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TIME OF FLIGHT MASS SPECTROMETRY FOR THE SPECIFIC IDENTIFICATION OF LOW LEVEL METABOLITES

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Presented at ASMS, Montréal, Canada, 8th-12th June 2003

INTRODUCTION

Oa-TOF-MS combined with HPLC is a powerful analytical tool because of the acquisition speed, available mass range and ion collection efficiency. Within many laboratories the high duty cycle of TOF is utilized for qualitative studies, generating full spectra at high mass accuracy (< 5 ppm) providing an extra degree of information that aids interpretation of the data. Within this study we show how technological advances have allowed the detection of previously unobserved metabolites supplying new information to the analytical chemist. 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 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 we have investigated the metabolism of 4-bromoaniline (4-BrA).

The qualitative profiling of the metabolites of 4-BrA in rat has been investigated. The animals were dosed at 50 mg/mL and urine samples were collected 0-6 hours, 6-12 hours, 12-24 hours and 24-48 hours post dosing. In addition post dosing bile samples were collected 0-12 hours, 12-24 hours and 24-48 hours. The high sensitivity of HPLC coupled to an oa-TOF has revealed a complex pattern of metabolism with a plethora of previously undetected minor metabolites. The combination of the distinctive bromine isotope pattern produced by the bromine substituent of 4-BrA with exact mass measurements have been used to conclusively identify these minor metabolites, in an attempt to obtain a more complete metabolic profile in urine and bile for 4-BrA.

The study has used an established oa-TOF MS system (Waters® Micromass® LCT™ Mass Spectrometer) and compared data acquired using a new benchtop oa-TOF-MS system (Waters Micromass LCT Premier™). The performance characteristics of TOF technology, are as follows, and the resolution capability of TOF technology is shown in Figure 3 for leucine enkephalin which is used as the reference mass for the data acquired.

- Elevated mass spectral resolution - W mode >10000 FWHM.
  V mode > 5000 FWHM.
- Exact mass measurements (<5 ppm RMS).
- Elemental composition determination of target analytes.
- Confidence in confirming target analytes help to identify unknowns (elemental composition calculator).
- Increased selectivity (nominal mass matrix interferences removed using exact mass chromatograms).
- High sensitivity - efficient 'non-scanning' instrument.
- Low level analyte detection.
- Full spectrum acquisition.

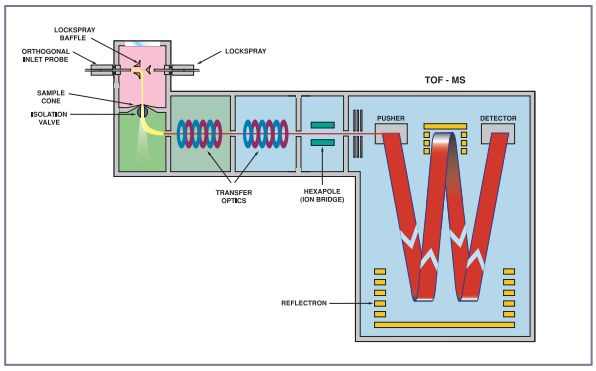

Figure 1. Schematic of oa-TOF.

- Dynamic range 4 orders of magnitude.

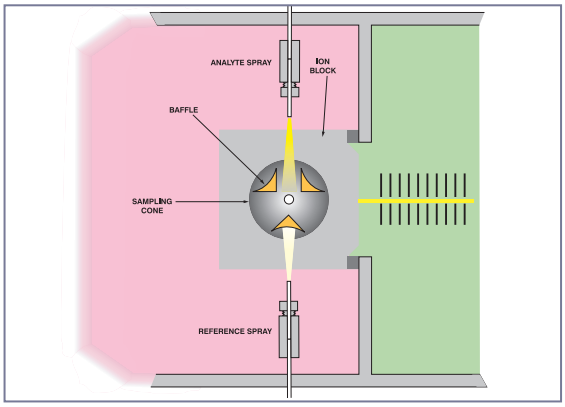

Figure 2. Schematic of LockSpray™.

