Metabolic Stability Screening Workflow using a Second Generation High Resolution Accurate Mass Benchtop Instrument

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Overview

Purpose: Demonstrate the application of a simultaneous acquisition and processing methodology to support relative quantitative / qualitative high throughput metabolic stability analysis.

Methods: Representative *in vitro* samples were prepared by incubation with liver microsomes for analysis by UHPLC-HRAM MS with simultaneous automatic processing

Results: The acquisition and processing of six representative compounds incubated in three species provided an immediate measure of the metabolic stability, or instability, as well as an initial determination of the primary metabolites formed from each compound. As a result, species comparisons for metabolite coverage could be quickly generated from the output results.

Introduction

In vitro metabolic stability screening performs a very important role in the drug discovery stage for compound selection in pharmaceutical companies. The screening is primarily supported by LCMS, which involves the monitoring of the disappearance of parent compounds, using selected reaction monitoring (SRM) on triple-quadrupole instruments. If moderate to high turnover is observed, separate metabolite identification experiments are then conducted to characterize the biotransformation products. In this study, we present a novel workflow using a high resolution accurate-mass benchtop mass spectrometer. This workflow combines relative metabolic stability and initial metabolite information from the same analysis. The high mass resolution with high scan speed data acquisition is compatible with UHPLC for high throughput screening.

Methods

Sample Preparation

Six model compounds were selected for this study. Compounds (3 µM) were incubated using human, dog and rat hepatic microsomes at nominal 0.5 mg protein/mL at 37˚ C for up to 60 minutes in the presence of NADPH. At 0, 5, 15, 30, 45 and 60 minutes, aliquots of the reaction mixture were transferred and mixed with quench solution. The quench solution consisted of reserpine, used as an internal standard, in acetonitrile. The samples were centrifuged, and supernatant was injected for analysis.

Liquid Chromatography

High throughput screening often utilizes rapid chromatographic conditions to achieve a higher sample throughput. For this study, a 2.5 minute UHPLC method was used to provide a rapid analysis. Samples (5µL) were injected onto a UHPLC system (Open Accela™ AS, 1250 Accela UHPLC pump) with chromatographic separation achieved with a Hypersil Gold 50 X 2.1mm, 1.9µm column. The column was maintained at 40˚ C throughout the analysis.

Table 1. LC Method

Time	Mobile Phase A	Mobile Phase B	Flow Rate (L/min)
0	98	2	600
0.25	98	2	600
2	2	98	600
2.1	2	98	600
2.2	98	2	600
2.5	98	2	600

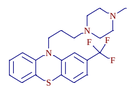
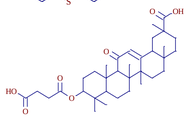
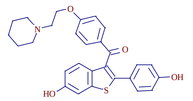
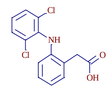
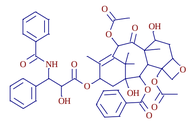
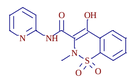
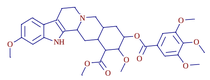
Mass Spectrometry

All quantitative and qualitative analysis was performed on a second generation Exactive™ benchtop Oribtrap™ mass spectrometer connected to an Accela LC system described above. The mass spectrometer was operated in positive ionization mode with HESI-II probe (Sheath Gas: 60, Auxiliary Gas: 15, Heater Temperature 450 ˚ C). Acquisition consisted of a full scan from m/z 200 to 900 with a resolution of 70,000 (FWHM @ m/z 200) followed by an all ions fragmentation (AIF) scan from 60 to 900 with a resolution of 35,000. The AIF scans were included to provide characteristic and diagnostic fragments to allow for initial determination of metabolism.

