Rapid Soft Spot Analysis using the SCIEX Routine Biotransform Solution

Rapidly Identify Top Metabolites with the SCIEX X500R QTOF System and MetabolitePilot™ 2.0 Software

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Key Challenges in Soft Spot Analysis and Metabolite ID

- Increasing productivity in early drug discovery with a higher-throughput soft spot identification assay
- Maintaining data quality for both qualitative and quantitative analysis in a high-throughput environment
- Extensive manual data interpretation to assign structures and identify soft spots

Key Features of the X500R QTOF System for Soft Spot Analysis

- Compact benchtop system featuring easy-to-use SCIEX OS for control and acquisition which enables quick setup and data acquisition even for non-mass spec experts
- Robustness with the renowned Turbo V™ source
- Independent calibrant delivery path for high accuracy and reliability in results even across large sample batches
- Intelligent and flexible data acquisition strategies featuring: SWATH® Acquisition for data independent acquisition (DIA) and real-time multiple mass-defect filtering (MDF) for information-dependent acquisition (IDA)
- Speed and Resolution – Ultra-high pressure liquid chromatography (UHPLC) with its narrower chromatographic peaks (~5 sec) are becoming standard in drug discovery and development labs. The X500R can keep up with UHPLC by collecting up to 100 MS/MS scans per second without compromising resolution
- Robust mass accuracy - Six heater drones throughout the TOF path maintain mass stability through longer runs

The SCIEX Routine Biotransform Solution featuring the X500R QTOF System and ExionLC™ with MetabolitePilot™ 2.0 software.

Key Features of MetabolitePilot™ 2.0 Software for Soft Spot Analysis

- Automatic structure proposals of metabolites are generated and presented with relative evidence for quick, easy review and confirmation
- Multiple peak finding strategies utilizing MS and MS/MS for both targeted and untargeted identification of drug related metabolites
- Advanced peak finding algorithms optimized for SCIEX accurate mass platforms and integrated MS/MS fragment interpretation and structural assignment tools
- High throughput batch processing (up to 200 rows) plus the ability to import batches from a spreadsheet and create processing methods on the fly
- Multiple sample correlation function for time course studies and inter-species comparison of metabolites

Introduction

Liver microsome studies are used to model hepatic oxidative metabolism *in vivo*. In early discovery, compounds susceptible to rapid clearance are typically avoided. Rational drug design can overcome the issue of rapid clearance and extend the therapeutic concentration of a drug. Structural information about the sites or 'soft spots' of oxidative metabolism is required to establish structure-metabolism relationships and guide medicinal chemistry efforts to overcome instability. For scientists working in this area, increasing sample throughput and minimizing the time spent on data processing and structural interpretation are important to accelerate early drug discovery.

In this tech note we employ the Routine Biotransform Solution featuring the X500R QTOF System with MetabolitePilot™ 2.0 software to study the oxidative metabolism of four model compounds. Data was acquired with both data dependent and data independent strategies and to increase sample throughput a short 50 mm column and a 5 minute gradient were used. Generic and predicted metabolite peak finding strategies were used in MetabolitePilot software and the automatic structure proposal feature was used to speed data review.

Experimental

Haloperidol, buspirone, midazolam and verapamil reference standard solutions (1 mg/mL) were purchased from Sigma-Aldrich. Rat liver microsomes (RLM) and Rapid Start NADPH regenerating system were purchased from Xenotech.

Sample Preparation Incubations

Compounds were first diluted in methanol to 100 mM. The concentration of organic solvent was 1% in the final incubations. Starting incubation volume was 500 µL and contained 1 mg/mL RLM, 10 µM substrate and 1.47 mM NADPH regenerating system in 100 mM potassium phosphate buffer pH 7.4. At 5, 15, 30, 60 and 90 minutes 50 µL aliquots of the mixture were taken and quenched with an equal volume of cold acetonitrile. The mixture was vortexed for 1 min and then centrifuged at 15000 rpm for 10 minutes. The supernatant was then analyzed on the X500R. The control sample (t=0) was prepared by quenching the microsomes first before adding substrate. RLM blank time point controls were prepared by incubating a mixture without substrate and taking aliquots at different time points.

