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EXACT MASS LC/MS/MS IDENTIFICATION OF DRUG METABOLITES IN A CASSETTE OF FOUR MICROSOMAL INCUBATIONS

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OVERVIEW

Purpose

- To provide rapid analysis of drug metabolites in a cassetted sample using exact mass-measured data (<5 ppm) for confirmation of predicted and unexpected metabolites at low concentration.
- To investigate the clearance of the parent drug and the appearance of its major Phase 1 metabolites over time.

Method

- Microsomal incubation at 37°C of 4 pharmaceutical-like compounds sampled at various time intervals over 45 minutes. Subsequent cassetting into groups of 4.
- Fast, reversed phase (C_{18}) chromatography and full-spectral TOF-MS, mass-measured by means of an external reference compound infused using a dual electrospray ion source.
- Automated exact mass MS/MS experiments on chromatographic peaks of interest.

Results

- 4 x analytical throughput.
- Positive confirmation of metabolite identification.
- Determination of rates of metabolism.

INTRODUCTION

In order to investigate both the disappearance of a parent drug and the appearance of its metabolites, current working practices (e.g. using a triple quadrupole mass spectrometer) generally require at least two analyses to be carried out on different samples. The clearance of a parent drug can be tracked easily using MRM type experiments on sub-1 µM samples as described herein. However, to fully investigate the appearance of its metabolites, especially those which are not predicted, full MS spectra must be employed. Since the sensitivity in full spectral mode of scanning mass spectrometers is relatively low, this requires a second sample to be created at a much higher incubation level (typically ~20 µM), which often has experimental implications in terms of metabolite formation.

The experiments described below utilize the high full spectral sensitivity afforded by the oa-TOF analyzer to enable such investigation to be carried out not only on a single, low concentration sample, but also on multiple analytes cassetted within that sample.

Using data-mining software to identify the analytes plays an important role in increasing throughput of this type of sample. In this case, such software was used to automatically create and run MS/MS experiments on the chromatographic peaks of any metabolites found. Using the exact-mass functionality of the system on these product ion spectra allows for fast, positive identification of isomeric metabolites which may have different retention times.

METHODS

Microsomal incubations were all carried out using a Tecan Genesis workstation. The four compounds shown in **Fig. 1** (propranolol, diazepam, dextromethorphan and verapamil) were individually incubated at 3µM with both rat and human liver microsomes. Following reaction at 37°C, aliquots were removed at 5, 15, 30 and 45 minutes. To quench each reaction, an equal volume of methanol was added.

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Following centrifugation at 4000 G for 30 mins, samples for each compound at each respective time point were pooled together for LC/MS/MS analysis. This provided analytical samples with 0.37 µM concentration.

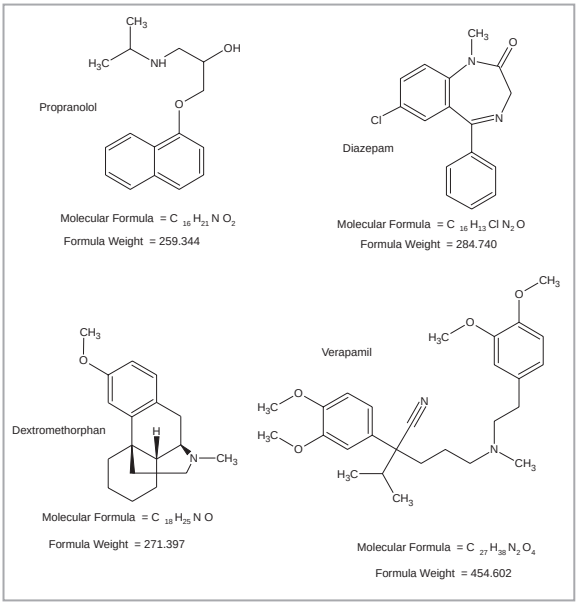

Figure 1. Drugs to be analyzed

All analyses were carried out using a benchtop hybrid quadrupole/oa-TOF mass spectrometer, operated with a resolving power of greater than 5,000 in electrospray positive ion mode. A dual ESI source (see **Fig. 2**) was used to simultaneously acquire sample and reference data to separate functions.

