STRAIGHTFORWARD *IN VIVO* METABOLITE IDENTIFICATION USING A COMBINATION OF ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY, NEW GENERATION QUADRUPOLE TIME OF-FLIGHT MASS SPECTROMETER, AND AUTOMATED DATA PROCESSING

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OVERVIEW

- The use of ACQUITY UPLC™ enabled metabolite identification to be carried out in a very fast and efficient manner. The peak capacity was greatly improved and also it enable us to obtain better chromatographic separation with little system optimisation
- The advantage of the Q-Tof Premier™ hybrid quadrupole time of flight mass spectrometer is the selectivity and sensitivity that can be achieved
- Exact mass measurement provided unequivocal results confirming all metabolites detected
- Automated data processing (MetaboLynx™ Application Manager for MassLynx™ Software) for metabolite identification is a vital step to handle large number of samples
- Automatic removal of false positives with exact mass data filters proves to be critical as it is a time-consuming task

INTRODUCTION

Different LC-MS strategies are used for metabolite identification, the two most popular being the use of ion trap or linear trap mass analyzers (with data-dependent type of experiments) or the use of Time-of-Flight or hybrid quadrupole Time-of-Flight technology – Q-Tof (with exact mass measurement of metabolites). In all cases, the challenge in metabolite identification is to get high quality chromatographic separation in a short time frame, good sensitivity for minor metabolites and a rapid and reliable metabolite identification process. Data processing appears as the most challenging part, and guesses often have to be made on metabolite molecular weights if metabolites are not visible in the MS trace, especially in complex biological matrices. In order to address these challenges, we present a combination of Ultra Performance Liquid Chromatography, a new generation Q-Tof mass spectrometer, and automated metabolite detection using a new set of exact mass algorithms. In vivo metabolism samples were obtained from rats administered verapamil orally at 30 mg/kg. Urine was collected, and samples from rats administered vehicle only were used as controls. The data demonstrated excellent metabolite separation. Mass accuracy was below 3 ppm RMS for all metabolites identified, including peaks that would have been highly saturated with conventional TOF technology. This is a major enhancement made possible by the Programmable Dynamic Range Enhancement pDRE) feature of the Q-Tof Premier. Beside excellent mass accuracy for a wide range of metabolite concentration, pDRE also provides a more reliable picture in terms of relative metabolite abundance. Automated metabolite detection using the new algorithms was extremely fast and straightforward: a very limited number of false positives were observed in urine, even when looking for minor metabolites (down to 0.1% of the major metabolites). Overall, this is a powerful approach for rapid and reliable metabolite identification in complex biological matrices, with no pre-requisite on potential routes of metabolism.

EXPERIMENTAL CONDITIONS

Sample Conditions

Lister Hooded rats were administered orally with one compound (Verapamil) (2 rats/ compound as a 1mL suspension in 10% acacia) at a dose of 30 mg/kg. Urine was collected on dry ice from 0 to 8 h after administration and was injected in LC/MS without any dilution.

LC Conditions

Solvent delivery system: ACQUITY UPLC™
Column: ACQUITY UPLC™ BEH C18 column 100 x 2.1 mm id, 1.7 µm particle size
Mobile phase A: Ammonium formate pH 9
Mobile phase B: Acetonitrile
Gradient: 0-2 min 100% A, 10 min 90% A, 20 min 60% A, 25-26 min 10% A, 26.1-30 min 100% A
Flow rate: 600 µL/min
Injection volume: 10 µL

MS Conditions

Mass spectrometer: Micromass® Q-Tof Premier™
Ionisation mode: Electrospray positive ion mode
Cone voltage: 30 V
Capillary voltage: 3 kV
Source temperature: 120 °C
Desolvation temperature: 450 °C
Lock mass: Leucine enkephalin m/z 556.2771, concentration 0.5 ng/uL

How does MetaboLynx work?

