THE INVESTIGATION OF BIOMARKERS OF NEPHROTOXICITY USING A METABONOMIC UPLC-MS APPROACH

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OVERVIEW

This poster describes a metabonomic study of mercuric chloride, a model nephrotoxin. The analysis was carried out using the Waters® Metabonomics System consisting of a Waters ACQUITY UPLC™ system coupled to a Waters® LCT Premier™ orthogonal acceleration time of flight (oa-TOF) mass spectrometer for the exact mass determination of any potential biomarkers. These masses were then searched against the exact mass database within the Waters MarkerLynx db™ Application Manager to aid identification of potential biomarkers of nephrotoxicity.

Figure 1. Waters Metabonomics® System

INTRODUCTION

Metabonomics is the study of the metabolic responses of mammalian systems to a toxic insult, environmental changes or disease and is complementary to genomics (concerned with DNA) and proteomics (concerned with proteins). These responses are time related and can show the onset of a toxic response or disease and recovery. This study investigates the changes to the concentrations of endogenous metabolites in urine over the time course of the study following the single dose of the model nephrotoxin mercuric chloride.

Comparison of the changes in urinary metabolite profiles compared to pre-dose and control animals, can then be used to detect and identify potential biomarkers of organ specific toxicity.

The cost of discovery and development of a drug is increasing dramatically; however, the number of approved new drug products is on the decline. Pharmaceutical companies, keen to find ways to accelerate the drug discovery process and minimize the late attrition of drug candidates are turning to metabonomics as a means of discovering biomarkers of potential toxicity.

EXPERIMENTAL

Animal Study

- Male Wistar-derived rats (n=5 per group), approx. 140g in weight acclimatised in metabolism cages for 3 days prior to treatment.
- Food and water were provided *ad libitum*.
- Group 1 were used as controls and were administered 0.9% (w/v) saline, at a dose volume of 10ml/kg.
- Group 2 were administered mercuric chloride at 2.0mg/kg as a single subcutaneous injection at a dose volume of 10ml/kg.
- Urine samples were collected daily for 9 days pre- and post-dose from control and dosed animals and stored at -20°C prior to analysis.

The samples were analyzed on the Waters Metabonomics System (figure 1) using the following conditions.

METHODS

UPLC™ Conditions

LC System:	Waters ACQUITY™ UPLC
Column:	ACQUITY UPLC™ BEH C18, 2.1 x100 mm, 1.7 µm
Column Temperature:	40°C
Flow rate:	600 µL/min
Mobile Phase A:	0.1% formic acid in water
Mobile Phase B:	0.1% formic acid in acetonitrile
Gradient:	Linear gradient, 0-20%B in 4mins, 20-95%B over 4-9mins
Injection volume:	3µL neat urine

MS Conditions

MS System:	Waters LCT Premier
Ion mode:	Electrospray positive and negative as separate acquisitions
Capillary voltage:	3000V positive, 2600V negative
Cone voltage:	35V
Source temp.:	120°C
Desolvation temp.:	350°C
Dwell time:	0.15sec
Inter-scan delay:	0.05sec

LockSpray Conditions

Reference:	Leucine enkephalin
Switching interval:	30 scans

Data Processing

The data from the control group (1) was processed against the mercuric chloride dosed group (2) using the MarkerLynx Application Manager. This allowed deconvolution, alignment and data reduction to give a table of mass and retention time pairs with associated intensities for all the detected peaks. This reduced data set was then visualized using principal components analysis (PCA) within MarkerLynx.

RESULTS

The PCA scores plot of component 1 against component 2 for the controls and mercuric chloride dosed rat urine samples are shown in figure 2. This shows the time related metabolite trajectory plot over the time course of the study after administration of a single dose of mercuric chloride. The dosed samples show the onset of changes to the urinary metabolite fingerprint as they move away from the controls (black crosses) with day 3 being the point of maximum disturbance. The trajectory then shows a return to normal by the end of the study.

