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Cyclosporin A-induced changes in endogenous metabolites in rat urine: a metabonomic investigation using high field ^{1}H NMR spectroscopy, HPLC-TOF/MS and chemometrics

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Abstract

The model nephrotoxin cyclosporin A was administered to male Wistar-derived rats daily for 9 days at a dose level of 45 mg/kg per day. Urine samples were collected daily and the excretion pattern of low molecular mass organic molecules in the urine was studied using ^{1}H NMR spectroscopy and HPLC-TOF/MS. Distinct changes in the pattern of endogenous metabolites, as a result of the daily administration of cyclosporin A, were observed by ^{1}H NMR from day 7 onwards. The NMR-detected markers included raised concentrations of glucose, acetate, trimethylamine and succinate and reduced amounts of trimethylamine-*N*-oxide.

In parallel studies by HPLC-TOF/MS a reduction in the quantities of kynurenic acid, xanthurenic acid, citric acid and riboflavin present in the urines was noted, together with reductions in a number of as yet unidentified compounds. In addition, signals resulting from the polyethylene glycol, present in the dosing vehicle, and cyclosporin A metabolites were detected by MS. However, these were excluded from the subsequent multivariate data analysis in order to highlight only changes to the endogenous metabolites.

Analysis of both the ^{1}H NMR and HPLC–MS spectroscopic data using pattern recognition techniques clearly identified the onset of changes due to nephrotoxicity.

Keywords: Metabonomics; Nephrotoxicity; Cyclosporin A; ^{1}H NMR spectroscopy; HPLC-TOF/MS; MS/MS; Pattern recognition; Biomarkers

1. Introduction

High field ^{1}H NMR spectroscopy of biofluids and tissues is a well established method for the detection of e.g. organ-specific toxic effects [1–11], or in

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the determination of differences between genders, strains or diurnal effects in experimental animals and man [12–14]. NMR spectroscopy provides an ideal technique for metabonomic analysis as it permits the rapid, multicomponent, analyses of samples such as urine and plasma to be performed with minimal sample pre-treatment. Recently, the use of HPLC–MS for the analysis of urine samples in metabonomic applications has also been described [15–18]. The data from both of these analytical techniques, when combined with multiparametric methods of data processing (e.g. principal components analysis, PCA) for chemometric "pattern recognition", can be used to identify, e.g. diagnostic compounds or groups of compounds that can be used as markers for the effects of toxins.

One area in which these techniques have been applied is to the investigation of nephrotoxicity, and we have recently described the results of an NMR and HPLC–MS/MS investigation on the effects of the proximal tubule nephrotoxin mercuric chloride on endogenous urinary metabolites in the rat [19]. In addition to mercuric chloride we have also examined the effects of another nephrotoxin, cyclosporin A. Cyclosporin A nephrotoxicity is well known in the clinic and is associated with reversible, dose-related increases in blood urea nitrogen and serum creatinine levels and depression of creatinine clearance [20]. Indeed approximately 80% of renal transplant patients are reported to suffer from some nephrotoxicity while using the drug [20]. In the rat the drug has been shown to cause a spectrum of toxicological effects, of which weight loss was the most striking [21] (explained by periods of anorexia) whilst other observations included markedly raised blood urea but a lesser rise in blood creatinine [21]. Here, in a study of the nephrotoxicity of cyclosporin A in the rat we provide a further illustration of the combined use of ^{1}H NMR spectroscopy and HPLC–MS for metabonomic investigations.

2. Experimental

2.1. Chemicals

Acetonitrile, HPLC grade, was purchased from Riedel-de Haën (Sigma Aldrich, GmbH) 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 (Poole, UK). Cyclosporin A, as "Neoral" (Sandoz Pharmaceuticals), batch number 250MFD0198 was used as supplied.

2.2. Animals and dosing

Male Wistar-derived rats ($n = 5$ per group) approx. 140 g in weight were allowed to acclimatise in metabolism cages for 3 days prior to treatment. Food and water was provided ad libitum. A dose level of 45 mg/kg per day of cyclosporin A was administered for 9 days. The compound was formulated in Neoral at a concentration of 50 mg/ml and a dose volume of 10 ml/kg was used. Control animals were administered 0.9% (w/v) saline, also at a dose volume of 10 ml/kg. Samples from both groups were collected for 9 days after dosing.

