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應用手冊

A Metabonomic Nephrotoxicity Study Using the ACQUITY UPLC/LCT Premier Platform

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Abstract

In this application note, we describe a metabonomic study of 3 compounds: mercuric

chloride (a model nephrotoxin); cyclosporin A (an immunosuppressant commonly used after organ transplant surgery); and gentamicin(an antibiotic). Both of the latter can induce nephrotoxicity upon chronic administration. Compounds which exhibit nephrotoxicity aretoxic or destructive to kidney cells and can result in reduced renal function.

Note: MarkerLynx has been replaced with Progenesis QI.

Benefits

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.

Introduction

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.

In this application note, we describe a metabonomic study of 3 compounds: mercuric chloride (a model nephrotoxin); cyclosporin A (an immunosuppressant commonly used after organ transplant surgery); and gentamicin(an antibiotic). Both of the latter can induce nephrotoxicity upon chronic administration. Compounds which exhibit nephrotoxicity aretoxic or destructive to kidney cells and can result in reduced renal function.

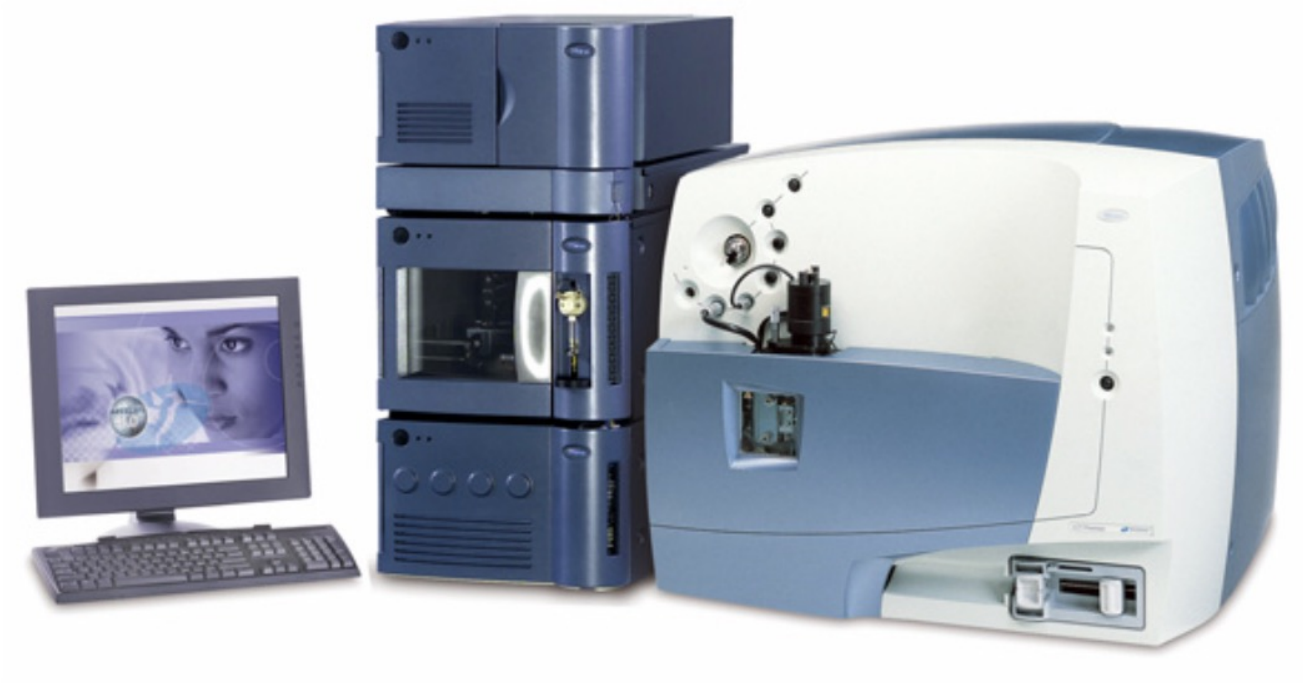
The analysis was carried out using the Waters Metabonomics MS System, consisting of the Waters ACQUITY UPLC System coupled to the Waters Micromass LCT Premier orthogonal acceleration time of flight (oa-TOF) mass spectrometer, for the exact mass determination of any potential biomarkers highlighted by the Waters MarkerLynx Application Manager for MassLynx Software.