Chromatography was achieved with a Waters® Alliance® 2695 Separations Module controlled through the MassLynx™ software.

LC/MS Analytical Conditions

Mass spectrometer:	Micromass® Q-Tof micro™ with LockSpray™ source
Software control:	MassLynx version 4.0
Ionization mode:	Positive ion electrospray (3.2 kV)
Cone voltage:	45 V
Acquisition time:	1 second
HPLC system:	Waters 2695 Separations Module
Column:	Waters Symmetry® C_{18} (3.5 µm x 2.1 x 50mm)
Injection volume:	20 µL
Mobile phase:	(A) Water w/ 0.1% formic acid (B) Acetonitrile w/ 0.1% formic acid
Flow rate:	200 µL/min
Gradient:	t = 0 mins, 2% B t = 4 mins, 98% B t = 5 mins, 98% B t = 5 mins, 2% B

Additional data processing and automatic set-up of MS/MS experiments was performed using MetaboLynx™ 4.0 software.

Exact Mass Measurement

The LockSpray unit interfaces directly with the ZSpray™ source of the Q-Tof micro, and consists of two electrospray probes operated at the same capillary voltage. A rotating baffle allows the flow from one or other sprayer to pass through to the sampling cone, and thus be analyzed independently by the mass spectrometer. The cone voltage and collision energy can be adjusted for the analyte and reference channels and the spectra from each stored in separate data functions. Thus, the possibility of interference and suppression effects due to post column introduction of a reference sample or 'lock mass' into the analyte flow is completely avoided.

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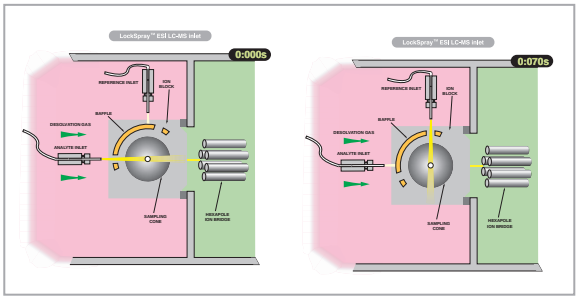

Figure 2. The LockSpray dual electrospray source in analyte and reference sample modes

RESULTS

Data files were exact mass-measured by comparison to a reference compound, Leucine Enkephalin ($[M+H]^+$ = 556.2771 Da) infused into the LockSpray reference channel. Mass chromatograms were extracted from the full spectrum (**Fig. 3**) and the spectra were used to verify the presence of a real parent drug peak by examining the exact mass, which was < 5 ppm or 2 mDa from the calculated value, as shown'in **Fig. 4**.

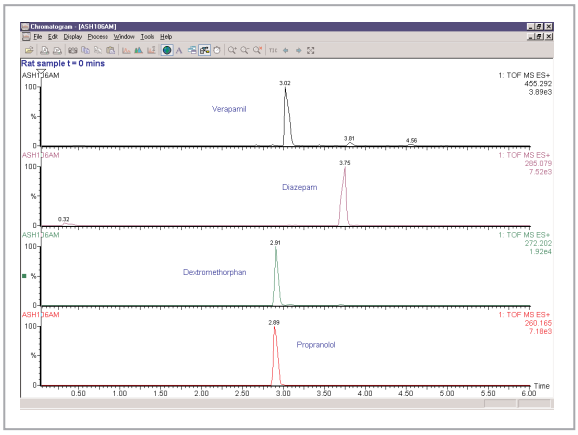

Figure 3. Extracted mass chromatograms of the four parent drugs in the t = 0 min sample