2.3. Sample collection and storage

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

2.4. ^{1}H NMR spectroscopy

One hundred μl of neat urine were buffered with 100 μl 0.2 M phosphate buffer/D_2O (pH 7.4) prior to 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 64k data points. Typically, 128 transients were collected with an acquisition time of 3.28 s (and a relaxation delay of 1 s). Solvent suppression was achieved by applying the standard Noesypresat pulse sequence (Bruker Biospin Ltd.) with secondary irradiation of the dominant water signal during the mixing time of 150 ms and the relaxation delay of 2 s. No line broadening function was applied prior to FT. Spectra were referenced to the internal reference standard TSP (sodium trimethylsilylpropionic acid [$^{2}H_4$], $\delta_{^{1}H} = 0.0$) dissolved in D_2O to provide a field-frequency lock.

2.5. Data analysis for ^1H 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 ($\delta_{^1H}$=0.0) prior to data-reduction into 245 spectral integral regions corresponding to the chemical shift range of $\delta_{^1H}$ 0.2–10 utilising AMIX (version 2.7.5, Analysis of mixtures, Bruker Spectrospin Ltd.). The region of $\delta_{^1H}$ 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.

In order to counteract separation on grounds of differences in dilution between urine samples, all spectra were then normalised, i.e. scaled, so that the total area for each spectrum had 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, Box 7960, SE 90719, Umeå, Sweden) and visualised 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 components analysis (PCA). The Spotfire program was used to visualise both the spectral data (reconstructed data-reduced spectra) and the output from SIMCA-P. Visualising 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 components analysis. PCA was performed using centred scaling. Detailed accounts of pattern recognition methods can be found in the literature [22].

2.6. HPLC–MS

For analysis by HPLC–MS 100 μl aliquots of the rat urine samples were centrifuged at 13,000 × *g* 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 QTof-micro™ (Manchester, UK) equipped with an electrospray source operating in either positive ion or negative ion mode. The source temperature was set at 120 °C with a cone gas flow of 50 l/h, a desolvation gas temperature of 250 °C and a desolvation gas flow of 500 l/h 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 throughout with a collision energy of 4 eV and a collision gas pressure of $\sim 2.8 \times 10^{-3}$ mbar argon. A lock-mass of leucine enkephalin at a concentration of 0.5 ng/μl, in 50:50 acetonitrile:water + 0.1% formic acid for positive ion mode ($[M + H]^+ = 556.2771$) and 1 ng/μl in 50:50 acetonitrile:water for negative ion mode ($[M - H]^- = 554.2615$), was employed at a flow rate of 30 μl/min 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® C183.5 μm column held at 40 °C (due to insufficient sample for day 8 of the study HPLC–MS analysis was not performed). The column was eluted with a linear gradient of 0–20% *B* over 0.5–4 min, 20–95% *B* 4–8 min 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; 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 1500 from 0 to 10 min, in positive and negative ion mode. The column eluent was split such that approximately 120 μl/min were directed to the mass spectrometer.

2.7. Data analysis for HPLC–MS

The LC–MS data were analysed using the Micromass MarkerLynx Applications Manager version 1.0. MarkerLynx incorporates a peak deconvolution package, which allows detection and retention time alignment of the peaks eluting in each chromatogram. 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 normalised intensities. The processed data list was either analysed by PCA within the MarkerLynx Applications Manager or exported for chemometric analysis using SIMCA-P (version10.0).

3. Results

3.1. Analysis of urine by ^1H NMR spectroscopy

All of the control animals presented normal ^{1}H NMR spectra over the duration of the study with only minor fluctuations in the concentrations of the endogenous metabolites. These changes in the control samples mainly related to variations in signals in the aromatic region of the spectrum, which reflected changes in the excretion of hippuric and 3-hydroxyphenylpropionic acid. As reported elsewhere, such variations are typically due to changes in gut microfloral metabolites [19,23–25] and did not preclude the observation of the effects of cyclosporin A.

In the case of cyclosporin A, despite daily administration of the drug, it was not until day 6 (144–160 h) that the visual appearance of the urinary ^{1}H NMR profiles was seen to diverge from those of the control animals. The detectable changes included slightly reduced concentrations of citrate and trimethylamine-*N*-oxide (TMAO) together with elevated concentrations of acetate and trimethylamine (TMA). The most marked changes were observed on days 7–9 and included a pronounced glucosuria together with high concentrations of acetate, succinate and TMA (Fig. 1). Lactate was observed in some samples whilst reduced concentrations of citrate, α-ketoglutarate and TMAO were noted for all animals in the study. These results are summarised in Table 1. The reduction in Krebs cycle intermediates has previously been attributed in the literature to reduced feeding [26] and this is supported by a stagnation in body weight increase over the time course of the study of the rats treated with cyclosporin A compared to the control animals (see Table 2).