Data Analysis

Control of the instrument for sample acquisition, automatic processing, and reporting of the results was all controlled by MetQuest 1.2 software. (Thermo Fisher Scientific, San Jose CA)

Figure 1: Compounds Used In The Study

Compound	Structure	m/z
Trifluoperazine		408.1716
Carbenoxolone		571.3629
Raloxifene		474.1734
Diclofenac		296.0240
Paclitaxel		854.3382
Piroxicam		332.0700
Reserpine (IS)		609.2807

Results

Automatic Processing of Data – Metabolite Detection

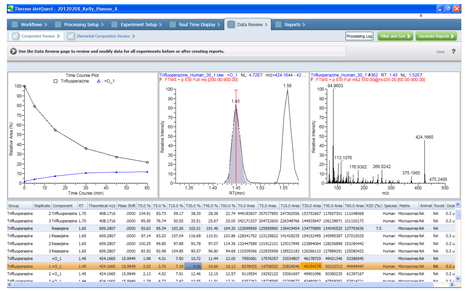
Acquired raw files were processed in parallel to acquisition using a targeted workflow. A list of common phase I biotransformations was applied with the application calculating the expected m/z values for each parent. Peaks were detected and integrated using a parameter-less peak integration approach which required no user input values. The software modeled each peak and, in the case of closely eluting positional isomers, split and modeled each of the overlapping members.

After peak detection and integration, detected peaks are grouped across timepoints by adjusting for retention time drift from injection to injection. The grouped components are assigned unique identifiers based on the proposed metabolic change and the relative formation of metabolites is calculated against the area of parent at time zero.

Automatic Processing – Data Review

Results were presented for analyst review in a review pane (Figure 2). The disappearance and formation of metabolites was displayed along with the integrated peak. The all ion fragmentation scan nearest the center of the peak was displayed as well. Within the review pane, integration was reviewed and in necessary, manually corrected. Poor / partial injections, based on internal standard peak area, were excluded from analysis.

Figure 2: Data Review Pane

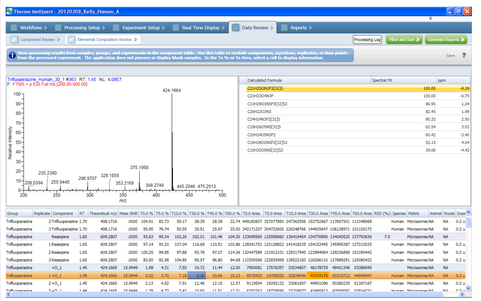


To simplify the review, the data was filtered so that only metabolites that reached at least 5% of parent area at time 0 were included. Those minor metabolites with a maximum relative formation below 5% were filtered out of the review and subsequent reporting.

Automatic Processing – Elemental Composition

Elemental compositions were predicted for all detected parent compounds and metabolites. The elements to use and the limits (maximum and minimum) were automatically calculated for each parent compound individually to achieve a better prediction with fewer false elemental compositions. Figure 3 shows the elemental composition predictions for the first oxidative metabolite of trifluoperazine. A total of ten possible compositions were provided with spectral fit scores from 100 to 39%. A score of 100% represents a perfect match of the observed data to a theoretical match. For this example, the best prediction was the correct formula with a 0.2 ppm error.

Figure 3: Elemental Composition



Results – Relative Stability Across Species

The results for the compounds studied are shown in Table 2. Relative stability of the six compounds varied from very stable (carbenoxolone, paclitaxel) to unstable (trifluoperazine, diclofenac). The relative stability of all six compounds in human liver microsomes are shown in Figure 4. Some compounds (diclofenac, raloxifene) displayed significant differences in stability across species. The variability in stability of diclofenac across the three species studied is shown in Figure 5. The analysis was quickly performed using the comma separated variable (csv) experiment based reports.