What Is Metabonomics?

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 work investigated the concentrations changes of endogenous metabolites in urine over the time course of

the study, following single dose or daily administration of known nephrotoxins.

Comparison of the changes in urinary metabolite profiles with pre-dose and control animals (receiving vehicle alone) can then be used to detect and identify potential biomarkers of organ-specific toxicity.



The Waters Metabonomics MS System.

Experimental

Animal Study

- Male Wistar-derived rats (n=5 per group), approx. 140 g in weight, were acclimatized 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 10 mL/kg
- Group 2 were administered mercuric chloride at 2.0 mg/kg as a single subcutaneous injection at a dose volume of 10 mL/kg
- Group 3 were administered cyclosporin A at 45 mg/kg/day, orally

- Group 4 were administered gentamicinat 60 mg/kg/day, subcutaneously, twice daily
- 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 MS System using the following conditions:

UPLC Conditions

LC system:	ACQUITY UPLC
Column:	ACQUITY UPLC BEH C_{18} 2.1 x 100 mm, 1.7 µm
Temperature:	40 °C
Flow rate:	600 µL/min
Phase A:	0.1% Formic acid in water
Phase B:	0.1% Formic acid in acetonitrile
Gradient:	Linear, 0–20% B in 4 min. 20–95% B from 4 to 9 min.
Injection volume:	5 µL neat urine

MS Conditions

MS system:	Waters Micromass LCT Premier
Ion mode:	Electrospray positive and negative as separate acquisitions
Capillary voltage:	3000 V positive, 2600 V negative
Cone voltage:	35 V

Source temp.:	120 °C
Desolvation temp.:	350 °C
Dwell time:	0.15 sec
Inter-scan delay:	0.05 sec

Lockspray Conditions

Reference:	Leucine enkephalin
Switching Interval:	30 scans

Data Processing

The data from each of the samples groups (2–4) were processed against the control group (1) 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 and Discussion

Mercuric Chloride

The table of mass and retention time pairs with associated intensities generated by MarkerLynx was exported to SIMCA-P v.10.0 multivariate data analysis software (Umetrics, Sweden) and a three-dimensional scores plot was generated. This shows the time-related metabolite trajectory plot over the time course of the study after the 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. Two of the animals were poor responders and hence their samples do not cluster with the rest of the samples from the same day. This is most apparent with days 2, 4, and 5 samples.

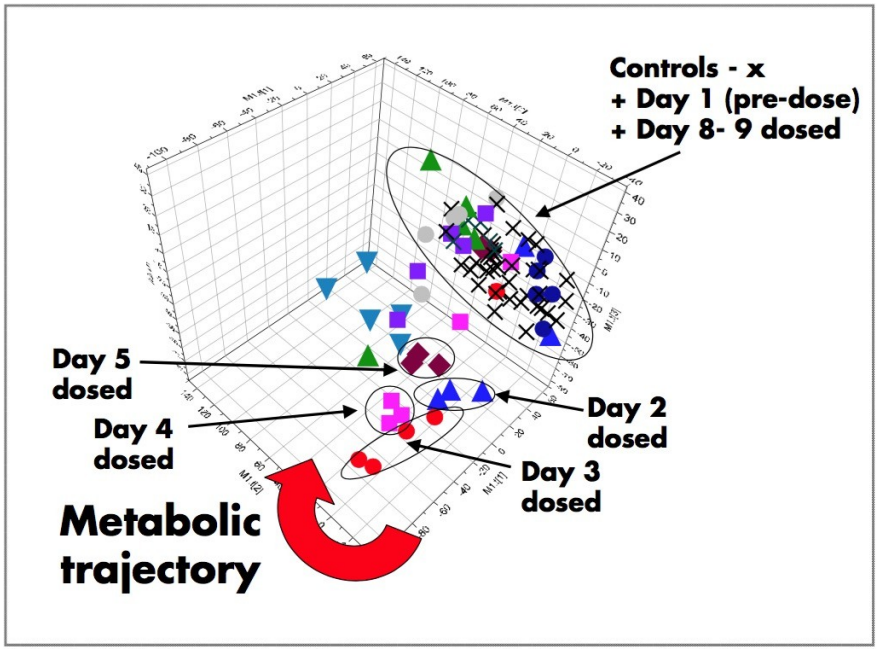


Figure 1. 3D positive ion scores plot for mercuric chloride samples.

