somatic or stem cell aging (replicative or genotoxic stress-induced senescence) (Figs 3-5).

FIGURE 1. Intelligent data acquisition strategy. Pictorial representation of intelligent data acquisition schemes for targeted peptide quantification using a targeted scanning window, target elution identification, and real-time product ion spectral acquisition. Both precursor and product ion spectral matching are performed to increase the selectivity of data acquisition.

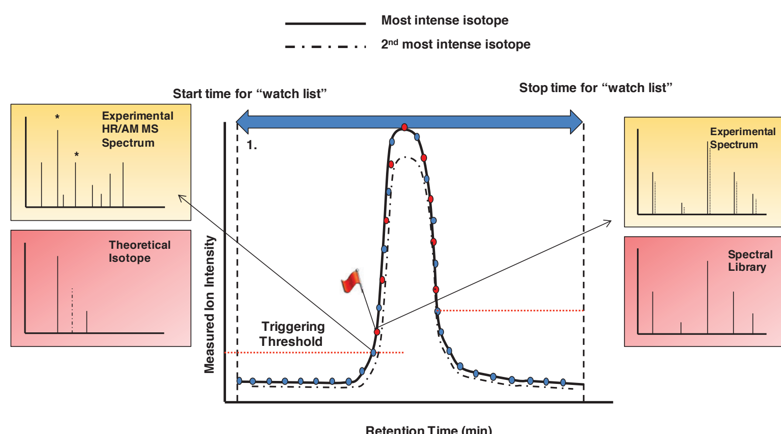
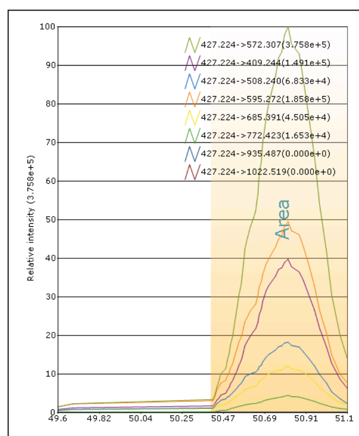


FIGURE 2. Extracted ion chromatograms of fragment ions from KESYSIYVKYK

A. Precursor ion charge state 3



B. Precursor ion charge state 2

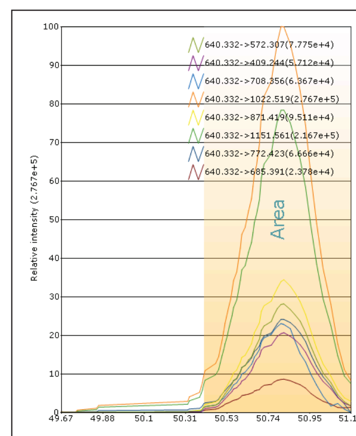


TABLE 1. Targeted proteins and peptides

Proteins	Peptide Sequence
>tr|H0Y765|H0Y765_HUMAN Histone-lysine N-methyltransferase MLL3 (Fragment) OS=Homo sapiens GN=MLL3 PE=2 SV=1	
	LM[Oxid]Q[Deamid]EVDR
>sp|P07305|H10_HUMAN Histone H1.0 OS=Homo sapiens GN=H1F0 PE=1 SV=3	
	GVGASGSFR
	YSDMIVAAIQAEK
>tr|Q5TEC6|Q5TEC6_HUMAN Histone cluster 2 H3 pseudogene 2 OS=Homo sapiens GN=HIST2H3PS2 PE=1 SV=1	
	EIAQEFK[Methyl]TDLR
	EIAQEFKTDLR
>sp|Q92769|HDAC2_HUMAN Histone deacetylase 2 OS=Homo sapiens GN=HDAC2 PE=1 SV=2	
	LGC[Carboxymethyl]FNLTVK
>sp|Q86X55|CARM1_HUMAN Histone-arginine methyltransferase CARM1 OS=Homo sapiens GN=CARM1 PE=1 SV=3	
	IVLDVGC[Carboxymethyl]GSGILSFFAAQ[Deamid]AGAR
>sp|Q9UQL6|HDAC5_HUMAN Histone deacetylase 5 OS=Homo sapiens GN=HDAC5 PE=1 SV=2	
	EKSK[di-Methyl]ESAIAS[Phosphoryl]TEVKLR
>sp|P68431|H31_HUMAN Histone H3.1 OS=Homo sapiens GN=HIST1H3A PE=1 SV=2	
	K[Acet]QLATK[Acet]AAR
>sp|P33778|H2B1B_HUMAN Histone H2B type 1-B OS=Homo sapiens GN=HIST1H2BB PE=1 SV=2	
	KESYSIYVYK
	IAGEASRLAHYNKR
	ESYSIYVYK
	AMGIMNSFVNDIFER
	AMGIMNSFVNDIFER[Methyl]
	AM[Oxid]GIMNSFVNDIFER
	AMGIM[Oxid]NSFVNDIFER
	AM[Oxid]GIM[Oxid]NSFVNDIFER
	AM[Oxid]GIM[Oxid]NSFVNDIFER[Methyl]
	STITSREIQ[Deamid]TAVRLLLPGELAK
	AM[Oxid]GIMNS[S_42]FVNDIFER
	AMGIM[Oxid]NS[S_42]FVNDIFER
	A[A_42]M[Oxid]GIMNSFVNDIFER
	A[A_42]MGIM[Oxid]NSFVNDIFER
	AMGIM[Oxid]NS[S_42]FVN[Deamid]DIFER
	AMGIM[Oxid]N[Deamid]S[S_42]FVNDIFER
	AM[Oxid]GIMNS[S_42]FVNDIFER[Methyl]
	AM[Oxid]GIM[Oxid]NS[S_42]FVNDIFER
	A[A_42]M[Oxid]GIM[Oxid]NSFVNDIFER
	A[A_42]M[Oxid]GIM[Oxid]NS[S_42]FVNDIFER
	AMGIMN[Deamid]SFVNDIFER
	AM[Oxid]GIMNSFVN[Deamid]DIFER
	AMGIM[Oxid]NSFVN[Deamid]DIFER
	AM[Oxid]GIMNSFVNDIFER[Methyl]
	AM[Oxid]GIM[Oxid]N[Deamid]S[S_42]FVNDIFER
	A[A_42]M[Oxid]GIM[Oxid]N[Deamid]SFVNDIFER
>sp|Q96A08|H2B1A_HUMAN Histone H2B type 1-A OS=Homo sapiens GN=HIST1H2BA PE=1 SV=3	
	HAVSEGTKAVTKYTSSK
	LLLPGELAK[Methyl]
	EIQTAVRLLLPGELAK
>sp|P62807|H2B1C_HUMAN Histone H2B type 1-C/E/F/G/I OS=Homo sapiens GN=HIST1H2BC PE=1 SV=4	
	KESYSVYVYK
	SRKESYSVYVYK
	KESYSVYVYKVLK
	ESYSVYVYK
>sp|Q93079|H2B1H_HUMAN Histone H2B type 1-H OS=Homo sapiens GN=HIST1H2BH PE=1 SV=3	
	PDPA[A_42]KSAPAPK
>sp|Q8IUE6|H2A2B_HUMAN Histone H2A type 2-B OS=Homo sapiens GN=HIST2H2AB PE=1 SV=3	
	HLQLA[A_42]VRN[Deamid]DEELNK
>sp|P16402|H13_HUMAN Histone H1.3 OS=Homo sapiens GN=HIST1H1D PE=1 SV=2	
	SET[T_42]APLAPTIPAPAEK
	S[S_42]ETAPLAPTIPAPAEK
>sp|Q9NQW5|PRDM7_HUMAN Probable histone-lysine N-methyltransferase PRDM7 OS=Homo sapiens GN=PRDM7 PE=2 SV=2	
	LK[K_68]LELR
>sp|P16403|H12_HUMAN Histone H1.2 OS=Homo sapiens GN=HIST1H1C PE=1 SV=2	
	SGVSLAALK
	KASGPPVSELITK
	SGVSLAALKK
	ASGPPVSELITK
	S[S_42]ETAPAAPAAAPPAEK
>sp|Q02539|H11_HUMAN Histone H1.1 OS=Homo sapiens GN=HIST1H1A PE=1 SV=3	
	K[Acet]PAKAAAAS[Phosphoryl]K[di-Methyl]K[Methyl]
	ALAAAGYDVEK
>sp|P56524|HDAC4_HUMAN Histone deacetylase 4 OS=Homo sapiens GN=HDAC4 PE=1 SV=3	
	ALAHR[Methyl]NLN[Deamid]HC[Carboxymethyl]ISSDPR
>sp|O14686|MLL2_HUMAN Histone-lysine N-methyltransferase MLL2 OS=Homo sapiens GN=MLL2 PE=1 SV=2	
	IIIS[S_42]SR
>tr|Q8TC04|Q8TC04_HUMAN Keratin 23 (Histone deacetylase inducible) OS=Homo sapiens GN=KRT23 PE=2 SV=1	
	LASYVEK
>tr|Q6FHM6|Q6FHM6_HUMAN NHP2 non-histone chromosome protein 2-like 1 (S. cerevisiae) OS=Homo sapiens GN=NHP2L1 PE=2 SV=1	
	AC[Carboxymethyl]GVSRPVIAC[Carboxymethyl]SVTIK
>sp|Q09472|EP300_HUMAN Histone acetyltransferase p300 OS=Homo sapiens GN=EP300 PE=1 SV=2	
	TSKNK[K_68]SSLS[S_42]R
>sp|Q6DN03|H2B2C_HUMAN Putative histone H2B type 2-C OS=Homo sapiens GN=HIST2H2BC PE=5 SV=3	
	AM[Oxid]GIMNSFLNDIFER
	AM[Oxid]GIMN[Deamid]SFLN[Deamid]DIFER
	AM[Oxid]GIM[Oxid]NSFLNDIFER
>sp|Q92522|H1X_HUMAN Histone H1x OS=Homo sapiens GN=H1FX PE=1 SV=1	
	YSQLVVETIR
>sp|P62805|H4_HUMAN Histone H4 OS=Homo sapiens GN=HIST1H4A PE=1 SV=2	
	TVTAMDVVYALK
	RKTVTAMDVVYALKR
	RKTVTAM[Oxid]DVVYALKR
	DNIQGITKPAIR
	DNIQ[Deamid]GITKPAIR
	DN[Deamid]IQGITKPAIR
	DNIQGITKPAIR[Methyl]
	DNIQGITKPAIRRLAR
	TVTA[A_42]M[Oxid]DVVYALK
	TVT[T_42]AM[Oxid]DVVYALK
	GLGK[Acet]GGAK[Acet]R
	GLGK[Acet]GGA[A_42]KR
	DAVTYTEHAKRK
	KTVTAMDVVYALK
	KTVTAM[Oxid]DVVYALK
	TVTAMDVVYALKR
	TVTAMDVVYALK[Methyl]R
	DNIQGITKPAIRR
	TVTAM[Oxid]DVVYALKR
	VFLEN[Deamid]VIR
	KTVTA[A_42]M[Oxid]DVVYALK
	KTVT[T_42]AM[Oxid]DVVYALK
	KT[T_42]VTAM[Oxid]DVVYALK
	K[Acet]TVTAM[Oxid]DVVYALK
	VFLENVIR[Methyl]
	TVTA[A_42]M[Oxid]DVVYALKR
	VFLENVIR[tri-Methyl]
	KTVTAMDVVYALKR
	DAVTYTEHAK
	ISGLIYEETR
	ISGLIYEETR[Methyl]
	ISGLIYEETR[tri-Methyl]
	RISGLIYEETR
>sp|Q09028|RBBP4_HUMAN Histone-binding protein RBBP4 OS=Homo sapiens GN=RBBP4 PE=1 SV=3	
	TVALWDLR
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	NDEELNKLLGK
>sp|P04908|H2A1B_HUMAN Histone H2A type 1-B/E OS=Homo sapiens GN=HIST1H2AB PE=1 SV=2	
	NDEELNKLLGR
	VTIAQGGVLPNIQ[Deamid]AVLLPK
	VTIAQGGVLPN[Deamid]IQAVLLPK
	VTIAQ[Deamid]GGVLPNIQAVLLPK
	VTIAQGGVLPN[Deamid]IQ[Deamid]AVLLPK
	VTIAQ[Deamid]GGVLPNIQ[Deamid]AVLLPK
	VTIAQGGVLPNIQ[Deamid]AVLLPK[Methyl]
	VTIAQGGVLPNIQ[Deamid]AVLLPKK
	VTIAQGGVLPN[Deamid]IQAVLLPKK
	VTIAQ[Deamid]GGVLPNIQAVLLPKK
	VTIAQGGVLPN[Deamid]IQAVLLPK[Methyl]
>sp|P16401|H15_HUMAN Histone H1.5 OS=Homo sapiens GN=HIST1H1B PE=1 SV=3	
	KATGPPVSELITK
	NGLSLAALKK
	ALAAGGYDVEK
	ATGPPVSELITK
>sp|Q13547|HDAC1_HUMAN Histone deacetylase 1 OS=Homo sapiens GN=HDAC1 PE=1 SV=1	
	MTHNLLLNYGLYR
	YGEYFPGTGDLR
>sp|Q8TEK3|DOT1L_HUMAN Histone-lysine N-methyltransferase H3 lysine-79 specific OS=Homo sapiens GN=DOT1L PE=1 SV=2	
	QLDGLAGLK[tri-Methyl]GEGSR
>sp|Q96QV6|H2A1A_HUMAN Histone H2A type 1-A OS=Homo sapiens GN=HIST1H2AA PE=1 SV=3	
	LLRKGNYAER
	SSRAGLQFPVGR
	HLQLAIR
	HLQ[Deamid]LAIR
	AGLQFPVGR
	AGLQFPVGR[Methyl]
	HLQLAIRN[Deamid]DEELNK
	LLGGVTIAQGGVLPNIQ[Deamid]AVLLPK
	LLGGVTIAQGGVLPN[Deamid]IQAVLLPK
	LLGGVTIAQ[Deamid]GGVLPNIQAVLLPK
>sp|Q71UI9|H2AV_HUMAN Histone H2A.V OS=Homo sapiens GN=H2AFV PE=1 SV=3	
	ATIAGGGVIPHIHK
>sp|O75367|H2AY_HUMAN Core histone macro-H2A.1 OS=Homo sapiens GN=H2AFY PE=1 SV=4	
	NC[Carboxymethyl]LALADDK
	SIAFPSIGSGR
	NC[Carboxymethyl]LALADDKK
	LEAIITPPPAK
	EFVEAVLELR
	NGPLEVAGAAVSAGHGLPAK
	GKLEAIITPPPAK
	AISSYFVSTMSSSIK
>sp|Q9P0M6|H2AW_HUMAN Core histone macro-H2A.2 OS=Homo sapiens GN=H2AFY2 PE=1 SV=3	
	AGVIFPVGR
>sp|Q16695|H31T_HUMAN Histone H3.1t OS=Homo sapiens GN=HIST3H3 PE=1 SV=3	
	STELLIRKLPFQR
	STELLIR
	YQKSTELLIR
	STELLIR[Methyl]
	EIAQDFK
	EIAQ[Deamid]DFK
	EIAQDFK[Methyl]TDLR
	EIAQ[Deamid]DFK[Methyl]TDLR
	EIAQDFK[di-Methyl]TDLR
	VTIM[Oxid]PKDIQLAR
	RYQKSTELLIR
	KS[S_42]APATGGVK
	K[Acet]SAPATGGVK
	K[K_56]SAPATGGVK
	K[Methyl]S[S_42]APATGGVK
	YRPGTVALR
>sp|P10412|H14_HUMAN Histone H1.4 OS=Homo sapiens GN=HIST1H1E PE=1 SV=2	
	K[Acet]ATGAATPK[tri-Methyl]K[di-Methyl]
	S[S_42]ETAPAAPAAPAPAEK