Table 2
Group mean body weight of rats ($n = 5$)

Day	Control rats weight (g) ± S.D.	Cyclosporin A treated rats weight (g) ± S.D.
1	139.8 ± 16.4	141.6 ± 12.1
2	145.6 ± 20.3	140.4 ± 11.7
3	150.2 ± 25.1	139.0 ± 13.4
4	154.6 ± 29.5	137.8 ± 17.5
5	162.6 ± 33.4	142.4 ± 21.9
6	172.4 ± 30.2	155.2 ± 19.1
7	182.6 ± 26.1	151.0 ± 14.0
8	189.6 ± 26.1	154.8 ± 14.1
9	199.4 ± 26.9	157.6 ± 16.5

A typical series of spectra from one animal, highlighting the aliphatic portion of the spectrum, taken over the time course of the study are shown in Fig. 1, whilst the metabolic trajectories, obtained via PCA of the respective spectra, for the dosed animals and controls are shown in Fig. 2a and b.

3.2. Analysis of urine by HPLC-TOF/MS

The samples from both control and dosed animals were analysed by reversed-phase gradient HPLC–MS in both positive and negative ionisation modes as detailed in the experimental section.

3.2.1. Positive ion mode

Initial results in positive ion mode showed marked differences between the control and dosed animals. However, further investigation of the ions responsible for this separation indicated that these differences were mainly due to the excretion of polyethylene glycol (PEG) originally present in the drug formulation, but absent from the control vehicle. The other major difference observed was due to the detection of metabolites of the drug itself. The total ion chromatograms

Table 1
Changes in the urinary profiles of animals dosed with cyclosporine A, compared to control animals

Days	Effect (changes in metabolite profile)	Histopathology
6+	Slightly raised acetate, TMA, lowered citrate	Proximal and distal convoluted tubule, thick ascending loop
7–9	Glucose, acetate, succinate, TMA↑↑ lactate↑ TMAO↓↓	

