Utilizing a Non-Targeted HR/AM-MS Method to Accelerate Quantitative Throughput for In-Vitro Metabolic Profiling

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Overview

Purpose: To evaluate the potential of high-resolution, accurate mass analysis for the quantitation of the target compounds in a metabolic stability assay with the simultaneous identification of metabolites present at various experimental time points.

Methods: In-vitro incubations for Buspirone, Verapamil, Diltiazem, Minaprine, Mirtazapine, and Omeprazole were performed at two concentrations (3µM and 300nM). A time-course study was designed to investigate the appearance of various Phase I metabolites and disappearance of parent compound. MS analysis was performed using a Thermo Scientific Q Exactive benchtop quadrupole Orbitrap mass spectrometer. The half-life of each compound was estimated using Thermo Scientific QuickCalc data review software and metabolite prediction and identification was performed using Thermo Scientific MetQuest metabolic screening software.

Results: Analysis for the 3µM incubation was performed and MS data was collected in full scan data dependent MS/MS mode for quantitation of target compounds and relative quantitation of metabolites. Metabolite profiles were plotted for signal response over time using MetQuest™ software. Analysis for the 300nM incubation was performed using an 8 minute shallow gradient. Metabolite identification at the lower concentration incubation and short LC analysis provided a strong correlation with the metabolite identification results at the 3µM incubation.

Introduction

Preliminary information regarding drug metabolism collected early in the ADME screening stage can be used to better optimize compound design before comprehensive metabolite identification is performed. High-resolution mass spectrometry can be used to simultaneously quantify the metabolism of the target compound and identify metabolites present in the sample as well collect data dependant MS/MS spectra, without significant MS method optimization. Analysis of fragmentation information for compound metabolites can provide additional confirmation for metabolite identification as well as supplemental information on metabolic soft spots in the target compound enabling more efficient drug design.

Methods

Sample Preparation
Six commercially available compounds were incubated at 37 °C in human and rat liver microsomes at 3µM and 300nM starting concentrations in a 100 mM $NaPO_4$, pH 7.4, 5 mM $MgCl_2$ buffer solution. The incubation was quenched at several time points between 0 and 45 minutes using Methanol: Acetonitrile (1:3, v:v) followed by centrifugation. Supernatants were then plated for LC analysis on a Q Exactive™ benchtop quadrupole Orbitrap mass spectrometer.

Liquid Chromatography

The sample sets were injected onto a Thermo Scientific Hypersil Gold C18, 2.1 x 150 mm, 3µm column. 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 0.5 minutes and then ramped to 70% B over 8 minutes, then ramped again to 95% B over 1 .5 minutes with a 0.25 minute hold at 98% before returning to the starting conditions at 2% B for 2.25 minute equilibration time.

The 300nM sample set was injected onto a C18, 2.1 x 50 mm, 3µm column. 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 0.5 minutes and then ramped to 70% B over 4 minutes, then ramped again to 95% B over 1 .5 minutes with a 0.25 minute hold at 98% before returning to the starting conditions at 2% B for 2.25 minute equilibration time.

All sample injections were completed using a Thermo Scientific Accela Open system with DLW (Dynamic Load and Wash) and with Accela™ 1250 pumps at a flow rate of 500µL/min.

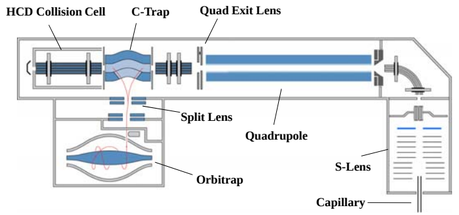
Mass Spectrometry

A Q Exactive benchtop high-resolution Orbitrap mass spectrometer was used in full scan mode, across a mass range of 170-1000 *m/z* at 70,000 resolution with a spectral speed of 3 Hz. Full scan mode was simultaneously coupled with data dependent MS/MS at 17,500 resolution and a spectral speed of 12 Hz to collect MS/MS spectral information for parent and metabolite compounds. Generic ion source conditions were used for all sample collection including vaporizer temp. 350°C, capillary temp. 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 software. Half-life calculations were performed, processed, reviewed, and reported using QuickCalc™ software (powered by Gubbs Inc., GMSU Gubbs™ Mass Spec Utilities, Atlanta, GA). Metabolite identification, time profiles, and data dependent MS/MS analysis were performed, processed, reviewed, and reported using MetQuest metabolic screening software. Structural identification was also performed using Thermo Scientific Mass Frontier spectral interpretation software.

