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Metabonomics with ^{1}H-NMR spectroscopy and liquid chromatography-mass spectrometry applied to the investigation of metabolic changes caused by gentamicin-induced nephrotoxicity in the rat

E. M. LENZ[1], J. BRIGHT[2], R. KNIGHT[3], F. R. WESTWOOD[3], D. DAVIES[3], H. MAJOR[4], & I. D. WILSON[1]

[1]*Department of Drug Metabolism and Pharmacokinetics,* [2]*Global Science and Informatics,* [3]*Safety Assessment, AstraZeneca, Mereside, Alderley Park, Macclesfield, UK and* [4]*Waters Corporation, Atlas Park, Simonsway, Manchester, UK*

Abstract

The model nephrotoxin gentamicin was administered to male Wistar-derived rats daily, for 7 days, at 60 mg kg^{-1} day^{-1}, subcutaneously, twice daily. Conventional clinical chemistry urinalysis showed a significant increase in N-acetyl-β-D-glucosaminidase (NAG) activity from day 3. At necropsy on day 9, clear histological damage to the kidney was noted with all animals showing a generally severe nephropathy primarily focused on the proximal convoluted tubules. The urinary excretion pattern of endogenous metabolites over the time course of the study was studied using a combination of ^{1}H-NMR spectroscopy and HPLC-TOF-MS/MS using electrospray ionization (ESI). Changes in the pattern of endogenous metabolites as a result of daily administration of gentamicin were readily detected by both techniques with significant perturbations of the urinary profile observed from day 7 onwards. The findings by ^{1}H-NMR included raised glucose and reduced trimethylamine N-oxide (TMAO). Changes in metabonomic profiles were observed by HPLC-MS in both positive and negative ESI. The MS data showed reduced xanthurenic acid and kynurenic acid, whilst neutral loss experiments also revealed a changed pattern of sulphate conjugation on gentamicin administration.

Keywords: *Metabonomics, nephrotoxicity, pattern recognition, gentamicin*

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Introduction

Metabonomic studies based on the use of high field ^{1}H-NMR spectroscopy of biofluids and tissues represents an established method for the study of toxicity (e.g. Gartland et al. 1989, Anthony et al. 1994, Foxall et al. 1996, Beckwith-Hall et al. 1998, Robertson et al. 2000, Lindon et al. 2003). NMR spectroscopy allows the multicomponent analyses of biological fluids such as urine to be carried out without the need for optimization of the analysis conditions for individual analytes, and with minimal sample preparation. The ^{1}H-NMR spectral fingerprints of biofluids typically comprise hundreds of endogenous organic metabolites, and disturbances in these

Correspondence: I. D. Wilson, Department of Drug Metabolism and Pharmacokinetics, AstraZeneca, Mereside, Alderley Park, Macclesfield SK10 4TG, UK. Email: Ian.Wilson@astrazeneca.com

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profiles compared with the basal metabolic state may reveal potential biomarkers of disease or toxicity. These changes are often characteristic of the affected organs and nature of the resulting toxicity as has been extensively described in the literature (e.g. Waters et al. 2001, Holmes & Antti 2002). Although potentially very complex, these metabolic fingerprints are amenable to analysis by pattern recognition techniques such as principal component analysis (PCA). More recently a number of groups have begun to report the use of HPLC-MS for metabonomic analysis, either alone (Plumb et al. 2002, 2003, Idborg-Bjorkman et al. 2003, Lafaye et al. 2003) or combined with concomitant ^{1}H-HMR spectroscopic studies (Lenz et al. 2004a, b). The combination of the two techniques appears to be very powerful as each methodology provides access to a different part of the urinary metabolic fingerprint. Here we describe the application of HPLC-MS and ^{1}H-NMR spectroscopy to the study of the effects of the nephrotoxin gentamicin which was undertaken as part of a series of experiments conducted in order to establish biomarkers of region specific toxic lesions in the kidney (Lenz et al. 2004a, b). Urinary levels of N-acetyl-β-D-glucosaminidase (NAG) and lactate dehydrogenase (LDH) activities and urinary glucose excretion were used as conventional urinalysis markers of nephrotoxicty (Whiting & Brown 1996) and light microscopy was used to evaluate histological damage to the kidney.

Materials and methods

Chemicals

Acetonitrile, HPLC grade, was purchased from Riedel-de Haën (Sigma Aldrich, Poole, UK); formic acid, Aristar grade, was purchased from BDH (Poole, UK); HPLC-grade water was purchased from Fisher Scientific (Loughborough, UK); leucine enkephalin was purchased from Sigma-Aldrich.

Animals and dosing

This study was planned in accordance with the standards of animal care and ethics described in Guidance on the Operations of the Animals (Scientific Procedures) Act 1986 issued by the UK Home Office and was conducted so that any clinical expression of toxicity remained within a moderate severity limit as described in guidelines agreed with the UK Home Office Inspector.

Male Wistar-derived rats ($n = 5$ per group) of approximately 140 g in weight were allowed to acclimatize in metabolism cages for 3 days before treatment. Food and water were provided *ad libitum*. A dose level of 120 mg kg^{-1} day^{-1} gentamicin, given as two separate doses of 60 mg kg^{-1} day^{-1} subcutaneously (s.c.), twice daily 8 h apart, was administered s.c. for 7 days. The compound was formulated in distilled water at a concentration of 12 mg ml^{-1} and a dose volume of 10 ml kg^{-1} was used. Control animals were administered 0.9% w/v saline, also at a dose volume of 10 ml kg^{-1}.

Sample collection and storage

Samples from both groups were collected daily at day −1 and daily thereafter for 9 days. A total of 45 samples/group were obtained. Urine samples were collected

overnight (16 h) in metabolism cages at ambient temperature. Following collection, samples were stored frozen at −20°C until analysis.