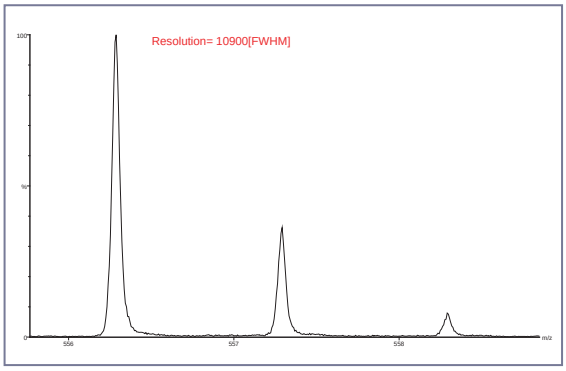

Figure 3. Oa-TOF-MS resolution illustration.

EXPERIMENTAL

HPLC Conditions

Column: Symmetry® 5 mm C_{18}, 250 X 4.6

Mobile Phase: 0.01 M ammonium formate (pH 7) (solvent A) methanol (solvent B)

Gradient: 0-10min:100%

10-35min: 0-60% B

35-40min: 60-80% B

40-50min: 0% B

Flow Rate: 1 mL/min (4:1 split to MS)

MS Conditions

MS= Oa-TOF

Capillary Voltage: 3000 kV

Ionization Mode: Positive electrospray

Resolution: 5500 V mode

Reference Lockmass: Leucine Enkephalin $[M+H]^+$= 556.2771

LockSpray Switch Time: 10 seconds

Acquisition Time: 1 spectra/second

Animal Dosing

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

RESULTS

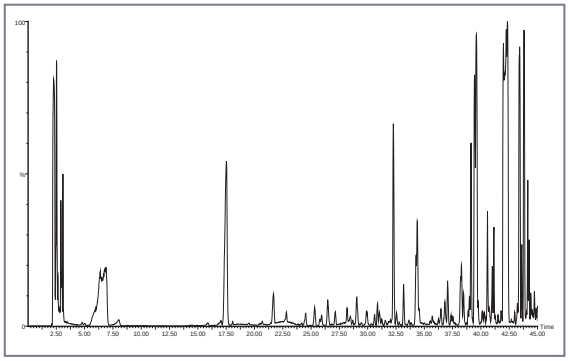

Figure 4. Positive ion mode BPI chromatogram for 0-6 hour bile sample from a 4-BrA dosed rat.

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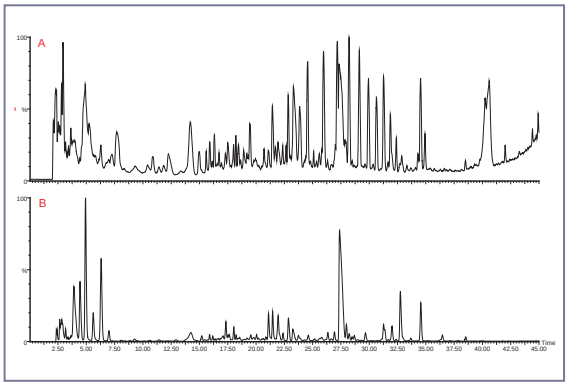

Figure 5. Positive ion mode total ion chromatogram (A) and UV chromatogram (B), for 0-12 hour urine sample from a 4-BrA dosed rat.

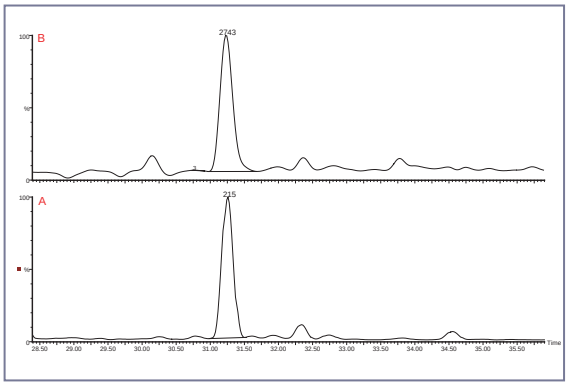

Figure 6. Positive ion mode expanded m/z 414 (4-BrA mono-hexose sulphamate metabolite) extracted mass chromatograms for 0-12 hour urine sample from a 4-BrA dosed rat, where A= LCT and B= LCT Premier.