MetaboLynx is a software application manager, which automatically detects putative biotransformations for expected and unexpected metabolites. The application manager runs samples scheduled for analysis by LC/MS and processes the resulting data (Figure 1). Results are reported via a 'Data Browser' that enables the chromatographic and mass spectroscopic evidence that support each automated metabolic assignment to be rapidly reviewed. The analytical strategy carried out in this poster is outlined below in Figure 2. It operates by comparing and contrasting each metabolised sample with a control sample—although unexpected metabolite searching may still be performed in the absence of a suitable control. Samples from *in vitro* incubations or *in vivo* dosing experiments can be quickly analysed by LC/MS, followed by a multidimensional data search which correlates retention time, m/z value, intensity and components from alternative detection technologies (e.g. diode array UV or radiochemical monitoring).

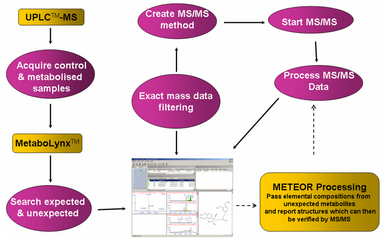

Figure 1. Shows description of analytical strategy used

Comparison of analyte data with the control sample allows filtering of matrix related peaks, which would otherwise produce an unmanageable list of false metabolite peaks. Isotopic cluster analysis can be built into the MetaboLynx automated processing method and is used to target potential metabolites with the desired isotope ratios. For example Cl or radiolabeled containing drugs/metabolites can be pinpointed, at low levels, within a complex matrix background to dramatically enhance specificity and increase confidence in metabolite identification.

Why exact mass metabolite identification and data processing?

- Exact mass measurements enable the elemental composition of detected peaks to be confirmed for 'known' drugs and their metabolites using both MS and MS/MS spectra.
- For unknowns the number of plausible elemental compositions may be restricted to a small number (or uniquely identified) with the aid of additional chemical information—e.g. the molecular formula of the parent drug and knowledge of possible metabolic pathways.

How does the exact mass data filter work?

This is an extremely accurate and specific filter. It is based on exact mass and mass deficiencies, which are specific to each parent drug compound of interest. Each parent drug has a specific number of elements (C,H,N,O, ...) which is known by the chemist.
Depending on the number of each one of the elements mentioned, the drug of interest will have a very specific mass deficiency. For example if we look at Verapamil, it contains the following elements; $C_{27}H_{38}N_2O_4$. This equates to a monoisotopic protonated mass of 455.2910 Da. If we take an alkyl group away (O-dealkylation, a common metabolic route) then the mass is shifted by -14.0157 Da leaving us with a monoisotopic mass of 441.2753 Da (Figure 2). If we now workout the delta mass difference for the 4 decimal places between Verapamil and its N-dealkylated metabolite, we are left with an exact mass deficiency of 0.1376–0.1219 = 0.0157 Da. Therefore, if we were to put a window of around 20 mDa we would be able to detect its O-dealkylated metabolite and exclude all other entries which fall outside this window.

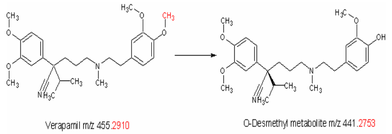

Figure 2. Shows the loss of the O-methyl group for Verapamil with the corresponding mass shift of 0.0157 Da

With this in mind, we can make the following hypotheses:

- All metabolites have masses within 0.25 Da of parent decimals.
- They are in general within 0.1 Da if there are no major cleavages leading to much smaller fragments (as an example, the biggest single phase II biotransformation, glutathione conjugation, will equate to a mass defect difference of 0.068 Da compared to the parent drug).
- Most metabolites will fall within a 180 mDa window of the parent compound, even if certain cleavages take place in the structure to yield smaller fragments.