A similar trajectory plot is observed in the negative ion mode MarkerLynx scores plot (Figure 2), with day 3 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.

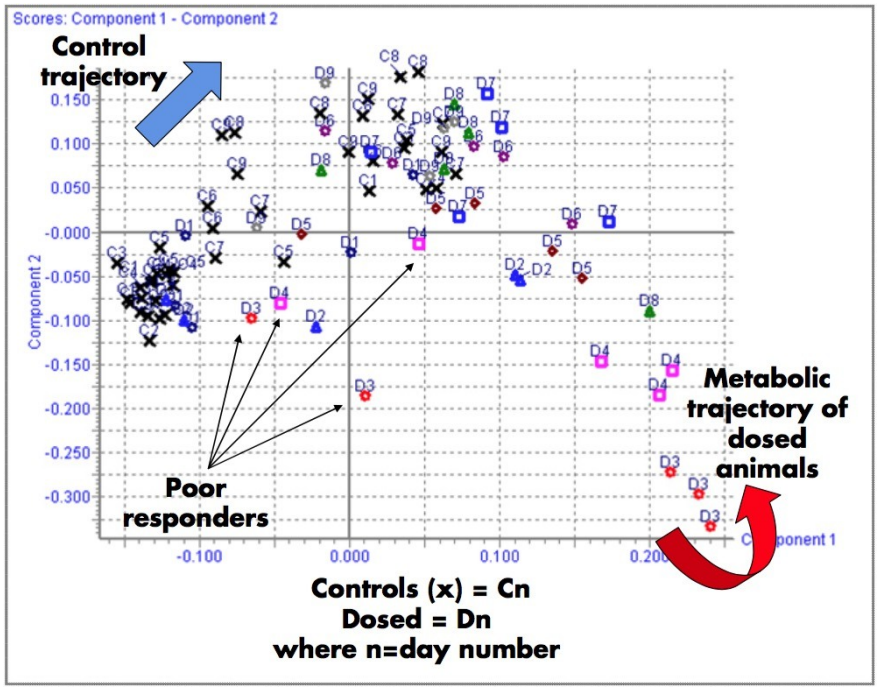


Figure 2. MarkerLynx PCA negative ion scores plot for mercuric chloride.

The masses and retention times for the metabolites responsible for the trajectory can be obtained from the

associated loadings plots (Figure 3).

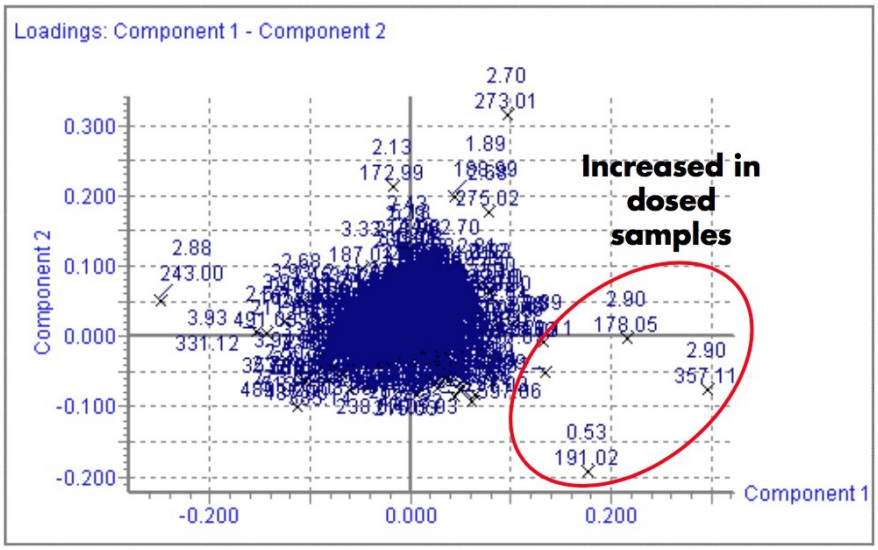


Figure 3. MarkerLynx PCA negative ion loadings plot for mercuric chloride.