Mass Spectrometry Data Collection

Data was collected on a SCIEX X500R QTOF System with SCIEX OS 1.2 using both IDA and SWATH® acquisition. TOF MS acquisition covered *m/z* 100 to 1000, with 100 ms accumulation time. Data independent acquisition was performed using SWATH® acquisition with 7 variable windows sized to cover Phase I pathways. Total scan time for the SWATH acquisition method was 325 msec. IDA data were acquired with dynamic background subtraction, intensity threshold 1000 cps, exclude isotope ± 3 Da, mass tolerance ± 50 mDa and 50 ms accumulation time.

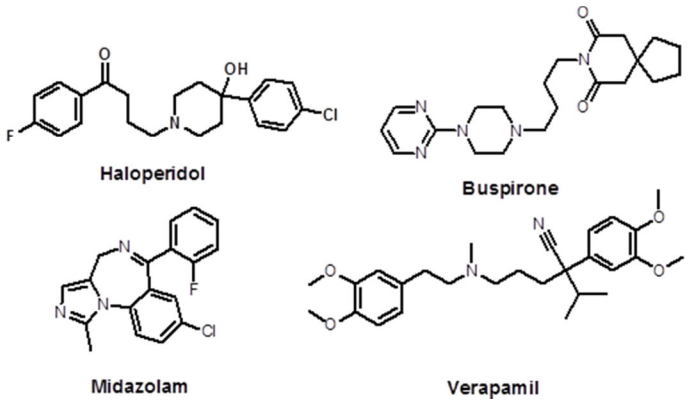

Figure 1. The molecular structures of the compounds used in this study.

Chromatography

Samples were chromatographed on a SCIEX ExionLC™ AD system using a Phenomenex Kinetex C18 column (2.0 x 50 mm), 2.6 µm. Elution was performed using a linear gradient from 5% to 40% B over 4 mins, then to 95% B at 4.5 min and held at 95% B until 5 min. The column was returned to 5% B at 5.25 min. Total run time was 6 minutes. Mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The flow rate was 400 µL/min, the column was kept at 40 °C and 2 µL was injected.

Data Processing

Data was processed in MetabolitePilot 2.0 software using the installed Phase I biotransformation list. A sample to control ratio of >3 was used. TOF MS was used to find metabolites using both the predicted and generic peak finding (mass defect filter on) algorithms. For the compounds haloperidol and midazolam the isotope pattern algorithm filter was also used. The LC-MS and MS/MS (XIC) threshold and MS/MS threshold was set to 3000 and 500 cps respectively. The Advanced MSMS filter was used for SWATH data processing.

Results

Each parent compound eluted between 2.9 and 3.7 minutes. An example chromatogram of the SWATH® Acquisition from the 30 min incubation of verapamil is shown in Figure 2A and 15 min incubation of buspirone in 2B. The verapamil chromatogram shows the parent, oxidized and de-methylated metabolite TOF MS XICs. Each peak has a width of approximately 5 seconds, and the variable window SWATH Acquisition approach allowed the collection of at least 12 data points across each peak. Each peak is well resolved using the 50 mm Kinetex C18 column and 5 min gradient. Similarly the oxidized metabolites of buspirone are also well resolved.

