

INTRODUCTION

The application area of drug metabolism analysis is extremely challenging. LC-MS is used due to its inherent sensitivity and selectivity. It has revolutionised areas of the drug discovery process, in particular lead optimisation, where tandem mass spectrometry is widely used to generate ADME information for new compounds. Quantitative data is usually provided by MRM experiments using tandem quadrupole mass spectrometers. One disadvantage is no qualitative information is available. The requirement to overcome this problem has led to a significant increase in the use of accurate mass measurements. LC coupled to orthogonal acceleration-time of flight (oa-TOF) mass spectrometers provides sensitive, accurate, qualitative and quantitative information from one analysis. The dynamic range of LC-TOF instruments has been improved, removing errors in quantification and accurate mass measurement at high metabolite concentrations. This allows mass defect to be utilised with confidence to distinguish drug-related metabolites from matrix components. Ultra Performance LC™ also provides faster analysis and improved chromatographic resolution. Here we present data using a UPLC and oa-TOF system with extended dynamic range capability that broadens the utility of this technology for metabolism studies. Rat microsomal incubations of 5 drug candidates were analysed. Due to the extensive data produced, only two drug candidates will be illustrated. Accurate mass measurement and mass defect filters were used to rapidly identify only drug-related metabolites.

EXPERIMENTAL

Mass spectrometer: Waters® Micromass® Q-TOF Premier™

Ionization mode: ESI+ at 3 kV;
Sample cone voltage: 40 V

Reference mass: Leucine enkephalin, [M+H]⁺ = 556.2771

Acquisition parameters: 100–1000 m/z;
0.2 second/spectrum;
0.1 second inter acquisition delay

Resolution: >8000 FWHM (V mode)

System: Waters® ACQUITY UPLC™

Column: Waters ACQUITY UPLC™ BEH C18 (100 mm x 2.1 mm, 1.7 µm particle size)

Column temperature: 45 °C Flow: 0.6 mL/min

Mobile phase: MeCN (B)
H₂O (0.1% HCOOH) (A)

Gradient: 0 min: 2% B
8.0 min: 98% B
10.0 min: 98% B
10.1 min: 2% B
13.0 min: 2% B

HPLC: Waters HT Alliance™ 2795

Column: Waters® Symmetry C₁₈ (100 mm x 2.1 mm x 3.5 µm)

Column temperature: 35 °C Flow: 0.3 mL/min

Mobile phase: MeCN (B)
H₂O (0.1 % Formic Acid) (A)

Gradient: 0–0.50 min: 2% B
0.5–20 min: 80% B
20–30 min: 80% B
30–30.5 min: 2% B
30.5–35 min: 2% B

Incubation

- Human microsomes/S9
- 37 °C shaking water bath
- Buffers (50–100 mM Tris or K Phosphate) @ pH 7.4
- NADPH regenerating system (NRS) in 2% NaHCO₃ (Sigma S-5761) solution containing: 0.5 mg/ml b-NADP (Sigma N-0505) 2.0 mg/ml Glucose-6-phosphate (Sigma G-7879) 1.5 units/ml Glucose-6-phosphate dehydrogenase (Sigma G-7877)
- Equal volume MeCN quench.
- Drug concentration 100 µl 0.1 mg/ml (final quench volume 2 mL)

RESULTS

In Figure 2 the UPLC/TOF extracted mass chromatograms for metabolites formed for the in vitro incubation of dextromethorphan are shown at T=15 mins, T=30 mins and T=60 mins time points. Presented in Figure 3 are the pDRE exact mass spectra for the demethylated metabolite formed for the in vitro incubation of dextromethorphan at 15 min, 30 min and 60 min. Figure 5 shows the pDRE exact mass spectra for the hydroxylated metabolite formed for the in vitro incubation

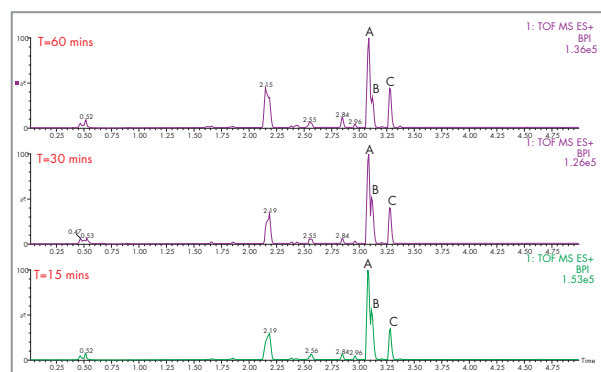


Figure 2. UPLC/TOF extracted mass chromatograms for metabolites formed for the in vitro incubation of dextromethorphan at time points T=15 mins, T=30 mins and T=60 mins.