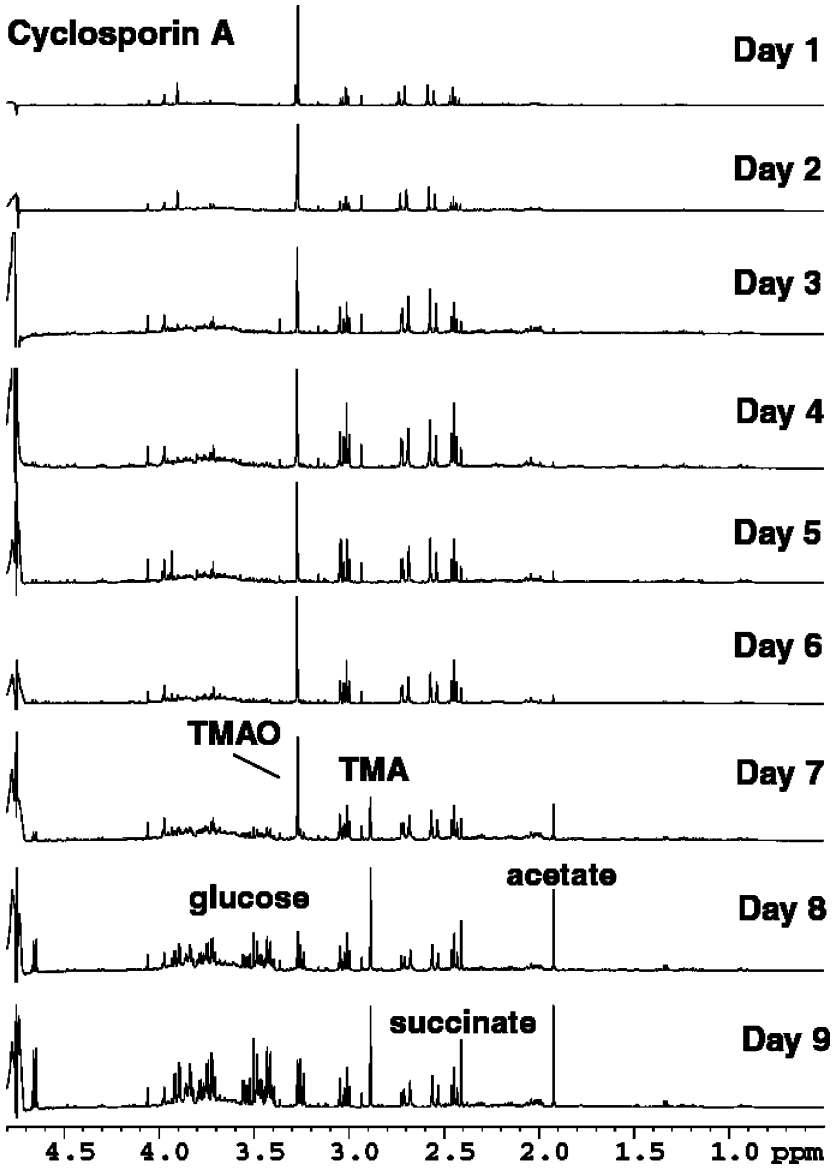

Fig. 1. Aliphatic regions from ^{1}H NMR spectra (days 1–9) of a rat dosed with cyclosporin A.

(TICs) from day 9 control and dosed animal with the cluster of PEG peaks eluting around 5.3 min are shown in Fig. 3a and b. The later eluting peaks in the dosed sample are principally due to cyclosporin A and its metabolites. These ions, once identified, were excluded from the PCA plots using the MarkerLynx software, thereby allowing changes in the endogenous metabolites to be observed.

The scores plot of PC 1 versus PC 2 for the positive ion data after exclusion of the signals due to PEG and xenobiotics is shown in Fig. 4. The most significant changes observed after dosing were a reduction in the intensity of the following ions from days 6 or 7 onwards: *m/z* 297, 372, 350, 377, 190, 319, 206, 267 and 245. The postulated elemental compositions for these ions are given in Table 3. The ions at *m/z* 190, 206 and