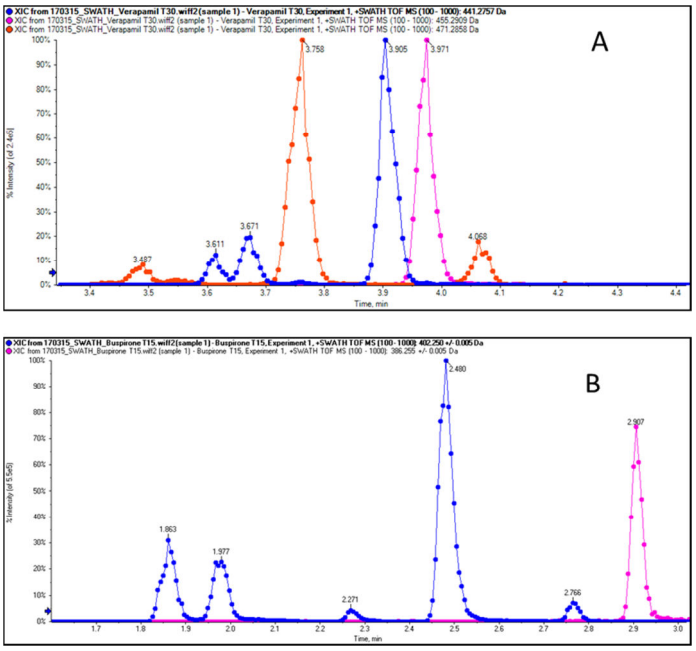

Figure 2. LCMS chromatogram from the SWATH® Acquisition of the 30 min incubation of verapamil (A) and 15 min incubation of buspirone (B). The verapamil chromatogram (A) shows the parent (pink), de-methylated (blue) and oxidized metabolites (red) are well resolved. Similarly the buspirone chromatogram (B) shows the oxidized metabolites (blue) are well resolved and resolved from the parent (pink).

Excellent mass accuracy and resolution were achieved in both MS and MS/MS scans (Figure 3). The high mass accuracy obtained in MS/MS scans greatly facilitates structure elucidation because it allows unambiguous assignment of elemental composition. As can be seen from the number of points across the peaks in figure 2 the data independent acquisition (SWATH Acquisition) is performed with enough speed to allow quantitation (>10 points) in both MS and MSMS at the resolution and mass accuracy seen in figure 3.

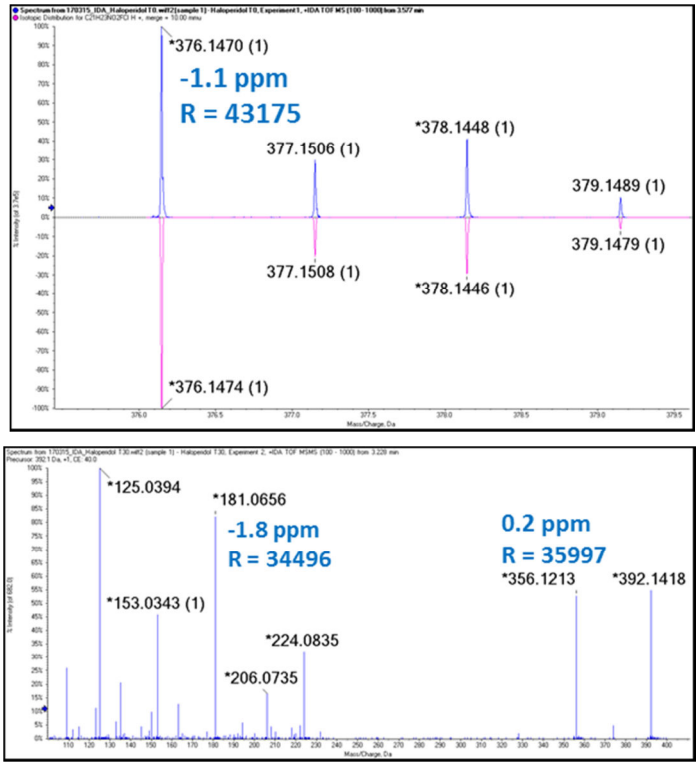

Figure 3. Mass accuracy and resolution in TOF MS and TOF MSMS. Panel A shows the MS scan of the haloperidol parent (blue trace) with a near perfect match of the theoretical isotope pattern (pink). The product ion spectrum of the major oxidized metabolite is shown in panel B with the ppm error and resolution for mass fragments 181.0656 ($C10H10FO2^+$) and 356.1213 ($C21H20ClFNO^+$). Excellent mass accuracy and > 30K resolution allow easy assignment of elemental composition, even to the product ions.

