Robust Ultra High Sensitivity Quantitative Analysis Using Low Flow LC/MS/MS and Reduced Phospholipid Matrix Effects. Myth or Reality?

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Overview

Purpose: Deliver significant sensitivity gains, reduced solvent use and less suppression for routine quantitative bioanalysis with minimal method development when compared with standard (high) flow LC methods.

Summary of Results: A simple reversed-flush trap method was developed for routine quantitation. No changes were made to the existing traditional high-flow LC gradient, eliminating the need for method development. The results were compared to traditional LC-MS/MS methods and the data supports the utility of a generic, simple to setup, robust and sensitive method using micro flow rates. For the compounds analyzed to date, the LLOQ sensitivity gain was 2-20x over traditional LC-MS/MS methods with one outlier. This is achieved in part by keeping the injection volumes consistent with the traditional LC method, e.g., if a 10µL injection is utilized for a LC method, the low-flow method incorporated a 10µL injection. Initial robustness testing has demonstrated excellent injection to injection reproducibility (Alprazolam-D5 CV ~7%) over 500 injections.

Introduction

The sensitivity gains in ESI analysis using cap/micro flow LC are well documented. However, these gains are neutralized in routine quantitative bioanalysis primarily due to reduced loading capacity as well as solvent delivery pump constraints, non-reproducibility of capillary columns, lack of easy to use leak proof fittings, and the slow speed of analysis. Most importantly, the limited sample volume loaded on the column negates the sensitivity gains delivered by an ESI source operating at maximum efficiency. Here, we present data showing increased sensitivity for the quantitative bioanalytical workflow for drugs in plasma using a single valve trap LC setup with no ESI source hardware changes needed to support the high efficiency cap/micro flow that enables robust methodology using currently available instrumentation.

Methods

Sample Preparation

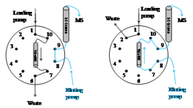
Human plasma extracts, acetonitrile precipitated (1:3) and further diluted with water + 0.1% formic acid (1:1), were used to prepare Standard Calibrators and Quality Controls containing a mixture of eight compounds plus four internal standards. For all compounds the standard curve range was 1 pg to 10,000 pg/mL and QCs were prepared at 25 pg, 250 pg and 2500 pg/mL. In both analysis approaches, traditional phospholipid ions (m/z 496, 524, 758.6, 786.6, 806.6) were monitored to gauge potential suppression effects at low flow rates. In order to ensure broad assessment of suppression differences, the matrix samples were infused and neat study samplers were injected. Therefore, there was matrix present in the ion source continously.

LC – Micro-Flow & High-Flow

The low-flow LC system was a Thermo Scientific Dionex Ultimate 3000 RSLCnano operating in Micro-Flow mode. The Micro-Flow method was run at 20 µL/min for analytical separation and 80uL/min for sample loading. The analytical column used for the Micro-Flow test was a Thermo Scientific Hypersil Gold 50 x 0.5 mm, 3 µm and the trap column was a Hypersil ™ Gold 10 x 1mm, 3 µm column. A single switch valve was used for this trap method.

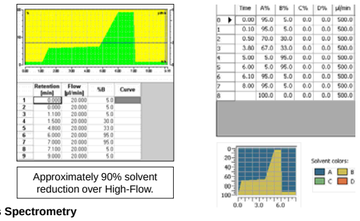
Nanoviper fittings were used to reduce dead-volume.

The traditional high flow LC system was comprised of Thermo Scientific Accela Open system with DLW (Dynamic Load and Wash) coupled to an Accela™ 1250 UHPLC pump at 500 µL/min. The column used was a Hypersil Gold 100 x 2.1 mm, 1.9 µm in a direct inject mode.



Mobile phases were water + 0.1% formic acid (v/v) and Acetonitrile + 0.1% formic acid (v/v). Identical gradients maintained for both assays with injection volumes of 10 µL.

LC Methods – (L) Micro-Flow and (R) Standard Flow



Approximately 90% solvent reduction over High-Flow.

Mass Spectrometry

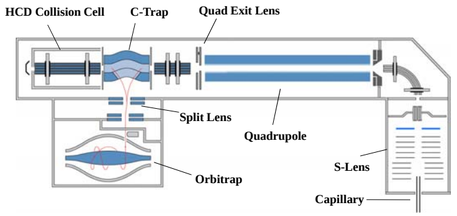
A Thermo Scientific Q-Exactive bench-top high resolution accurate mass Orbitrap mass spectrometer was used in full scan mode to monitor phospholipids and in targeted SIM mode for routine quantitation at resolutions of 70,000 or 140,000 (based on m/z 200) yielding spectral speeds of 3 and 1.5 Hz, respectively.

Generic ion source conditions were used for all sample collection including vaporizer temp. The micro-flow method source conditions were: spray voltage 4kV, vaporizer temp 200°C, capillary temp. 325°C, sheath gas 40 units, and AUX gas 5 units. The standard flow method source conditions were generic source settings of: spray voltage 4kV, vaporizer temp 475°C, capillary temp. 325°C, sheath gas 60, AUX gas 20, and sweep gas of 1 unit. The instrument was calibrated in positive ion mode before sample acquisition using Pierce LTQ Velos ESI Positive Ion Calibration Solution.