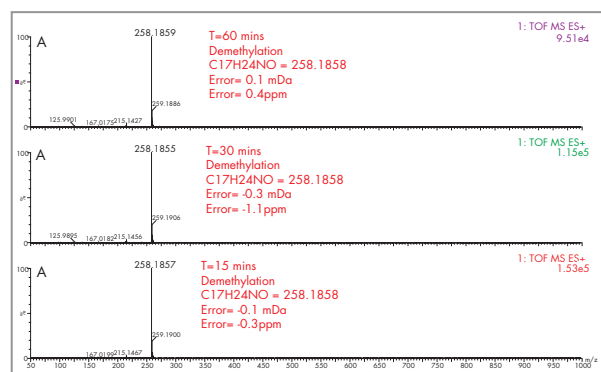


Figure 3. pDRE exact mass spectra for the demethylated metabolite formed for the in vitro incubation of dextromethorphan at time points 15 min, 30 min and 60 min.

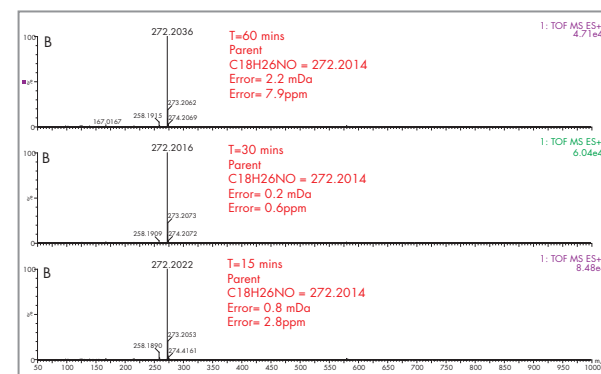


Figure 4. pDRE exact mass spectra for the in vitro incubation of the parent dextromethorphan drug at time points 15 min, 30 min and 60 min.

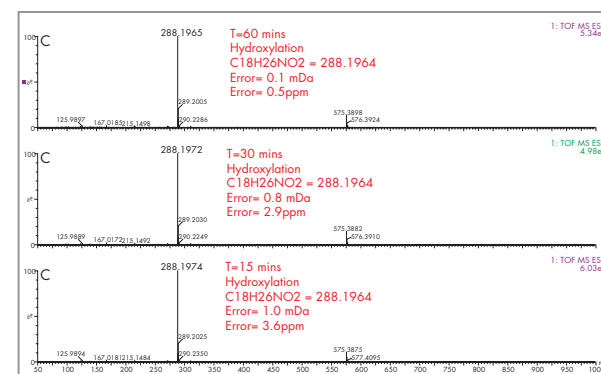


Figure 5. pDRE exact mass spectra for the hydroxylated metabolite formed for the in vitro incubation of dextromethorphan at time points 15 min, 30 min and 60 min.

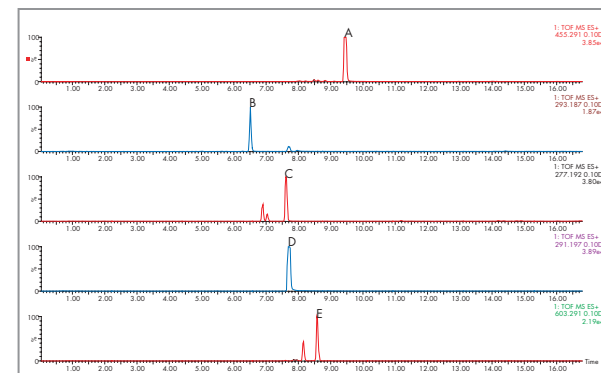


Figure 6. HPLC/TOF/MS extracted mass chromatograms for metabolites formed for the in vitro incubation of verapamil.

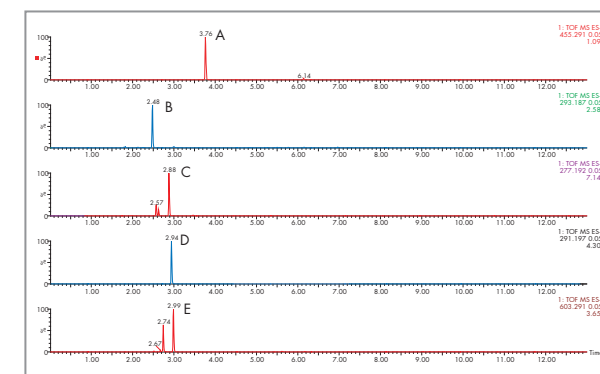


Figure 7. UPLC/TOF/MS extracted mass chromatograms for metabolites formed for the in vitro incubation of verapamil.

