A Metabolite ID Workflow on a Small Footprint Benchtop Q-TOF Mass Spectrometer with Automated Software Structure Generation

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ABSTRACT

In this study the SCIEX Routine Biotransform Solution with the new X500 Series QTOF high-resolution, accurate mass system and the new MetabolitePilot™ 2.0 software were used to perform metabolite ID and identify metabolic soft spots on four different compounds. Rat liver microsome incubations were used generate metabolites of buspirone, haloperidol, midazolam and verapamil. Samples were chromatographed using a 50 mm column and a short 4.5 minute gradient and data were acquired using both information dependent (IDA) and data independent (SWATH®) acquisition techniques. The data were processed using MetabolitePilot™ 2.0 software to speed metabolite assignment.

INTRODUCTION

Early drug discovery microsomal stability assay is used to determine drug candidate compounds' metabolic clearance, as well as finding metabolic soft spot on molecular structures. These assays are typically done separately, and even sequentially, on a normal resolution LC/MS system. In a high-throughput environment with premium lab space, combination of the two assays can improve productivity significantly. In this study, data acquired on a new small footprint TOF system, X500R QTOF, in combination with new MetabolitePilot™ 2.0 metabolite identification software for data processing demonstrate the quantitative/qualitative (microsomal stability/Met ID) combined workflow.

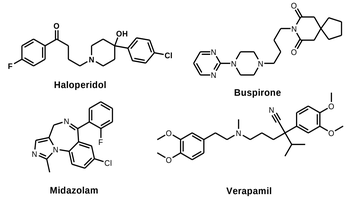


Figure 1. The structures of the compounds used in this study of metabolic soft spot analysis.

METHODS

Sample Preparation:

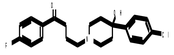
1. Incubations were performed using rat liver microsomes from Xenotech at 1 mg/mL protein concentration.
2. The Xenotech RapidStart NADPH regenerating system was used at a final concentration of 1.47 mM NADPH, in 100 mM potassium phosphate buffer, pH 7.4.
3. The incubation reaction volume was 500 µL and the final compound concentration was 10 µM.
4. At 5, 15, 30, 60 and 90 minutes a 50 µL aliquot was removed for processing.
5. Samples were quenched with and equal volume of ice-cold ACN.
6. The mixture was vortexed for 1 min, and then centrifuged at 15000 rpm for 10 min.
7. The supernatant (2 µL) was subject to LC/MS analysis on X500R system.

MS Data Collection:

- SCIEX X500R QTOF System with SCIEX OS 1.2
- IDA: Threshold 1000 cps, with DBS; Top 6 ions; exclude isotope ±3 Da; mass tolerance ±50 mDa; 50 ms accumulation
- SWATH® acquisition: 7 compound dependent variable SWATH windows sized to bracket Phase I metabolic pathways
- Total scan time for the SWATH method was 350 ms

The SCIEX routine Biotransform solution consisting of the X500R QTOF System and Exion AD LC.

Figure 2. SCIEX OS SWATH method setup window for haloperidol. Seven variable SWATH windows were used to cover the expected Phase I biotransformations.



Start Mass	Stop Mass	Biotransformation
295.15	345.15	Di-demethylation Demethylation Parent, +2, -2 Oxidation, hydrolysis Di-oxidation
344.15	358.15	
357.15	371.15	
370.15	382.15	
381.15	399.15	
398.15	416.15	
415.15	465.15	

LC Conditions:

- SCIEX Exion AD system
- 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 mins until 5.0 mins. A – H_2O, B – ACN 0.1% CH_2O_2

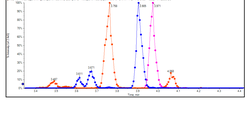
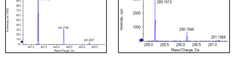


Figure 3. LC-MS chromatogram from the SWATH acquisition of a RLM sample of verapamil. The pink trace is the parent compound, red trace oxidized metabolites and blue trace is demethylated metabolites. The average peak width is ~5 seconds, and each peak has 10-12 points The resolution of the demethylated parent was >37 000 and MS/MS was >30 000 for the characteristic 289.19 ion.



Data Processing with MetabolitePilot 2.0:

Peak Finding Algorithms:
- TOF MS peak finding: predicted metabolites, isotope pattern (haloperidol, midazolam) and generic peak finding with mass defect filter (-30 to +20 mDa)

Biotransformations
- Phase I

Cleavage
- Break 2 bonds

Figure 4. An example of the cleavage metabolites and reference MS/MS spectrum from the processing method used for buspirone.

RESULTS

After processing, the results table was sorted based on peak area percentage (TOF MS XIC) to show the top metabolites formed for each species at each time point. An example table list is shown below for the buspirone 30 minute time point.

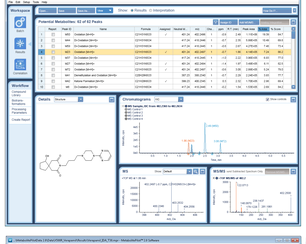


Figure 5. The results workspace of MetabolitePilot 2.0 software displaying results of the top ten metabolites of buspirone at the 30 minute timepoint. The XIC trace above shows the mono-oxidized products.