Sample analysis

In addition to the ^{1}H-NMR spectroscopy and HPLC-MS analysis reported in detail here, the samples were also subjected to conventional clinical chemical analysis and histopathology. The plasma was analysed for urea on days −1, 4 and 9 while urine samples collected overnight on days 1, 2, 3, 4, 6 and 8 were analysed for urinary glucose (UGLU), urinary lactate dehydrogenase (ULDH) and N-acetyl–D-glucosaminidase (UNAG). Urine volumes were determined for all samples and all results are expressed as the total amount of analyte excreted during the overnight urine collection period. All analyses were performed on a Hitachi Modular Clinical Chemistry Analyser using standard reagents provided by Roche for urea, glucose and LDH. NAG was determined by a means of colorimetric method provided by PPR Diagnostics Ltd.

The kidneys were excised from all animals during necropsy at the end of the study period. Following fixation in buffered 10% formalin and processing to wax blocks, serial transverse step sections were prepared. For each organ, individual sections were stained with haematoxylin and eosin and by the periodic acid-Schiff method. They were examined by light microscopy

^{1}H-NMR spectroscopy

Aliquots of undiluted urine (100 µl) were buffered with 100 µl 0.2 M phosphate buffer/D_2O (pH 7.4) before analysis by NMR spectroscopy. Urinalysis was carried out on a Bruker DRX500 NMR spectrometer operating at 500 MHz ^{1}H resonance frequency. ^{1}H-NMR spectra were acquired at 30°C, with 90° pulse widths over a spectral width of 9980.04 Hz into 64 000 data points. Typically, 128–512 transients were collected with an acquisition time of 3.28 s (and a relaxation delay of 1 s). The pulse recycle time of 4.28 s was considered adequate for the qualitative analysis performed here. Solvent suppression was achieved by applying the standard Noesypresat pulse sequence (Bruker Biospin Ltd., Coventry, UK) with secondary irradiation of the dominant water signal during the mixing time of 150 ms and the relaxation delay of 1 s. No line broadening function was applied before FT. Spectra were referenced to the internal reference standard TSP (sodium trimethylsilylpropionic acid [$^2H_4$], $\delta 1_H = 0.0$) dissolved in D_2O. The D_2O was used to provide a field-frequency lock for the NMR spectrometer.

Data analysis for ^{1}H-NMR spectroscopy

All ^{1}H-NMR spectra were manually corrected for phase and baseline distortions within XWINNMR™ (Version 2.6, Bruker Spectrospin Ltd.). Spectra were referenced to TSP (δ_{1H} 0.0) before data reduction into 245 spectral integral regions corresponding to the chemical shift range of δ_{1H} 0.2–10 utilizing AMIX (version 2.7.5, Analysis of MIXtures, Bruker Spectrospin Ltd.). The region of δ_{1H} 4.52–6.0 was set to zero to remove the effects of variations in the presaturation of the water resonance in all NMR spectra, and to alleviate cross-relaxation effects in the urea signal via solvent exchanging protons. Integration into bins (or buckets) across the

spectral regions of 0.04 ppm was performed automatically in AMIX. To counteract separation on grounds of differences in dilution between urine samples, all spectra were then normalized, i.e. scaled, so the total area for each spectrum has the same value.

The resulting data matrix (peak integral values/bins per sample) was analysed by pattern recognition methods within SIMCA-P (Version 8, UMETRICS AB, Umeå, Sweden) and visualized using 'Spotfire' (Spotfire DecisionSite 6.2 version 6.2.0).

Following the processing of the NMR spectra by AMIX, data analysis was performed using various techniques including principal component analysis (PCA). The Spotfire program was used to visualize both the spectral data (reconstructed data-reduced spectra) and the output from SIMCA-P. Visualizing the spectra in this way aided the identification of unusual spectra and individual peaks in the spectra, which increased or diminished over time. SIMCA-P was used to perform the principal component analysis. PCA was performed using centred scaling.

HPLC-MS

For analysis by HPLC-MS, 100-µl aliquots of rat urine samples were centrifuged at 13 000 rpm for 5 min at room temperature and the supernatant liquid removed and transferred to autosampler vials for analysis. Chromatography was performed on a Waters Alliance® HT HPLC system equipped with a column oven. The HPLC system was coupled to a Waters Micromass Q-Tof micro™ (Manchester, UK) equipped with an electrospray source operating in either positive- or negative-ion mode. The source temperature was set at 120°C with a cone gas flow of 50 l h^{-1}; a desolvation gas temperature of 250°C and a desolvation gas flow of 500 l h^{-1} were employed. The capillary voltage was set at 3.2 kV for positive-ion mode and 2.6 kV in negative-ion mode and the cone voltage to 30 V. A scan time of 0.4 s with an inter-scan delay of 0.1 s was used for all MS acquisitions with a collision energy of 4 eV and a collision gas pressure of about 2.8×10^{-3} mbar argon. The collision energy was increased to between 12 and 25 eV for the MS/MS experiments. A lock-mass of leucine enkephalin at a concentration of 0.5 ng μl^{-1}, in 50:50 acetonitrile:water +0.1% formic acid for positive-ion mode ($[M+H]^+ = 556.2771$) and 1 ng μl^{-1} in 50:50 acetonitrile:water for negative-ion mode ($[M\text{-}H]^- = 554.2615$), was employed at a flow rate of 30 µl min^{-1} via a LockSpray interface. Data was collected in centroid mode, the lock spray frequency was set at 5 s and the lock mass data was averaged over 10 scans for correction.