FIGURE 3. Differential abundance of nuclear fraction histones and histone modification proteins in mesenchymal stem cells with acute DNA damage (2 hr treatment with bleomycin) and DNA damage-induced cellular senescence (cellular recovery after 5 days of post-bleomycin treatment).

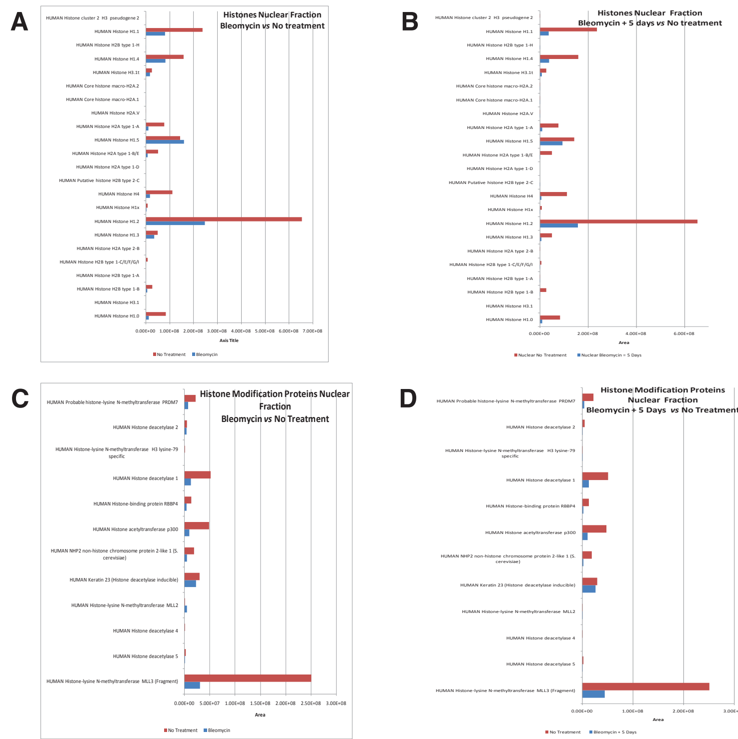


FIGURE 4. Differential abundance of chromatin associated histones and histone modification proteins in mesenchymal stem cells with acute DNA damage (2 hr treatment with bleomycin) and DNA damage-induced cellular senescence (cellular recovery after 5 days of post-bleomycin treatment).