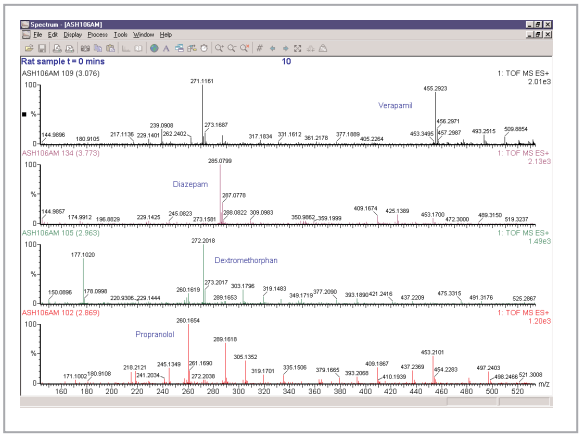

Figure 4. Exact mass-measured spectra of each parent drug

The disappearance of the parent drugs was then investigated by plotting mass-chromatograms of each component at the various time intervals. Software integration of these chromatograms was then performed (**Fig. 5**).

Figure 5. Integrated mass chromatograms showing the disappearance of Verapamil over time

Manual interrogation of these data shows that the major phase 1 metabolites (hydroxylation and demethylation) are present as expected and confirmed by the exact mass spectra.

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So the same processing was then carried out on each of the metabolites - **Fig. 6** below shows the integrated mass chromatograms of the 2 x demethylation of dextromethorphan ($C_{16}H_{21}NO$), $[M+H]^+$ = 244.1701 Da. These results were then plotted to show the changes in parent and metabolite concentration over time for each of the four drugs, as shown below in **Fig. 7**.

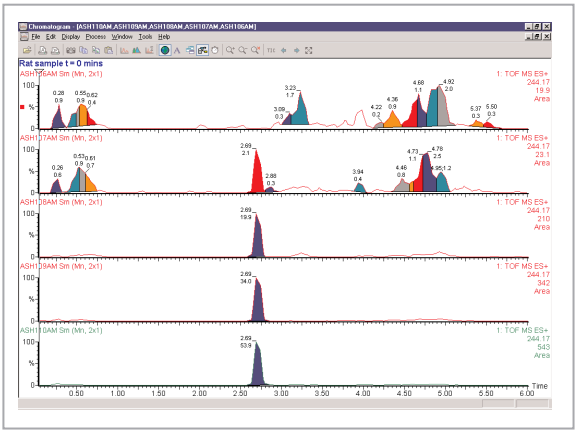

Figure 6. Appearance of the 2 x demethylation metabolite of Dextromethorphan

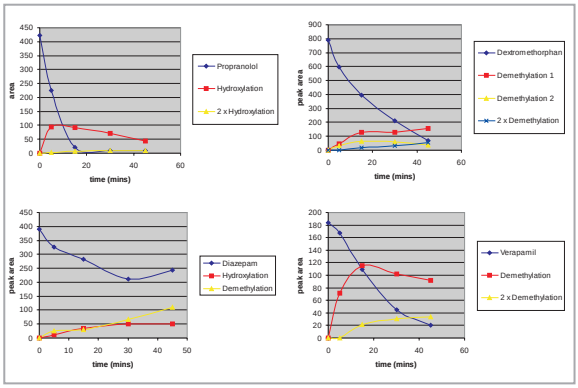

Figure 7. Metabolic clearance of the four parent drugs and the appearance of their major Phase 1 metabolites

Automated Metabolite ID and MS/MS Setup

The data was also scrutinized automatically using MassLynx 4.0 MetaboLynx metabolite search algorithm. It was set up to search for all expected metabolites as described above. However, by plotting 10 Da window mass chromatograms, any peaks which appear in the analyte but not the control samples would be noted. Using the exact mass-measured spectra for these peaks, a suggestion of any relation to the parent drug can be made.