A 10 µl aliquot of rat urine was injected onto a 2.1 mm × 10 cm Symmetry® C18 3.5 µm column held at 40°C. The column was eluted with a linear gradient of 0–20% B over 0.5–4 min, 20–95% B over 4–8 min and the composition was held at 95% B for 1 min then returned to 100% A at 9.1 min at an eluent flow rate of 600 µl min^{-1}, where A = 0.1% formic acid (aq.) and B = 0.1% formic acid in acetonitrile. A 'purge-wash-purge' cycle was employed on the autosampler, with 90% aqueous methanol used for the wash solvent and 0.1% aqueous formic acid used as the purge solvent, this ensured that the carry-over between injections was minimized. The mass spectrometric data was collected in full-scan mode from *m/z* 50 to 850 from 0 to 10 min, in positive- and negative-ion mode. The column eluent was split such that approximately 120 µl min^{-1} were directed to the mass spectrometer.

Data analysis for HPLC-MS

The LC-MS data were analysed using the Micromass MarkerLynx Applications Manager version 1.0. MarkerLynx incorporates a peak detection and deconvolution package and also performs retention time alignment of the peaks across the samples. The data was combined into a single matrix by aligning peaks with the same mass/retention time pair together from each data file in the dataset, along with their associated normalized intensities. The processed data list was either analysed by PCA within the MarkerLynx Applications Manager or exported for chemometric analysis using SIMCA-P (version 10.0). PCA was performed in SIMCA-P using centred scaling.

Results and discussion

Histopathology and clinical chemistry

In addition to metabonomic analysis, the effects of gentamicin were examined using a range of conventional techniques, including histopathological examination of the kidneys and standard clinical chemical analysis of urine and plasma, to confirm the extent and sites of nephrotoxicity.

Clinical chemistry analysis showed that plasma urea values were similar for controls and gentamicin treated rats on days −1 and 4 but, by day 9, the mean plasma urea was significantly elevated ($p < 0.001$) at 15.4 ± 3.5 (5) mmol l^{-1}, compared with 4.4 ± 0.2 (5) mmol l^{-1} for controls. Urinalysis also showed that the nephrotoxicity was progressive with significant increases in urinary NAG being observed from day 3, and glucose and LDH activity increasing from day 4. The most marked increase in all these parameters was observed on day 8 (Figure 1). These changes are consistent with severe kidney toxicity.

Clear histological damage to the kidney was noted with all animals showing a generally severe nephropathy primarily focused on the proximal convoluted tubules and characterized by a marked epithelial necrosis. This and other zones of the tubules showed protein casts, epithelial basophilia with attenuation of the basophilic epithelium and some tubular dilatation. There was some associated interstitial inflammatory cell infiltration. These data are summarized in Table I.

Analysis of urines by ^{1}H-NMR spectroscopy

Changes in the urinary profiles of animals dosed with gentamicin, compared with control animals, are discussed below (and summarized in Table II). In the case of the control animals, some fluctuations in the concentrations of endogenous metabolites were observed, particularly for signals in the aromatic region of the spectrum. These were subject to unpredictable variation due to changes in the excretion of hippuric and 3-hydroxyphenylpropionic acids. Such changes, however, reflect changes in the metabolism of the gut microflora (Phipps et al. 1997, 1998) rather than the biochemistry of the animals themselves. It should be noted, however, that there was a change in the control rat profile over time due to decreasing concentrations of trimethylamine-N oxide (TMAO) and betaine over the time course of the study. For the first 7 days of the study, there were few major changes in the urinary metabolite profiles of the dosed animals apart from a decrease in TMAO and a gradual increase

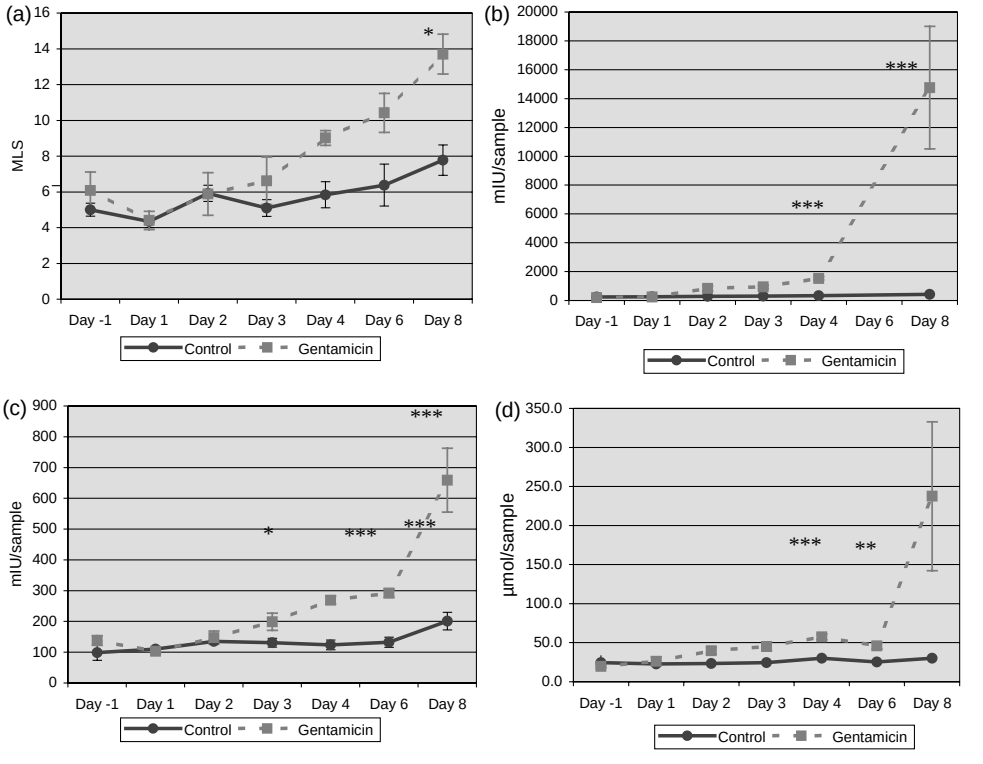

Figure 1. Group mean ± SE ($n = 5$) for control (circle and solid line) and gentamicin (square and dashed line) dosed rats. Shown are the results for (a) urine volume, (b) LDH, (c) NAG and (d) glucose. Where the SE bars appear missing then the data falls within the symbols. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control values on the same day).

in the concentrations of citrate. However, the day 8 samples were characterized by marked glucosuria, together with slightly raised lactate concentrations. Glucosuria was equally pronounced on day 9, together with slightly raised lactate and N-acetyl glycoprotein excretion. Effects were also noted on the excretion of the microflora-

Table I. Assessment of kidney histopathology following gentamicin treatment.

