APPLICATION OF MASS DEFECT FILTERING - INFORMATION DEPENDANT ACQUISITION (MD-IDA) IN THE ANALYSIS OF *IN-VITRO* SAMPLES FOR METABOLITE SOFT-SPOT IDENTIFICATION

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INTRODUCTION

The continued drive to accelerate the drug discovery process is increasing the desirability to understand candidate biotransformation in a shorter time frame following synthesis. The identification and elucidation of metabolites or the soft-spot of the structure can give chemists a better understanding of the structure-metabolism relationship (SMR) and this information can aid candidate selection.

The use of real time mass defect filtering (MDF) on information dependant acquisition (IDA) scans allows the generation of simultaneous selective MS and MSMS data in one injection. The combination of high selectivity and sensitive high resolution mass spectrometry (HRMS) with faster chromatographic run times and improvements in semi-automated commercial software has allowed qualitative analysis and elucidation of the top 3 metabolites to be completed in a time frame compatible with early-stage drug discovery.

Here we present soft-spot data for benchmark compounds in liver microsomes from various species obtained from short chromatographic gradients with data capture using the MD-IDA approach. The presence/absence of relevant metabolites compared to a more conventional metabolite identification approach is discussed.

METHODS

Incubation Conditions

Pooled liver microsomes (final protein concentration of 0.5 mg/mL), 0.1 M phosphate buffer pH 7.4 and test compound (final substrate concentration 3 µM) were incubated at 37 °C prior to initiation by the addition of NADPH (final concentration 1 mM). Each compound was incubated for 0 and 45 min at 37°C in a thermomixer (700 rpm). The reactions were stopped by the addition of acetonitrile. The incubation plates were centrifuged and the supernatants were diluted 1:1 (v/v) with water prior to analysis by HRMS.

Two compounds were incubated in each species, dextromethorphan and verapamil in human and dog and diazepam and diphenhydramine in mouse and rat. A total of 9 sets of incubations were run for each compound with 3 replicates in three different assays.

Analytical Conditions

Samples were analysed by HRMS over a mass range of m/z 50 – 550 with mass defect- triggered IDA (MDF – IDA)[1] on a system consisting of an Agilent™ 1290 Infinity Binary HPLC and Pump Column Oven (Agilent Technologies, Cheshire, UK), HTS PAL DLW autosampler (CTC Analytics, Zwingen, Switzerland), and an Sciex 6600 TTOF (Sciex, Warrington, UK). The analysis was performed using the following solvent system and gradient.

Column	Cortecs T3 (2.7 µm) 2.1 x 100 mm (Waters Ltd, Herts, UK)			
Column Temp	60 °C			
Injection Vol.	10 µL			
Mobile Phase A	10mM Ammonium Formate + 0.1% Formic Acid (aq)			
Mobile Phase B	Acetonitrile + 0.1% Formic Acid			
Gradient Profile	Time (min)	Flow Rate (µL/min)	% Mobile Phase A	% Mobile Phase B
	0.00	800	100	0
	0.10	800	100	0
	3.50	800	45	55
	4.00	800	5	95
	4.60	800	100	0
	5.00	800	100	0

Table 1: Chromatographic conditions on Sciex 6600 TTOF

One replicate for each test compound in human liver microsomes was also run by MS^e over a mass range of m/z 150 to 1000 on a HRMS system consisting of an Acquity™ Binary Solvent Manager (BSM), Acquity™ 4 position heated column manager, Acquity™ PDA detector, 2777 Ultra High Pressure Autosampler and a Xevo G2-S QTof mass spectrometer (Waters, Herts, UK) as comparison to Cyprotex's conventional metabolite identification approach. The analysis was performed using the following solvent system and gradient.

Column	2.1 x 100 mm C18 ACQUITY UPLC® HSS T3 1.8µm (Waters Ltd, Herts, UK)			
Column Temp	60 °C			
Injection Vol.	10 µL			
Mobile Phase A	10mM Ammonium Formate + 0.1% Formic Acid (aq)			
Mobile Phase B	Acetonitrile + 0.1% Formic Acid			
Gradient Profile	Time (min)	Flow Rate (µL/min)	% Mobile Phase A	% Mobile Phase B
	0.00	400	98	2
	0.50	400	98	2
	10.00	400	45	55
	11.00	400	5	95
	12.00	400	5	95
	12.20	400	98	2
	15.00	400	98	2

Table 2: Chromatographic conditions on Waters Xevo G2-S QTOF

Metabolites were identified by processing samples through MetaSense™ [2], a vendor neutral metabolite profiling software suitable for processing both Waters and Sciex data. In all processed samples the top 3 metabolites were identified above 1% of total drug related material.