Data Analysis

Data was acquired using Xcalibur 2.2 with DCMSLink to control the RSLCnano front end. Data was processed using Thermo Scientifoc LCQuan 2.7 quantitation software.

FIGURE 1. Q Exactive Instrument Schematic



Results

Figure 2: LLOQ: Micro-Flow vs High-Flow

Standard calibrators and quality controls were analyzed using both micro-flow and standard flow LC methods for the same compound mixture prepared in human plasma extracts. The table below summarizes the LLOQ for each LC method using a +/- 20% accuracy cut-off, the gain achieved using micro-flow and the R^2 values.

Analyte	Flow Rate	LLOQ (pg/mL)	Gain (Micro/ High)	R^2
Alprazolam	Micro	5	2x	0.9940
	High	10		0.9909
Clonazepam	Micro	5	20x	0.9943
	High	100		0.9889
Clopidogrel	Micro	5	10x	0.9905
	High	50		0.9904
Ketoconazole	Micro	5	2x	0.9914
	High	10		0.9963
Oxycodone	Micro	5	10x	0.9907
	High	50		0.9908
Paroxetine	Micro	5	2x	0.9947
	High	10		0.9939
Reserpine	Micro	10	-2x	0.9896
	High	5		0.9919
Verapamil	Micro	5	2x	0.9924
	High	10		0.9888

* all linear weighting, 1/X^2

The LLOQ for each analyte was determined by using a +/- 20% cut-off for accuracy. In addition, out of the four replicates, ≥50% of the standards at the LLOQ must meet this criteria. The gain is not based on pure signal. As the summary table indicates, the improvement seen using the Micro-Flow method ranged from 2-20x. Reserpine (-2x), was the single outlier. The reason for this observation is under investigation.

Figure 3. Quality Control Summary – Micro-Flow and Standard-Flow LC Methods

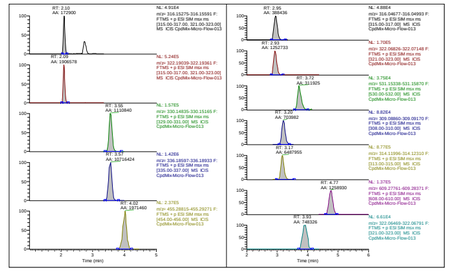
Quality Controls were measured under Micro-Flow and Standard-Flow LC conditions. Calculated concentrations were produced using standard calibrators prepared in precipitated human plasma extracts and using LCquan ™2.7 for data processing. Measured values are reported for all QCs even if they fall below the LLOQ and no QC was dropped even if accuracy was >15% from nominal. Standard accuracy was used for reporting R^2 and adopted the +/-20% cut-off for LLOQ and +/-15% cut-off for all other standards.

Micro-Flow				
Component Name	Level	Avg Calc Conc	Avg % Diff	% CV
Alprazolam	QC-L	25.5	1.88	2.06
	QC-M	254	1.54	4.75
	QC-H	2309	-7.65	2.26
Clonazepam	QC-L	23.8	-4.88	4.03
	QC-M	247	-1.29	4.38
	QC-H	2156	-13.8	1.92
Clopidogrel	QC-L	26.3	5.13	8.45
	QC-M	260	4.16	4.19
	QC-H	2181	-12.8	3.40
Ketoconazole	QC-L	23.9	-4.43	6.18
	QC-M	259	3.45	4.51
	QC-H	2288	-8.48	4.78
Oxycodone	QC-L	22.5	-10.0	4.05
	QC-M	223	-10.9	3.43
	QC-H	2228	-10.9	0.523
Paroxetine	QC-L	22.3	-10.8	3.28
	QC-M	252	0.929	2.23
	QC-H	2216	-11.4	1.55
Reserpine	QC-L	24.0	-4.09	2.93
	QC-M	261	4.21	0.757
	QC-H	2027	-19.0	0.965
Verapamil	QC-L	26.7	6.59	4.79
	QC-M	241	-3.60	5.60
	QC-H	2055	-17.8	7.57

High-Flow				
Component Name	Level	Avg Calc Conc	Avg % Diff	% CV
Alprazolam	QC-L	24.9	-0.355	4.79
	QC-M	272	8.65	2.55
	QC-H	2005	-19.8	4.24
Clonazepam	QC-L	33.8	35.2	23.4
	QC-M	246	-1.42	12.0
	QC-H	2026	-19.0	12.0
Clopidogrel	QC-L	5.43	-78.3	19.6
	QC-M	206	-17.8	14.6
	QC-H	2629	5.15	2.10
Ketoconazole	QC-L	17.7	-29.0	21.7
	QC-M	231	-7.65	7.98
	QC-H	1967	-21.3	2.21
Oxycodone	QC-L	33.9	35.7	1.96
	QC-M	247	-1.26	1.86
	QC-H	2020	-19.2	1.29
Paroxetine	QC-L	28.4	13.6	4.07
	QC-M	257	2.62	10.9
	QC-H	2209	-11.6	11.9
Reserpine	QC-L	281	12.5	6.46
	QC-M	2122	-15.1	2.58
	QC-H	26.4	5.50	8.58
Verapamil	QC-L	271	8.28	10.7
	QC-M	1990	-20.4	3.81
	QC-H	27.0	8.02	11.7

FIGURE 4. Micro-Flow representative chromatogram – Standard 100 pg/mL (10 µL injection, analytical flow rate of 40 µL/min)

Compounds are in the following order (Top to Bottom): Left Column: Oxycodone, Oxycodone-d6, Paroxetine, Paroxetine-d6, and Verapamil. Right Column: Clonazepam, Clonazepam-D6, Ketoconazole, Alprazolam, Alprazolam-d5, Reserpine, and Clopidogrel.