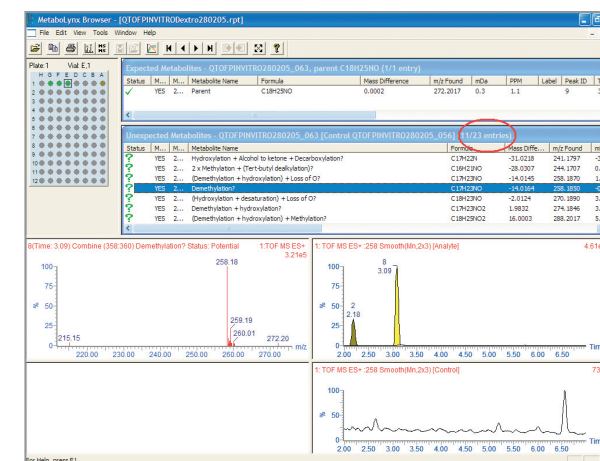


Figure 8. Metabolynx report browser illustrating application of post processing mass defect data filter.

of dextromethorphan at 15 min, 30 min and 60 min. The HPLC/TOF/MS extracted mass chromatograms for metabolites formed for the in vitro incubation of verapamil are presented in Figure 6. The corresponding UPLC/TOF/MS extracted mass chromatograms can be seen in Figure 7. An illustration of the Metabolynx report browser and application of the post processing mass defect data filter is contained in Figure 8.

DISCUSSION

The parent drugs verapamil and dextromethorphan were incubated in vitro using human S9 microsomes. These incubations were utilised to test both hardware and software

enhancements for determining metabolic fate of drugs. The schematic of a Q-TOF Premier in Figure 1 shows the DRE lens, incorporation of this lens allows the analytical applicability of oa-TOF to be enhanced. This new development has enabled extended dynamic range up to 4 orders of magnitude, providing both qualitative and quantitative capabilities with good exact mass measurements for highly concentrated peaks. Previously at high ion counts a shift to lower mass would occur, giving incorrect mass measurement and less confidence in elemental composition determination. Selection of spectra at low ion counts would provide excellent mass measurement and high confidence in the resultant data, however this could be a laborious task. Even at high ion counts, pDRE allows either the peak top, or the whole peak to be averaged to generate an accurate mass spectrum. This improves the ease with which data can be generated and processed. Also since no detector saturation is taking place, a true profile of the route and rate of metabolism will be obtained. The impact of dynamic range enhancement can be seen in Figure 2 where the UPLC/TOF BPI chromatograms for metabolites formed, for the in-vitro incubation of dextromethorphan are shown at T=15 mins, T=30 mins and T=60 mins time points. A true chromatographic profile is observed. The accurate mass spectra obtained for the demethylated (A) and hydroxylated metabolites (C), can be seen for three time point intensities in Figures 3 and 5 respectively. In each case the spectra comprised of a single spectrum, generated from the top of the peak. Intensities of up to 150000 counts per ion accumulation are illustrated, where no detector saturation has been observed and mass measurement errors of typically less than 3 ppm have been obtained. The corresponding spectra produced for the remaining parent dextromethorphan are shown in Figure 4. pDRE has enabled accurate mass measurement to be obtained routinely over a suitable concentration range for true measurement of metabolite formation and profiling.

As well as improving mass measurement performance, chromatography technology has also been enhanced by using ultra performance liquid chromatography. In Figures 6 and 7, the HPLC and UPLC/TOF extracted mass chromatograms for metabolites formed for the in vitro incubation of verapamil are presented. The HPLC data was acquired as part of a previous study.¹ It is shown that not only is analysis time

reduced by greater than 50%, but also chromatographic resolution is also improved. Metabolite isomers can now be chromatographically resolved. Improved chromatographic resolution also adds further opportunity to obtain higher quality MS data, reducing the need for multiple simultaneous MS experiments.

An illustration of the Metabolynx report browser and application of the post processing mass defect data filter is contained in Figure 8. The mass defect property of the parent drug will be maintained within a narrow mass range window, for both phase I and phase II biotransformations. The ability to acquire enhanced quality accurate mass spectra provides the opportunity to exploit mass sufficiency and deficiency. Application of a +/- 7 mDa mass defect window has enabled the number of unexpected metabolites found to be reduced to 11 from 23. This post processing step enabled immediate removal of 12 non drug matrix related unexpected metabolites to be removed.

CONCLUSION

- Analysis time reduced by at least 50% using a generic method UPLC method.
- Improved chromatographic resolution has been obtained routinely.
- pDRE extends the dynamic range for which accurate mass measurement can be made to 4 orders, enabling specific routine route and rate determination.
- Oa-TOF can be used for quantitative and qualitative analyses.
- Mass measurement errors of < 3ppm have been obtained routinely at high ion intensities.
- The highly specific nature of accurate mass measurement enables elemental compositions to rapidly be determined and therefore absolute confidence in producing the correct identification of metabolites formed.
- Application of a post processing mass defect data filter enables rapid exclusion of non drug related unexpected metabolites.

References

- Simultaneous determination of route and rate metabolism using LC-TOF. (Poster presentation at PBA 2004 Florence).

Figure 1. Q-ToF Premier Schematic.