Data were processed in MetabolitePilot™ 2.0 software with the auto-assign option and using the t = 0 sample and RLM blank as controls. Each results table was then sorted based on MS peak area to identify the top metabolites formed at each time point. The auto-assign function of MetabolitePilot software will generate structural proposals for metabolites with: one or more cleavages, one biotransformation and metabolites with a combination of one cleavage and one biotransformation. This feature helps to speed up metabolite assignment because the user only needs to review and confirm the proposed metabolite structure using the MS/MS interpretation tool. An example of the interpretation workspace is shown in figure 4 (page 5) for the major oxidized metabolite of midazolam. There are several possible sites of oxidation and each candidate is represented with a line in the structure candidates tab. The top structure from the assignment algorithm is shown as rank 1 and the other possibilities are shown below. The strength of the evidence of the other candidates is shown relative to rank 1 and is represented as a histogram. In the case of

1'-hydroxymidazolam the metabolite structure was confirmed by matching the MSMS spectrum with one previously reported.[2] However, not all candidates can be distinguished based on MSMS, such as oxidation on an aromatic ring and are presented with the same relative evidence (blue histogram, rank 3) and are best represented with a Markush structure.

The top 5 metabolites found for each compound after 30 minutes are shown in Table 1 (page 6). Buspirone metabolites included: 6'-OH, 3'-OH and di-oxidations, verapamil included: N- and O-demethylation and C-N bond cleavage, haloperidol included: pyridine oxidation, C-N bond cleavage and oxidation and midazolam metabolites included: di- and tri-oxidation.

There were no differences observed in the top 10 metabolites found by either IDA or SWATH® Acquisition for each compound. However as observed previously[1], there were differences in the number of MSMS spectra acquired between the two techniques for metabolites 11 to 20 (based on TOFMS peak intensity) for each compound. Not all metabolites in this intensity range had associated MSMS spectra with IDA acquisition while 100% of the metabolites had MSMS spectra when using SWATH Acquisition. The absence of product ion spectra for some metabolites would require reacquisition of the sample targeting the specific metabolite precursor ion. Another advantage of SWATH Acquisition is the collection of isotope pattern information in the product ion spectrum, which can be used for metabolite peak finding (figure 5).

The correlation workspace was used to compare and confirm metabolites across multiple time points using the processed results tables. After selecting the samples for correlation the software automatically tabulates the abundance of each metabolite across all samples and overlays all chromatographic, MS, and MS/MS data. Figure 6 shows an example correlation plot for the oxidized metabolites of buspirone from 5 to 90 mins and shows the correlation in a linear plot. A bar graph and table views are also available in the correlation workspace.

Conclusions

Rapid detection and characterization of major metabolites is required for lead optimization in fast paced drug discovery environments. Rat liver microsome incubations of haloperidol, verapamil, buspirone and midazolam have shown that the X500R QTOF system combined with MetabolitePilot™ 2.0 software is a powerful solution for rapid soft spot analysis. Both IDA and variable window SWATH Acquisition techniques are suitable for high throughput acquisition and SWATH Acquisition offers the benefit of collecting product ion spectra for all detectable metabolite precursor ions and capturing product ion isotope pattern information. Data processing is speeded by the automatic structure proposal feature and integrated MS/MS fragment interpretation tool that performs fragment assignments all in a single integrated workspace.