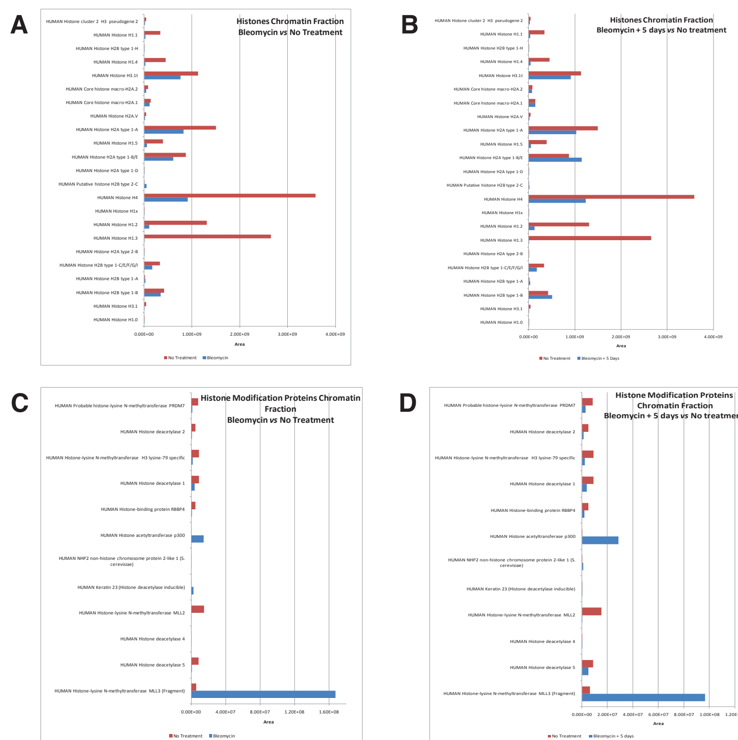
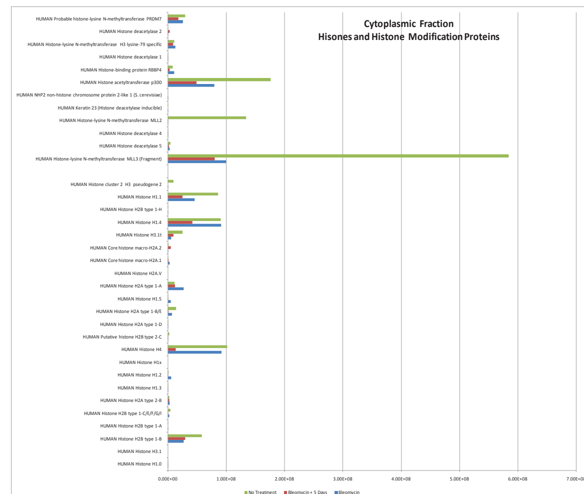


FIGURE 5 Differential abundance of cytoplasmic fraction histones and histone modification proteins in mesenchymal stem cells with acute DNA damage (2 hr treatment with bleomycin) and DNA damage-induced cellular senescence (cellular recovery after 5 days of post-bleomycin treatment).



Conclusion

- We have developed a real-time, intelligent acquisition strategy for HR/AM global targeted quantification of histones, histone PTMs, and histone modification enzymes in primary human stem cells upon acute DNA damage and during drug-evoked senescence.
- Assessment of the histone H1, H2a, H2B, H4, and H3 family member abundance in the soluble nuclear fraction of the cells subjected to genotoxic drug-induced senescence (5 days after exposure to bleomycin), demonstrated that the dynamics of histone binding in the senescent cells changes significantly (Figure 3B). Our data also suggest that the composition of the nucleosomal particles undergoes dramatic changes upon senescence. We have observed significant reduction in chromatin-bound histone H4 and all of the members of the H1 family (Figure 3B). These data suggest that with reduction of H1 histone family members in the chromatin of senescent cells, the overall compaction of chromatin fiber decreases dramatically.
- The representation of macro H2A histone does not change with senescent-specific transformation. Levels of H2B type1-A and type1-B histones also do not change significantly. These data suggest histone type specificity in chromatin dynamics upon reaching a senescent state.
- A dramatic increase in chromatin-bound HAT p300 and HMT MLL3 correlates well with the loss of histone 1 family members from the chromatin. These data support the hypothesis that relaxation of chromatin compaction in senescent cells might be a leading cause of increased transcriptional noise (transcriptional leakage) upon senescence.

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A Phospho-Peptide Spectrum Library for Improved Targeted Assays

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Overview

Purpose: Our long-term goal is quantification of peptides phosphorylated at specific sites. We explore ways of designing appropriate targeted methods.

Methods: We acquire MS/MS spectra on synthetic phospho-peptides and compile them into a spectrum library.

Results: Side-by-side spectrum comparisons of peptide sequences modified at different residues reveal diagnostic fragments which can inform targeted assay development.

Introduction

The profound and diverse effects of protein phosphorylation have created a keen interest in their characterization and quantification. However, they present unique challenges. Fragmentation of phosphorylated peptides is less predictable than unmodified peptides, making more difficult the prediction of reliable fragment ions to monitor in targeted assays. The presence of multiple isoforms that are not chromatographically resolved present an additional complication in that one must rely on specific product ions to distinguish between different modified forms since all forms will share the same intact mass. To find diagnostic transitions, we turned to empirical observation of synthetic phospho-peptides.

Methods

Sample Preparation

Synthetic peptides were prepared for 25 unique sequences containing between 2 and 6 different phosphorylation sites for each (Figure 1). They were mixed into pools of 8 peptides such that no two modified forms of the same sequence were mixed together.

Mass Spectrometry

Collision-induced dissociation (CID) and higher-energy C-trap dissociation (HCD) tandem mass spectrometry (MS/MS) spectra were acquired on a Thermo Scientific™ LTQ Orbitrap™ mass spectrometer. Several spectra for each peptide were acquired by sampling across the whole elution profile.

Data Analysis

Spectra were collected in a spectrum library that creates an averaged spectrum from all observations from one peptide at each charge state and activation type. A software tool was developed to predict and locate b- and y-type ions for all peptide isoforms in the library spectra.

FIGURE 1. Synthetic peptides and their modifications. Red letter indicates a phosphorylation site. A consolidated spectrum is built in the library when there are at least five spectra acquired for a peptide. Some peptides were observed at multiple charge states.

Phospopeptide sequence	Consolidated spectrum CID +2	CID +3	CID +4	HCD +3
AGGKPSQSPSQEAAGEAVLGAK		✓		
AGGKPSQSPSQEAAGEAVLGAK				
AGGKPSQSPSQEAAGEAVLGAK				
AGGKPSQSPSQEAAGEAVLGAK				
ETTTSPKKYYLAEK		✓		
ETTTSPKKYYLAEK				
ETTTSPKKYYLAEK				
ETTTSPKKYYLAEK				
ETTTSPKKYYLAEK				
FGESDTENQNNK	✓			
FGESDTENQNNK				
FGESDTENQNNK				
FSDQAGPAIPTSNSYSK	✓	✓		
FSDQAGPAIPTSNSYSK				
FSDQAGPAIPTSNSYSK				
GRRSPSPGNSPSGR		✓		
GRRSPSPGNSPSGR				
GRRSPSPGNSPSGR				
ILSDVTHSAVFGVPASK	✓	✓		
ILSDVTHSAVFGVPASK				
ILSDVTHSAVFGVPASK				
IQPSSPPPNHPNNHLFR		✓	✓	
IQPSSPPPNHPNNHLFR				
IQPSSPPPNHPNNHLFR				
LQTVHSIPLTINK	✓	✓		
LQTVHSIPLTINK				
LQTVHSIPLTINK				
LRSADSENALSVQER	✓	✓		
LRSADSENALSVQER				
LRSADSENALSVQER				
RLSSTSLASGHSVR		✓		
RLSSTSLASGHSVR				
RLSSTSLASGHSVR				
SFNGSLKNVAVDELSR	✓	✓		
SFNGSLKNVAVDELSR				
SFNGSLKNVAVDELSR				
SNSTSSMSSGLPEQDR	✓			
SNSTSSMSSGLPEQDR				
SNSTSSMSSGLPEQDR				
SNSTSSMSSGLPEQDR				
SNSTSSMSSGLPEQDR				
SNSTSSMSSGLPEQDR				
SNSTSSMSSGLPEQDR				
SGGQRHSPLSQR		✓		
SGGQRHSPLSQR				
SGGQRHSPLSQR				
SPGPSSPKEPLLFSR	✓	✓		✓
SPGPSSPKEPLLFSR				
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SQSDIFSR	✓			
SQSDIFSR				
SQSDIFSR				
SRNSPLLER		✓		
SRNSPLLER				
SRNSPLLER				
SRTPPSAPSQSR	✓	✓		
SRTPPSAPSQSR				
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SSSFREMDGQPER	✓			
SSSFREMDGQPER				
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SSSFREMDGQPER				
SSSPTQYGLTK	✓			
SSSPTQYGLTK				
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SSSPTQYGLTK				
STFHAGQLR	✓			
STFHAGQLR				
STFHAGQLR				
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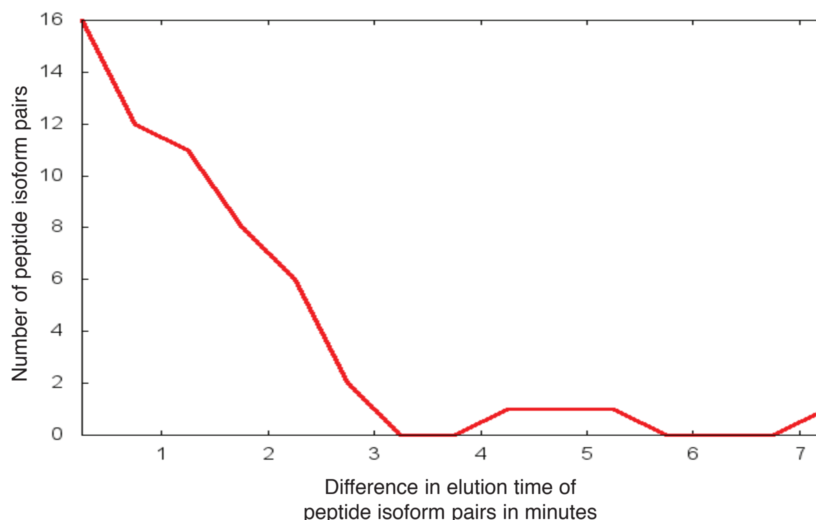
STLVLHDLLK				
STLVLHDLLK				
STVASMMHR	✓			
STVASMMHR				
STVASMMHR				
VKEEGYELPYNPATDDYAVPPPR		✓		✓
VKEEGYELPYNPATDDYAVPPPR				
VKEEGYELPYNPATDDYAVPPPR				
VQTTPPPAVQGQK	✓			
VQTTPPPAVQGQK				
VQTTPPPAVQGQK				
YIEDEDYYK	✓			
YIEDEDYYK				
YIEDEDYYK				

Results

Retention Time Does Not Resolve Most Isobaric Peptides

We compared the retention time of each peptide to its other modified forms. The majority of peptides eluted within a minute of each other on a 60-minute gradient, which may not be enough separation to confidently differentiate one from another (Figure 2).