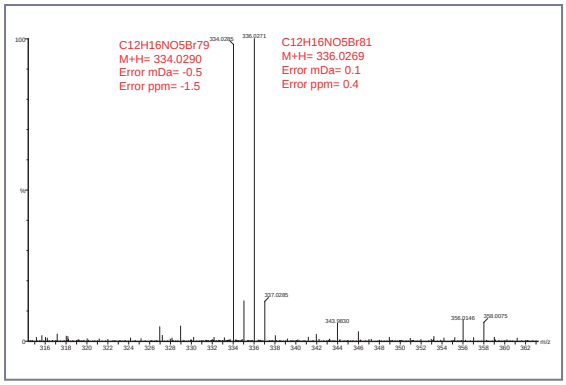

Figure 7. Positive ion mode exact mass spectrum for mono-hexose metabolite determined to be present in 0 -12 hour urine sample from a 4-BrA dosed rat.

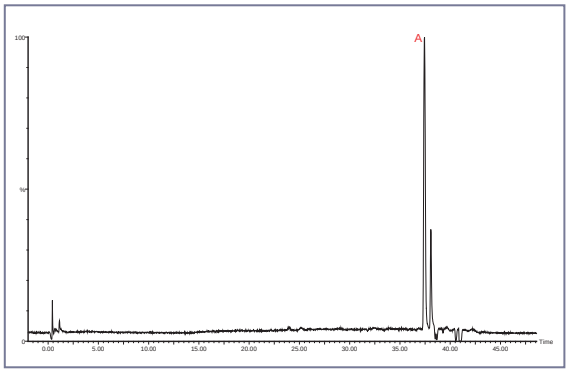

Figure 8. Positive ion mode m/z 214 extracted mass chromatogram for 0-6 hour bile sample from a 4-BrA dosed rat.

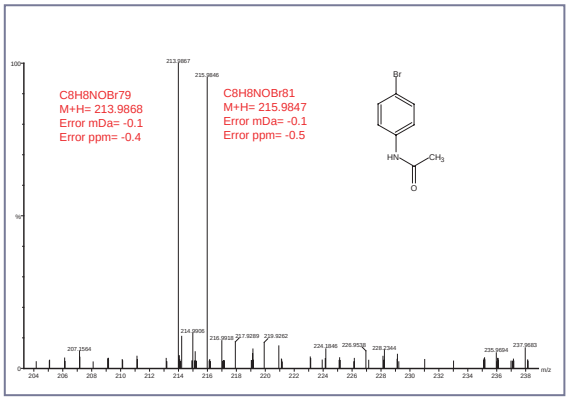

Figure 9. Positive ion mode exact mass spectrum for the N acetyl metabolite of 4-BrA determined to be present in 0-6 hour bile sample from a 4-BrA dosed rat.

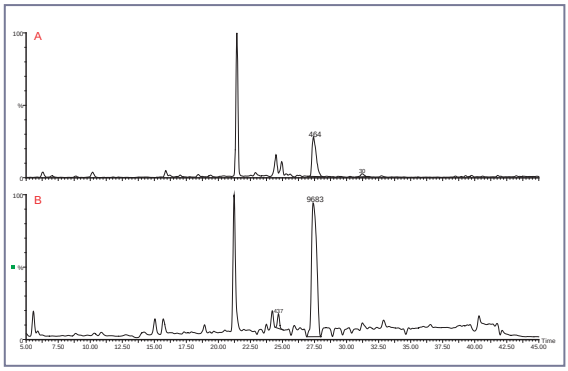

Figure 10. Positive ion mode m/z 267 extracted mass chromatograms for 0-12 hour urine sample from a 4-BrA dosed rat, where A= LCT and B= LCT Premier.

