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Bioanalytical Strategy for *in vitro* Metabolite Screening with Exact Mass Using the Q-Tof micro

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Abstract

This application note presents an automated bioanalytical approach using exact mass in combination with an automated software package, MetaboLynx, for *in vitro* metabolite screening.

Introduction

In metabolism studies, it is vital to understand how a particular drug is absorbed, distributed, metabolised, and eliminated by the body. Metabolite identification is a very important part of the drug discovery/development process, because early detection and identification of major and toxic metabolic routes can help to fine-tune drugs that go on to development. Metabolism is not an easy task and it can be extremely complex; that is why speed and accuracy of results are vital in order to make the right decisions at this stage.

Mass spectrometry is a well established technique for pharmacokinetic and metabolism studies, due to the fact that it is sensitive, fast, and robust. Exact mass measurement technologies, such as quadrupole time of flight (Q-Tof) MS, offer accurate data and high sensitivity, which are very important for metabolite identification. Use of exact mass data in the determination of metabolite structures allows medicinal chemists to make the necessary "structural tuning" to achieve the desired compound biological activity. Moreover, as the demand to screen a large number of compounds in drug discovery increases, bottlenecks in data processing can often result. Therefore, there is a need to develop an automated approach that can cope with the number of candidates analysed in the drug discovery process. In this paper, we present an automated bioanalytical approach using exact mass in combination with an automated software package, MetaboLynx, for *in vitro* metabolite screening (Figure 1). A range of different compound incubates in microsomes at 5 μ M will be shown. We will show that the MetaboLynx Software algorithm enables the identification of expected and unexpected metabolites from a control sample comparison. Moreover, we will also show how we can automate the entire process of metabolite detection and MS/MS method creation for subsequent sample analysis for identification purposes. Finally, we will show how enhanced mass measurement accuracy in the assay results may increase sample throughput and improve decision-making steps. This is especially applicable in the discovery phase, where some "compound structural tuning" may be required to achieve the pharmacokinetic and metabolism effects required for a specific novel structure.
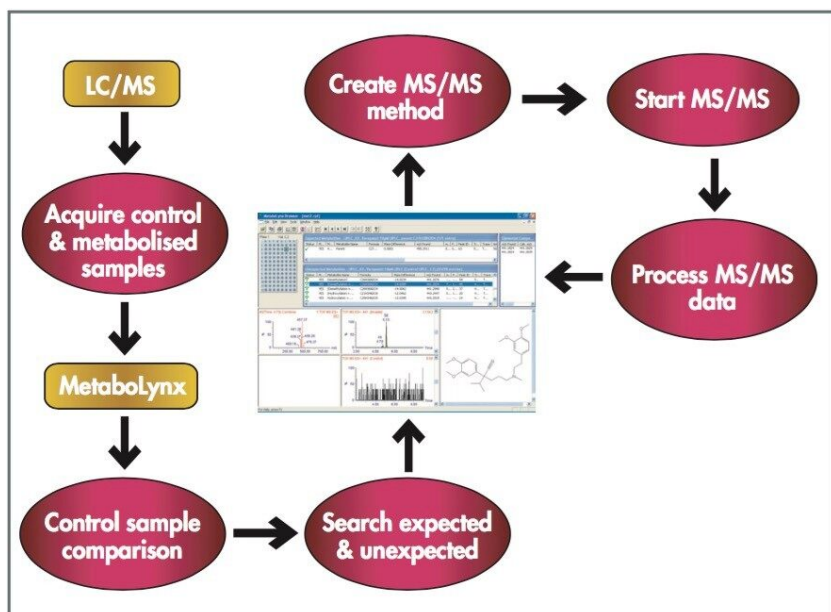
Figure 1. Bioanalytical strategy for in vitro metabolite screening using LC-MS/MS.