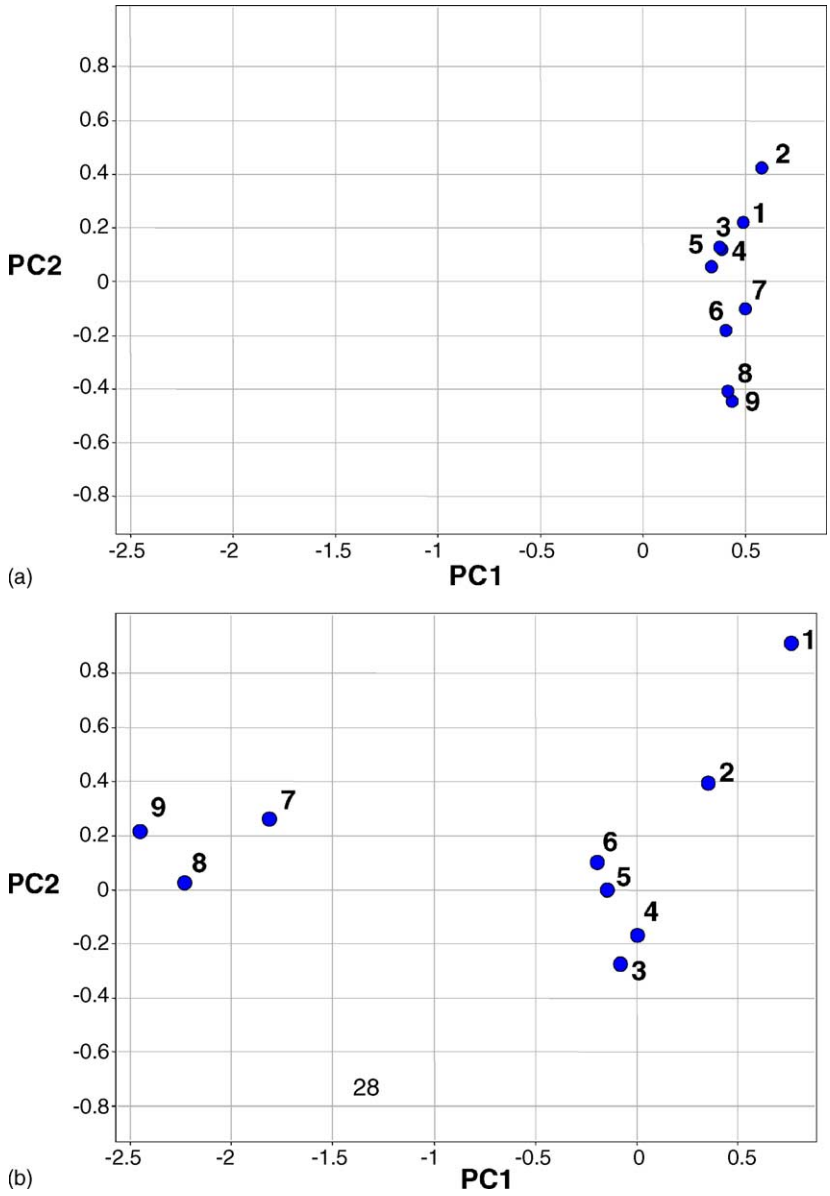

Fig. 2. Mean trajectory plot showing the ^{1}H NMR for (a) control groups and (b) cyclosporin A-treated animals.

377 were from kynurenic and xanthurenic acids and riboflavin respectively. It should be noted that some mass measurements are lower than expected due to detector saturation.

A further series of ions was also observed to increase after dosing, peaking at day 3. These seemed to be related to a polyethylene glycol type species (from their elemental compositions) but were not from the dosing vehicle as they were also observed in the controls and pre-dose samples.

As observed in the ^{1}H NMR spectra there were variations, in both control and dosed animals, over the time-course for gut microflora-related metabolites (e.g. hippuric acid etc.).

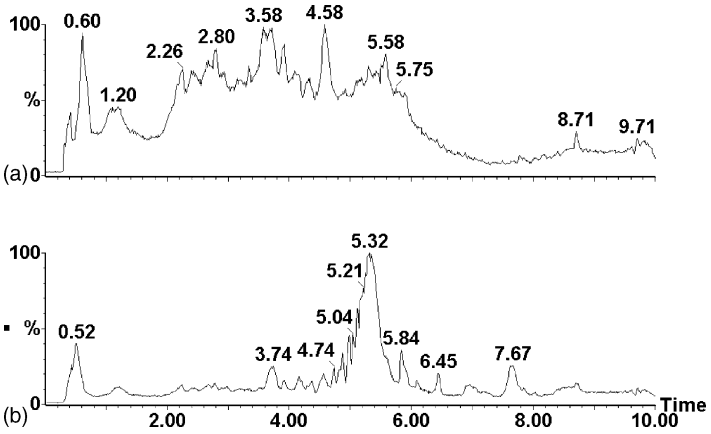

Fig. 3. Positive ion HPLC-TICs for (a) a day 9 control and (b) a day 9 dosed animal.

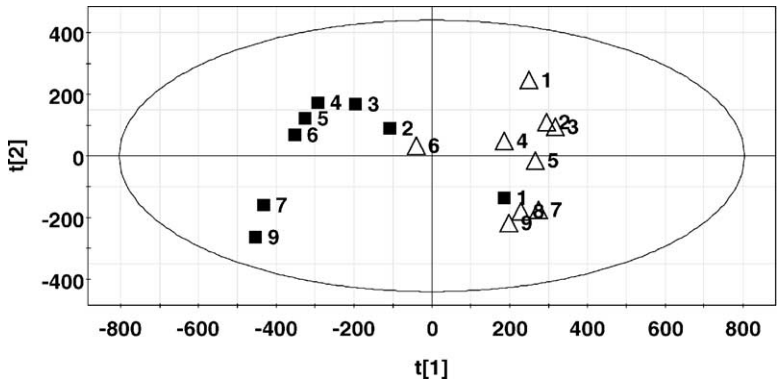

Fig. 4. Mean positive ion scores plot (PC 1 vs. PC 2) after removal of PEG peaks and xenobiotics, open triangles, control animals; solid squares, dosed animals.

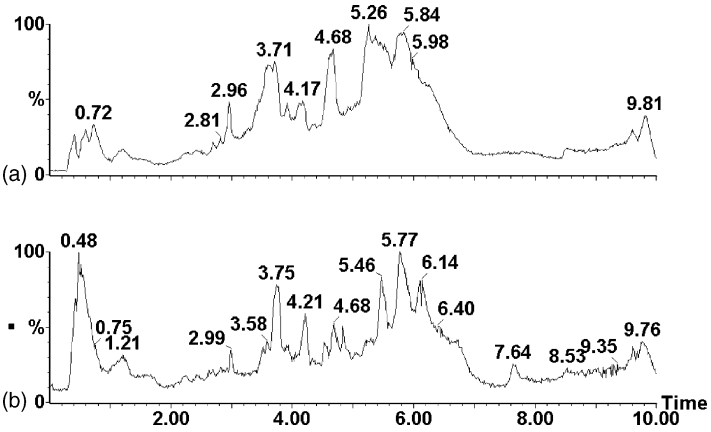

Fig. 5. Negative ion HPLC-TICs for (a) a day 9 control and (b) a day 9 dosed animal.