Figure 4: Stability of Six Compounds in Human Liver Microsomes

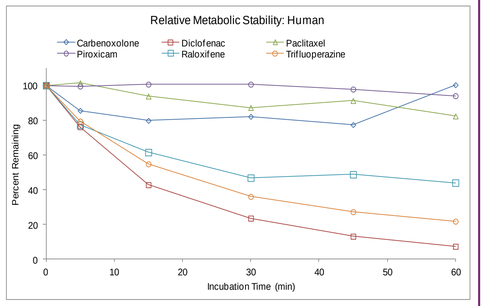


Table 2: Results of Stability Screening

Compound	Species	Percent Remaining 0 min	5 min	15 min	30 min	45 min	60 min
Carbenoxolone	Dog	100	112	109	107	121	123
	Human	100	86	80	82	77	100
	Rat	100	98	84	86	87	92
Diclofenac	Dog	100	106	96	96	94	84
	Human	100	76	43	24	13	7
	Rat	100	76	57	34	19	12
Paclitaxel	Dog	100	108	92	93	89	92
	Human	100	101	94	87	91	82
	Rat	100	103	106	103	101	103
Piroxicam	Dog	100	92	75	90	92	85
	Human	100	100	101	101	98	94
	Rat	100	94	93	93	91	63
Raloxifene	Dog	100	58	17	3	1	1
	Human	100	77	62	47	49	44
	Rat	100	67	43	23	16	12
Trifluoperazine	Dog	100	32	5	1	1	1
	Human	100	79	55	36	27	22
	Rat	100	22	3	1	0	0

Results – Relative Stability Across Species

The differential stability of diclofenac was largely due to the lack of formation of a primary oxidative metabolite in dogs as could be seen by analysis of the detected metabolites. (Figure 6).

Figure 5: Stability Differences Across Species - Diclofenac

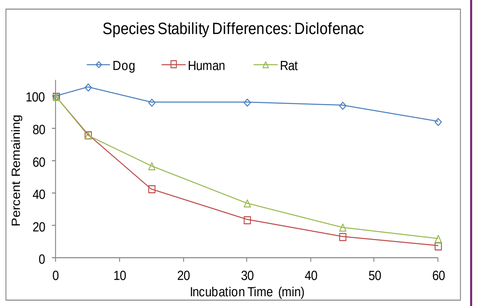
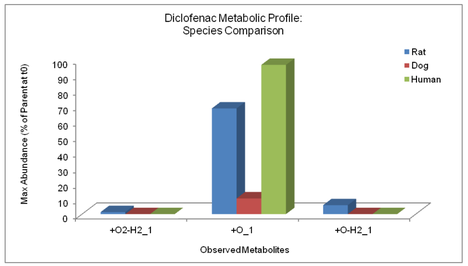


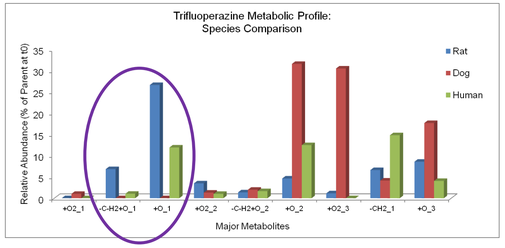
Figure 6: Metabolite Species Comparison - Diclofenac



Metabolite Comparison – Species Coverage

In order to assure coverage of expected human metabolites in toxicological animal studies, it is important that species selected should adequately reflect the expected metabolite profile. This coverage of expected human metabolites can be assisted by using the output from our screening results. The analysis of metabolites across species for trifluoperazine is shown in Figure 7. The major metabolites from each species are represented and their levels in all three species are plotted. From this it can be quickly seen that dog liver microsomes do not significantly form one important human oxidative metabolite (+O_1) that is well represented by rats. In addition, a demethylated + oxidative metabolite was also not observed in dogs but present in rats. From this assessment it can be determined that rat provides coverage of the top four human hepatic microsomal metabolites formed from trifluoperazine (+O_1, +O_2, -CH^2_1, and +O_3)

Figure 7: Metabolite Species Comparison - Trifluoperazine



Conclusions

The rapid simultaneous acquisition and processing of relative quan/qual data in DMPK screening allows for more information to be gathered from early studies. Utilizing a high resolution accurate mass platform for this work allowed a very easy setup with minimal tuning and provided accurate mass data on both parent compounds and metabolites that allowed the assignment of elemental composition. All ion fragmentation data provided diagnostic fragment ions that further supported the identification of related metabolites.

Overall, this approach provided multiple benefits including:

- Maximized sample use by providing more data than simple parent stability
- Identified species which provide adequate coverage of human metabolism
- Determined stability differences in species that may translate into PK