Animal	Proximal convoluted	Pars recta	Loop	Thick ascending	Distal convoluted	Collecting duct
1	***1	**2	–	–	–	–
2	****1	***2	–	***3	***2	–
3	****1	**2	–	*3	**2	–
4	****1	***2	–	**3	**2	–
5	****1	***2	–	**3	**2	–

Severity of tubular epithelial cell degeneration, necrosis and regeneration graded subjectively minimal (*), mild (**), moderate (***) or severe (****).

Character of histopathological changes: 1, predominantly degeneration/necrosis although some regenerating basophilic tubular epithelial cells were present: 2, degeneration/necrosis, and regeneration characterized by basophilic tubular epithelial cells, present together; or 3, predominantly basophilic regenerating tubular epithelial cells).

Table II. Observed perturbations in the urinary metabolite profiles by ^{1}H-NMR spectroscopy after administration of gentamicin to the rat.

Days	Effect (changes in metabolite profile)
3+	Increasing citrate, α-KG, DMG, decreasing TMAO and betaine
8–9	glucose ↑↑, lactate, citrate, N-acgp, unidentified singlet at 1.39 ppm↑ TMAO and betaine ↓↓

derived aromatics that were somewhat reduced in dosed animals compared with controls. However, these reductions are most likely due to the antibiotic activity of the compound rather than nephrotoxicity. Similar effects have been observed in studies designed to investigate the effects of microfloral metabolism in the rat (Williams et al. 2002). Typical ^{1}H-NMR spectra for samples obtained predose and on day 9 for a gentamicin-dosed animal are shown in Figure 2.

PCA of the data obtained on these urine samples was performed as described in the materials and methods section. The resulting loadings/scores plots are shown in Figure 3 for both control and dosed animals. As this figure shows, the trajectory followed by the dosed animals begins to diverge from the controls on day 5 of administration. By day 8/9 of the study the metabolic profiles of the urine samples from the gentamicin-dosed animals had completely diverged from those of the control group. The markers responsible for the differences between control and test animals, determined from the scores plot were increased glucose, lactate and the unassigned singlet at 1.39 ppm together with decreased betaine and TMAO. These changes in metabolite profile are typical of those observed for nephrotoxins (e.g. Gartland et al.

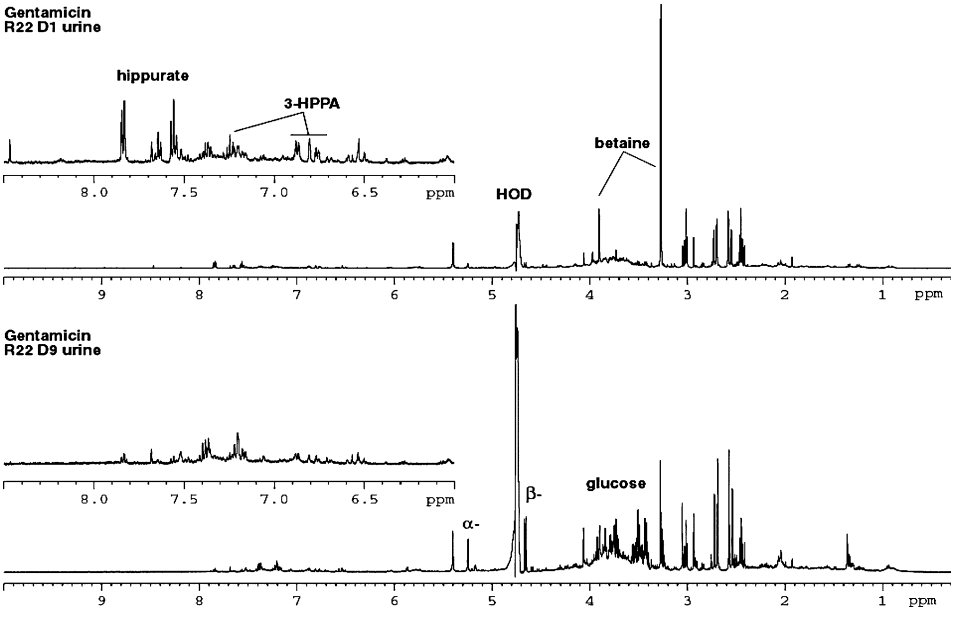

Figure 2. ^{1}H-NMR spectra of rat urine obtained from the same gentamicin-dosed animal for predose (upper) and day 9 (lower) of the study highlighting the relevant difference. The aromatic region of each spectrum is shown in the expansion.