The most significant ions reported by MarkerLynx as showing an increase after dosing with mercuric chloride were *m/z* 191.0185 ($C_6H_7O_7$ -0.7 mDa, -3.5 ppm) at RT 0.53 minutes, identified as citric acid and *m/z* 178.0500 ($C_9H_8NO_3$-0.4 mDa, -2.3 ppm) at RT 2.90 min, identified as hippuricacid. The other significant ion highlighted in the loadings plot, *m/z* 357.11 at 2.90 min is the dimerof hippuricacid. Further ions identified by= MarkerLynx are tabulated later (Figures 10 and 11).

Cyclosporin A and Gentamicin

The cyclosporin A PCA scores plot generated in MarkerLynx is shown in Figure 4. Here we can see that there is clear separation of the controls (black crosses) and pre-dose samples (dark blue circles) from all of the other samples.

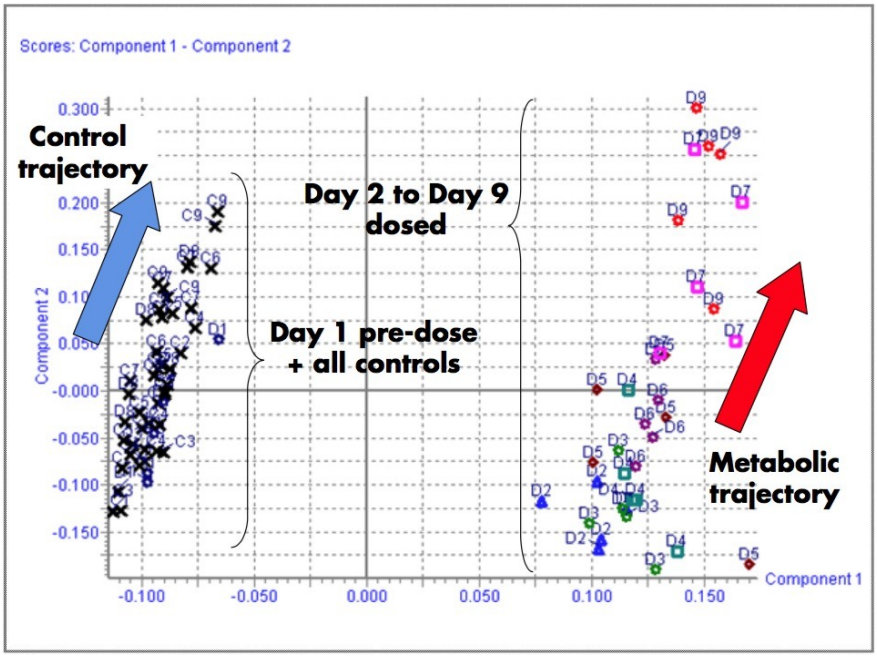


Figure 4. MarkerLynx PCA positive ion scores plot for cyclosporinA.

Examination of the TIC chromatograms (Figure 5) of urines from the same animal from day 1(pre-dose) and day 2 (after dosing) shows a cluster of intense peaks eluting around 4.5 to 5.5 minutes. These peaks, unsurprisingly, are reported in MarkerLynx as the most significant differentiators between the two groups of samples.