Table 3
HPLC–MS data obtained in positive ionisation mode

Retention time (min)	Measured mass (Da)	Calculated mass (Da)	Proposed elemental composition $(M+H)^+$	Comments and postulated identity
Principal positive ions showing a decrease after dosing				
2.28	297.1460	297.1450	$C_{14}H_{21}N_2O_5$	Day 6 onwards
2.82	372.2380	372.2386	$C_{19}H_{34}NO_6$	Day 6 onwards
3.46	350.0882	350.0876	$C_{16}H_{16}NO_8$	Day 7 onwards, glucuronide (MS/MS)
3.90	377.1472	377.1461	$C_{17}H_{21}N_4O_6$	Day 7 onwards, riboflavin
4.58	149.0590	149.0603	$C_9H_9O_2$	Reduced after dosing
		149.0562	$C_4H_9N_2O_4$	
3.93	190.0435	190.0504	$C_{10}H_8NO_3$	Day 7 onwards, kynurenic acid
4.55	319.1303	319.1310	$C_{19}H_{20}O_3Na$	Day 3 onwards
3.53	206.0385	206.0453	$C_{10}H_8NO_4$	Day 7 onwards, xanthurenic acid
1.05	267.1372	267.1345	$C_{13}H_{19}N_2O_4$	Day 7 onwards
1.08	245.1634			Day 7 onwards
4.50	285.0839	285.0835	$C_{10}H_{13}N_4O_6$	Reduced in all dosed samples, xanthosine?
Principal positive ions showing an increase after dosing				
4.37	481.2597	481.2625	$C_{20}H_{42}O_{11}Na$	Peaked at day 3, PEG Na
4.15	437.2327	437.2363	$C_{18}H_{38}O_{10}Na$	Peaked at day 3, PEG Na
3.92	393.2103	393.2101	$C_{16}H_{34}O_9Na$	Peaked at day 3, PEG Na
3.92	371.2290	371.2281	$C_{16}H_{35}O_9$	Peaked at day 3, PEG
3.65	349.1859	349.1838	$C_{14}H_{30}O_8Na$	Peaked at day 3, PEG Na
3.35	305.1598	305.1576	$C_{12}H_{26}O_7Na$	Peaked at day 3, PEG Na
Principal positive ions showing a large variation during study				
3.64	397.0748	397.0802	$C_{18}H_{18}N_2O_6K$	Hippuric acid dimer K salt
3.64	105.0313	105.0340	C_7H_5O	Hippuric acid fragment
3.64	180.0605	180.0660	$C_9H_{10}NO_3$	Hippuric acid
3.64	202.0444	202.0480	$C_9H_9NO_3Na$	Hippuric acid Na salt

3.2.2. Negative ion mode

Typical results for the TICs obtained for the negative ionisation mode HPLC–MS of control and cyclosporin A-dosed rats on day 9 of the study are shown in Fig. 5. with the PC 1 versus PC 2 scores plot shown in Fig. 6. PCA revealed that only the days 6–9 dosed samples showed a separation from the control group. The most significant ion in the loadings plot (not shown) was *m/z* 245 from 3-hydroxyphenylpropionic acid sulphate, which varied considerably during the study. The

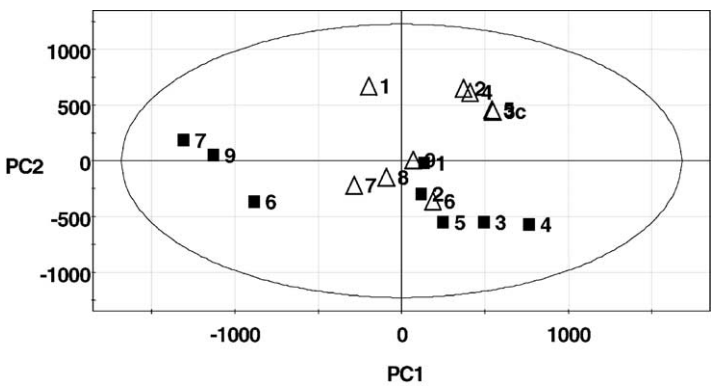

Fig. 6. Mean negative ion scores plot (PC 1 vs. PC 2), open triangles, control animals; solid squares, dosed animals.