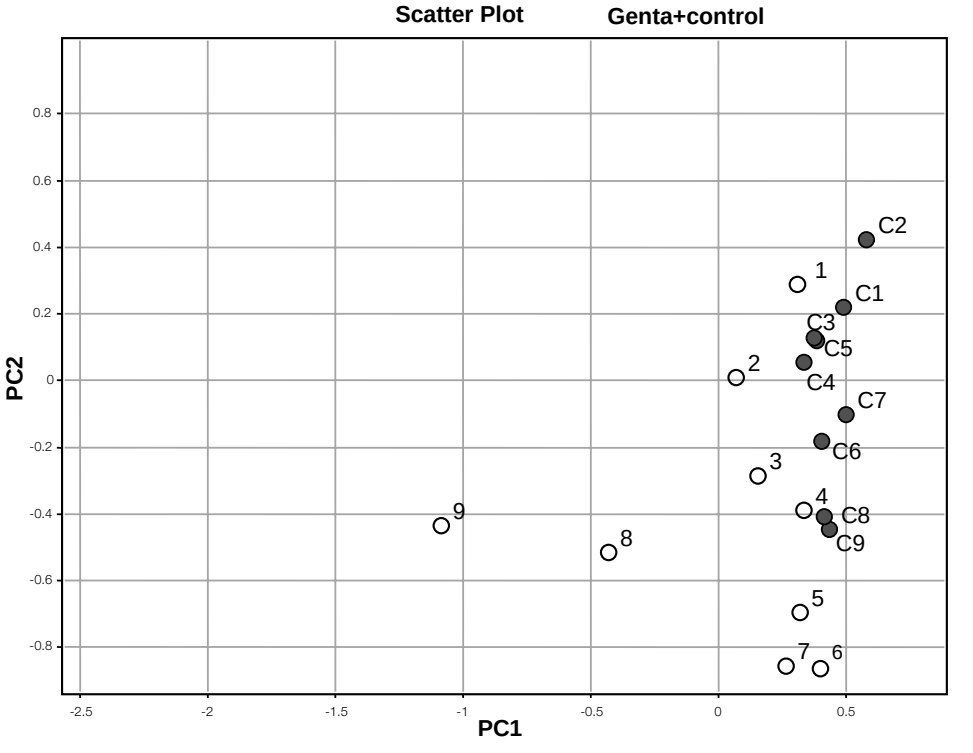

Figure 3. Combined mean trajectory plot for control (solid circles) and gentamicin-dosed (open circles) rats. The numbers by the symbols refer to the day of the study (e.g. C1–9 = control group, 1–9 = gentamicin-dosed group). Note the strong divergence of the dosed animals for days 8 and 9 compared with the control animals.

1989, Anthony et al. 1994, Beckwith-Hall et al. 1998, Robertson et al. 2000, Lindon et al. 2003) and are consistent with the clinical chemistry and histopathology observed here.

Analysis of urines by HPLC-MS

As described in the experimental section all of the urine samples incurred in this study were analysed by HPLC-MS with both positive and negative electrospray ionization. As noted with ^{1}H-NMR spectroscopy above, pronounced changes were seen in the urinary metabolite profiles for both modes of ionization by day 9 of dosing. Typical positive total ion current (TIC) chromatograms for a day 9 control and treated rat are shown in Figure 4a and b while the negative-ion TICs from day 9 control and treated animals are shown in Figure 4c and d, respectively. As also noted by NMR spectroscopy, some unpredictable variation was observed in the results obtained for control animals due to variations in the excretion of hippuric acid and 3-hydroxyphenylpropionic acid over the course of the study.

Pattern recognition via PCA was then performed on both positive and negative ESI data. The mean metabolic trajectory plots obtained are shown in Figure 5a and b, respectively. These show that the dosed animals began to deviate from the controls from day 3 onwards (although the changes to some metabolites did not begin to occur

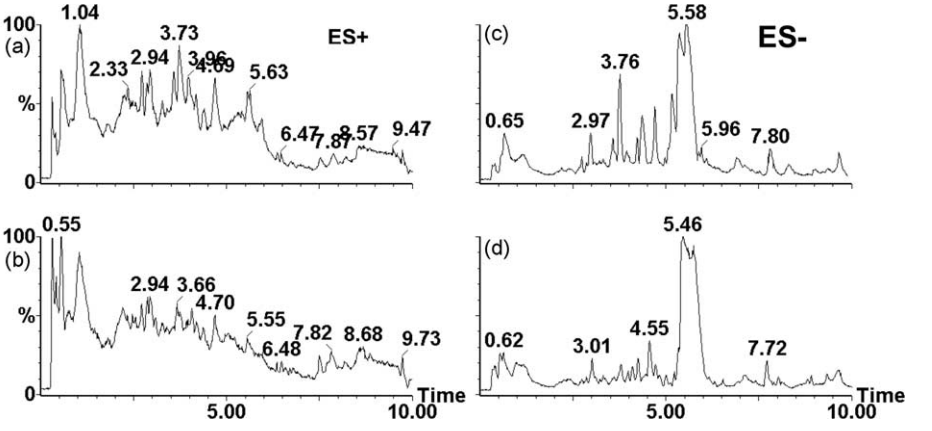

Figure 4. Typical HPLC-MS TIC data for (a) positive ESI day 9 control, (b) positive ESI day 9 gentamicin-dosed animals, (c) negative ESI day 9 control and (d) negative ESI day 9 gentamicin-dosed animals.

until day 5 or later). Not all of the compounds detected were associated with endogenous compounds however, and on analysis of these samples in positive ESI, we noted the presence of ions corresponding to the administered drug and its metabolites. The gentamicin complex consists of three closely related components, gentamicin C_1,