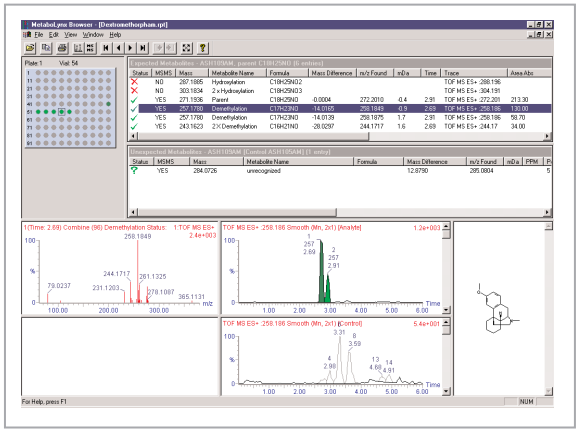

Figure 8. The MetaboLynx 4.0 browser window - shown here displaying the results of a search for metabolites of Dextromethorphan.

Following a search, MetaboLynx can set up and run a MS/MS experiment automatically. The molecular ions from the spectra of any suspected to be metabolites are added to a list of precursor ions, together with their retention times in a product ion scan function. If required, this function can be automatically acquired upon completion of the metabolite search, so the sample is injected a second time, the gradient started and MS/MS spectra acquired.

It was noted that dextromethorphan has two possible demethylation sites, and the MS chromatogram shows two peaks, each containing a molecular ion whose exact mass corresponds to the elemental composition of one demethylated metabolite.

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The MS/MS spectra created by MetaboLynx's method allow differentiation of the two peaks by their fragmentation. That of the first, larger peak is shown in **Fig. 9**. It is immediately apparent that the major production corresponds to the red fragment shown in **Fig. 10 (a)** rather than any possible fragment from **Fig. 10 (b)**. This, again, is confirmed by the measured mass being within 2 mDa of the calculated mass of this fragment.

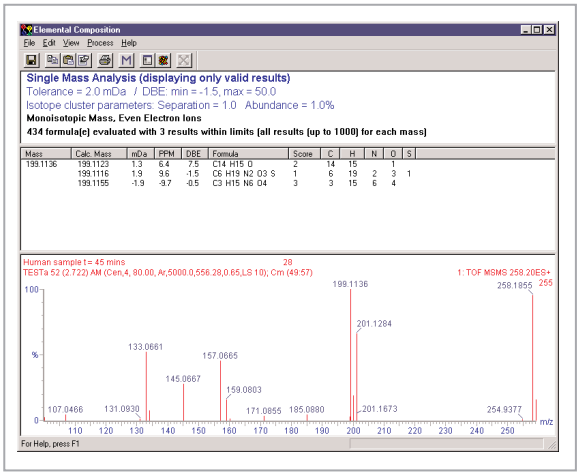

Figure 9. Exact mass spectrum of the first peak from the demethylation of Dextromethorphan, along with it's possible elemental compositions.

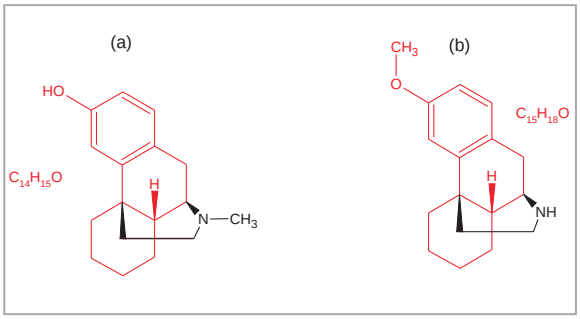

Figure 10. The two possible forms of demethylated Dextromethorphan. The red fragment in (a) clearly corresponds to the spectrum above.

CONCLUSION

- Full spectral data acquired by oa-TOF analysis can be used to identify parent drugs and their metabolites in low concentration, complex mixtures, thus allowing for much faster analysis times.
- The LockSpray source enables exact mass MS and MS/MS data to confirm the elemental composition, and therefore greatly facilitate structural elucidation and positive identification.
- MetaboLynx software can be used to automate such analyses and provide a large amount of information from little sample consumption.

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