Table 4
HPLC–MS data obtained in negative ionisation mode

Retention time (min)	Measured mass (Da)	Calculated mass (Da)	Proposed elemental composition $(M-H)^-$	Comments and postulated composition
Principal negative ions showing a decrease after dosing				
3.48	326.0893	326.0876	$C_{14}H_{16}NO_8$	$C_{14}H_{17}NO_8$ glucuronide, day 7 →
2.93	357.0836	357.0822	$C_{15}H_{17}O_{10}$	$C_{15}H_{18}O_{10}$, day 7 →
1.25	191.0138	191.0192	$C_6H_7O_7$	Citric acid?, day 7 →
Principal negative ions showing a large variation during study				
5.20	245.0119	245.0120	$C_9H_9O_6S$ (loses SO_3)	3-Hyroxyphenylpropionic acid sulphate
5.86	245.0121	245.0120	$C_9H_9O_6S$ (loses SO_3)	3-Hyroxyphenylpropionic acid sulphate
3.66	178.0443	178.0504	$C_9H_8NO_3$	Hippuric acid
5.50	275.0253	275.0225	$C_{10}H_{11}O_7S$ (loses SO_3)	$C_{10}H_{12}SO_7$, ↑ from day 6
5.65	211.9975	212.0018	$C_8H_6NO_4S$	Indican
5.18	273.0105	273.0069	$C_{10}H_9O_7S$ (loses SO_3)	Ferulic acid sulphate

mass chromatogram of the *m*/*z* 245 ion was very broad and showed two apexes resulting in two entries in the loadings plot. Other ions showing a significant variation were *m*/*z* 178 and *m*/*z* 212 from hippuric acid and indican, respectively.

Three ions, at *m*/*z* 326, 357 and 191 (possibly citric acid), were observed to decrease from day 7 onwards. The *m*/*z* 326 and 357 ions are as yet unidentified however, from its fragmentation pattern the 326 Da ion was a glucuronide. The various negative ions showing the maximum variation during the study, together with their proposed elemental compositions, are given in Table 4.

4. Discussion

Analysis of the urines by both ^{1}H NMR spectroscopy and HPLC-TOF/MS provided a rapid means of detecting toxin-induced lesions in the kidney of the dosed animals. Both techniques revealed a slow onset of toxicity with major changes only apparent from day 7 onwards. For ^{1}H NMR spectroscopy the most prominent changes were the appearance of first acetate and TMA, and then glucose and lactate in the urine, together with changes in the Krebs cycle intermediates citrate, α-ketoglutarate and succinate. These observations, particularly the excretion of large amounts of glucose, are typical of those observed by ^{1}H NMR for proximal tubular nephrotoxicity (e.g. see [1,4]), and were confirmed by histopathological investigations on the kidneys of these animals (data not shown).

However, some of the changes, such as those relating to Krebs cycle intermediates and the appearance of acetate, may relate to the reduced weight gain seen with the dosed animals relative to the controls. Such effects have been reported to be due to reduced feeding [26]. We have also noted such changes in animals in a number of studies where the only toxicological finding was reduced weight gain (unpublished observations).

The results of the HPLC–MS study gave complementary information to that provided by ^{1}H NMR spectroscopy, with both techniques showing broadly the same time course but via a different range of markers. In addition to changes in endogenous metabolites the MS results revealed both the presence of drug metabolites and PEG resulting from the Neoral dose vehicle. Neither of these different sources of interference were observed by NMR spectroscopy because they were not present in the samples at sufficient concentration to be easily detectable. This example highlights both the advantages and disadvantages that can result from the potentially much greater sensitivity of MS-based techniques compared to NMR, and highlights the care with which data from such studies must be interpreted. However, once recognised as such these interferences were readily removed from the PCA analysis so that they were not considered as potential markers of cyclosporin A nephrotoxicity. The value of the combined NMR and HPLC–MS analysis

is clear from the fact that the overlap between the two sets of markers detected is limited.

5. Conclusions

The NMR and HPLC–MS analysis of urine samples obtained from rats administered cyclosporin A at 45 mg/kg per day for 9 days revealed the time course of the onset of nephrotoxicity via a range of changes in the fingerprint of endogenous metabolites. In addition, HPLC–MS detected compound and dose-related material in the samples (metabolites and PEG). Changes in urine composition were observed from day 6 onwards, increasing in intensity as the duration of the study increased.

