

## INTRODUCTION

A comparison of the response and chromatographic separation obtained using conventional HPLC and UPLC™ coupled with oa-ToF has been performed. Utilising UPLC technology and columns packed with particles <2 µm, high efficiency separations can be achieved at high flow rates, allowing superior resolution and sensitivity to be obtained in a shorter analysis time. UPLC generates chromatographic peaks that are typically only several seconds in width, which places demands upon the detection technique to acquire data at an appropriate rate. The fast duty cycle of oa-ToF technology allows 20 spectra/second to be acquired generating sufficient sampling points across a peak to produce a representative response. An application area has been selected, where previous studies have been performed to illustrate the reduced retention times, improved chromatographic resolution and increased MS response.

When a potential pharmaceutical product has been developed, ADME data has to be generated for molecules with potential therapeutic activity. The rate and route of metabolism of the potential compound within the body is required to be determined. As part of a series of studies aimed at developing a better understanding of the factors governing the metabolic fate of xenobiotics, the metabolism of 4-bromoaniline (4-BrA) has been investigated. Using a newly developed bench top oa-ToF mass spectrometer (Figure 1) previously unobserved metabolites were detected supplying new information to the analytical chemist. The high sensitivity of HPLC- coupled to an oa-ToF revealed a complex pattern of metabolism with a plethora of previously undetected minor metabolites. The integral LockSpray™ (as shown in Figure 1) source enabled real time exact mass measurements to be used to identify these minor metabolites in an attempt to obtain a more complete metabolic profile in urine and bile for 4-BrA. Due to the many endogenous and xenobiotic metabolites present, the complete chromatographic separation has not been achieved with conventional chromatography, resulting in long analysis times.

Using UPLC, faster analysis times and better sensitivity have been achieved than was obtained using conventional HPLC.

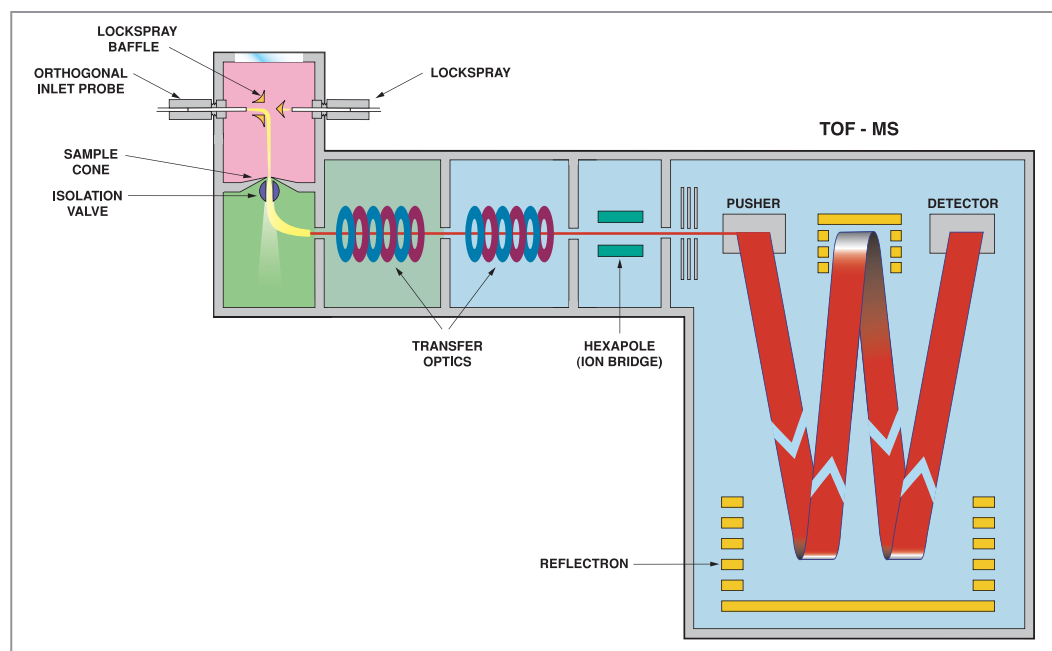


Figure 1. Schematic of dual resolution geometry LCT Premier™ oa-ToF (W mode > 10000 FWHM).