Experimental

MS Conditions

Mass spectrometer:	Waters Micromass Q-Tof micro
Ionisation mode:	ESI +ve ion
Capillary voltage:	3.2kV
Cone voltage:	35V
Source temperature:	120 °C
Desolvation temperature:	280 °C

Acquisition mass range:	50–800 amu
Lock mass:	Leucine Enkephalin *m/z* 556.2771

HPLC Conditions

Solvent delivery system:	Waters Alliance 2795
Column:	Waters Atlantis dC$_{18}$ 150 x 2.1 mm id, 3.5 μm
Flow rate:	300 μL/min
Mobile phase A:	Water + 0.1% Formic acid
Mobile phase B:	Acetonitrile + 0.1% Formic acid
Injection volume:	10 μL

Gradient

Time(min)	A%	B%	Flow(μL/min)
0.00	90.0	10.0	0.3
1.50	90.0	10.0	0.3
10.00	15.0	85.0	0.3
10.50	90.0	10.0	0.3
13.00	90.0	10.0	0.3

Samples

Rat liver microsomes with a protein content of 0.5 mg/mL were used to incubate verapamil, midazolam, and

dextromethorphan at 5 µM level. The reaction was stopped by adding one part of ice-cold acetonitrile with two parts of sample after a 60 minute incubation. Then, the sample was centrifuged at 15,000 rpm and the supernatant was collected for subsequent LC-MS/MS analysis.

Results and Discussion

Mass measurement accuracy levels of 2.5 ppm RMS were obtained for all incubated compounds and putative metabolites (Table 1). All major expected and unexpected metabolites for the compounds incubated were identified by the use of an automated processing algorithm, MetaboLynx (Figure 2). Subsequent MS/MS acquisitions were performed after detection of xenobiotics for structural identification (Figure 3).

Compound Name	m/z observed	m/z theoretical	mDa +/-	ppm +/-
Verapamil	455.2877	455.2910	3.3	7.2
M-14	441.2749	441.2753	0.4	0.9
M-14	441.2766	441.2753	1.3	2.9
M-28	427.2622	427.2597	2.5	6.0
M-164	291.2059	291.2073	1.4	4.6
Dextromethorphan	272.2007	272.2014	0.7	2.6
M-14	258.1852	258.1858	0.6	2.5
M-14	258.1866	258.1858	0.8	3.2
Midazolam	326.0869	326.0860	0.9	2.7
M+16	342.0812	342.0809	0.3	0.9
M+16	342.0804	342.0809	0.5	1.7
Average mDa			1.2	
RMS ppm				2.5