RESULTS AND DISCUSSION

- With the use of the exact mass data filter tool (Figure 3) the number of entries in the unexpected table on the MetaboLynx Browser were reduced from 136 to 39 'real entries' (Figure 4). All metabolites detected showed mass measurements of RMS = 2.83 ppm (Table 1)
- UPLC™ coupled with Q-Tof technology provided an excellent separation and a short analysis time for in-vivo samples (Figure 5)
- Low level and high level metabolites were detected and identified with excellent exact mass accuracies (Figure 6 and 7)

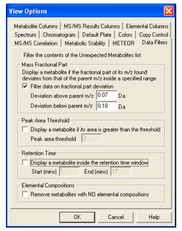

Figure 3. Shows the exact mass data filter applied to the data set in the browser

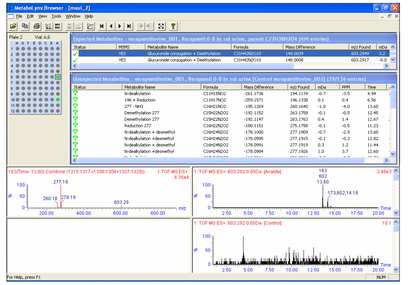

Figure 4. Shows the MetaboLynx Browser with all of the Verapamil metabolites in urine detected

Metabolite Name	Formula	Mass Difference	Mzi Found	mDa	PPM	Time	Area Abs
Parent	C27H38N2O4	-0.0038	455.2872	-3.8	-8.3	16.2	661
N-dealkylation	C11H15NO2	-261.1736	194.1174	-0.7	-3.5	4.94	27
198 + Reduction	C11H17NO2	-259.1571	196.1338	0.1	0.4	6.56	11.5
277 - NH3	C16H21NO2	-195.1269	260.164	-1	-4	13.6	157.4
Demethylation 277	C15H23N2O2	-192.1152	263.1758	-0.1	-0.5	12.45	14.2
Demethylation 277	C15H23N2O2	-192.1147	263.1763	0.4	1.4	12.67	23.1
Reduction 277	C16H25N2O2	-180.1151	275.1758	-0.1	-0.5	11.23	12.7
N-dealkylation + desmethyl	C18H24N2O2	-178.1083	277.1909	-0.7	-2.5	13.6	1526.6
N-dealkylation + desmethyl	C18H24N2O2	-178.0995	277.1915	-0.1	-0.3	12.82	299.8
N-dealkylation + desmethyl	C18H24N2O2	-178.0991	277.1919	0.3	1.2	11.44	5
N-dealkylation + desmethyl	C18H24N2O2	-178.0984	277.1926	1	3.7	12.6	465.6
N-dealkylation	C17H26N2O2	-164.0846	291.2084	-0.8	-2.9	13.74	2251.7
291 - Desmethyl + hydroxylation	C16H24N2O3	-162.104	293.187	0.5	1.7	12.18	20.5
291 + hydroxylation	C17H26N2O3	-148.0885	307.2025	0.4	1.2	10.84	31.9
2x desmethyl + reduction	C25H32N2O4	-30.0472	425.2439	-0.2	-0.5	14.46	17.4
2 x desmethyl + reduction	C25H32N2O4	-30.0462	425.2448	0.8	1.9	14.33	8.9
2 x Demethylation 455	C25H34N2O4	-28.0317	427.2592	-0.5	-1.1	15.1	24.9
2 x Demethylation 455	C25H34N2O4	-28.0318	427.2592	-0.5	-1.1	15.23	18.5
2 x Demethylation 455	C25H34N2O4	-28.0317	427.2592	-0.5	-1.1	15.33	39.9
Desmethyl 453	C21H30N2O8	-16.0829	439.208	0	0	10.31	45.9
Desmethyl 453	C21H30N2O8	-16.0828	439.2081	0.1	0.2	10.39	23.6
Desmethyl 453	C21H30N2O8	-16.0811	439.2099	1.9	4.3	11.06	7.7
Desmethyl 453	C26H34N2O4	-16.0223	439.2586	-1.1	-2.4	14.78	7.5
Desmethyl 453	C26H34N2O4	-16.0314	439.2586	-0.1	-0.1	15.53	11
Desmethyl 453	C26H34N2O4	-16.0313	439.2587	0	0.1	14.91	9.5
Desmethyl 455	C26H36N2O4	-14.0197	441.2713	-4	-9.1	16.01	676.5
Desmethyl 455	C26H36N2O4	-14.016	441.275	-0.3	-0.7	15.45	47.9
Desmethyl 455	C26H36N2O4	-14.0159	441.2751	-0.2	-0.5	15.26	23.4
Glucuronidation 277	C22H32N2O8	-2.0663	453.2226	-1.1	-2.4	10.58	723.3
Glucuronidation 277	C22H32N2O8	-2.0669	453.2241	0.4	1	11.43	53.8
Glucuronidation 277	C22H32N2O8	-2.0667	453.2242	0.5	1.2	11.25	20.9
Glucuronidation 277	C22H32N2O8	-2.0666	453.2243	0.6	1.4	10.66	379.8
Desmethyl + hydroxylation 455	C26H36N2O5	1.9787	457.2707	0.5	1	13.89	81.8
Desmethyl + hydroxylation 455	C26H36N2O5	1.9798	457.2707	0.5	1	15.53	44.8
Desmethyl + hydroxylation 455	C26H36N2O5	1.9801	457.2711	0.9	1.9	13.26	32.4
Hydroxylation 455	C27H38N2O5	15.9948	471.2858	-0.1	-0.2	13.32	21.9
Hydroxylation 455	C27H38N2O5	15.9951	471.286	0.1	0.3	14.02	125.5
Hydroxylation 455	C27H38N2O5	15.9963	471.2873	1.4	3	15.62	15.9
Hydroxylation 455	C27H38N2O5	15.9978	471.2889	3.0	6.2	15.74	36.1
2 x Hydroxylation 455	C27H38N2O6	31.9883	487.2793	-1.5	-3.1	14.91	7.3
Glucuronide conjugation + Deethylation	C31H42N2O10	148.0039	603.2849	3.2	5.3	13.8	94.3
Glucuronide conjugation + Deethylation	C31H42N2O10	148.0009	603.2917	0	-0.1	14.18	34.2
Glucuronide conjugation + Demethylation	C30H40N2O10	162.0175	617.3085	1.1	1.8	13.69	12.9
					RMS = 2.83		