FIGURE 1. Q Exactive benchtop high-resolution Orbitrap Instrument Schematic



Results

Half-life calculations were performed for each compound in both human and rat liver microsomes at 3µM and 300nM concentrations using QuickCalc software. (Figure 2)

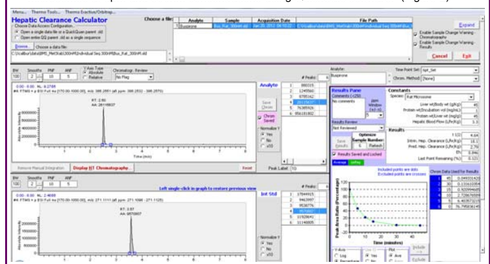


FIGURE 2. QuickCalc Hepatic Clearance Calculator. Analyte and internal standard chromatography as well as half-life and clearance calculations are easily reviewed and modified in a single view.

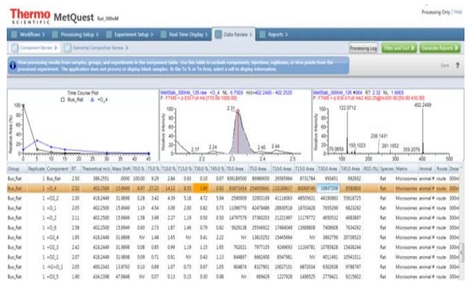
Half-life calculations were compared at both 3µM and 300nM for each compound and each species. (Table 1) Higher compound dosing concentrations are generally used to provide metabolite identification but often can affect compound half-life when compared to the concentration at typical dosing levels for most *in-vitro* metabolic stability assays. High dosing concentrations can also introduce potential issues with compound solubility and reagent costs. Therefore two separate experiments are often performed to characterize compound half-life and identify potential compound metabolites.

Table 1. Calculated results for t ½ of incubations at 3µM and 300nM for both human and rat species.

Species	Rat		Species	Human	
Compound	$t^{1/2}$ (min) 300nM	$t^{1/2}$ (min) 3uM	Compound	$t^{1/2}$ (min) 300nM	$t^{1/2}$ (min)3uM
Minaprine	8.97	20.9	Minaprine	57.8	17.6
Diltiazem	4.72	4.46	Diltiazem	22.3	46.6
Omeprazole	16.6	37.2	Omeprazole	64.1	25.9
Verapamil	12.3	17.5	Verapamil	12.7	23.7
Buspirone	4.64	4.03	Buspirone	9.67	22.1
Mirtazapine	17	27	Mirtazapine	56	56.8

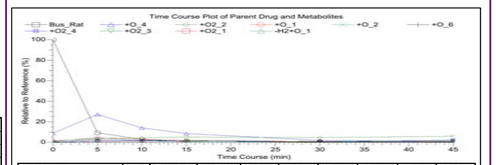
The capability to combine parent compound quantitation for half-life determination and preliminary metabolite identification in a single metabolism and LC/MS experiment provides the potential for a cost effective and streamlined workflow for metabolic profiling. MetQuest metabolic screening software was used to identify the metabolites for each compound at 3µM and 300nM and in both rat and human liver microsomes. (Figure 3)

FIGURE 3. MetQuest metabolic screening software for Buspirone at 300nM dosing concentration in rat liver microsomes. Parent disappearance and metabolite appearance is plotted, chromatography is displayed and metabolite results can be filtered for easy review. Available MS/MS spectra is also displayed for both parent and metabolite.