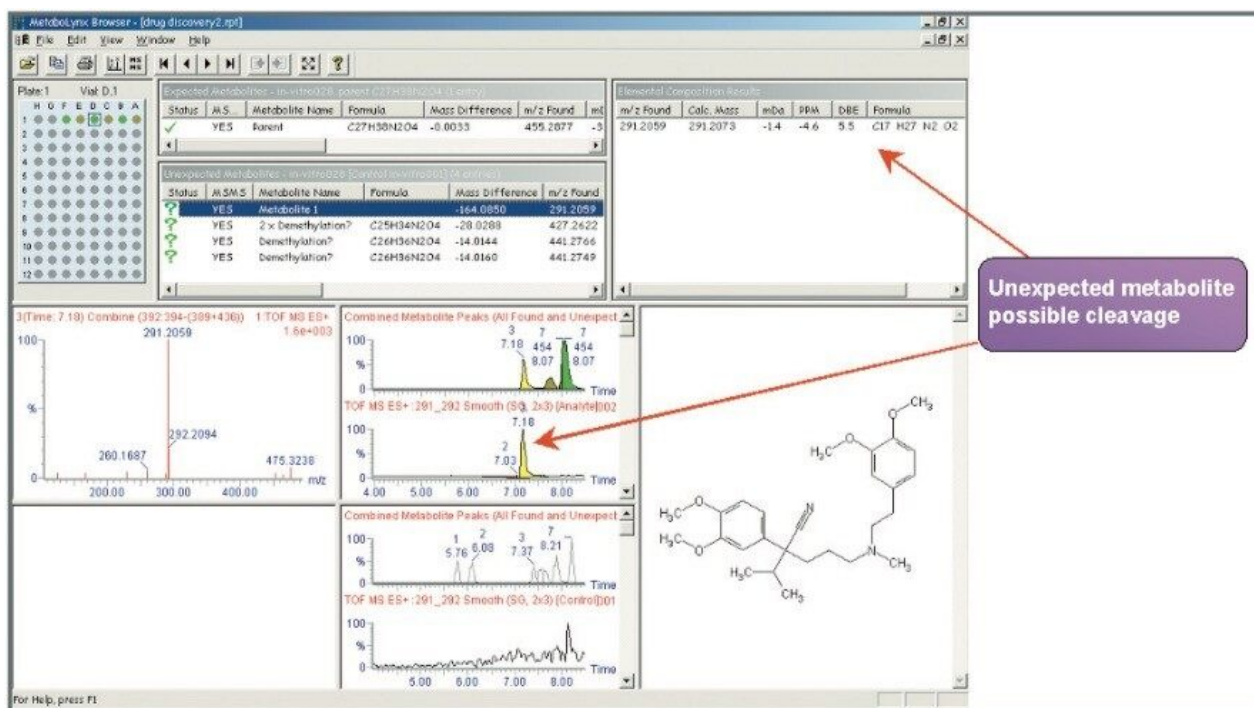Table 1. Mass accuracies for major metabolites.

Figure 2. MS MetaboLynx results for all drugs incubated, including expected and