Simultaneous PK Analysis and Metabolite Identification Using Novel Mass Spectrometry Acquisition Techniques

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SUMMARY

Metabolite identification and quantification are important parts of the drug development process. To date LC/MS has had to rely on chromatographic separation and mass-to-charge ratio for selectivity while LC/MS/MS can give an additional dimension through MRM pairs or Precursor or NL scans. Accurate mass can help discriminate against many chemical interferences but even infinite resolution cannot discriminate between true isobars with the same chemical formulae such as the multiple oxidations or dealkylations possible for many compounds. With high speed differential mobility spectrometry (DMS), another dimension of separation, compatible with fast chromatography is now available, that may be able to discriminate between those isobars. We will demonstrate separation of isobars using synthetic metabolites. Additionally, the potential for lowered background from mobility spectrometry was investigated. Initial data showing the promise of cleaner background using DMS will be demonstrated with an in vitro incubation of nefazodone using rat liver microsomes. Another approach to metabolite ID uses data-independent acquisition. Here, we present data from a unique data-independent HRMS acquisition approach called SWATH (Sequential Windowed acquisition of All THeoretical fragment ion spectra) for simultaneous Qual/Quant analysis.

INTRODUCTION

Many interfering compounds can be encountered when working on samples in a pharmacokinetics laboratory. Some interferences come from mobile phases and columns, others come from the sample matrix, and, depending on the chromatography, isobars can also behave like interferences. One of the goals of early ADME is high throughput which typically means using faster chromatography which can, in turn, lead to co-eluting peaks, especially for very similar compounds such as the positional isomers that can occur from oxidative metabolites or the multiple demethylations possible from compounds such as verapamil. Our goal was to show separation of these isobaric compounds that cannot be separated on any existing or theoretical mass spectrometer. We will demonstrate separation in DMS for three oxidations of carbamazepine and two demethylation metabolites of verapamil.

While working on the data to show separation of compounds with the same formula, it was found that the majority of the compounds we'd worked with moved further into "negative COV space" the higher the separation voltage (SV) became (see figure 2). When optimizing the transmission of Nefazodone in the DMS, it moved to positive COV space, inspiring us to try running the samples incubated with rat liver microsomes to see if there would be better signal-to-noise. The incubations were run while ramping COV and then, when optimal DMS conditions were found, the samples were re-analyzed using the best SV/COV combination. The chromatogram from data collected with DMS on was nearly identical to the extracted ion chromatogram of the known metabolites and showed higher signal for some metabolites. The preliminary work shown here shows some promise for finding metabolites in complex matrices.

MATERIALS AND METHODS

Samples:

DMS—Synthetic oxidative metabolites of carbamazepine—CBZ (2-hydroxy CBZ, 3-hydroxyCBZ and 10,11-CBZ epoxide and two verapamil demethylations—Norverapamil and O-desmethylverapamil from Toronto Research Chemicals were used to illustrate DMS separation of isobaric metabolites. To test whether DMS could minimize background, Nefazodone was incubated at 10 µM with rat liver microsomes. The samples were then quenched with ACN, lyophilized, and reconstituted in 5% ACN/H_2O for analysis.

SWATH—Bromociptine was incubated in rat liver microsomes to an initial concentration of 10 µM. The samples were diluted to concentrations of 1, 0.1 and 0.01 µM with mobile phase. Bromocriptine incubations t = 0 and t = 60 min were analyzed.

HPLC Conditions:

DMS—A Shimadzu Prominence LC system with a Waters Acquity C18, 2.1×50 mm, 1.7 µm column at 40˚ C using water (A) and acetonitrile (B) each with 0.1% formic acid for mobile phases was used. Gradients and injection volumes were adjusted as appropriate.

SWATH—A Shimadzu Prominence UFLC XR using a Kinetex C18 (2.6 µm) 2×50 mm column were used.

MS Conditions:

DMS—A prototype AB Sciex TripleTOF® 5600+ LC/MS/MS system equipped with SelexION™ technology was used with a DuoSpray™ source and electrospray ionization. The data presented here were analyzed on only one system. The system was operated in the information-dependant acquisition mode for all samples. The separation of isobars was done with a one minute gradient acquisition (2 min. total/sample) and the separation of incubated samples used a 9 minute gradient (12 min. total/sample).

SWATH—A TripleTOF® 5600+ system employing information-independent SWATH™ acquisition and information dependent (IDA) analyses of microsomal incubates were carried out in positive ion mode. A TOF MS scan (m/z 100 to 1000, TOF accumulation of 40 ms) was followed with either a TOF MS/MS under IDA conditions or a suite of 22 or 40 SWATH experiments with a Q1 isolation window set at 25 Da covering the mass range of 100 – 1000 Da with a total cycle time of 1.2 s. Both dynamic background subtraction and compound-specific mass defect was used as preferential criteria for precursor candidate selection within the IDA method. The collision energy of 35 ± 15V with was used in both approaches. The resulting data were processed with research grade metabolite identification software.