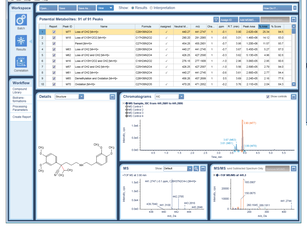


Figure 6. The results workspace of MetabolitePilot 2.0 software displaying results of the top ten metabolites of verapamil at the 30 minute timepoint. The XIC trace above shows the mono-demethylated products.

The number of metabolites reported with a peak area > 1% of the total (TOF MS XIC) was tabulated for each metabolite at each time point and for each acquisition technique. There was consistent agreement between the number of metabolites found using each data acquisition technique for the compounds haloperidol, midazolam, buspirone and verapamil (±13%) across the time points.

	Time Point	Metabolite Candidates with Peak Area >1%	
		IDA	SWATH
Haloperidol	5	13	13
	15	15	12
	30	17	15
	60	14	14
	90	15	15
Midazolam	5	--	--
	15	17	16
	30	23	23
	60	16	21
	90	21	22
Verapamil	5	--	--
	15	14	14
	30	17	17
	60	16	15
	90	17	15
Buspirone	5	20	18
	15	22	19
	30	22	19
	60	24	21
	90	26	22

Table 1. The number of metabolites identified in each RLM sample using both data dependent (IDA) and data independent acquisition techniques (SWATH®)

MetabolitePilot 2.0 contains a new automated structure generation feature to enable a more automated and faster soft spot identification workflow and to minimize the time spent performing manual structural assignments. Using the protonated adduct MetabolitePilot will assigned structures automatically for metabolites with one or more cleavages, metabolites with one biotransformation and for metabolites with combination of one cleavage and one biotransformation. Figure 7 shows an example of the MSMS interpretation and assignment of a metabolite of haloperidol that was auto assigned. There were 2 oxidized metabolites of haloperidol found with peak area >1%. There are four possible sites of oxidation: the chlorophenyl ring, the piperidine ring, the alkyl chain, or the fluorophenyl ring.

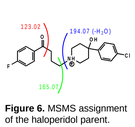


Figure 6. MSMS assignment of the haloperidol parent.

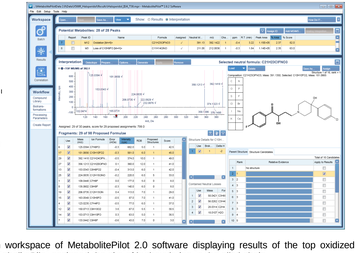


Figure 7. The interpretation workspace of MetabolitePilot 2.0 software displaying results of the top oxidized metabolite of haloperidol. MetabolitePilot assigned the site of hydroxylation to the alkyl chain.

The oxidized metabolite (392.1422) with the highest peak area was observed at 3.22 mins and the parent at 3.58 mins. In the MSMS spectrum of this oxidized metabolite the 2 most intense fragment ions observed were *m/z* 125.0394 and 181.0656. The presence of the un-shifted fluorophenyl ring fragment and the shifted (+16) alkyl fluorophenyl ring fragment localizes the hydroxylation to the alkyl chain. The 194.07 fragment was also observed indicating that the chlorophenyl ring and the piperidine ring were not modified.
The structural possibilities considered by MetabolitePilot are shown in the structural candidates pane and each one is ranked (blue histogram) for the user to review and confirm.

SWATH® acquisition collects MSMS for all detectable analytes, while IDA acquisition may not trigger MSMS for all potential metabolites. An example was seen in the data set for haloperidol. A minor oxidized metabolite (<0.14%) at 3.14 minutes was found in both the IDA and SWATH datasets. The MSMS spectrum was not collected with IDA but was with SWATH. The presence of the 123.06 and 165.07 fragments help to confirm oxidation on either the chlorophenyl ring or the piperidine ring.

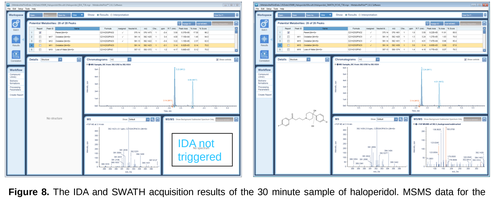


Figure 8. The IDA and SWATH acquisition results of the 30 minute sample of haloperidol. MSMS data for the minor oxidized metabolite at 3.14 mins was not collected with IDA acquisition but was with SWATH acquisition helping assign the site of oxidation to either the chlorophenyl ring or the piperidine ring.

Figure 9. The correlation workspace of MetabolitePilot 2.0 software displaying results of the Buspirone incubation experiments. Shown on the correlation plot are the results for the parent compound, N-oxide, 6'-OH, 5,6'-di-OH and 3'-OH.

CONCLUSIONS

The SCIEX Routine Biotransform solution with the SCIEX X500R QTOF system and new MetabolitePilot 2.0 software has been demonstrated to be an effective platform for small molecule Met ID using both IDA and SWATH® acquisition workflows.

TRADEMARKS/LICENSING

AB Sciex is doing business as SCIEX.

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