RESULTS

The mean, standard deviation (SD) and co-efficient of variation (CV) of peak area response were calculated for each observed metabolite. In the human and dog samples the CV was less than 10% for all metabolites; a greater variation of CV was observed in the metabolites seen in mouse and rat, further validation samples will be carried out to aim to improve this variation.

The most abundant metabolite formed for each compound in the 4 species was compared against the parent response in the control to obtain a pass criteria for the assay based on metabolite formation in the absence of a full clearance profile. These compounds will be used in future assays as benchmark compounds for confirmation that the assay performed as expected. Table 3 shows the top 3 metabolites observed for dextromethorphan and verapamil in the human liver microsomes.

Species	Test Compound	Biotransformation	Retention Time (Min)	Average Total % Area
Human	Verapamil	Parent	3.09	2.58
		Parent - $C_{10}H_{12}O_2$	2.48	62.4
		Demethylation	3.05	31.0
		Demethylation + Oxidation	2.90	4.05
	Dextromethorphan	Parent	2.60	44.0
		Demethylation	1.95	2.47
		Demethylation	2.57	50.7
		Oxidation	2.73	2.91

Table 3: Top 3 metabolites detected in human liver microsomes for verapamil and dextromethorphan

Results obtained using the shorter chromatographic gradient were compared against Cyprotex's conventional metabolite identification approach. The reported top 3 metabolites were the same following both sets of analytical conditions and the calculated CV was less than 20% for all observed metabolites between the two data sets. Figure 1 shows combined extracted chromatograms (XIC) for both data.

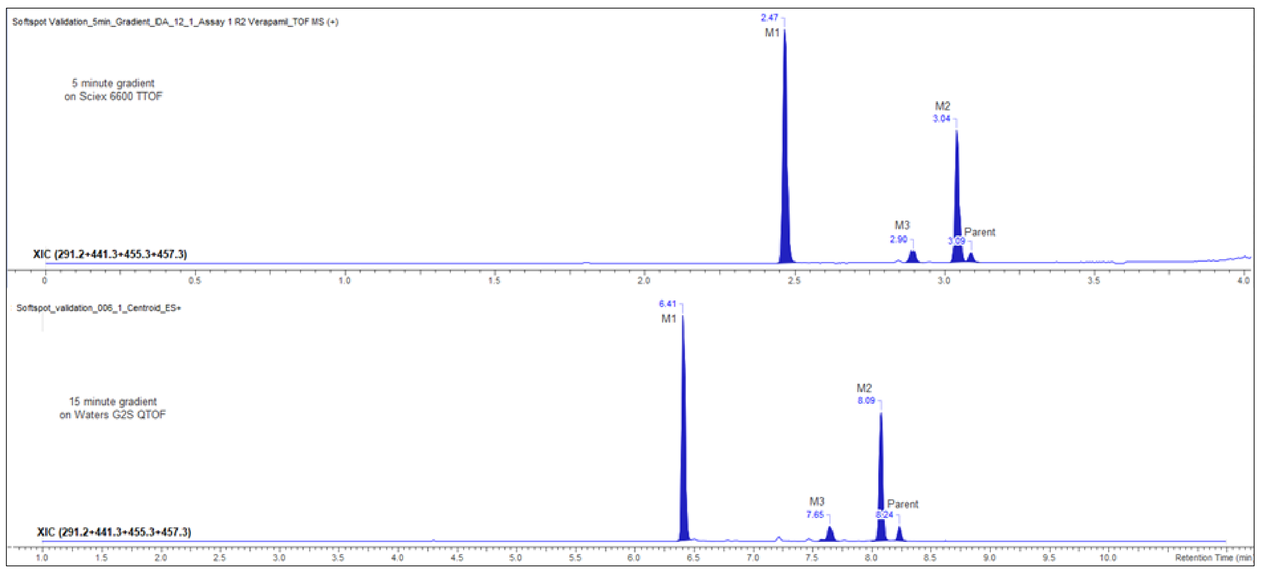

Figure 1: Comparison of results from 5 minute gradient on Sciex TTOF and 15 minute gradient on Waters QTOF

MetaSense™ software is able to assign the structures of metabolites using both the generated MSMS data and a probability scoring system which utilises the physical-chemical properties of the analyte. During this work the proposed structures assigned by the software were confirmed by expert review and compared against the metabolites found in literature. Figure 2 shows structural elucidation for one of the metabolites of verapamil