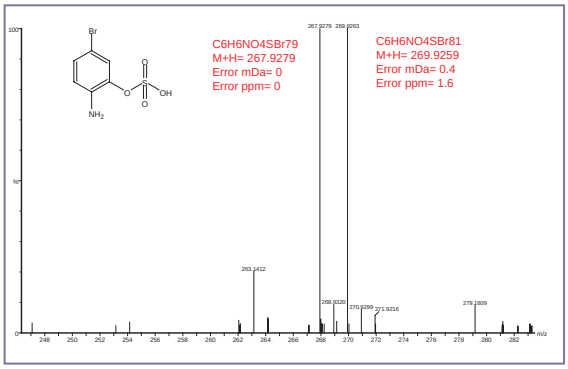

Figure 11. Positive ion mode exact mass spectrum for the hydroxy sulphate metabolite of 4-BrA determined to be present in 0-12 hour urine sample from a 4-BrA dosed rat.

ELEMENTAL COMPOSITION	M-H	MASS MEASURED	ERROR mDa	Error ppm	RETENTION TIME	METABOLITE
$C_{12}H_{14}NO_{10}S^{79}Br$	441.9444	441.9453	0.9	2	20.91	SULPHATE-GLUCURONIDE
$C_{12}H_{14}NO_{10}S^{81}Br$	443.9426	443.9438	1.5	3.4		
$C_6H_6NO_4S^{79}Br$	265.9123	265.9136	1.3	5	27.5	HYDROXYLATION + SULPHATE
$C_6H_6NO_4S^{81}Br$	267.9102	267.9115	1.3	4.8		
$C_8H_6NO_3^{79}Br$	241.9453	241.9454	0.2	0.6	32.51	OXALINIC ACID
$C_8H_6NO_3^{81}Br$	243.9433	243.9424	-0.8	-3.3		
$C_{13}H_{15}N_2O_4S^{79}Br$	372.9858	372.9859	0.1	0.4	34.47	MERCAPURATE
$C_{13}H_{15}N_2O_4S^{81}Br$	374.9837	374.9833	-0.4	-1.0		
$C_{14}H_{16}NO_8^{79}Br$	403.9981	403.9978	-0.3	-0.3	31.88	GLUCURONIDE + N-ACETYL
$C_{14}H_{16}NO_8^{81}Br$	405.9961	405.9963	0.2	0.7		
$C_6H_6N^{79}Br$	169.9606	169.9615	1	5.7	32.69	PARENT
$C_6H_6N^{81}Br$	171.9585	171.9582	-0.3	-1.6		
$C_8H_8NO^{79}Br$	211.9771	211.9713	0.2	0.9	38.52	N-ACETYL
$C_8H_8NO^{81}Br$	213.9691	213.9692	0.1	0.6		

Table 1. Examples of exact mass measurement for urinary metabolites of 4 bromoaniline from a 4-BrA dosed rat.

ELEMENTAL COMPOSITION	M-H	MASS MEASURED	ERROR mDa	Error ppm	RETENTION TIME	METABOLITE
$C_{12}H_{14}NO_{10}S^{79}Br$	441.9444	441.9453	0.9	2	20.91	SULPHATE -GLUCURONIDE
$C_{12}H_{14}NO_{10}S^{81}Br$	443.9426	443.9438	1.5	3.4		
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$C_{13}H_{15}N_2O_4S^{81}Br$	374.9837	374.9833	-0.4	-1.0		
$C_{14}H_{16}NO_8^{79}Br$	403.9981	403.9978	-0.3	-0.3	31.88	GLUCURONIDE + N-ACETYL
$C_{14}H_{16}NO_8^{81}Br$	405.9961	405.9963	0.2	0.7		
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$C_8H_8NO^{79}Br$	211.9771	211.9713	0.2	0.9	38.52	N-ACETYL
$C_8H_8NO^{81}Br$	213.9691	213.9692	0.1	0.6		

Table 2. Examples of exact mass measurement for bile metabolites of 4 bromoaniline in a 4-BrA dosed rat.