## UPLC CONDITIONS

UPLC System: Waters® ACQUITY UPLC™  
 Column: Waters ACQUITY UPLC™ BEH C<sub>18</sub> (100 mm x 2.1 mm, 1.7 µm)  
 Mobile phase: 0.01 M ammonium formate (pH 7)(A): methanol (B)  
 Gradient: 0–1 min: 5% B; 1–10 min: 20% B; 10–13 min: 20% B; 13–14 min: 30% B; 14–19 min: 30% B; 19.1–27 min: 5% B  
 Column temperature: 40 °C  
 Flow rate: 0.5 mL/min

## HPLC CONDITIONS

HPLC System: Waters Alliance® HT 2795  
 Column: Waters Symmetry® C<sub>18</sub> (250 mm x 4.6 mm, 5 µm)  
 Mobile phase: 0.01 M ammonium formate (pH 7)(A): methanol (B)  
 Gradient: 0–10 min: 100% A; 10–35 min: 0–60% B; 35–40 min: 60–80% B; 40–50 min: 0% B  
 Column temperature: 27 °C  
 Flow rate: 1 mL/min–split (4:1)

## MS CONDITIONS

MS= LCT Premier™ oa-ToF  
 Capillary Voltage: 3000 V (+VE) and 2600 V (-VE)  
 Ionisation mode: Positive and Negative electrospray  
 Resolution: 5500 FWHM (V mode) and 12000 FWHM (W mode)  
 Reference Lock mass: Leucine Enkephalin [M+H]<sup>+</sup>= 556.2771  
 Leucine Enkephalin [M-H]<sup>-</sup>= 554.2615  
 LockSpray switch time: 10 spectra HPLC  
 30 spectra UPLC  
 Acquisition time: 1 spectra/second HPLC  
 1 spectra/ 0.2 seconds UPLC

## ANIMAL DOSING

- 4-BrA dissolved in ethanol/water 50:50 at 50 mg mL<sup>-1</sup>
- Nominal dose level 50 mg kg<sup>-1</sup>
- Sample collected post dose
- Urine 0–12 hrs, 12–24 hrs and 24–48 hrs
- Bile 0–6 hrs, 6–12 hrs, 12–24 hrs and 24–48 hrs

## RESULTS

### Metabolite Identification

Presented in Figure 2 is an example of the high standard of chromatography that can be achieved using HPLC and Symmetry column technology for metabolite identification studies. Where the negative ion electrospray BPI chromatogram obtained for the analysis of the 0–12 hour urine sample of 4-bromoaniline dosed rat is presented. The same sample was also analysed using UPLC and the corresponding BPI chromatogram is shown ion Figure 3. From the HPLC and UPLC analyses the m/z 469 extracted mass chromatograms are illustrated in Figure 4, along with the overlaid expanded chromatography obtained with HPLC and UPLC for the brominated metabolite isomer B. Figure 5 shows the exact mass spectrum obtained for the 4-bromoaniline hydroxy sulphate metabolite A, the major metabolite determined to be present.