FIGURE 2. Histogram of retention time separation of pairs of isobaric phosphopeptides on a 60-minute gradient. For example, the peptide LQTVHSIPLTINK phosphorylated at position 3 (T) eluted just 3 seconds before the same peptide phosphorylated at position 6 (S).



Library Consolidated Spectra Illustrate Diagnostic Peaks

The spectrum library combines all observed spectra for a peptide, keeping only those peaks that are common to the majority of spectra. This is a distinct advantage over using a single observation to design targeted assays as it accounts for variability seen even in high-intensity fragment ions (Figure 3).

The library also provides a mechanism for finding and storing fragment ions that differ between isobaric peptides. Ideally, one could predict which y-ions will shift with the different location of a phosphorylation. Figure 4 illustrates such an example. The y_8 ion is the only one predicted to differ between the two isoforms of singly-phosphorylated STFHAGQLR. The y_8^{2+} ion is observed in both spectra. Since this is not the case for all peptides, we considered several alternative strategies for differentiating between isoforms.

FIGURE 3. Variability of fragmentation. (A) The library's consolidated spectrum built from the seven spectra observed. (B–D) Examples of three of the observed CID spectra, precursor charge +2 for peptide ETTTSPKKYYLAEK (phosphorylated serine). The observed spectra are shown as processed by the library (noise peaks removed). The consolidated spectrum contains only the peaks shared across the observed spectra.

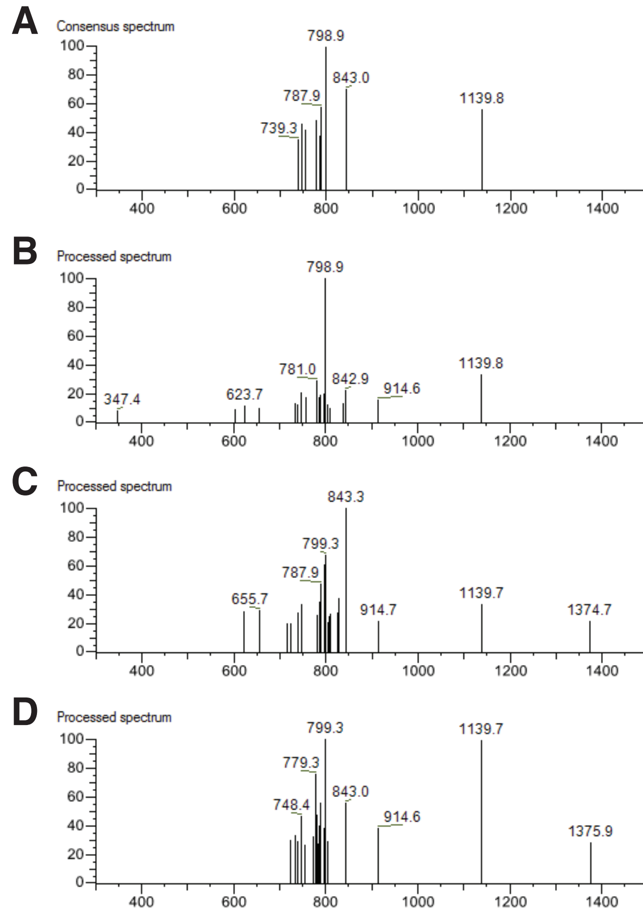
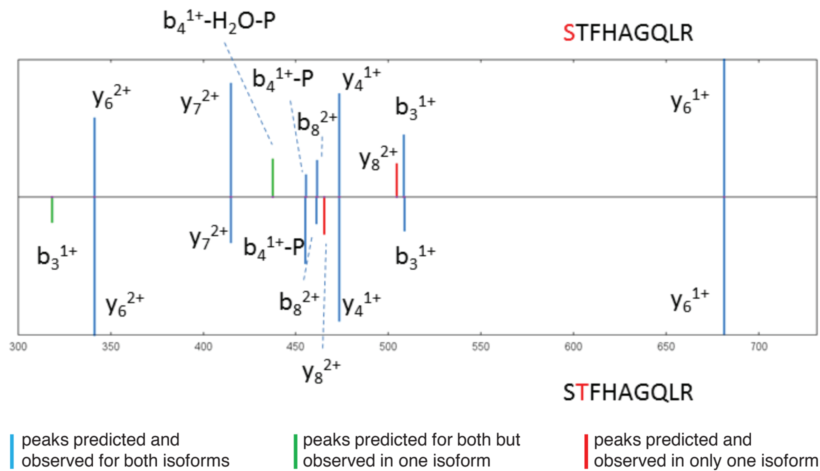


FIGURE 4. Library spectra for two isoforms of peptide STFHAGQLR. Red S or T in the peptide sequence indicates the site of phosphrylation. Peaks are color-coded according to which isoforms they are common to. One peak is unique to each isoform. Some peaks predicted in both forms are only observed in one.



Adjusting Normalized Collision Energy can Produce Diagnostic Peaks

Fragmenting a peptide at different normalized collision energies (NCE) can result in different observed peaks. When our initial spectra did not yield any diagnostic peaks, we tried different NCE values. Figure 5 illustrates one peptide at three different collision energies, each with a slightly different fragmentation pattern. For lower NCE values we see more diagnostic peaks unique to this isoform. This trend was common for many but not all of the 43 peptides examined.

In The Absence of Diagnostic Peaks

We find that theoretically diagnostic peaks specific to one modified isoform are not always observed. In those cases, the library reveals less-easily predicted differences. Figure 6 illustrates an example of how different relative intensities can distinguish between isoforms even if all of the same peaks are observed in both spectra.

FIGURE 5. Library spectra for one isoform of peptide FGESDTENQNNK acquired at different normalized collision energies (NCE). Peaks are color-coded according to which isoforms they are common to. Blue peaks are predicted for both isoforms. Red peaks are predicted only for this isoform (modified at serine [S], position 4).

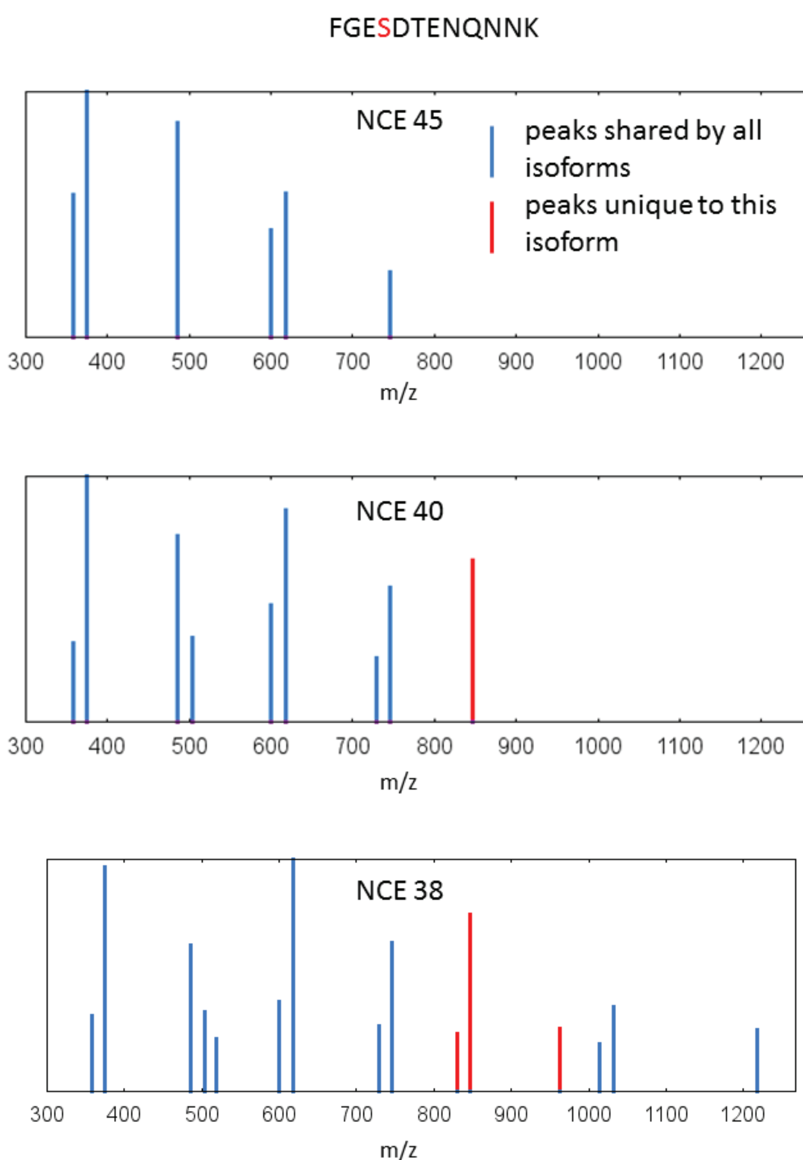
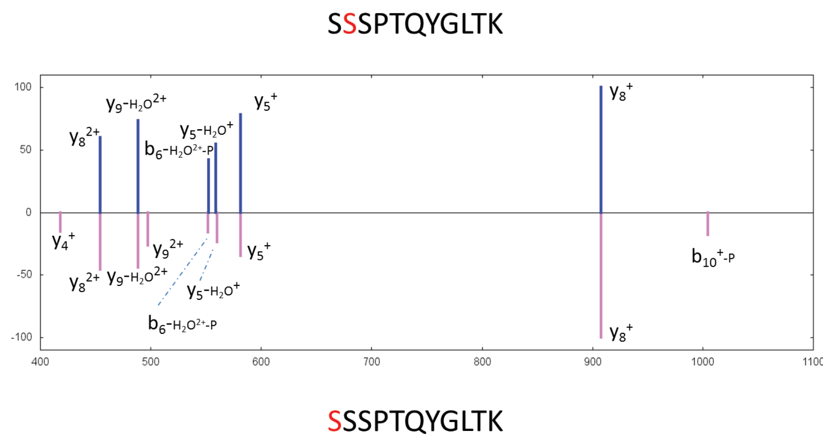