Sample analysed	Number of brominated metabolites identified				
	0-6hrs	6-12hrs	0-12hrs	12-24hrs	24-48hrs
+ve ion Rat Urine	-	-	46	28	8
-ve ion Rat Urine	-	-	60	33	10
+ve ion Rat Bile	14	16	-	10	0
-ve ion Rat Bile	19	23	-	21	2

Table 3. Summary of the number of brominated metabolites detected.

A base peak ion chromatogram for a 0-6 hour bile sample from a 4-BrA dosed rat is shown in Figure 4, the plethora of major and minor components present can be seen. The corresponding total ion chromatogram and UV chromatogram for a 0-12 hour urine sample from the rat is presented in Figure 5. From Figure 6 the increase in sensitivity achieved using a new benchtop oa-TOF MS system is shown, where the response for the same metabolite is compared for the old and new mass spectrometer. The expanded m/z 414 extracted mass chromatograms illustrate a ten-fold increase in the integrated peak area observed, for the minor novel mono-hexose sulphamate metabolite. An illustration of the exact mass measurement achieved is shown in Figure 7, where the exact mass spectrum for the mono-hexose urine metabolite from a 4-BrA dosed rat is presented. The distinctive bromine isotope fingerprint is shown and mass accuracy maintained within 5 ppm. In 0-6 hour bile taken from a 4-BrA dosed rat, the major metabolite was the formed by the N acetyl species, the extracted m/z 214 mass chromatogram is shown in Figure 8, along with the corresponding exact mass spectrum obtained in Figure 9. The determined elemental composition and mass measurement error obtained are also

illustrated. In Figure 10 the response obtained for the major hydroxy sulphate metabolite in 0-12 urine of rat dosed with 4-BrA is shown, where the area response for LCT and LCT Premier are compared. The exact mass spectrum obtained and the mass measurement errors obtained for the hydroxy sulphate metabolite is shown in Figure 11. Exact mass measurement within 5 ppm was obtained. In Tables 1 and 2 examples of some of the metabolites identified in urine and bile of rat dosed with 4-BrA are shown. The number of brominated metabolites detected in the respective samples is illustrated in Table 3.

DISCUSSION

Using oa-TOF technology it has been possible to identify over 60 major and minor metabolites in one single analysis of urine of rat dosed with 4-BrA, using negative mode electrospray ionization oa-TOF LC/MS for metabolites. In positive ion mode over 46 major and minor metabolite were identified. In addition, the previously unstudied metabolic profile obtained from bile of rat dosed with 4-BrA was determined. In negative ion mode twenty-three brominated metabolites were detected and in positive ion mode sixteen brominated metabolite species were determined to be present in one single analysis. The ability to acquire full spectra over a wide mass range with exact mass measurement, enables major and minor components to be identified in one analysis with increased confidence. Within the urine samples provided the 4-BrA hydroxy sulphate metabolite was identified as the major component, where as in the 0-6hr bile sample initially the hydroxy N-acetyl metabolite formed the major metabolite. In the bile 6-12hr sample the N-acetyl cysteinyl conjugate formed the major metabolite. The initial metabolic fate studies of 4-BrA were performed using an established oa-TOF-MS system, the study has been repeated and compared with the results obtained using a new benchtop oa-TOF-MS system, the schematic diagram is shown in Figure 1, and the LockSpray schematic shown in Figure 2. Initial studies show that more brominated metabolite species have been determined to be present. The response obtained indicates at least a ten-fold increase in sensitivity.

CONCLUSION

- Oa-TOF technology enables large numbers of major and minor unknown metabolites to be identified in one analysis.
- Accurate mass measurement within 5 ppm is routinely achieved.
- Further metabolites have been detected using the new benchtop oa-TOF-MS system.
- The new oa-TOF system was at least ten times more sensitive than the LCT system.
- The high sensitivity of HPLC coupled to an oa-TOF has revealed a complex pattern of metabolism with a plethora of previously undetected minor metabolites.
- Exact mass measurements have been used to identify these minor metabolites in an attempt to obtain a more complete metabolic profile in urine and bile for 4-BrA

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