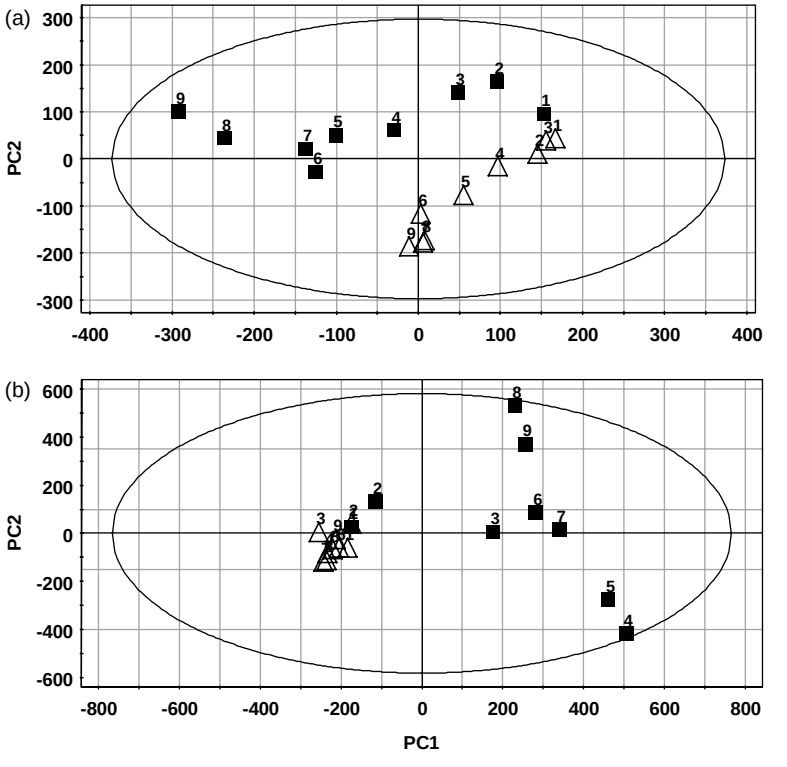

Figure 5. Combined mean HPLC-MS data for (a) positive ESI trajectory plots for control (open triangles) and gentamicin-dosed animals (solid squares) and (b) for negative ESI (open triangles) and gentamicin-dosed (solid squares).

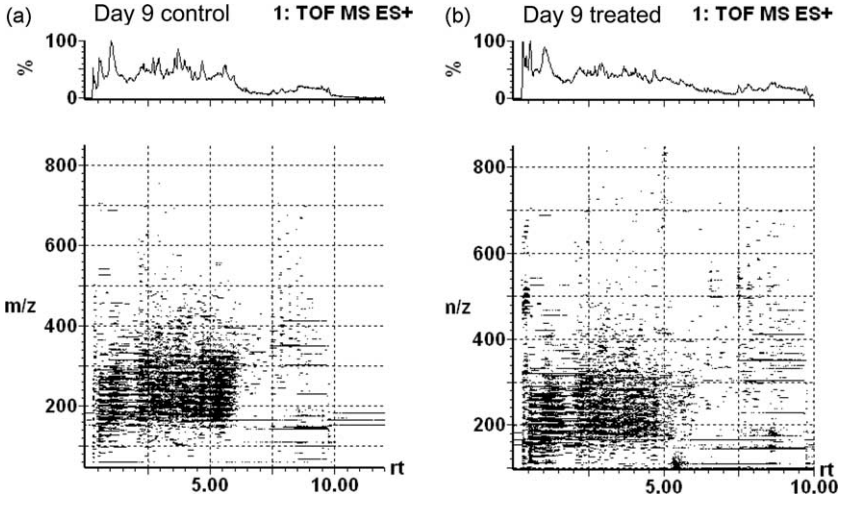

Figure 6. Typical positive-ion TIC (upper) and two-dimensional plots of mass versus retention time (lower) for typical day 9 (a) control and (b) gentamicin-dosed animals.

C_2 and C_{1a} and, for obvious reasons, the mass spectral peaks from these drug molecules and their corresponding metabolites were excluded from the PCA analysis of the data. This exclusion process was set up automatically within the MarkerLynx software. Two-dimensional plots of mass versus retention time for typical day 9 control and dosed animals, obtained by positive ESI, are shown in Figure 6a and b. Analysis of these data using PCA revealed that dosing with gentamicin was associated with the elevation or reduction of a number of compounds in positive ESI mode. The main species highlighted in the PCA analysis as being reduced after dosing with gentamicin were xanthurenic acid (*m/z* 206) and kynurenic acid (*m/z* 190), which

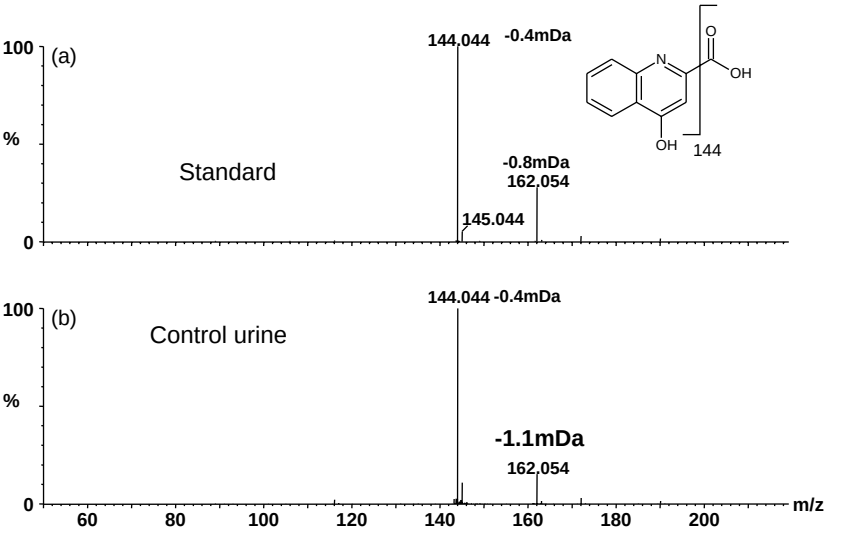

Figure 7. HPLC-MS/MS product ion spectra for the ion at *m/z* 190 from (a) a kynurenic acid standard and (b) a control urine, respectively.

Table III. Positive-ions increased after administration of gentamicin to the rat.