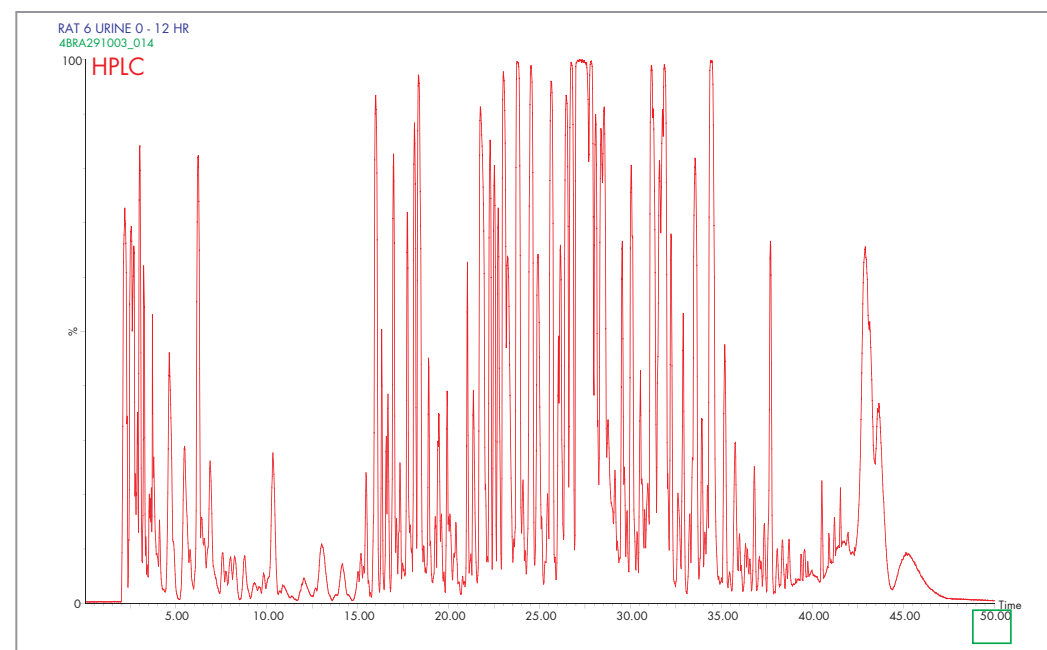


Figure 2. Negative ion electrospray base peak ion chromatogram (BPI) for the analysis of 0–12 hr urine of 4 bromoaniline dosed rat using HPLC oa-ToFMS.

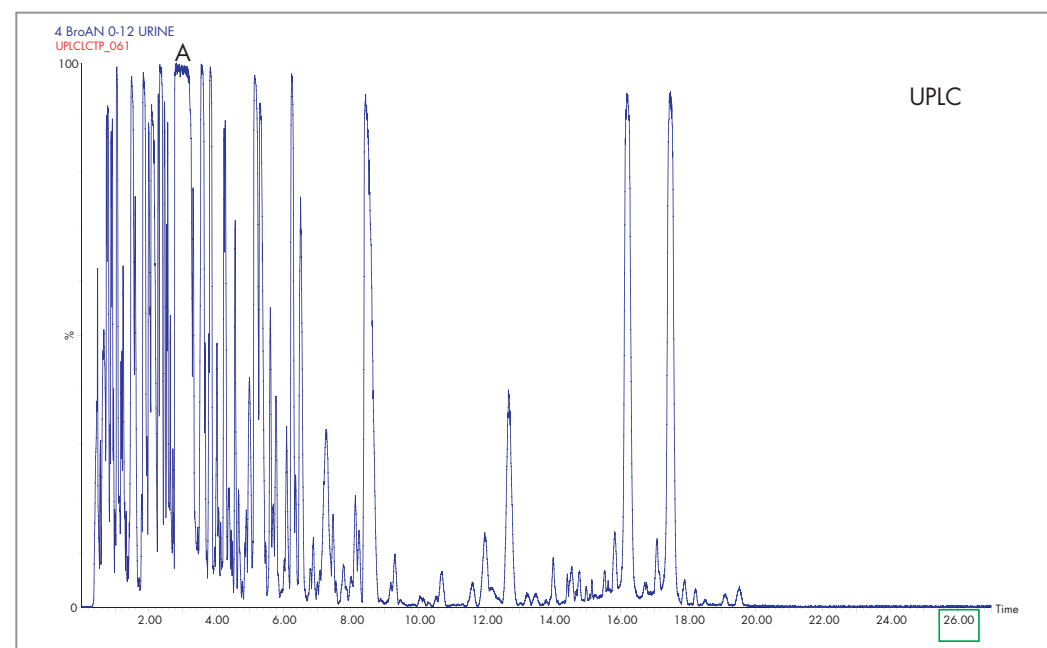


Figure 3. Negative ion electrospray base peak ion chromatogram (BPI) for the analysis of 0–12 hr urine of 4 bromoaniline dosed rat using UPLC oa-ToFMS.