FIGURE 6. Library spectra for two isoforms of peptide SSPTQYGLTK, phosphorylation sites in red. Both are consolidated from CID observed spectra, precursor +2, 19 observations for modification at position 2 (above) and 24 observations for modification at position 1 (below). Very similar fragmentation was seen for these isobaric peptides. Only the y_{10} ion will differ in mass between them and it is not observed in either. However, three predicted b- or y-ions are seen in one form and not the other. The relative intensities are also substantially different.



Conclusion

A spectrum library of synthetic phospho-peptides provides an invaluable starting point for designing targeted assays, particularly for distinguishing isobaric peptides.

- A library spectrum comprised of several observations outperforms a single observed spectrum by identifying consistent peaks and summarizing variability.
- Library spectra are a convenient mechanism for finding and storing diagnostic differences in fragmentation between isobaric peptides.
- Some NCE values will produce more diagnostic peaks than others.
- When no diagnostic peaks are observed, the relative intensities of peaks can distinguish between isoforms.

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Improving Label-Free Quantification of Plasma and Serum Proteins Using a High-Resolution Hybrid Orbitrap Mass Spectrometer

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Overview

Assessing the differences between MS1- and MS2-based label-free relative quantification in a complex plasma matrix using a novel real-time, intelligent acquisition strategy for high-resolution, accurate-mass (HR/AM) global targeted quantification.

Introduction

Label-free mass spectrometry (MS) is an increasingly preferred method for biomarker discovery workflows applied to serum and plasma samples. Given the right conditions, label-free relative quantification is cleaner, simpler, and higher throughput. Resulting differential analysis from these label-free discovery experiments often leads to targeted analyses for verification. High resolution and mass accuracy are critical components to successfully identifying and quantifying peptides in a label-free experiment. Here we present a real-time intelligent acquisition strategy for HR/AM targeted quantification and compare it to relative quantification from MS1 full scan spectra, and introduce a strategy that enables higher confidence in both qualitative and quantitative results in the label-free discovery runs. We propose using HR/AM MS and MS/MS schemes in conjunction with validated spectral libraries for automated method building, data acquisition, verification, and quantification in real-time using novel acquisition schemes.

Methods

Sample Preparation

A protein mixture consisting of eight proteins — cytochrome c (horse), α-lactalbumin (bovine), serum albumin (bovine), carbonic anhydrase (bovine), ovalbumin (chicken), α-S1-casein (bovine), α-S2-casein (bovine), β-casein (bovine) — was prepared at equimolar ratios. The eight non-human proteins were analyzed at 100 fmol on column in a "neat" background as well as 100 fmol on column spiked into a human plasma matrix of 1ug on column. The eight proteins were also investigated in the human plasma matrix at varying amounts ranging from 0.5 to 500 fmol each protein on column.

MS Data Acquisition and Analysis

All samples were digested with trypsin and analyzed on a Thermo Scientific™ Q Exactive™ mass spectrometer equipped with a Thermo Scientific™ Nanospray Flex Ion Source . Data was acquired in two steps to simulate traditional workflows. Initial experiments employed unbiased data-dependent MS/MS acquisition resulting in peptide/protein identification as well as building of a spectral library. These initial data-dependent runs were run on both the "neat" conditions of the eight protein mix (without the plasma matrix), and then on a 100 fmol level (each protein) on column in a plasma matrix of (1 μg plasma on column). These initial data-dependent runs were searched against a modified human database containing the eight additional proteins. The combined results from the discovery experiments were used to build a local spectral library consisting of precursor and product ion *m/z* values and relative abundance distribution as well as relative retention time values. A highly multiplexed, targeted protein list was created from the spectral library and used for automated data acquisition and processing real-time to facilitate changes to the acquisition scheme. For full description of acquisition method and scheme, please visit poster 131 on Tuesday, by Prakash *et. al*.[1]

Thermo Scientific™ Proteome Discoverer™ version 1.3 and Thermo Scientific™ Pinpoint™ version 1.3 software packages were used to analyze both the qualitative and quantitative data. The spectral library resulting from initial runs was used to create a targeted inclusion list and reference information to perform qual/quan determination in real time. Data were acquired and peptide coverage and relative quantification were measured for each of the eight standard proteins. All samples were run in triplicate.

Results

Intelligent Real-Time Data Acquisition

The discovery experiments were performed in an unbiased data-dependent acquisition for the eight protein mixtures in "neat" conditions as well as in a complex plasma matrix. From these initial results, 170 target peptides from the eight proteins were used to build the spectral database, Figure 1. These 170 targets were built into a spectral library look-up table that was used in real-time state modeled acquisition. The look-up table includes the precursor *m/z* values for the defined charge state as well as the expected retention time window, which are used to initiate product ion spectral acquisition based on the presence of multiple precursor isotopes during the expected elution window (Figure 2).

FIGURE 1. Histogram showing the number of targeted peptides with confident MS2 peak area quantification per femtomolar level of protein mixture. The bars represent the number of confident areas quantified out of the potential 170 targets on the spectral library look-up table.

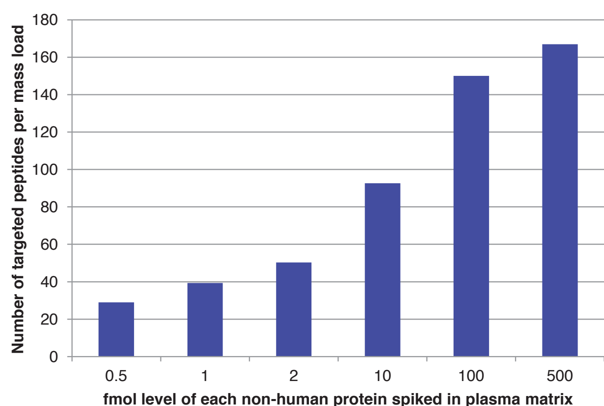
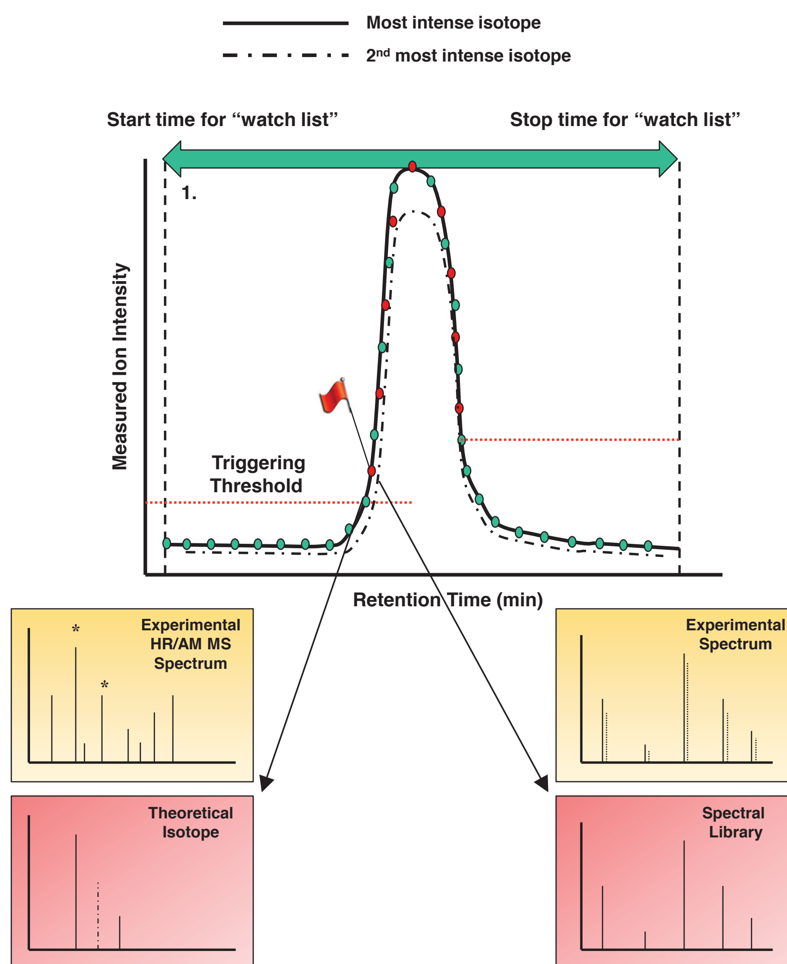


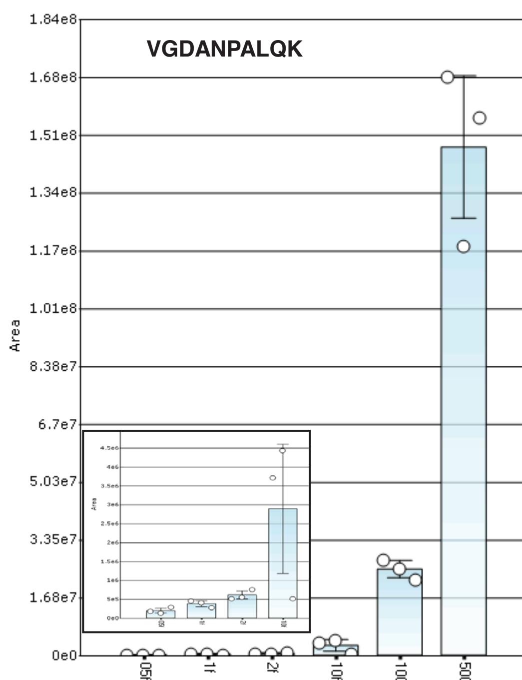
FIGURE 2. Pictorial representation of high IQ data acquisition schemes for targeted peptide quantification using a targeted scanning window, target elution identification, and real-time product ion spectral acquisition. Both precursor and product ion spectral matching is performed to increase the selectivity of data acquisition.