Metabolite identification was processed for each parent compound set and the results filtered to display the top ten most intense metabolites identified for each. Metabolites for each compound are listed by intensity with the modification type and chromatographic retention time correlated to each result. Due to the generic liquid chromatography method used to characterize the diverse compound set, not all metabolite peaks are symmetrical or fully resolved from one another; but as a result of the high resolving power used for sample analysis (70,000) coupled with an accurate mass of less than 5 ppm deviation from theoretical, metabolites could be identified confidentially even with less than ideal chromatography. Reports were generated for each compound set in both PDF and Excel formats displaying the disappearance of parent compound as well as the appearance of each individual metabolite over the time course of the experiment (Figure 4).

FIGURE 4. Example MetQuest metabolic screening report summary for parent and metabolite percentage over time for Buspirone at 300nM in rat liver microsomes. Top: Time course plot of parent drug and metabolites. Bottom: Component by % Parent at Time Zero



Metabolite Name	RT (min)	Theoretical m/z	Mass Shift	0.0 min	5.0 min	10.0 min	15.0 min	30.0 min	45.0 min
Bus_Rat	2.50	386.2551	0.0000	100.00	9.29	2.84	0.93	0.10	0.07
+O_4	2.32	402.2500	15.9949	8.97	27.23	14.12	8.55	1.80	0.92
+O2_2	2.30	418.2449	31.9898	0.28	3.42	4.39	5.16	4.72	5.94
+O_1	2.05	402.2500	15.9949	1.19	4.54	3.09	2.00	0.82	0.73
+O_2	2.11	402.2500	15.9949	1.58	3.99	2.27	1.19	0.50	0.50
+O_6	2.58	402.2500	15.9949	0.60	2.73	1.87	1.46	0.79	0.82
+O2_4	1.95	418.2449	31.9898	NV	1.48	1.65	NV	0.41	2.22
+O2_3	2.42	418.2449	31.9898	0.08	0.85	0.99	1.19	1.15	1.65
+O2_1	2.07	418.2449	31.9898	0.09	0.71	0.91	NV	0.43	1.13
-H2+O_1	2.05	400.2343	13.9793	0.10	0.89	1.07	0.73	0.67	1.05
+O3_5	1.90	434.2398	47.9848	NV	0.07	0.13	0.15	0.30	0.98

The top five most abundant metabolites for each compound were compared at both 3µM and 300nM for samples in both human and rat liver microsomes. (Table 2 and Table 3) Although metabolite ratios can be affected by the dosing concentration of the parent compound, the metabolites identified in the 3µM experiment correlate strongly to the metabolites identified in 300nM dosing experiment. The correlation between metabolites at both concentrations demonstrates the ability to obtain preliminary metabolite identification at lower parent compound dosing levels while simultaneously providing quantitation of parent compound for half-life calculations.

Furthermore data dependent MS/MS scans collected during full scan quantitation experiments allow for the possible identification metabolic soft spots located in the structure of the parent compound. By comparing the fragmentation profile of the parent compound at T0 to the data dependent MS/MS scans collected for each identified metabolite, modifications to the parent compound structure can be isolated to specific regions. As an example, the structural fragmentation of Buspirone was calculated using Mass Frontier™ and subsequently used to locate possible areas of modification by comparison of metabolite fragmentation spectra (Figure 5).

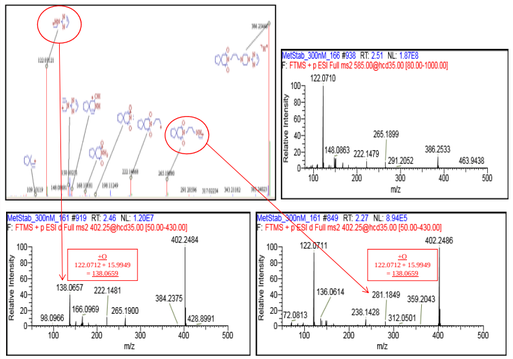
Table 2. Top five most intense metabolites in rat liver microsomes detecting using MetQuest software.