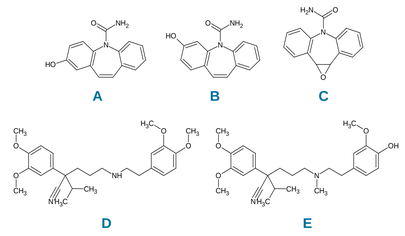

Figure 1. Compounds used to test the power of DMS to sepa-rate isobars. A = 2-OH-CBZ, B = 3-OH-CBZ,C = 10,11-CBZ epoxide (all with molecular formula $C_{15}H_{12}N_2O_2$), D = norverapa-mil, and E = *p*-O-desmethylverapamil (formula = $C_{26}H_{36}N_2O_4$).

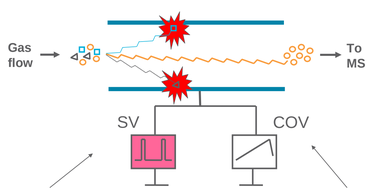

Figure 2. SelexION™ Technology uses a planar geometry to which the term "Differential Mobility Spectroscopy" or DMS can be applied. The Separation Volt-age (SV) radially displaces ions towards one electrode or the other depending upon their high and low field mobility characteristics. Compensation Volt-age (COV) restores the ion trajectories for a given compound, allowing them to transit through the DMS device and enter the mass spectrometer.

RESULTS

For the best separations, the DMS was tuned using isopropanol as modifier to optimize the transmissions of the five compounds shown in Figure 1. Samples of the individual compounds and also mixes of the isomers of hydroxycarbamazepine (OH-CBZ) and desmethylverapamil were run under the optimized DMS conditions for each individual analyte using a short gradient to simulate coelution. Figures 3 shows the transmission of the 3 OH-CBZs when run under conditions optimal for the transmission of 2-OH-CBZ. Figure 4 shows the transmission of both desmethylverapamils using optimized conditions for p-O-desmethylverapamil.

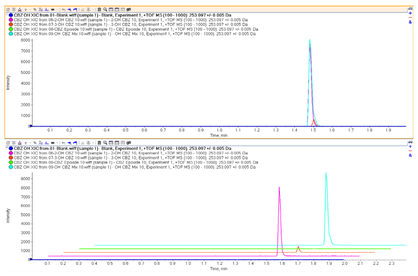

Figure 3. Transmission of the three CBZ metabolites with molecular formulae of $C_{15}H_{12}N_2O_2$ by a method optimized for 2-OH CBZ. The upper trace shows all the overlaid XICs and the bottom shows them in a pseudo-3D view. There is a small (7%) trans-mission of 3-OH CBZ (red trace) when the system is optimized for 2-OH CBZ.

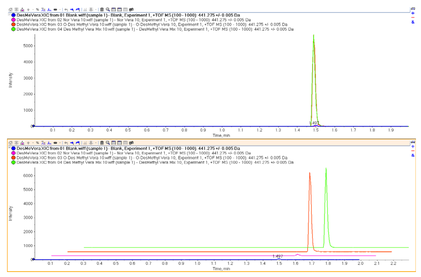

Figure 4. Transmission of the norverapamil and *p*-O-desmethyl-verapamil using a method optimized for *p*-O-desmethylverapamil. The transmission of norverapamil is essentially the same as that of the blank.

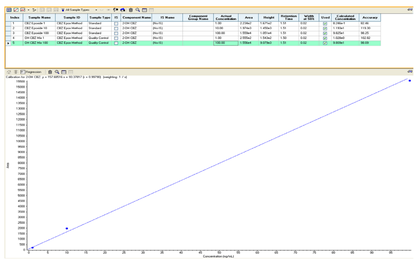

Figure 5. Quantification of 2-OH CBZ from 1-100 ng/mL. Samples with a mix of all three CBZ oxidations were also run at 1 and 100 ng/mL for each oxide. They're shown in the table as QC samples. If the other oxides were essentially removed from the chromatogram by the DMS, the % accuracies should be about the same for the standards and the QCs (which is, indeed, the case).

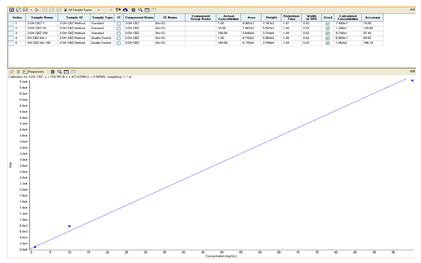

Figure 6. Quantification of CBZ epoxide from 1 to 100 ng/mL. Recovery of the QC sample (see figure 5 caption) again show that the DMS was able to give enough separation that the other two oxides were essentially ignored.