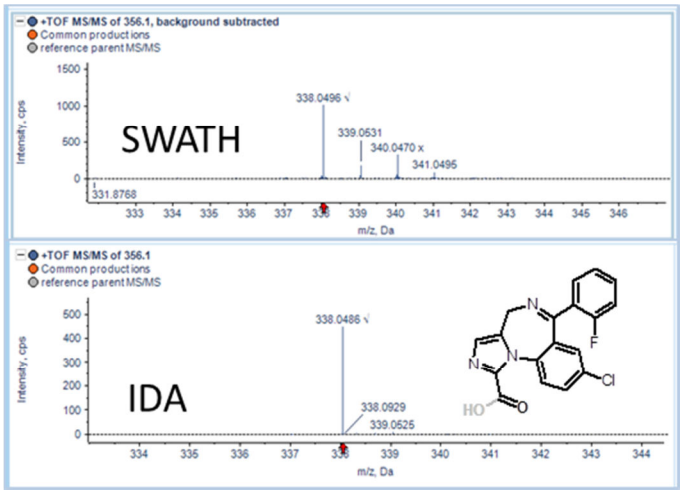

Figure 5. An example of the isotope pattern information collected in the product ion spectrum using SWATH® Acquisition (Top) versus IDA (Bottom). The 338.0492 product ion of the 1'-midazolam carboxylic acid metabolite (*m/z* 356.0597) is shown.

References

1. Comparison of Information-Dependent Acquisition, SWATH, and MS^{All} Techniques in Metabolite Identification Study Employing Ultrahigh-Performance Liquid Chromatography–Quadrupole Time-of-Flight Mass Spectrometry. Xiaochun Zhu, et al. Analytical Chemistry 2014 86 (2), 1202-1209
2. Sensitive and specific determination of midazolam and 1-hydroxymidazolam in human serum by liquid chromatography–electrospray mass spectrometry. P Marquet et al. Journal of Chromatography B: Biomedical Sciences and Applications, Volume 734, Issue 1, 1999, Pages 137-144.

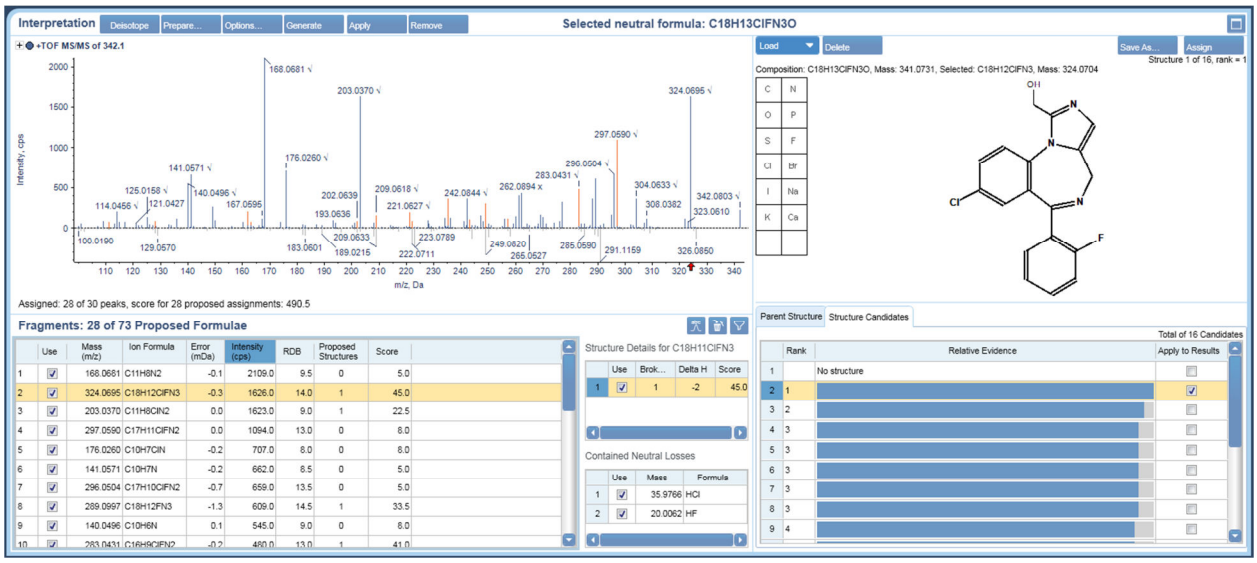

Figure 4. The interpretation workspace of MetabolitePilot™ 2.0 software showing data from the 15 minute incubation of midazolam. There are 2 mono-oxidized products among the 10 major metabolites at this time point, the product ion spectrum and structure candidates tab is shown for the 3.12 min mono-oxidized metabolite. There are many possible sites of oxidation on the midazolam structure that could represent the metabolite at this retention time, each regioisomer is represented as a line in the structural candidates table and the evidence for each proposal is represented relative to the top proposal (1'-hydroxymidazolam). The MSMS spectrum of the proposed 1'-hydroxymidazolam metabolite matches one previously reported.[2]

Figure 6. MetabolitePilot™ 2.0 software correlation workspace with an example correlation plot for the oxidized metabolites of buspirone from 5 to 90 min incubation time points. The correlation is shown in a linear plot with overlays of chromatographic, MS, and MS/MS data, bar graph and table views are also available in this correlation workspace.