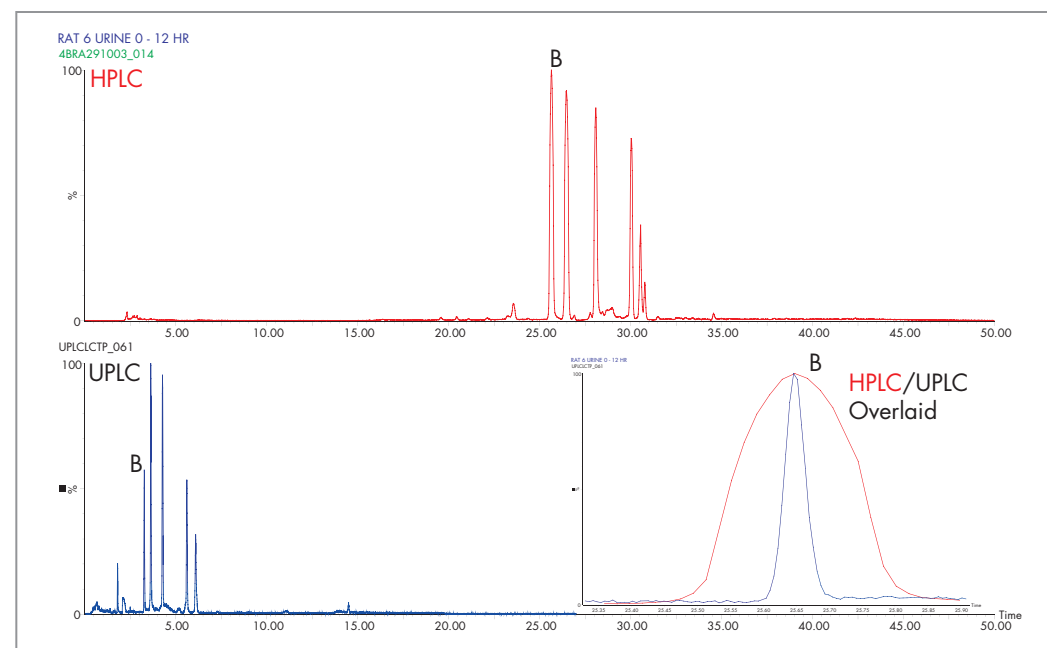


Figure 4. Negative ion electrospray m/z 469 extracted mass chromatograms for the analysis of 0–12 hr urine of 4 bromoaniline dosed rat using UPLC/HPLC oa-ToFMS and the overlaid UPLC/HPLC chromatographic peaks obtained for metabolite B.