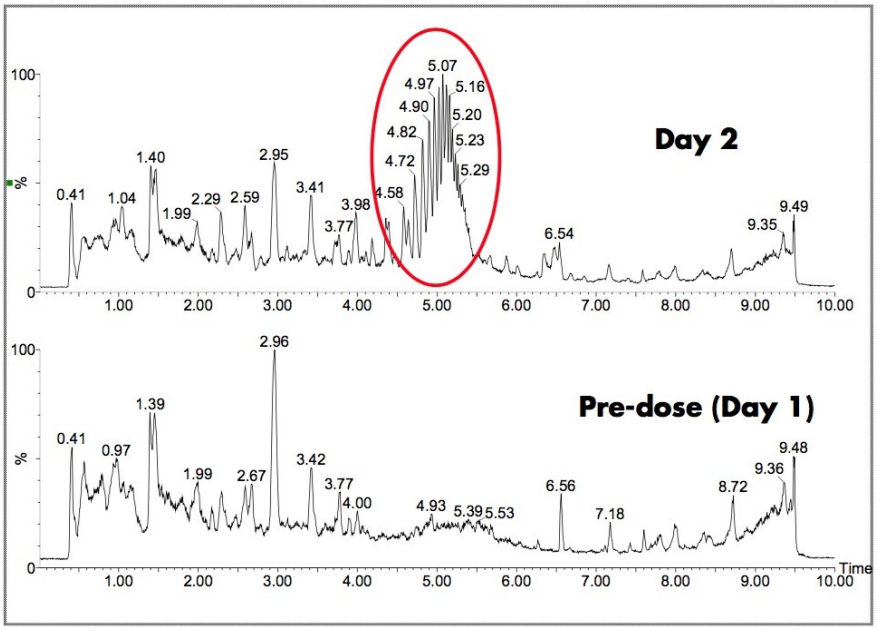


Figure 5. TIC chromatograms from day 1 (pre-dose) and day 2 after dosing with cyclosporin A.

Examination of the trend plots (Figure 6), which are a display of peak intensity against sample number, show that these peaks are not endogenous and were subsequently found to be part of the dosing vehicle. This particular peak was reported in MarkerLynx as *m/z* 652.4109 at a retention time of 4.76 minutes and corresponds to the ammonium adduct of a polyethylene glycol ($C_{26}H_{62}NO_{15}$, error -1.0 mDa, -1.6 ppm). Undesired contributions from either the dosing vehicle or xenobiotics can be readily excluded within MarkerLynx and new PCA plots generated.

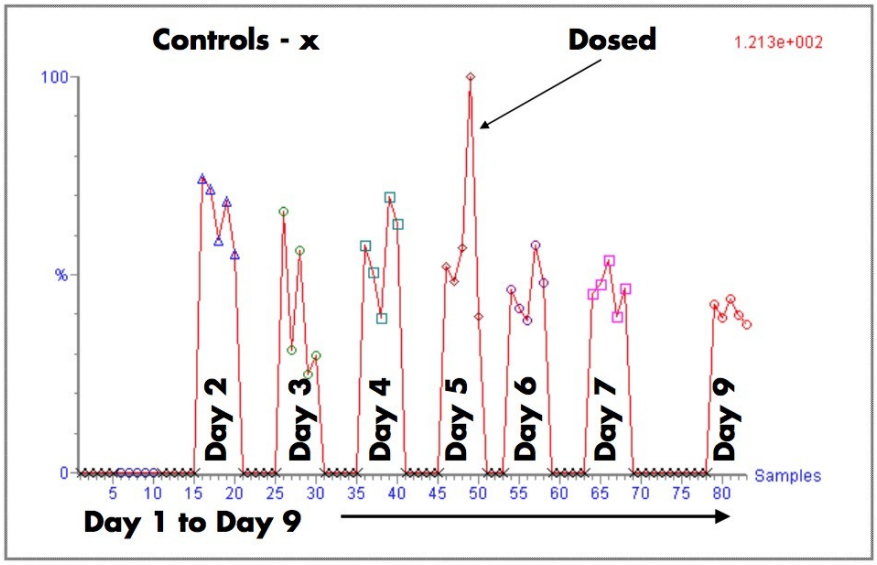


Figure 6. Trend plot for component found to be part of the dosing vehicle.

Typical trend plots for endogenous metabolites showing an increase and decrease after dosing are shown in Figure 7.