Table 1. A summary of the top 5 major metabolites observed from rat liver microsomes incubations (30 min) of the test compounds shown in figure 1.

Peak ID	Name	Formula	Neutral Mass	m/z	ppm	R.T. (min)	Peak Area	% Area	% Score
				Buspirone					
M50	Oxidation [M+H]+	C21H31N5O3	401.2423	402.2496	-0.9	2.49	1.11E+06	19.4	84.7
M44	Di-Oxidation [M+H]+	C21H31N5O4	417.2373	418.2446	-0.7	2.35	5.99E+05	10.5	69.8
M59	Di-Oxidation [M+H]+	C21H31N5O4	417.2373	418.2445	-0.8	2.67	4.27E+05	7.5	73.4
M23	Oxidation [M+H]+	C21H31N5O3	401.2424	402.2497	-0.7	1.86	4.14E+05	7.2	86.2
M37	Di-Oxidation [M+H]+	C21H31N5O4	417.2372	418.2445	-1.0	2.22	3.96E+05	6.9	77.0
				Verapamil					
M77	Loss of CH2 [M+H]+	C26H36N2O4	440.2675	441.2747	-0.1	3.90	2.62E+06	25.3	94.5
M18	Loss of C10H12O2 [M+H]+	C17H26N2O2	290.1992	291.2065	-0.8	3.01	1.46E+06	14.1	83.0
	Parent [M+H]+	C27H38N2O4	454.2828	455.2901	-0.7	3.98	1.20E+06	11.6	93.7
M63	Loss of CH2 [M+H]+	C26H36N2O4	440.2672	441.2745	-0.7	3.67	5.45E+05	5.3	87.0
M62	Loss of CH2 and CH2 [M+H]+	C25H34N2O4	426.2517	427.2590	-0.3	3.62	5.13E+05	5.0	92.8
				Haloperidol					
M15	Loss of 22.0423 [M+H]+	C21H17ClFNO	353.0978	354.1051	-1.3	3.92	1.62E+06	38.5	71.9
	Parent [M+H]+	C21H23ClFNO2	375.1400	376.1473	-0.4	3.58	8.37E+05	19.9	96.2
M2	Loss of O and C10H9FO+Desaturation [M+H]+	C11H12ClN	193.0656	194.0729	-1.1	1.84	2.70E+05	6.4	62.5
M9	Loss of 197.0974 [M+H]+	C10H7FO2	178.0427	179.0500	-1.6	3.11	2.66E+05	6.3	55.5
M5	Loss of C11H12ClNO+Desaturation [M+H]+	C10H9FO	164.0635	165.0708	-1.2	2.99	1.52E+05	3.6	93.7
				Midazolam					
M21	Di-Oxidation [M+H]+	C18H13ClFN3O2	357.0676	358.0748	-1.3	2.87	7.89E+05	21.3	83.0
M5	Di-Oxidation [M+H]+	C18H13ClFN3O2	357.0679	358.0752	-0.4	2.21	2.74E+05	7.4	89.6
M11	Tri-Oxidation [M+H]+	C18H13ClFN3O3	373.0623	374.0696	-1.7	2.47	2.60E+05	7.0	77.2
M2	Tri-Oxidation [M+H]+	C18H13ClFN3O3	373.0623	374.0696	-1.7	2.01	2.45E+05	6.6	82.2
M18	Di-Oxidation [M+H]+	C18H13ClFN3O2	357.0679	358.0752	-0.4	2.70	2.06E+05	5.6	75.7

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