Table 1. Shows the results table for all 43 metabolites detected with its corresponding exact mass and elemental composition information. The overall mass error when taking all metabolites detected into account was 2.83 ppm RMS

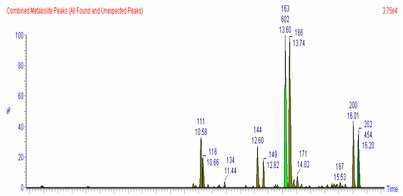

Figure 5. Shows the combined XIC trace for all metabolites detected. Excellent resolution was achieved by the use of UPLC™

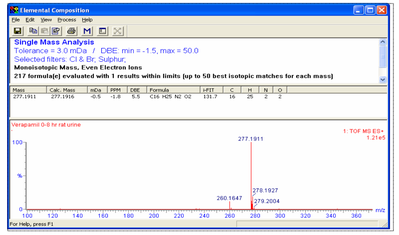

Figure 6. Shows a high signal for the one of the N-dealkylated +desmethyl metabolite at retention time 13.6 min with very good exact mass measurement of –1.8 ppm

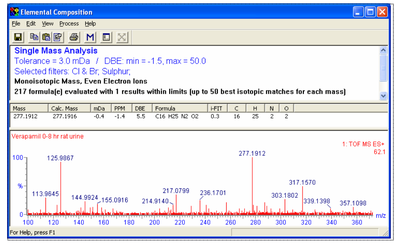

Figure 7. Shows a very low signal for one of the N-dealkylated +desmethyl metabolite at retention time 11.44 min with very good exact mass measurement of –1.4 ppm

CONCLUSIONS

- Exact mass data filters in MetaboLynx provides an easy and fast analytical strategy for unequivocal metabolite identification
- pDRE enables excellent dynamic range (4 orders) for different concentration levels with excellent exact mass measurements
- UPLC™ is the perfect combination with Mass Spectrometry in Metabolism Studies as it provides speed of analysis, better chromatographic resolution and increased sensitivity

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