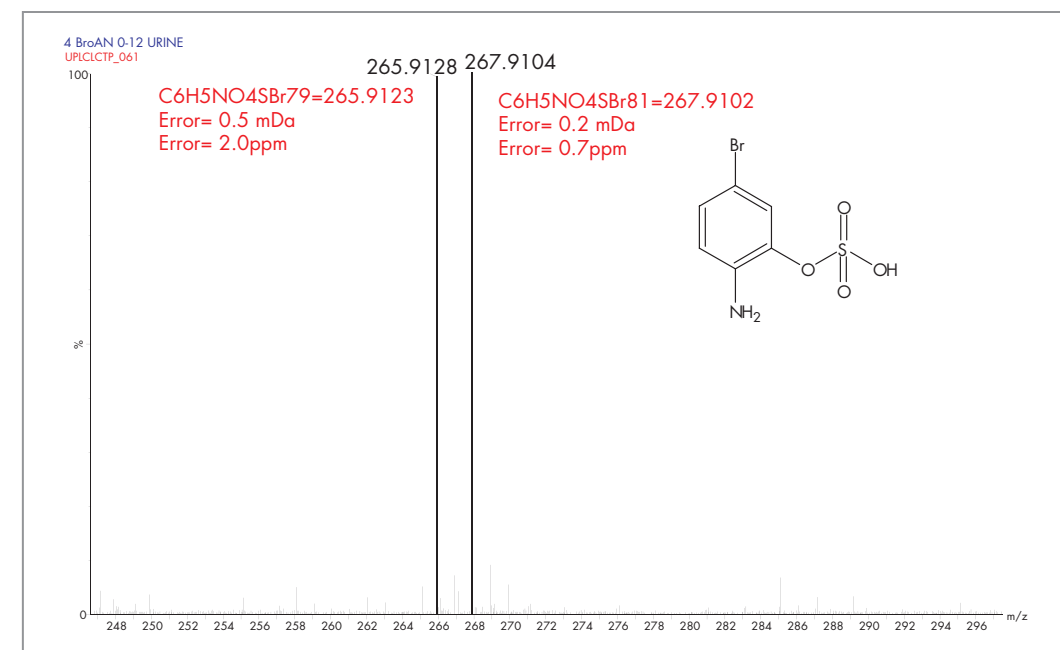


Figure 5. Exact mass spectrum for the 4-bromoaniline hydroxy sulphate metabolite A obtained with UPLC oa-ToFMS.

## DISCUSSION

The results illustrate the comparisons of UPLC and HPLC combined with oa-ToF-MS for metabolite identification. In metabolite identification analysis, the sample make up consists of hundreds of components ranging from polar to non-polar species. As can be seen from Figure 2 excellent chromatographic separation can be achieved using HPLC, although, an analysis time of fifty minutes is required. In Figure 3 it can be seen that the same sample of rat dosed with 4-bromoaniline analysed by UPLC, the speed of analysis has been reduced to less than twenty minutes, equating to a sixty percent reduction in analysis time. It can be seen from Figure 4 where a series of metabolites isomers have been selected, for both the UPLC and HPLC oa-ToF-MS analyses, for those analyses performed using UPLC it is clear that chromatographic resolution has been maintained. From these examples it can be seen that a lower plate height is produced using 1.7 µm particles and a 2.1 mm id column at flow rates of 0.6 mL/min leading to improved the peak efficiency resulting in narrower chromatographic peaks. With improved peak efficiency comes better resolution, due to narrower chromatographic peaks and hence an increase in peak capacity. As can be seen from Figure 4, an example of a UPLC and HPLC oa-ToF-MS extracted mass chromatograms corresponding to metabolite isomer B has been expanded and overlaid. The metabolite B the base peak widths were 0.48 minutes (HPLC) and 0.12 minutes (UPLC), illustrating a four-fold decrease in baseline peak width. By reducing the peak width this has been utilized to enable faster analysis times whilst maintaining resolution. For this metabolite identification study, three time course periods were analysed as well as the control, this was completed in 80 minutes using UPLC compared to 200 minutes previously obtained with HPLC. Oa-ToF-MS enables fast acquisitions to be performed at 20 spectra per second allowing sufficient points across the superior efficient narrow peaks obtained with UPLC oa-ToF-MS acquisition. An example of the specificity that can be obtained with exact mass measurement is shown in Figure 5, where both bromine isotopes elemental compositions for the 4-bromoaniline hydroxy sulphate metabolite were determined within 2 ppm. In this study unknowns are being profiled. The combination of low analyte detection with full spectra acquisition and the highly specific nature of exact mass is required to produce complete confidence in analyte identification. UPLC and oa-ToF-MS offers an unequalled means of increasing sample throughput with full exact mass spectral information being acquired.

## CONCLUSION

- UPLC oa-ToF-MS analysis for metabolite identification has been performed with a 60% increase in speed of analysis.
- For metabolite identification UPLC data indicating a four-fold decrease in baseline peak width has been shown enabling time efficient analysis to be achieved without compromising resolution.
- Increased peak efficiency has produced narrower chromatographic peaks and therefore improved peak resolution.
- The LCT Premier with integral LockSpray and independent reference sampling enables the routine acquisition of highly specific data in combination with UPLC.
- Exact mass measurement with <3 ppm error is achieved routinely.