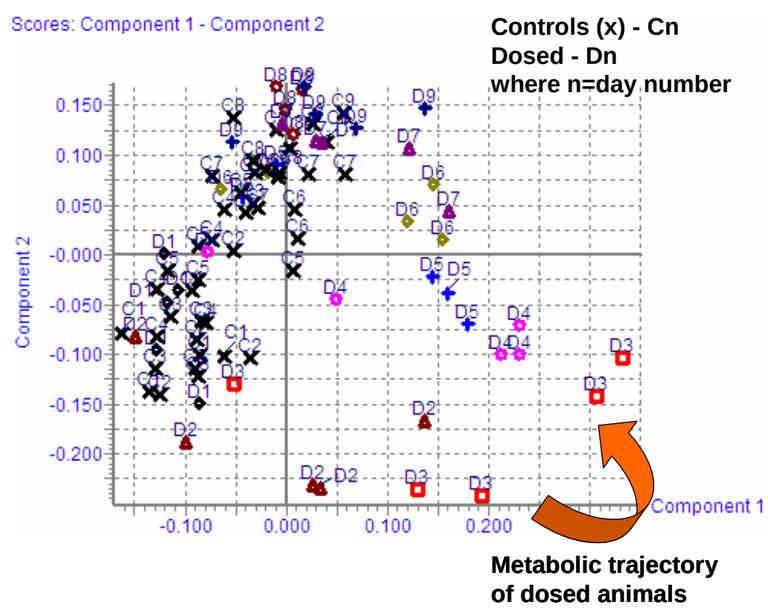

Figure 2. MarkerLynx PCA scores plot for positive ion analysis of urine samples from control and mercuric chloride dosed rats.

A similar trajectory plot is observed in the negative ion MarkerLynx scores plot (figure 3) with day 3 again being the point of maximum disturbance. Two animals were observed to be poor responders throughout the study but by day 9 the urinary profiles of all the dosed animals had returned to control levels.

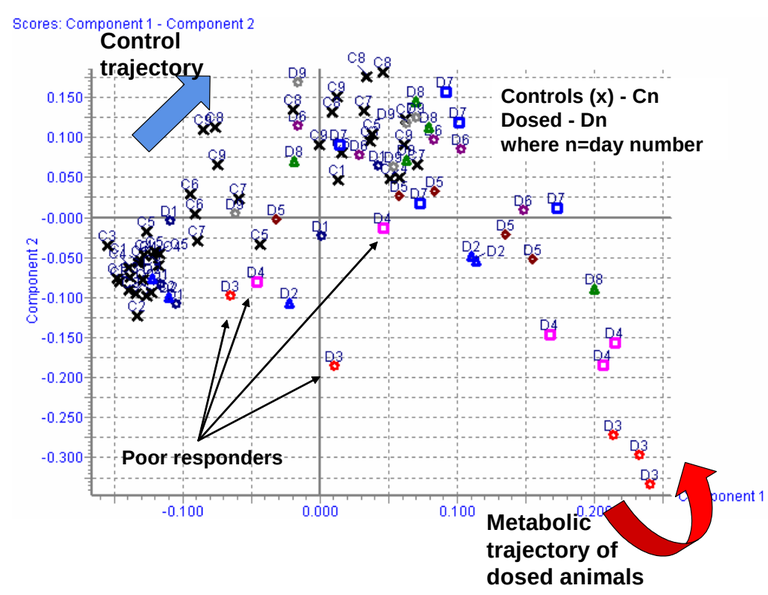

Figure 3. MarkerLynx PCA negative ion scores plot

The principal ions responsible for the metabolic trajectory away from the controls were determined from the associated loadings plot (see figure 4).