Our current MS-based biomarker discovery studies employ label-free quantification of proteomic data using MS1 extracted ion chromatograms (XIC). In our typical discovery experiments, we get MS1 quantification at signal threshold levels as low as $1e^5$. However, when employing powerful software such as Pinpoint software, we can verify that some of the MS1 quantification may be false positives (demonstrated by loss of multiple isotope peaks or isobaric contaminants).

In this study, we compare the novel real-time state modeled acquisition based MS2 quantification with full scan MS1 (XIC) based quantification (Figure 3). As a general observation, we see that the quality of the MS1 quantification for peptides above 2 femtomoles, the number of targeted peptides we quantify with confidence, is on-par with the MS2-based quantification. However, below 2 femtomoles, the quantity and quality of peptides quantified based on MS1 XIC, drops to nearly 50% of MS2. There is 25–50% false positive quantification at the 0.5–1 fmol level peptides.

FIGURE 3. MS2 Peak area as a function of mass load and their corresponding variance per run for peptide VGDANPALQK. Inset: Expanded view of the 0.5 to 10 fmol level.



Figures 4 and 5 demonstrate the increased confidence that is attained with real-time state-modeled MS2 data quantification. The advantage of this acquisition scheme is evident at levels of proteins and peptides below 10 fmol on column (or at signal thresholds below 1 e^4). There are cases in our study where both MS1 and MS2 do not quantify a species; however, it is important to note that there are significantly fewer false-positives with MS2 quantification.

FIGURE 4. Peak profiles for peptides identified at each femtomolar level, for MS1 quantification (A, C, E) and for MS2 quantification (B, D, F). (A) MS1 peak profiles for peptide VGDANPALQK. (B) MS2 peak profile view of VGDANPALQK. Insets (A) and (B) are expanded views of 0.5 to 2 fmol level. (C) and (D) MS1 and MS2 peak profiles, respectively, for peptide LGEYGFQNALIVR. Insets (C) and (D) are expanded views of 0.5 to 2 fmol level. (E) and (F) MS1 and MS2 peak profiles, respectively, for peptide FALPQYLK. Inset (E) shows an expanded view of probable isobaric contamination from neighboring peak. Inset (F) shows MS2 confidence, 10 fmol level quantification. (G) and (H) MS1 and MS2 peak profiles, respectively, for peptide DM[Oxid]PIQAFLLYQEPVLGPVR. Inset (H) shows an expanded view of peak profile for the product ions for the representative peptide at 10 fmol level.

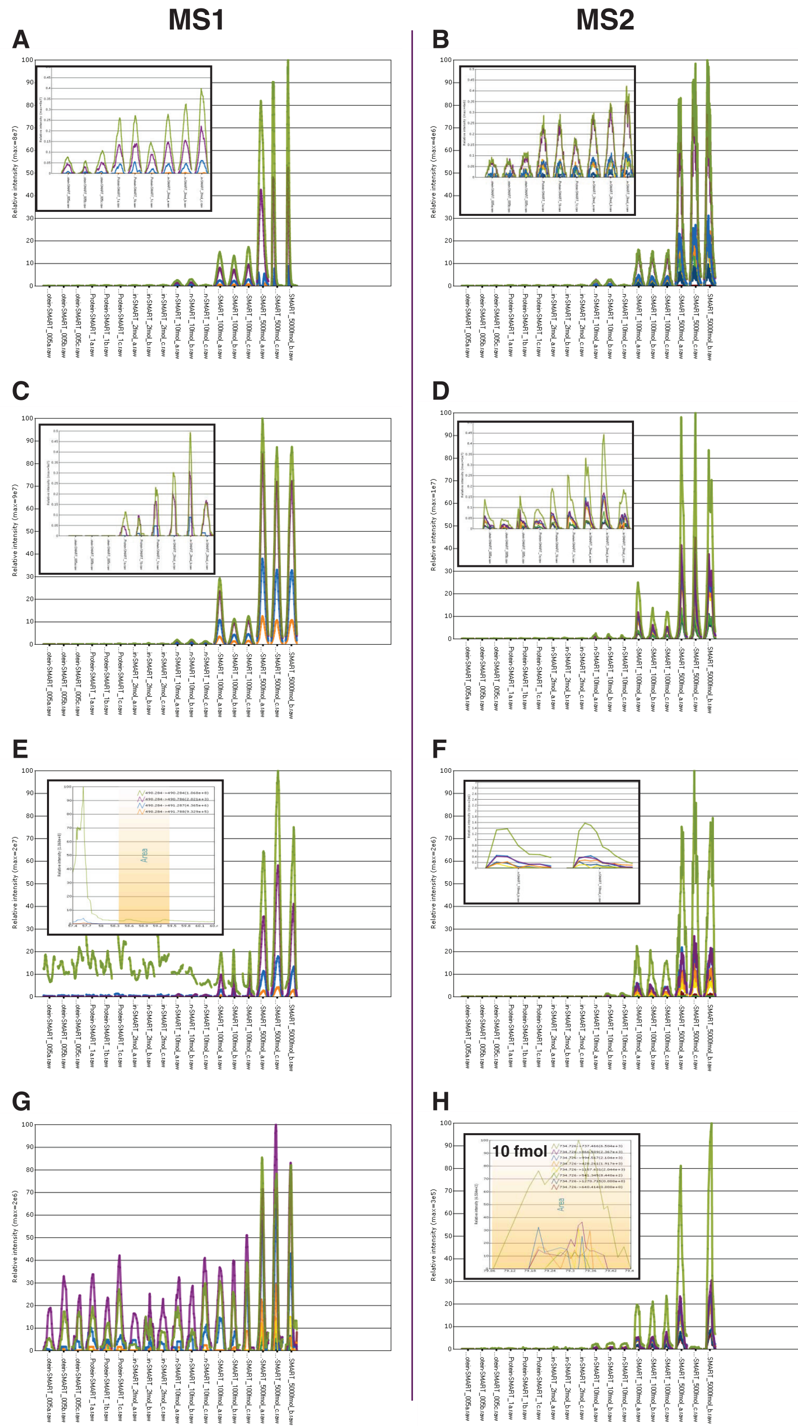
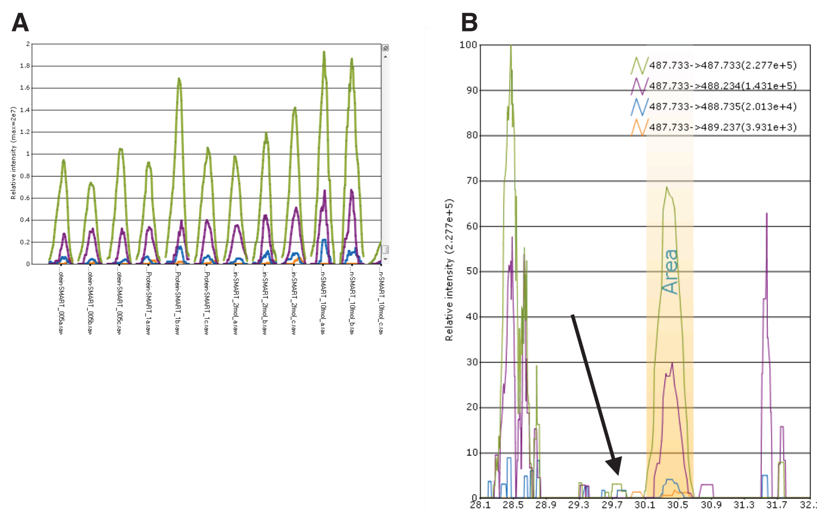


FIGURE 5. (A) False positive MS1 peak profiles for peptide DLGEEHFK at 0.5 to 10 fmol level. (B) XIC for 487.733 isotopes, illustrating probable isobaric contaminants; (arrow) points to observed elution time for peptide DLGEEHFK at higher levels.



Conclusion

- The development of the real-time state modeled data acquisition provides quantification of peptide species at lower concentrations and lower signal thresholds (below 1 e^4).
- Real-time state modeled data acquisition results in at least 50% more identifications than MS1 quantification below 10 fmol.
- This novel data acquisition scheme provides higher sensitivity and selectivity of peptides in a label-free complex matrix – ideal for a biomarker discovery workflow.

References

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Detection of Cellular Response to an in vitro Challenge with Bacterial Gram-Negative Lipopolysaccharides (LPS) in Peripheral Blood Mononuclear Cells (PBMCs) for Biomarker Research

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Overview

Purpose: To find differentially expressed marker proteins for sepsis in an *in vitro* model environment.