Retention time (min)	Measured mass (Da)	Calculated mass (Da)	Elemental composition of $[M+H]^+$ ion	Comment
1.16	215.0181			co-eluting interfering peak at *m/z* 215.13
2.95	255.0868	255.0842	$C_8H_{11}N_6O_4$	does not readily fragment
		255.0882	$C_{13}H_{11}N_4O_2$	MS/MS spectrum very weak

declined in concentration from day 5 onwards. The identity of these species was confirmed by HPLC/MS/MS product ion analysis of the putative biomarkers and comparison with authentic standards. The LC/MS/MS product ion spectra from *m/z* 190, from a kynurenic acid standard and a control urine are shown in Figure 7a and b. The two principal ions observed to increase in positive-ion ESI after dosing were those for *m/z* 215 and *m/z* 255, eluting at 1.16 min at 2.95 min, respectively. Both of these ions increased from day 3 but had returned to pre-dose levels by the end of the study. The identity of these species remains unknown. Other ions that showed a decrease after dosing were an ion of *m/z* 149 (from day 3 onwards), identified as cinnamic acid by HPLC-MS/MS, and an ion of *m/z* 220 (from day 7 onwards) postulated to be pantothenic acid. Additional ions at *m/z* 255, 267 and 297 that were seen to decrease after dosing remain unidentified. These data for increasing and decreasing compounds are summarized in Tables III and IV, which also provide postulated elemental compositions.

The principal ions showing an increase after dosing in negative-ion (*m/z* 261, 275, 332 and 330) were all sulphated species and showed an increase from Day 3 onwards. The structures of these species are currently unknown but their postulated elemental compositions are given in Table V.

The principal ions showing a decrease after dosing were either sulphated (*m/z* 243, hydroxycinnamic acid sulphate; *m/z* 189, resorcinol sulphate; *m/z* 173, phenol sulphate and *m/z* 219, an unknown sulphate) or glucuronidated (*m/z* 283). The structures of some of these metabolites were confirmed by MS/MS product ion analysis and an example of this is shown in Figure 8a and b for resorcinol and phenol sulphates, respectively. A continuing problem with the confirmation of the identity of

Table IV. Positive-ions decreased after administration of gentamicin to the rat.

Retention time (min)	Measured mass (Da)	Calculated mass (Da)	Elemental composition of $[M+H]^+$ ion	Postulated identity
3.59	206.0434	206.0453	$C_{10}H_8NO_4$	xanthurenic acid[a]
3.95	190.0499	190.0504	$C_{10}H_8NO_3$	kynurenic acid[a]
1.02	267.1320	267.1345	$C_{13}H_{19}N_2O_4$	$C_{13}H_{18}N_2O_4$
5.57	255.0653	255.0657	$C_{15}H_{11}O_4$	$C_{15}H_{10}O_4$
2.35	297.1447	297.1450	$C_{14}H_{21}N_2O_5$	$C_{14}H_{20}N_2O_5$
2.73	220.1194	220.1185	$C_9H_{18}NO_5$	pantothenic acid
4.70	149.0618	149.0603	$C_9H_9O_2$	cinnamic acid[a]
5.64	285.0746	285.0763	$C_{16}H_{13}O_5$	$C_{16}H_{12}O_5$
		285.0723	$C_{11}H_{13}N_2O_7$	$C_{11}H_{12}N_2O_7$

[a]Confirmed by LC/MS/MS against standards.

Table V. Negative-ions increased after administration of gentamicin to the rat.

Retention time (min)	Measured mass (Da)	Calculated mass (Da)	Elemental composition of $[M-H]^-$ ion	Postulated identity
4.36 and 5.13	261.0049	261.0069	$C_9H_9SO_7$	$C_9H_{10}SO_7$
	181.0504	181.0501	$C_9H_9O_4$	loss of SO_3
5.07	275.0207	275.0225	$C_{10}H_{11}SO_7$	$C_{10}H_{12}SO_7$
	195.0656	195.0657	$C_{10}H_{11}O_4$	loss of SO_3
4.40	332.0420	332.0440	$C_{12}H_{14}NO_8S$	$C_{12}H_{15}NO_8S$
	252.0852	252.0872	$C_{12}H_{14}NO_5$	loss of SO_3
4.74	330.0288	330.0284	$C_{12}H_{12}NO_8S$	$C_{12}H_{13}NO_8S$
	250.0702	250.0715	$C_{12}H_{12}NO_5$	loss of SO_3

many of these compounds is that authentic standards are not available for them, making unequivocal structure determination difficult.

The detection of changes in the sulphation pattern of the urinary metabolites is an interesting observation as sulphates represent a class of compounds that can be readily detected and identified by MS using neutral loss experiments. Examples of the MS/MS chromatograms obtained from a Q-Tof instrument for the neutral loss of 80 (sulphate) from day 9 control and treated urines are shown in Figure 9a and b, respectively. The MS/MS chromatograms reveal a clear change in the pattern of sulphate conjugates present in the urine of dosed and control animals. The significance of this observation is not yet clear, and perhaps reflects changes in the production of diet-derived aromatic compounds (such as, for example, phenol sulphate) by the gut microflora as a result of the antimicrobial action of gentamicin, rather than a change in renal function. However, the use of such neutral loss experiments to interrogate the sample provides another illustration of the way in which