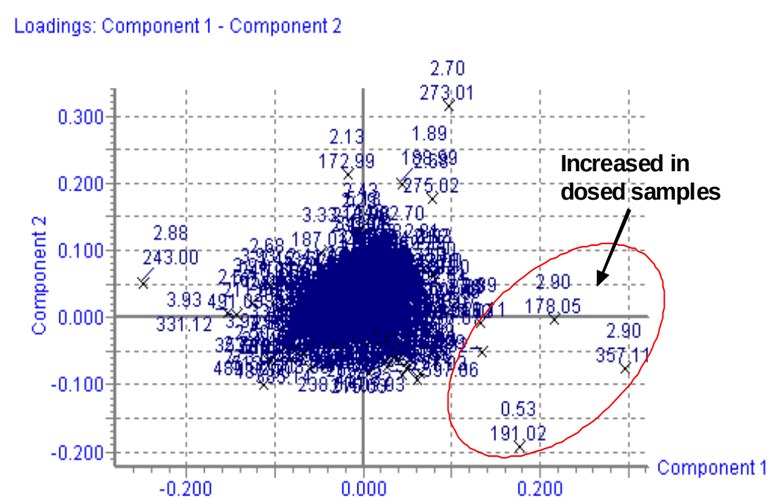

Figure 4. MarkerLynx PCA negative ion loadings plot

The database searching facility within MarkerLynx db allows potential identification of the markers to be determined based on exact mass and/or retention time. An example of the exact mass searching of the positive ion markers table is shown in figure 5.

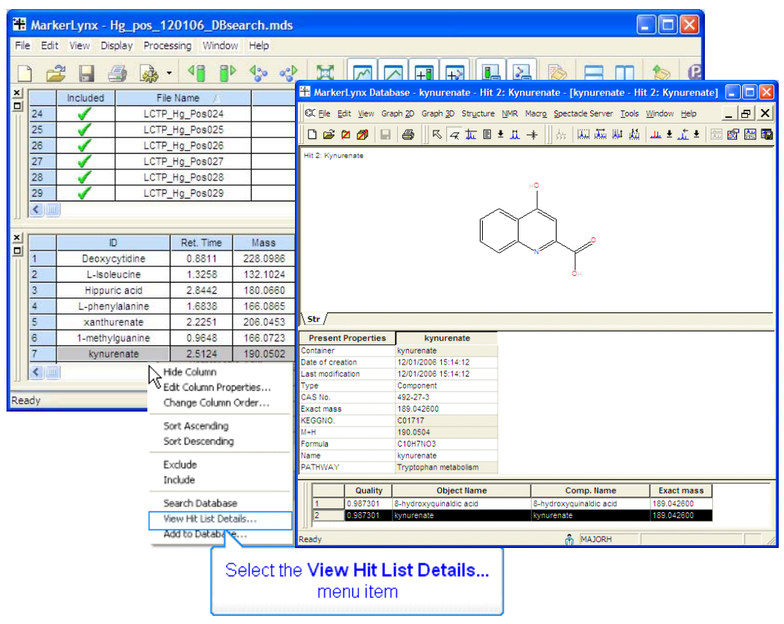

Figure 5. MarkerLynx db database search results

The ions showing the most significant changes after dosing are tabulated below in figures 6 and 7 for the positive ion and negative ion respectively.

Principal Ions Increased After Dosing

RT min	Measured Mass	Calculated $[M+H]^+$	Error mDa	Error ppm	Elemental composition (neutral species)	Postulated identity
0.53	215.0168	215.0168	0.0	0.0	$C_6H_7O_7Na$	citric acid Na salt
1.33	132.1024	132.1025	-0.1	-0.8	$C_6H_{13}NO_2$	leucine/iso-leucine
1.68	166.0865	166.0868	-0.3	-1.8	$C_9H_{11}NO_2$	phenylalanine
2.85	243.0747	243.0746	0.1	0.4	$C_6H_{15}N_2O_6P$	5-phosphonooxy-L-lysine