Methods: Blood from healthy volunteers is treated with toxic ligands secreted by gram-negative bacteria. PBMCs are isolated, reduced, alkylated, digested with trypsin. The resulting peptides are analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Differential peptides are identified by Thermo Scientific™ Proteome Discoverer™ software and compared using Thermo Scientific™ Pinpoint™ software.

Results: Full scan quantification of several hundred relevant kinase and pathway specific proteins generated from over 4000 identified proteins.

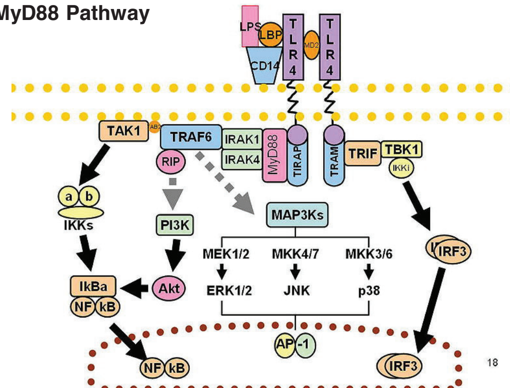
Introduction

Gram-negative bacteria, and a major component, lipo-polysaccharides (LPS), are associated with sepsis. In this study, we look at global protein profiling of mononuclear cells from LPS-challenged whole blood.

Mononuclear cells are easy to collect and have little of the protein dynamic range difficulties associated with plasma. In addition, they are responsive to many immune state conditions, making them ideal targets for biomarker discovery experiments. Using an *in vitro* stimulation using a whole blood system directly in tubes used for the isolation allows for a highly facile method for looking for changes in either secreted proteins in the plasma fraction or for quick-onset protein changes in the PBMC cell fraction.

As most gram-negative sepsis infections are from *E. coli*, we chose the corresponding LPS. LPS from rod shaped bacteria stimulates the specific Toll like receptor 4(TLR4) in the MyD88 pathway. Toll like receptors are part of the innate immune response pathways.

FIGURE 1. MyD88 Pathway



Cascades in this pathway involve many signaling events that are either proteolytic cleavages, phosphorylations or other modifications. The large number of human proteins and their associated post-translational modifications (PTM) represent a challenge for MS-based biomarker discovery. To this effect, the simplest sample preparation techniques will provide the most reproducible results.

Methods

Sample Preparation

Blood samples from a healthy single donor were collected into BD Vacutainer™ CPT Cell Separation Tubes (Becton Dickinson) in accordance with IRB approval. Buffers and stimulant solutions were injected directly into the blood collection tubes using a 1 mL syringe with a 27 ga needle. Control tubes had 200 μL of phosphate buffered saline added and were prepared in parallel to the stimulated tubes. LPS-EB Toll Like Receptor 4 Ligand (InvivoGen, San Diego, CA) was added to a concentration of 100 ng/mL (Low stim) and 10 μg/mL (High stim) of whole blood.

After incubation at 37 ºC for 30 min the cells were isolated according to the manufacturer's instructions for a total exposure time of 60 min. Rinsed cell pellets (~2 mg) were denatured in 350 µL of 8M Urea 300 mM Tris-HCl 2.5% n-propanol 10 mM Dithiothreitol, reduced/alkylated, diluted to 2 mL with 50 mM Tris, 5 mM CaCl2 and digested overnight with 20 µg of Pierce Trypsin Protease, MS Grade.

Liquid Chromatography

Peptide retention time standards were added and the samples were loaded into 96-well plates onto a Thermo Scientific™ EASY-nLC™ 1000. Separations were done on a Thermo Scientific™ Dionex™ PS-DVB trap column, (5 µm particle, 300Å pore, 150 µm x 12 cm) connected in a "vented t" configuration to a 5 µm particle, 200Å pore C18AQ 100 µm x 50 cm packed tip resolving column in a Thermo Scientific™ Nanospray Flex™ Ion Source on a hybrid Thermo Scientific™ Orbitrap Velos Pro™ MS. Stepped Flow and gradient from 4-50%B at 650 nL/min over 205 min. Buffer A is 2% Methanol 0.2% formic acid, water(v/v). Buffer B is 10% water, 10% isopropanol, 80% acetonitrile 0.2% formic acid (v/v), all solvents are Thermo Scientific™ Optima™ LC-MS grade.

Portions of each of the TLR4 digests (Low and High stim) pooled for library creation were fractionated into 12 fractions of 1.8 ml, on a 4.6 mm x 25 cm PS-DVB column 8 µm particle 300A pore, buffer A: 100 mM ammonium formate, 58 mM ammonium hydroxide, Buffer B: 29 mM ammonium hydroxide in 91% acetonitrile 9% water (v/v), using a flow rate of 1 mL/min in a gradient to 45% B (Figure 2).

Mass Spectrometry

For mass spectrometric analysis, a data-dependent top 25 method has been used . Full MS scans acquired at a resolution of 100,000 using a 1e6 target value, with dependent scans analyzed in the linear ion trap with normal scan resolution. Uncharacterized charge states and + 1 charge states are rejected. Chromatography phase triggering with monoisotopic fitting was used with a peak width of 40 s and a minimum peak threshold of 3.5e4. The maximum inject time allowed for MS/MS scans was set to 100 ms. Dynamic exclusion is turned on using a peak width of 60 s.

Data Analysis

Full-scan comparisons were made using Pinpoint software, and MS/MS spectra were processed by Proteome Discoverer software using The Mascot® search engine. Two different peptide identification strategies were used. The simple search method (Figure 3) only searches for high-confidence, tryptic peptides and phosphopeptides. The more complex search strategy (Figure 4), breaks the PTM search strategy into multiple nodes, where small groups of PTMs, likely to occur on the same peptide, are searched in each node. This allows for higher-confidence assignments due to the reduced size of each database, albeit at an increased search computational time. Pathway information was processed using Thermo Scientific™ ProteinCenter™ software (not shown). Pinpoint software allows for the import of spectral libraries which can be obtained from data from both unfractionated and fractionated samples provided the chromatography in all samples is reproducible and retention times are consistent.

FIGURE 2. High pH reverse phase fractionation for library peptide fractionation

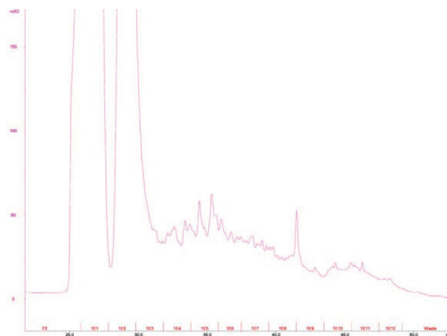


FIGURE 3. Search workflow for protein phosphorylation.

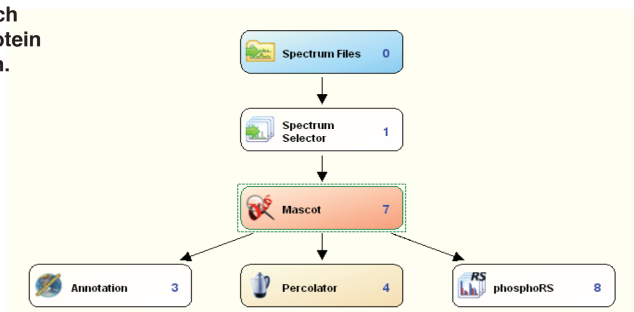


FIGURE 4. Search workflow for multiple modifications. Searches are broken up into groups of most likely to occur modifications. This search strategy is computationally intensive and works best with the high-resolution Orbitrap-analyzed MS2. Many modifications such as ubiquitination, oxidations and deamidations, semi-tryptic, and different databases can be searched even by other search engines without compromising the integrity of the results.

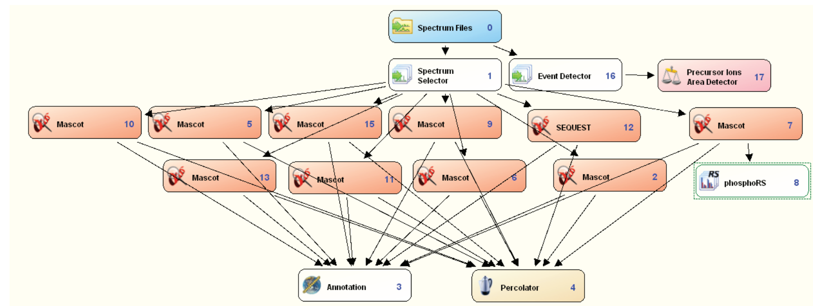
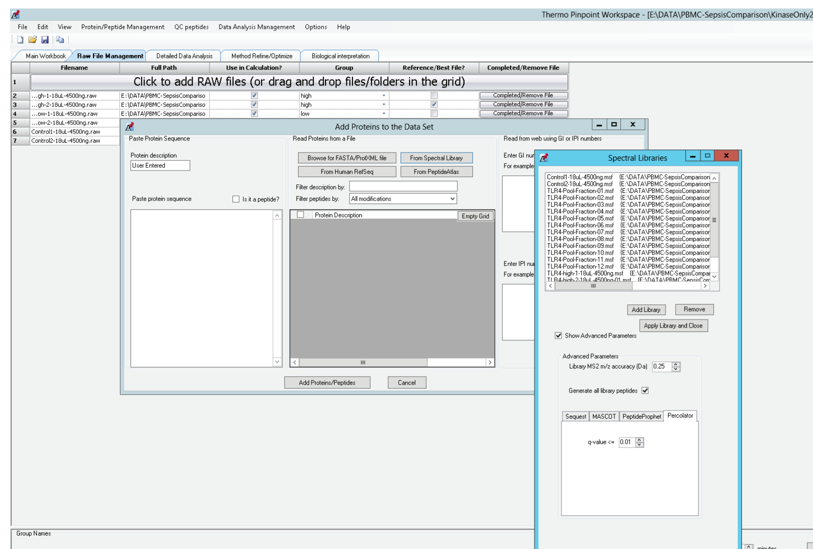


FIGURE 5. Results from different search strategies and any fractionation can then be brought into Pinpoint software through the spectral library function.