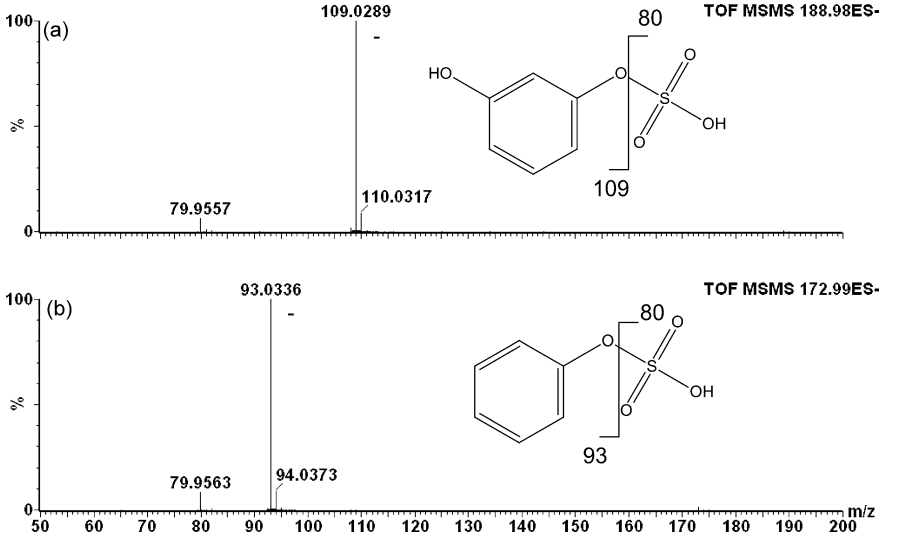

Figure 8. HPLC-MS/MS product ion spectra from a control urine showing the loss of sulphate from (a) resorcitol phenol and (b) phenol sulphates, respectively.

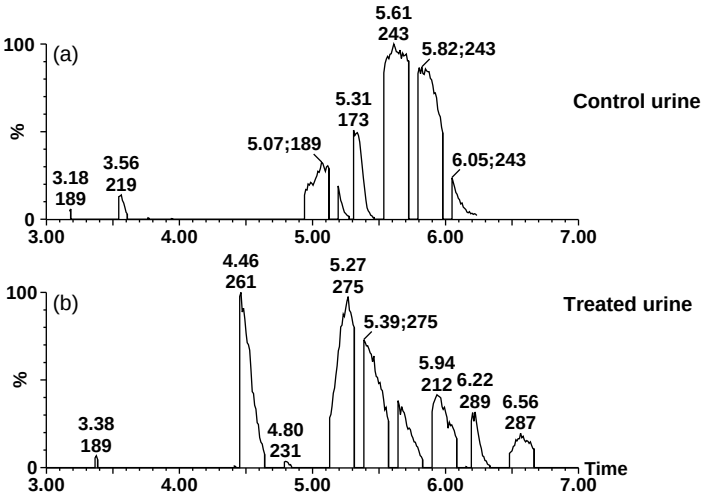

Figure 9. Ion chromatograms for urine obtained by monitoring the neutral loss of 80 Da (sulphate) from urine samples obtained on P9 for (a) control and (b) a gentamicin-dosed animal, respectively.

HPLC-MS can be used to detect differences between test and control samples in metabonomic studies.

These negative-ion data, including postulated elemental compositions, are summarized in Tables V and VI.

As we have shown previously for mercuric chloride and cyclosporin-induced nephrotoxicity (Lenz et al. 2004a, b), the results obtained for both ^{1}H-NMR and HPLC-MS showed a similar time course of changes in urinary composition following administration of gentamicin, with increasing effects seen as the dosing progressed and the maximum observed changes on day 9. The changes in the ^{1}H-NMR spectra for the gentamicin-dosed animals were consistent with proximal tubular damage as highlighted in previous ^{1}H-NMR investigations (e.g. Gartland et al. 1989, Anthony et al. 1994). However, whilst the time course of these changes was similar the markers on which these separations from control values were observed were different. Perhaps the most obvious example is that of glucose which was the most obvious marker of toxicity for the NMR spectroscopy but which, because of its poor ionization

Table VI. Negative-ions decreased after administration of gentamicin to the rat.

Retention time (min)	Measured mass	Calculated mass	Elemental composition of $[M-H]^-$ ion	Postulated identity
5.50	242.9911[a]	242.9963	$C_9H_7O_6S$	hydroxycinnamic acid sulphate
	163.0397	163.0395	$C_9H_7O_3$	loss of SO_3
4.79	188.9853	188.9858	$C_6H_5O_5S$ (loses SO_3)	resorcinol sulphate
4.69	283.0799	283.0818	$C_{13}H_{15}O_7$	$C_{13}H_{16}O_7$
	175.0252	175.0243	$C_6H_7O_6$	fragment – glucuronide
3.68	218.9953	218.9963	$C_7H_7O_6S$	$C_7H_8O_6S$
0.66	191.0178	191.0192	$C_6H_7O_7$	citric acid
5.31	172.9899	172.9909	$C_6H_5O_4S$ (loses SO_3)	phenol sulphate

[a]Measurement lower than expected due to detector saturation.

characteristics in ESI was not noted as significant for the HPLC-MS result. Similarly, markers such as xanthurenic acid and kynurenic acid that were prominent in the positive ESI HPLC-MS results were not significant for NMR. This study further highlights the care which must be taken in examining the data to remove, as far as possible, drugs and their metabolites (and dosing excipients e.g. polyethylene glycol (PEG) where appropriate, see also Lenz et al. 2004a) from consideration in the pattern recognition process to avoid the detection of spurious markers that merely reflect the administration of the compound(s) under study rather than genuine biomarkers of toxicity. In addition, the potential pharmacological effects of the drug on the endogenous metabolite profile, in this case the putative effect of gentamicin on suppressing the gut microflora, also need to be taken into account and excluded from the data used for PCA.

Conclusions

Whilst as yet not widely adopted for metabonomic investigations, it is becoming increasingly clear that HPLC-MS-based techniques show great promise for this type of work. The complementary nature of the NMR and HPLC-MS results seen in previous studies is further confirmed by this investigation and there seems little doubt that the combined methodologies will be widely used for such studies. Such a combination clearly provides a much more comprehensive picture of the metabolic changes resulting from a toxic insult than either method of analysis alone.

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