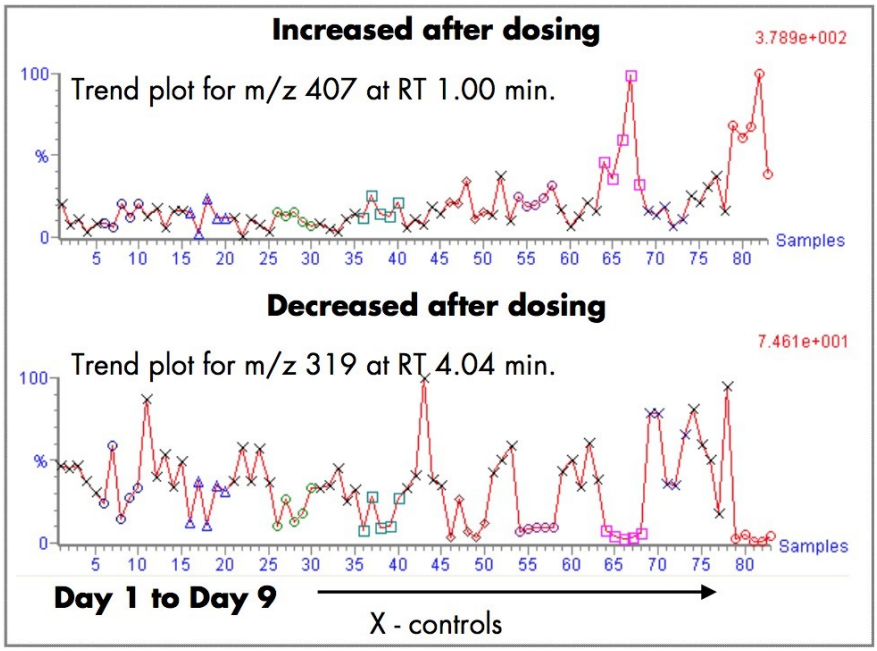


Figure 7. Trend plots for an endogenous metaboilte increasing after dosing (upper) and decreasing after dosing (lower).

The PCA scores plots was regenerated after exclusion of the PEG peaks from the dosing vehicle and is shown in Figure 8.

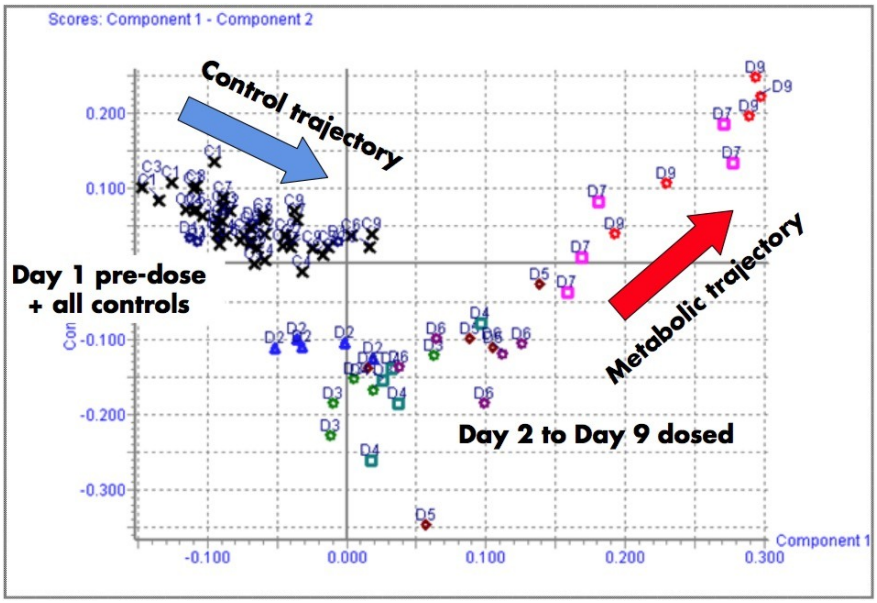


Figure 8. MarkerLynx PCA positive ion scores plot for cyclosporin A after removal of PEG contribution.

The scores plot shows a metabolic trajectory away from the controls as a result of the toxic insult from the daily

dosing of cyclosporin A. A similar trajectory plot was observed for the negative ion data (not shown). Tables of results listing the metabolites which showed the greatest perturbation are shown later.

The MarkerLynx positive ion scores plot for the urine from gentamicin-dosed animals is shown in Figure 9 and displays a trajectory away from normal. The most significant endogenous metabolites responsible for the differentiation in both positive and negative are listed in the tables (Figures 10 and 11).