References

[1] K.P.R. Gartland, F.W. Bonner, J.K. Nicholson, Mol. Pharmacol. 35 (1988) 242–250.
[2] M.L. Anthony, J.C. Lindon, C.R. Beddell, J.K. Nicholson, Mol. Pharmacol. 46 (1994) 199–211.
[3] B.M. Beckwith-Hall, J.K. Nicholson, A.W. Nicholls, P.J. Foxall, J.C. Lindon, S.C. Connor, M. Abdi, J. Conelly, E. Holmes, Chem. Res. Toxicol. 11 (1998) 260–272.
[4] E. Holmes, J. Shockcor, Curr. Opin. Drug Discov. Dev. 3 (2000) 72–78.
[5] P.J.D. Foxall, E.M. Lenz, G.H. Neild, J.C. Lindon, I.D. Wilson, J.K. Nicholson, Ther. Drug Monit. 18 (1996) 498–505.
[6] P.J.D. Foxall, J.M. Singer, J.M. Hartley, G.H. Neild, M. Lapsley, J.K. Nicholson, R.L. Souhami, Clin. Cancer Res. 3 (1997) 1507–1518.
[7] J.K. Nicholson, D.P. Higham, J.A. Timbrell, P.J. Sadler, Mol. Pharmacol. 36 (1989) 398–404.
[8] J.A. Timbrell, R. Draperand, C.J. Waterfield, TEN 1 (1994) 4–15.
[9] J.A. Timbrell, C.J. Waterfield, R.P. Draper, Comp. Haemat. Int. 5 (1995) 112–119.
[10] D.G. Robertson, M.D. Reily, R.E. Sigler, D.F. Wells, D.A. Paterson, T.K. Braden, Toxicol. Sci. 57 (2000) 326–337.
[11] E.M. Lenz, I.D. Wilson, J.A. Timbrell, J.K. Nicholson, Biomarkers 5 (2000) 424–435.
[12] C.L. Gavaghan, I.D. Wilson, J.K. Nicholson, FEBS Lett. 530 (2002) 191–196.
[13] C.L. Gavaghan, E. Holmes, E. Lenz, I.D. Wilson, J.K. Nicholson, FEBS Lett. 484 (2000) 169–174.
[14] C.L. Gavaghan, J.K. Nicholson, I.D. Wilson, FEBS Lett., submitted for publication.
[15] R.S. Plumb, C.L. Stumpf, M.V. Gorenstein, J.M. Castro-Perez, G.J. Dear, M. Anthony, B.C. Sweatman, S.C. Connor, J.N. Haselden, Rapid Commun. Mass Spectrom. 16 (2002) 1991–1996.
[16] H. Idborg-Björkman, P.-O. Edlund, O.M. Kvalheim, I. Schuppe-Koistinen, S.P. Jacobsson, Anal. Chem. 75 (2003) 4784–4792.
[17] A. Lafaye, C. Junot, B.R. Gall, P. Fritsch, J.C. Tabet, E. Ezan, Rapid Commun. Mass Spectrom. 17 (2003) 2541–2549.
[18] R. Plumb, J. Granger, C. Stumpf, I.D. Wilson, J.A. Evans, E.M. Lenz, Analyst 128 (2003) 819–823.
[19] E.M. Lenz, J. Bright, R. Knight, I.D. Wilson, H. Major, Analyst, in press.
[20] W.M. Bennett, J.P. Pulliam, Ann. Intern. Med. 99 (1983) 851–854.
[21] M.J.G. Farthing, M.L. Clark, Biochem. Pharmacol. 30 (1981) 3311–3316.
[22] E. Holmes, H. Antti, Analyst 127 (2002) 1549–1557.
[23] A.N. Phipps, B. Wright, J. Stewart, I.D. Wilson, Pharm. Sci. 3 (1997) 143–146.
[24] A.N. Phipps, B. Wright, J. Stewart, I.D. Wilson, Xenobiotica 28 (1998) 527–537.
[25] R.E. Williams, H.W. Eyton-Jones, M.J. Farnworth, R. Gallagher, W.M. Provan, Xenobiotica 32 (2002) 783–794.
[26] B.C. Sweatman, J.A. Manini, C.J. Waterfield, Toxicology 164 (2001) 225.