Results

In order to allow the detection of differentially expressed proteins and peptides, instrumentation should provide enough quantitative full-scan measurements while simultaneously providing MS/MS fragmentation data to allow sequencing of as many peptides as possible. In this experiment, ca 4000 proteins were identified over 95% confidence containing >250 phosphorylations, >150 ubiquitinylations, peptide oxidations (other than methionine) that were inserted into the Pinpoint spectral library. Pinpoint software has the advantage in that it allows for a selected protein/peptide library import. In this example, all kinases identified in the library can be selected for analysis. Once selected, proteins that are up or down regulated can be highlighted and verified using Pinpoint software (Figures 6, 7). In addition to protein class selection, specific proteins of interest (Figure 8) TGF beta and pathways specific components, (Figure9) MAPKKK 15, can be selected and analyzed for quantification.

FIGURE 6. Quantification of the +2 charge state of peptide GDDTPLHLAASHGHR from integrin-linked protein kinase gil4758606 [Homo sapiens] A) Comparison of Control, Low and High Stimulation B) Alignment of all isotopes.

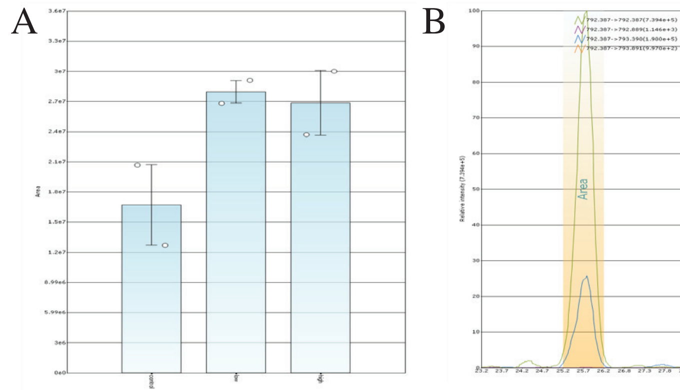


FIGURE 7. View of the replicates of sample quantifications from the first two isotopes (792.387, green: 792.889, blue) of the peptide GDDTPLHLAASHGHR in Control, Low and High stimulation experiments (pairs, left to right).

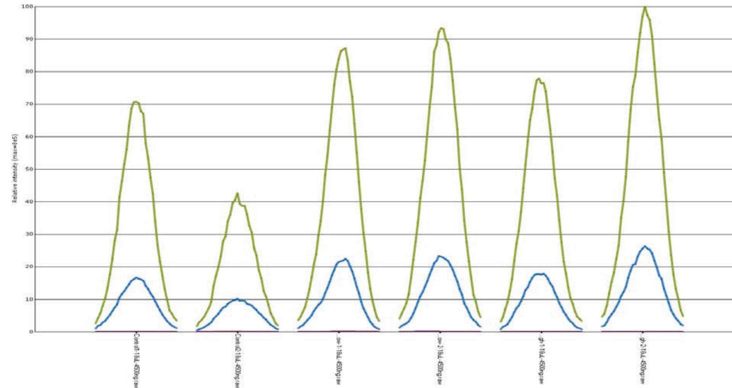


FIGURE 8. Signal Pathway proteins up-regulated from LPS stimulation. Peptide GGEIEGFR from transforming growth factor beta-1 precursor gil63025222 [Homo sapiens].

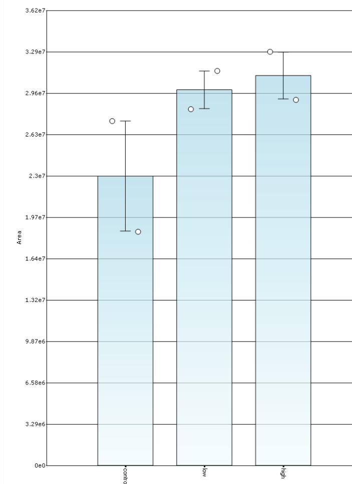
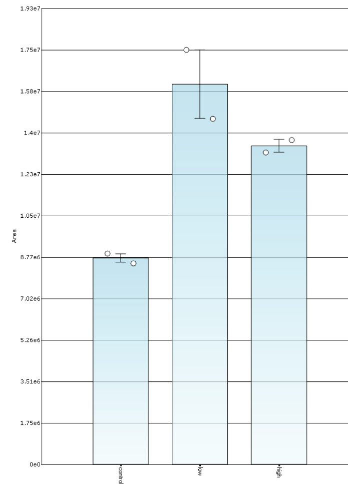


FIGURE 9. MyD88 signal pathway proteins up-regulated from LPS stimulation. Peptide QILEGLK from mitogen-activated protein kinase kinase kinase 15 gil282847398 [Homo sapiens].



Conclusion

- The *in vitro* whole blood PBMC stimulation model combined with a simple sample preparation workflow strategy allows for a facile and reproducible method of testing cell signaling in the immune response from human research samples.
- Spectral Library creation from simple fractionation techniques allows for a large number of different PTMs to be identified without using any specific PTM enrichment strategy.
- Pinpoint software allows for targeted quantification in even full scan discovery modes in complex sample mixtures.
- Future work will be to utilize the library and initial data to generate a targeted list of peptides for selected reaction monitoring (SRM) workflows on both Thermo Scientific™ TSQ Vantage™ and Thermo Scientific™ Q Exactive™ instruments. These will be to address the low level library peptides that were difficult to quantify in this workflow, but are of interest in the biological pathways that were shown to be up- and down-regulated in these samples and are also correlated with sepsis.

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Translational Research Webinars

WB64292: Routine Measurement: New Methods and Workflows for Translational Clinical Research

Mass spectrometry (MS) has been universally applied for biomarker discovery, but only recently is becoming a fixture in clinical research labs. New MS technologies have accelerated the collection of high quality, deep data from complex samples, but workflows for analyzing and efficiently processing these data have lagged behind the hardware improvements. In this webinar we will discuss complete workflows that integrate data from discovery and targeted quantification experiments to streamline and simplify translational clinical research.

From Biology to Routine Measurement: New Methods and Workflows for Translational Clinical Research

WB64305: Biomarker Discovery: Translating Proteomics into Clinical Diagnostics

In this webinar Dr Eleftherios P. Diamandis and Dr. Andrei P. Drabovich of Mount Sinai Hospital will discuss integration of -omics technologies for a selection of biomarker candidates, and focus on mass spectrometry as a principal technique for qualitative and quantitative analysis of proteins. Proteomic profiling of tissues, proximal fluids and cell lines as well as development of quantitative selected reaction monitoring (SRM) assays will be discussed.

Biomarker Discovery: Translating Proteomics into Clinical Diagnostics

WB64307: Clinical Applications of LC-MS: Development and Research Application of a Highly Sensitive LC-MS Method for Quantification of a Cholesterol Protein in Plasma

The topic of this webinar is Proprotein convertase subtilisin/kexin type 9 (PCSK9) a key player in the regulation of circulating low-density lipoprotein cholesterol (LDL-C). Both the distinct forms observed in plasma and posttranslational modifications (PTMs) described in cell-based studies are likely to affect its function and thereby LDL-C levels. ELISA kits are available for quantification, but inherently lack the discriminative power to resolve isoforms and PTMs. To address this issue, and given the complexity and wide dynamic range of the plasma proteome, we have developed and applied a Mass Spectrometry ImmunoAssay-Selected Reaction Monitoring (MSIA-SRM) method to quantify PCSK9. This web seminar will explain the development and validation of the method, including the sample preparation techniques.

Applications of LC-MS: Development and Research of a Highly Sensitive LC-MS Research Method for Quantification of a Cholesterol Protein in Plasma

WB64011: LC/MS - More Analytically Sensitive and Specific for Subtyping Proteins in Clinical Research

Protein subtyping by LC/MS offers unique advantages. It is not limited by antibody availability and is able to identify the entire proteome at a single analysis with high sensitivity and specificity. We have successfully developed a novel method for subtyping amyloid proteins using the Thermo Scientific™ Q Exactive™ Mass Spectrometer. This clinical research method offers high sensitivity and specificity for identifying amyloid proteins and will be the focus of the discussion in this video.

LC/MS - A More Analytically Sensitive and Specific Method for Subtyping of Amyloid Proteins in Clinical Research

WB64010: Development of Multiplexed MSIA (Mass Spectrometric Immunoassay)-SRM Methods for Proteins Associated with Alzheimer's Disease and Application to Plasma Samples

One of the biggest challenges in the translation of mass spectrometry (MS)-based biomarkers into the development of clinical research methods is the lack of fully developed tools and workflows to deliver the throughput specificity, sensitivity, and robustness needed for clinical applications. Immunoenrichment coupled to selected-reaction monitoring (MSIA-SRM) allows high-throughput, quantitative and highly selective detection of clinically important proteins in plasma, serum and cerebrospinal fluid. In this video, the crucial parameters that must be considered when developing MSIA-SRM assays are discussed, along with how this method has been applied to the study of Alzheimer's Disease plasma samples in a series of studies.

Development of Multiplexed MSIA (Mass Spectrometric Immunoassay) - SRM Methods for Proteins Associated with Alzheimer's Disease and Application to Plasma Samples

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