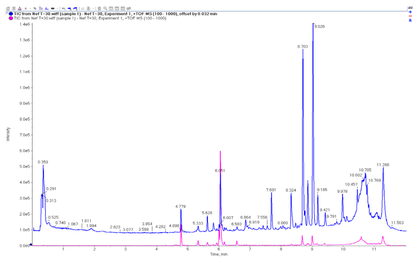

Figure 7. Overlaid chromatograms of DMS in transparent mode (blue trace), and DMS on (pink trace). It is clear that the back-ground is much lower in with DMS on for this analyte.

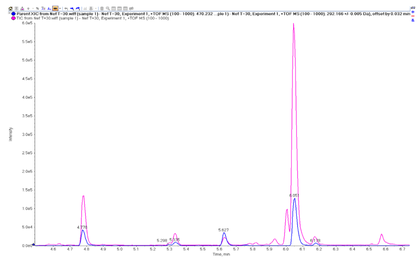

Figure 8. Overlaid chromatograms of the XICs of the major metabolites (intensities above ≈1% of total area) DMS in transparent mode (blue trace), and the un-extracted chromatogram with DMS on (pink trace) zoomed in to the area where the metabolites are eluting. The two chromatograms look remarkably similar. It is interesting to note that in addition to how clean the chromatogram with DMS on is, it is also higher in intensity possibly due to less interference from co-eluting peaks.

SWATH™ Acquisition
(Sequential Windowed acquisition of All THeoretical fragment ion spectra)

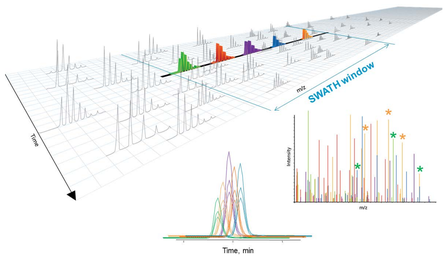

Figure 9. The non-specific fragmentation data collection strategy generates an unbiased record of all fragments of all precursors within one SWATH™ isolation window. Fragments that belong to the a given precursor will have the same chromatographic profile as that precursor.

Novel 2-Dimensional Algorithm for SWATH™ Metabolism Data Processing (PCVG data filtering strategy)

PCVG is an unsupervised, intuitive method that assigns a large number of variables to a smaller number of groups that can be more easily understood and manipulated. This simplifies interpretation since there are usually considerably fewer groups than variables and group representatives substitute well the behaviour for any variable within a particular group.

- The pre-processed aligned multiplexed fragment ion spectra undergo principal component analysis (PCA)
- PCVG analyzes the PCA loadings values to find correlated variables
- PCVG uses the chromatographic profile of an LC peak to group the variables
- Target PCVG routine filters out the groups of variables that do not correlate with the target LC peak profile
- This processing can find correlated signal in any mass range of any experiment

Total Metabolite Coverage and MS/MS Coverage

Peak ID	Name	m/z	R.T. (min)	IDA Occurrence 0.1 uM	IDA Occurrence 1 uM	IDA Occurrence 10 uM	SWATH Occurrence 0.1 uM	SWATH Occurrence 1 uM	SWATH Occurrence 10 uM
	Parent	654.2276	4.09	×	√	√	×	√	√
	Parent	654.2311	4.56	×	√	×	√	√	√
C1	Loss of Br->H+Tri-Oxidation	624.3052	2.59	×	√	√	×	√	√
C2	Loss of Br->H+Di-Oxidation	608.3077	3.1	×	√	√	×	√	√
C3	Di-Oxidation	686.2209	3.42	×	√	√	√	√	√
C4	Di-Oxidation	686.219	3.56	√	√	√	×	√	√
C5	Hydroxy	670.2265	3.68	×	√	√	√	√	√
C6	Di-Oxidation	686.2186	3.69	√	√	√	√	√	√
C7	Hydroxy	670.2235	3.89	√	√	√	√	√	√
C8	Hydroxy	670.2242	4.42	×	√	√	√	√	√
	Summary	Found Metabolites		3	10	9	6	10	10
		MS/MS available		3	10	9	6	10	10

Table 1. Metabolites found using IDA and SWATH™ acquisition method for bromocriptine incubated at 0.1, 1and 10 µM concentrations.

The numbers of metabolites found by the IDA and SWATH methods are comparable with the SWATH method finding a few more. For this specific example, the MS/MS coverage (a spectrum was found without having to re-acquire) was 100% across all concentrations for both methods. In our experience with many cases, however, IDA does not always trigger MS/MS on all metabolites whereas SWATH does.