unexpected metabolites with exact mass.
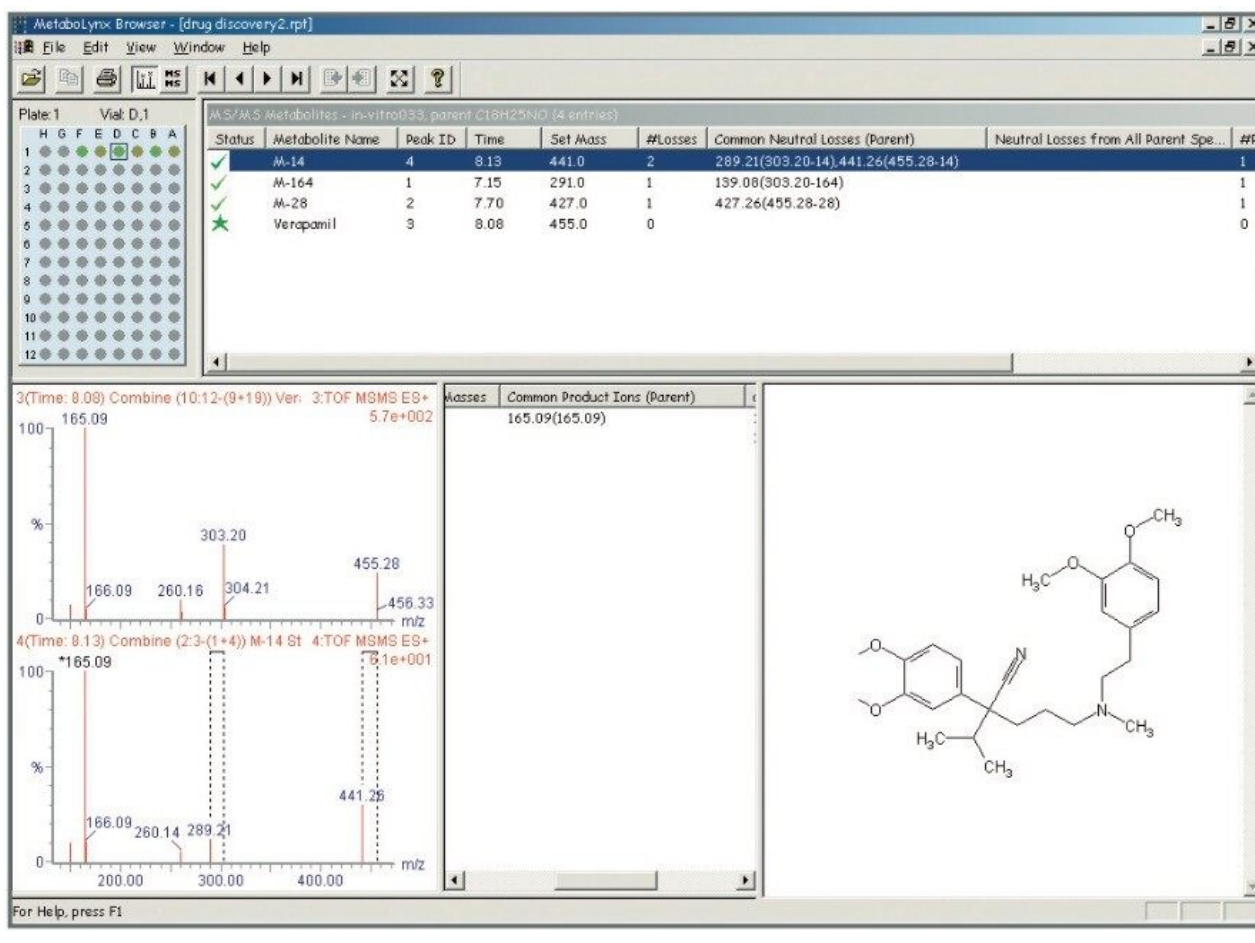
Figure 3. MS/MS MetaboLynx results for all drugs incubated and metabolites with exact mass.