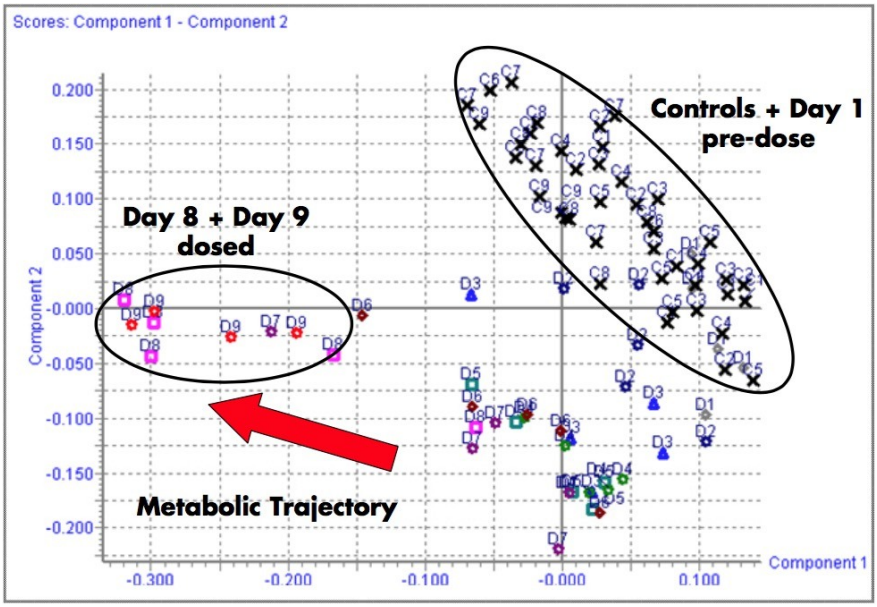


Figure 9. MarkerLynx PCA positive ion scores plot for gentamicin.

Principal Ions Increased After Dosing

RT	$HgCl_2$ Measured Mass	Cyclosporin Measured Mass	Gentamicin Measured Mass	Calculated $[M+H]^+$	Elemental Composition (neutral species)	Postulated Identity
0.59	215.0168	215.0159		215.0168	$C_6H_7O_7Na$	citric acid Na salt
0.89	407.0446	407.0435		407.0438	$C_{12}H_{15}O_{14}Na$	glucuronide Na adduct
0.88	215.0168	215.0162	215.0168	215.0168	$C_6H_7O_7Na$	citric acid Na salt
0.88	210.0628	210.0612		210.0614	$C_6H_{11}NO_7$	
2.85	243.0747	243.0740	243.0755	243.0746	$C_6H_{15}N_2O_6P$	
6.50	343.2952	343.2942		343.2961	$C_{19}H_{38}N_2O_3$	

Principal Ions Decreased After Dosing

RT	$HgCl_2$ Measured Mass	Cyclosporin Measured Mass	Gentamicin Measured Mass	Calculated $[M+H]^+$	Elemental Composition (neutral species)	Postulated Identity
0.65	212.1034		212.1037	212.1035	$C_9H_{13}N_3O_3$	dideoxycytidine
0.85	228.0982	228.0981		228.0984	$C_9H_{13}N_3O_4$	deoxycytidine
1.39		267.1332	267.1357	267.1345	$C_{13}H_{18}N_2O_4$	
1.91	297.1448		297.1461	297.1450	$C_{14}H_{20}N_2O_5$	
2.23	206.0453	206.0435	206.0463	206.0453	$C_{10}H_7NO_4$	xanthurenic acid
2.52	190.0504	190.0486	190.0512	190.0504	$C_{10}H_7NO_3$	kynurenic acid
2.59	328.1033	328.1017	328.1047	328.1032	$C_{14}H_{17}NO_8$	glucuronide of $C_8H_9NO_2$
2.75		164.0701	164.0718	164.0712	$C_9H_9NO_2$	methyldioxyindole
3.30	194.0816	194.0803	194.0823	194.0817	$C_{10}H_{11}NO_3$	phenylacetylglycine
4.03		297.1435		297.1450	$C_{14}H_{20}N_2O_5$	
3.91	319.1275	319.1261		319.1270	$C_{14}H_{19}N_2O_5Na$	Na adduct of 297

Figure 10. Table of most significant positive ions reported by MarkerLynx.