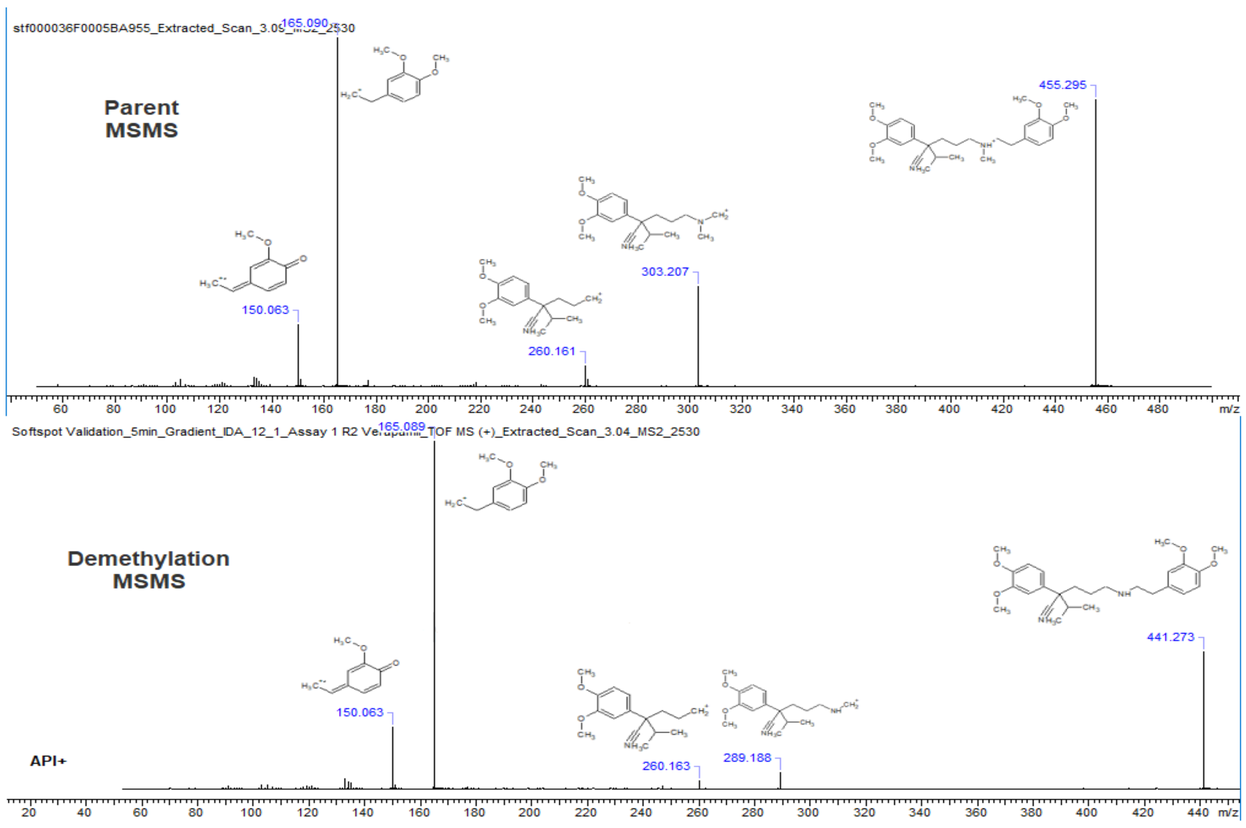

Figure 2: Structural assignment of main fragment ions for verapamil and the associated demethylation metabolite.

CONCLUSIONS

MDF-IDA scans are a powerful and accurate method for obtaining simultaneous high resolution MS and MSMS data in one injection. Advancements in column chemistry and UPLC systems allows greater separation and resolution of chromatographic peaks over a shorter run time. Advancements in MS scan capabilities allows simultaneous acquisition of MS full scan and MS/MS data without loss of data quality.

Advancements in elucidation tools within metabolite software packages assist the end user in establishing the location of any biotransformations more rapidly. MetaSense™ software is a powerful vendor neutral software package with many functionalities; further development and testing is required with both MassLynx and Sciex data to confirm both the capabilities and the report functionality.

References

1. ABSciex (2014) – Xenobiotic Metabolism Workflows in Drug Discovery & Development
2. Lee. R – ACD Labs Application Note – Automation and Effective Data Sharing for Metabolite Identification