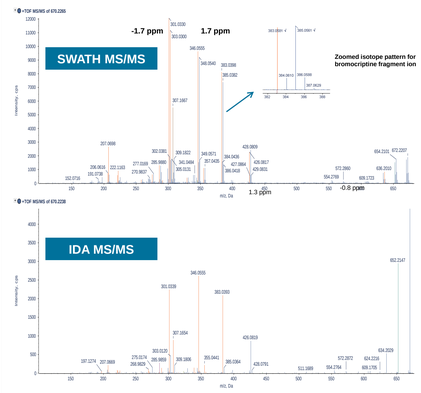

Figure 10. SWATH™ and IDA MS/MS spectra for one of the hydroxy metabolites (RT=3.68 min) in the 10 µM bromocriptine incubations.

MS/MS Quality and Sensitivity Comparison

The MS/MS spectrum from the SWATH acquisition method shows a similar MS/MS fragment pattern and intensities when compared to the targeted IDA spectrum. However, the SWATH MS/MS spectrum has the additional benefit of retaining the isotope pattern reflecting wider Q1 (25-Da) isolation window. For compounds that have distinctive isotope patterns, e.g., Cl- or Br-containing compounds such as Clozapine or Bromocriptine, etc., retaining the isotope pattern information in the product ion spectrum can be very helpful for tracking specific structural modifications when doing structural or soft spot analysis. The mass accuracies were very similar in both methods as well.

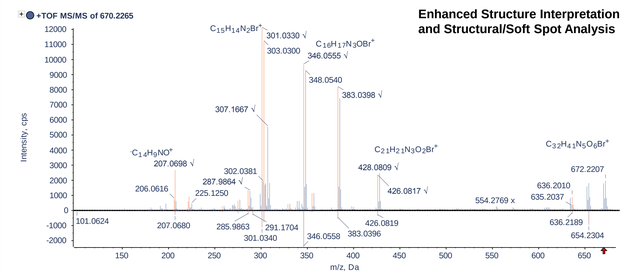

Figure 5. TOF MS/MS spectra of hydroxy metabolite of Bromocriptine (from the 10 uM level)

Using the SWATH MS/MS spectrum and the Interpretation portion of the MetabolitePilot™ software helped us to propose a structure for the hydroxy metabolite at 3.68 min. Since the Br isotope pattern carries over into this SWATH MS/MS spectrum we used that information to localize where the change probably occurred and to eliminate places where it couldn't have occurred. We had previously interpreted the spectrum of the parent (the inverted spectrum). When compared to the hydroxy metabolite's spectrum, all fragments with the bromine isotope pattern matched those found to be from the lower left portion of the molecule (shown in red in the structure—the Br-containing rings) indicating the hydroxylation was not on that portion of the molecule. The biggest shared common fragment (m/z 511.1707) allowed us to predict that the hydroxylation is probably one of the three available carbons of the pyrrolidine ring.

CONCLUSIONS

DMS has been shown to have the potential to be an additional orthogonal separation technique in addition to those currently available in LC/MS and LC/MS/MS. Figures 3 and 4 illustrate this the potential by showing the removal of isobaric interferences for two compounds and a few of their metabolites. The removal was essentially quantitative as shown in Figures 5 and 6.

Very early work illustrating the potential for DMS to provide a cleaner background, possibly with more signal due to suppression of interferences is shown in Figures 7 and 8 while Table 1 shows that at least the major metabolites found by methods with and without DMS (at least for Nefazodone) were the same. This will require much more study.

Information independent data collection with the SWATH strategy yielded data sets where both TOF MS and TOF MS/MS data were available at all times. A fast, robust, and reliable approach for the deconvolution of multi-component fragment ion spectra acquired within one SWATH window has been established. This approach was applied within a research version of MetabolitePilot™ software for the identification and structure information in support of the drug metabolism studies.

SWATH benefits for metabolite identification workflow

1) Generic MS method(s) that are simple and easy to build
2) Comprehensive fragment coverage and the ability to re-interrogate data without having to re-inject the sample
3) Improved MS/MS quality and sensitivity for better structure elucidation and soft spot analysis
4) Seamless Qual/Quant workflows with enhanced Qual and Quant when compared to information-dependant workflows

FUTURE WORK

This was preliminary work, done on one prototype instrument. Certainly more analytes and more replicates for each analyte must be investigated. However the results seem to encourage further investigation. Similarly, the data from the Nefazadone *in vitro* incubations indicate that further investigation may be warranted into the use of DMS for lowering background when running more complicated *in vivo* samples.