		Rat Liver Microsomes				
		M1	M2	M3	M4	M5
Buspirone	3uM	+O	+O	+O2	+O	+O
	300nM	+O	+O2	+O	+O	+O
Diltiazem	3uM	-C-H2	-C-H2	-C2-H4	-C2-H2	-C2-H4
	300nM	-C-H2	-C2-H4	-C2-H2	-C2-H4	-C3-H6
Minaprine	3uM	-C2-H2	-C2-H2	-C-H2+O	-C-H2+O	-H2+O
	300nM	-C2-H2	-C2-H2	-C-H2+O	-C-H2+O	-H2
Mirtazapine	3uM	+O	-H2+O2	+O	-H2+O2	-C-H2
	300nM	+O	-H2+O2	-C-H2	+O	+O
Omeprazole	3uM	+O	-H2	+O	-C-H2	-C-H2
	300nM	+O	+O	-C-H2	+O	+O
Verapamil	3uM	-C-H2	-C-H2	-C2-H4	+O	-C2-H4
	300nM	-C-H2	-C2-H4	-C-H2	-C-H2+O	-C2-H4

Table 3. Top five most intense metabolites in human liver microsomes detecting using MetQuest software.

		Human Liver Microsomes				
		M1	M2	M3	M4	M5
Buspirone	3uM	+O	+O	+O	+O	+O
	300nM	+O	+O	+O	+O	+O2
Diltiazem	3uM	-C-H2	-C2-H4	-C-H2	+O	-C-H2+O
	300nM	-C-H2	-C-H2	-C2-H4	-C2-H2-O	-C2-H4
Minaprine	3uM	+O	-C-H2+O	-C2-H2	-C-H2+O	-H2+O
	300nM	+O	-C-H2+O	-H2	-C2-H2	-H2
Mirtazapine	3uM	-C-H2	-C-H2	+O	+O	+O
	300nM	-C-H2	+O	+O	+O	-H2+O2
Omeprazole	3uM	+O	-H2	+O	-C-H2	+O
	300nM	+O	+O	-C-H2	+O	+H2+O
Verapamil	3uM	-C-H2	-C-H2	-C2-H4	+O	-C-H2
	300nM	-C-H2	-C2-H4	-C-H2	-C-H2	-C-H2+O

Figure 5: Fragmentation assignment of Buspirone and metabolites at 300nM in human liver microsomes. Top Left: Mass Frontier software fragmentation assignment. Top Right: Data dependent MS/MS scan of Buspirone at T0, displayed in MetQuest software. Bottom Left: Data dependent MS/MS scan of metabolite peak at RT 2.46, displayed in MetQuest software. Bottom Right: Data dependent MS/MS scan of metabolite peak at RT 2.27, displayed in MetQuest software.



The data dependent MS/MS spectrum collected for Buspirone in 300nM rat liver microsomes at T0 is compared to the fragmentation pattern calculated by Mass Frontier software for fragment confirmation. Data dependent MS/MS scans for metabolites identified in MetQuest software are then reviewed for exact mass modifications of the known fragmentations of the parent compound. The hydroxylation of several parent fragments can clearly be identified with less than 1 ppm mass deviation from the theoretical mass. The addition of data dependent MS/MS scanning of compound metabolites can help to identify regions of modification on the parent compound at an early stage in the drug discovery process allowing for more effective design of future drug candidate compounds and further streamlining early metabolic profiling.

Conclusion

- Full scan high-resolution, accurate mass LC/MS allows for accurate quantitation of parent compounds for half-life profiling.
- Instrument performance allows for potential dosing at lower concentration levels while preserving simultaneous quantitation and metabolite identification.
- The implementation of data dependent MS/MS scanning enables structural information about compound metabolites and potential regions of biotransformation.

Acknowledgements

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