Principal Ions Decreased After Dosing

RT min	Measured Mass	Calculated $[M+H]^+$	Error mDa	Error ppm	Elemental composition (neutral species)	Postulated identity
0.65	212.1034	212.1035	-0.1	-0.5	$C_9H_{13}N_3O_3$	dideoxycytidine
0.85	228.0982	228.0984	-0.2	-0.9	$C_9H_{13}N_3O_4$	deoxycytidine
1.31	242.1143	242.1141	0.2	0.8	$C_{10}H_{15}N_3O_4$	5-methyl-2-deoxycytidine
2.23	206.0453	206.0453	0.0	0.0	$C_{10}H_7NO_4$	xanthurenic acid
2.52	190.0504	190.0504	0.0	0.0	$C_{10}H_7NO_3$	kynurenic acid
3.30	194.0816	194.0817	-0.1	-0.5	$C_{10}H_{11}NO_3$	phenylacetylglycine

Figure 6. Principal ions changing in positive ion

Principal Ions Increased After Dosing

RT min	Measured Mass	Calculated $[M-H]^-$	Error mDa	Error ppm	Elemental composition (neutral species)	Postulated identity
0.48	215.0321	215.0320	0.1	0.5	$C_5H_{13}O_7P$	methylerythritolphosphate
0.53	191.0185	191.0192	-0.7	3.7	$C_6H_8O_7$	citric acid
0.53	173.0082	173.0086	-0.4	2.3	$C_6H_4O_6$	aconitic acid
0.74	145.0129	145.0137	-0.8	-5.5	$C_5H_6O_5$	alpha ketoglutaric acid
1.19	117.0181	117.0188	-0.7	-6.0	$C_4H_6O_4$	succinic acid
2.90	178.0500	178.0504	-0.4	-2.2	$C_9H_9NO_3$	hippuric acid
3.35	192.0657	192.0661	-0.4	-2.1	$C_{10}H_{11}NO_3$	phenylacetylglycine

Principal Ions Decreased After Dosing

RT min	Measured Mass	Calculated $[M-H]^-$	Error mDa	Error ppm	Elemental composition (neutral species)	Postulated identity
2.12	172.9904	172.9909	-0.5	-2.9	$C_6H_6O_4S$	phenol sulphate
2.28	230.0118	230.0123	-0.5	-2.2	$C_8H_9NO_5S$	
2.86	242.9953	242.9963	-1.0	-4.1	$C_9H_8O_6S$	hydroxycinammic acid sulphate
3.93	165.0545	165.0552	-0.7	-4.2	$C_9H_{10}O_3$	hydroxyphenylpropionic acid

Figure 7. Principal ions changing in negative ion

DISCUSSION

The ability to routinely obtain exact mass measurements of <3ppm, when used in conjunction with a searchable database, greatly simplifies the identification of potential biomarkers. These identities however need to be confirmed by comparison against standards.

Citric acid, part of the TCA or Krebs cycle, was observed to show the most significant increase after dosing. In positive ion, kynurenic acid and xanthurenic acid, end products of the trytophan catabolism cycle, both decreased after dosing.

The ions at m/z 178.05 and 245.02, observed in negative ion mode, were from hippuric acid and 3-hydroxyphenyl propionic acid. These showed a considerable variation throughout the study in both the controls and dosed samples. This accounts for some of the spread in the control data and it has been shown elsewhere that changes in the excretory pattern of these metabolites is associated with changes in the metabolism of gut microflora[1,2].

CONCLUSIONS

- UPLC/TOF MS in conjunction with principal components analysis has been shown to be a valuable tool in the study of renal toxins.
- The changes to the endogenous urinary metabolite concentrations have been used to track the onset of nephrotoxicity over time.
- The enhanced chromatographic resolution and increased sensitivity of UPLC coupled with the fast acquisition rates and sensitivity of the TOF mass spectrometer allowed the detection of low level metabolites.
- Exact mass measurements have been used to facilitate data base searching for identification of the potential biomarkers, although their biological significance still needs to be determined.
- UPLC/TOF MS based metabonomics has the potential to be an integral part of toxicological screening and lead compound selection in the pharmaceutical industry and should help to minimise late stage attrition of candidate drugs.

REFERENCES

[1] Phipps A.N. et al *Pharmaceutical Sciences* 3, 143-146 (1997)

[2] Phipps A.N. et al *Xenobiotica* 28(5), 527-537 (1998)

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