The linear gradient utilized for the traditional LC Standard-Flow method was directly transferred to the Micro-Flow LC method with an additional 1-minute hold to compensate for flushing the trap column. The Standard-Flow method run time was increased by one minute to compensate for the flush step.

FIGURE 5. High-Flow representative chromatogram – Standard 100 pg/mL (10 µL injection, analytical flow rate of 500 µL/min)

Compounds are in the following order (Top to Bottom): Left Column: Oxycodone, Oxycodone-d6, Paroxetine, Paroxetine-d6, and Verapamil. Right Column: Clonazepam, Clonazepam-D6, Ketoconazole, Alprazolam, Alprazolam-d5, Reserpine, and Clopidogrel.

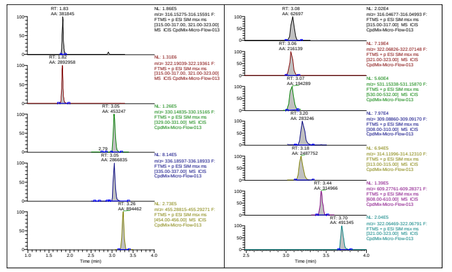
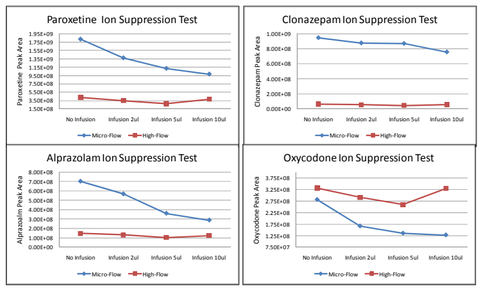


Figure 6. Ion Suppression Test

Ion suppression effects were investigated under Micro-Flow and Standard-Flow LC conditions. For this experimental setup a neat Standard mixture was prepared at 100 ng/mL. Ten microliter (10µL) was injected while Tee-Infusing precipitated human plasma (1:3 acetonitrile). Infusion flow rates of the extracted matrix were zero, 2 µL, 5 µL, and 10 µL/min.

Analyte	Flow	Blank	No Infusion of matrix	Infusion of matrix 2ul	Infusion of matrix 5ul	Infusion of matrix 10ul
Oxycodone	Micro	0	280,512,996	166,390,775	135,375,600	127,650,957
	High	0	330,964,625	290,799,261	259,132,534	329,191,343
Paroxetine	Micro	0	1,820,691,827	1,372,468,222	1,119,092,095	984,310,228
	High	0	427,434,318	349,660,603	276,519,523	384,534,017
Verapamil	Micro	0	947,430,503	876,700,676	870,365,398	757,313,379
	High	0	434,966,926	368,107,221	406,770,700	419,858,982
Clopidogrel	Micro	0	655,984,091	541,468,656	515,529,087	491,253,463
	High	0	361,309,888	330,467,243	300,048,640	339,991,504
Clonazepam	Micro	0	442,219,373	332,725,726	155,041,498	118,430,135
	High	0	63,854,904	56,053,157	46,440,915	57,567,712
Alprazolam	Micro	0	703,177,916	568,124,244	360,452,289	287,811,884
	High	0	146,584,063	131,280,208	104,252,912	125,637,373
Ketoconazole	Micro	0	82,806,775	88,712,167	95,468,826	90,842,707
	High	0	43,579,301	43,630,254	51,355,451	45,283,041

The graphs below display the peak area intensity difference for representative analytes while infusing precipitated human plasma extracts. Peak areas were compared under both Micro-Flow and Standard-Flow. Besides one outlier, Oxycodone, peak intensity was higher under Micro-Flow LC conditions.



Conclusion

- Micro-Flow LC provide an improvement in sensitivity which directly translated into a gain in LLOQ peak responses of 2 to 20x in 7 out of 8 compounds tested (one outlier - Reserpine).
- Micro-Flow LC did not require a change in injection volume, MS hardware changes, and more importantly there was no need to change the LC gradient when converting from the High-Flow LC method.
- Ion suppression testing, while in its preliminary stages, has hinted at better signal intensity under Micro-Flow conditions while introducing plasma extracts. This can lead to reduced suppressing effects from phospholipids which will be investigated further.
- Additional tests will include lower flow rates, smaller ID columns, isolating matrix components, normalizing infusion/LC flow rates and ion source comparison tests.

Acknowledgements

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