Furthermore, there was an interesting finding for verapamil. After processing the data with MetaboLynx, an unexpected metabolite with *m/z* 291.2059 was detected by the control sample comparison algorithm (Figure 2). Under further examination on the browser, there was not a chromatogram present in the control sample with this mass. Exact mass measurements of 4.6 ppm revealed that it was a metabolite corresponding to the loss of $C_{10}H_{12}O_2$ from verapamil. Therefore, a postulated structure was proposed (Figure 4). Next, both verapamil and the putative metabolite at *m/z* 291.2059 were analysed by LC-MS/MS. Excellent MS/MS data with exact mass was obtained which helped to confirm the metabolite and elucidate its structure by comparing several common fragments on both parent drug and metabolite (Figure 5).
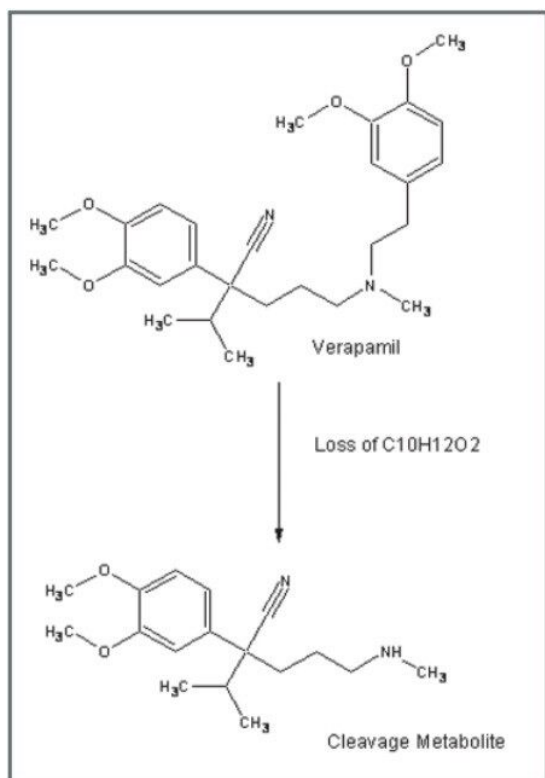
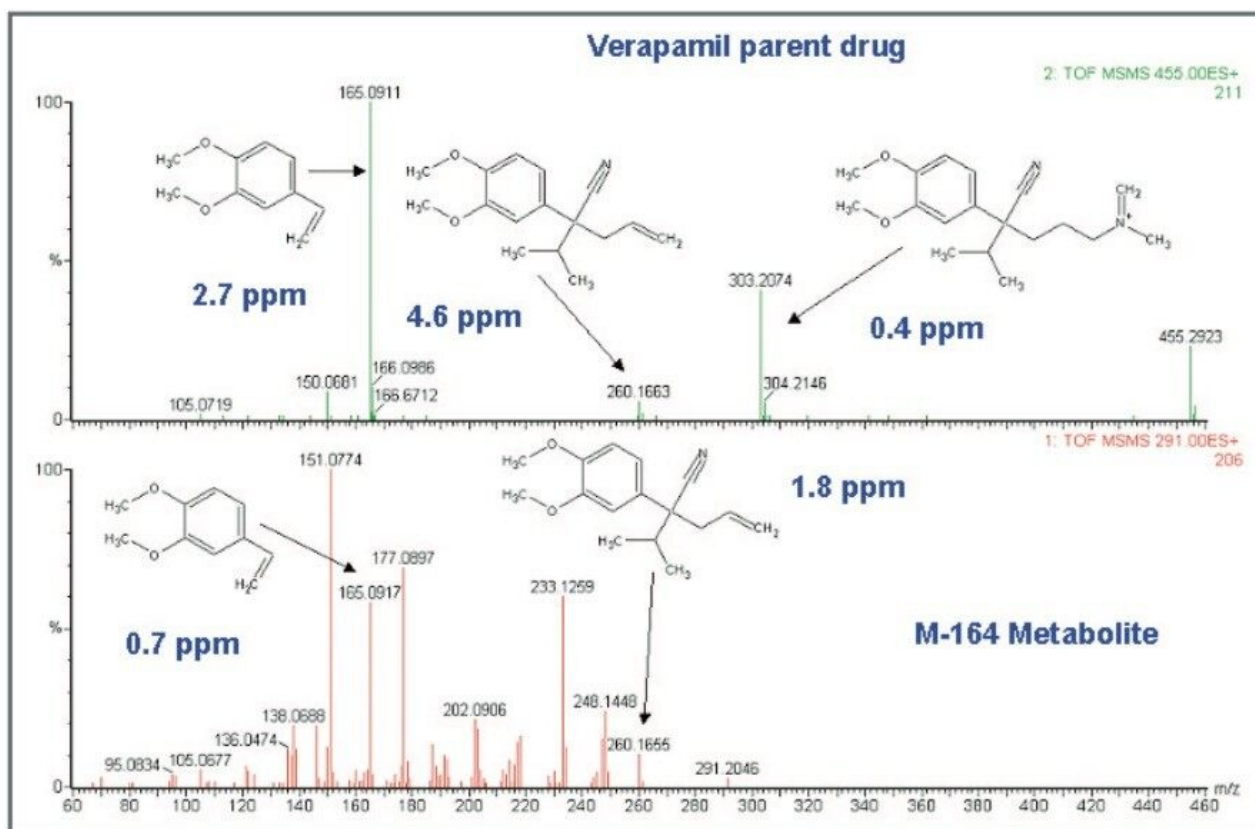*Figure 4. Proposed biotransformation route for metabolic cleavage.*

Figure 5. MS/MS exact mass data for verapamil and M-164 metabolite.

Conclusion

This strategy allowed an automated fast turnaround of samples, not only in the analysis time but also in the identification process, which can be the real bottleneck. Specifically, it allowed us to search for unexpected metabolites using the control comparison MetaboLynx algorithm with exact mass, making the whole process more accurate.

Exact mass in MS and MS/MS experiments together with Double Bond Equivalences is a major advantage in the identification process because it provides confidence in the results obtained and enables us to eliminate false positives.

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