Principal Ions Increased After Dosing

RT	$HgCl_2$ Measured Mass	Cyclosporin Measured Mass	Gentamicin Measured Mass	Calculated $[M-H]^-$	Elemental Composition (neutral species)	Postulated Identity
0.48	215.0321	215.0313	215.0307	215.0320	$C_5H_{13}O_7P$	methylerythritol phosphate
0.53	191.0185	191.0185	191.0189	191.0192	$C_6H_8O_7$	citric acid
0.53	173.0082	173.0076		173.0086	$C_6H_6O_6$	aconitic acid
0.74	145.0129			145.0137	$C_5H_6O_5$	alpha ketoglutaric acid
0.92	405.0295	405.0278		405.0281	$C_{12}H_{13}O_{14}Na$	Na adduct of m/z 383
1.19	117.0181			117.0188	$C_4H_6O_4$	succinic acid
1.78	350.0553	350.0538		350.0546	$C_{12}H_{17}NO_9S$	
2.40		261.0063	261.0064	261.0069	$C_9H_{10}SO_7$	homovanillic acid SO_4
2.67	326.0876	326.0868		326.0876	$C_{14}H_{17}NO_8$	glucuronide of $C_8H_9NO_2$
2.70	273.0064	273.0063	273.0069	273.0069	$C_{10}H_{10}O_7S$	ferulic acid sulphate
2.90	178.0500	178.0486		178.0504	$C_9H_9NO_3$	hippuric acid
3.35	192.0657			192.0661	$C_{10}H_{11}NO_3$	phenylacetylglycine
3.35	407.1230	407.1216		407.1223	$C_{13}H_{28}O_{12}S$	

Principal Ions Decreased After Dosing

RT	$HgCl_2$ Measured Mass	Cyclosporin Measured Mass	Gentamicin Measured Mass	Calculated $[M-H]^-$	Elemental Composition (neutral species)	Postulated Identity
1.87	188.9852		188.9859	188.9858	$C_6H_6O_5S$	sulfocatechol
2.12	172.9904		172.9908	172.9909	$C_6H_6O_4S$	phenol SO_4
2.28	230.0118		230.0121	230.0123	$C_8H_9NO_5S$	
2.86	242.9953	242.9953	242.9963	242.9963	$C_9H_8O_6S$	SO_4 of $C_9H_8O_3$ hydroxycinammic acid
3.93	165.0545			165.0552	$C_9H_{10}O_3$	hydroxyphenylpropionic acid
3.93	331.1169	331.1179	331.1169	331.1182	$C_{18}H_{20}O_6$	dimer of 165
3.94	353.1002	353.0996		353.1001	$C_{18}H_{19}O_6Na$	Na adduct of 165 dimer
3.94	211.0600	211.0597	211.0604	211.0606	$C_{10}H_{12}O_5$	
4.20	209.0447		209.0447	209.0450	$C_{10}H_{10}O_5$	

Figure 11. Table of most significant negative ions reported by MarkerLynx.

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]

In the gentamicinstudy, the change in the metabolic profile of the dosed samples could also be as a result of the antibiotic activity of the gentamicin, affecting the gut microflora metabolism. The elemental compositions and exact mass measurements of the major species changing after dosing of all the compounds are tabulated in Figures 10 and 11.

Citric acid, part of the TCA or Krebs cycle, was observed to show the most significant increase after dosing of all the compounds. In positive ion mode, the species showing the most significant decrease after dosing were kynurenic acid and xanthurenic acid, end products of the trytophan catabolism cycle.

Conclusion

UPLC/TOF MS, in conjunction with principal components analysis using the MarkerLynx Application Manager,

has been shown to be a valuable tool in the study of renal toxins. 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 thus facilitating the discovery of more potential biomarkers.

The changes to the endogenous urinary metabolite concentrations have been used to track the onset of nephrotoxicity over time. The interactive MarkerLynx browser has allowed unwanted contributions from dosing vehicle and xenobiotics to be readily excluded. Exact mass measurements have been used to identify some of the potential biomarkers, although their biological significance is yet to be determined. The identity of the other potential markers needs to be confirmed, their position in the metabolic pathways located, and their toxicological significance evaluated.

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., Wright B., Stewart J. and Wilson I.D. *Pharmaceutical Sciences* 3, 143–146 (1997).
2. Phipps A.N., Wright B., Stewart J. and Wilson I.D. *Xenobiotica* 28(5), 527–537 (1998).

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