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Short Communication

A pattern recognition approach to the comparison of PMR and clinical chemical data for classification of nephrotoxicity*

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Introduction

In recent years the authors have explored the application of high resolution proton nuclear magnetic resonance (PMR) spectroscopic analysis of various body fluids to the investigation of metabolic disorders, drug metabolism and toxicity states in man and animals [1]. In particular urine samples obtained from rats in experimental, nephrotoxicity states have been examined [2–5], as well as a clinical case of acute renal failure [6]. PMR spectroscopy is well suited to the study of toxicological events, as multi-component analyses on biological materials can be made simultaneously, without any bias imposed by the experimenters' expectations of toxin-induced metabolic changes. Quantifiable changes in metabolite patterns often give not only information on the location and severity of a toxic lesion, but also insights into the underlying biochemical features of the toxic process [2–5]. These patterns are often unique for different toxin types and hence can be used as a basis for their classification. PMR spectra of biological materials are very rich in information which is carried in the overall pattern of the metabolite resonances. However, the information contained in the PMR metabolite patterns is often extremely complex, and subtle biochemical alterations may be lost even in extensive conventional quantitative and statistical analysis of the spectral data. The authors have, therefore, used a computer-based pattern recognition (PR) approach to analyse the PMR urine analysis data obtained in various experimental nephrotoxicity states, the object being to compare the accuracy of the classifications derived from PMR data with those derived from clinical chemistry data.

Experimental

Animals and treatments

Male Fischer 344 rats were housed individually in metabolism cages and allowed free access to food and tap water in well ventilated animal rooms with regular light cycles. Urine was collected over ice during the 24-h period prior to, and 0–8, 8–24 and 24–48 h after, dosing nephrotoxins (see Table 1 for the strain of rats used, treatments given, dose route and the vehicles used).

Conventional clinical chemistry

Urine was tested for glucose by the hexokinase method, the urinary enzymes *N*-acetyl-β-D-glucosaminidase (NAG) and γ-glutamyl-

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Table 1
Rat strain, doses and vehicles employed for the administration of the six nephrotoxins

Treatment	Rat strain (*n*)	Dose	Vehicle
Control	Fischer 344 (5)	—	corn oil
HCBD	Fischer 344 (5)	200 mg kg^{-1}	corn oil
Control	Fischer 344 (3)	—	saline
PAP	Fischer 344 (3)	100 mg kg^{-1}	saline
CrO_4	Fischer 344 (5)	20 mg kg^{-1}	saline
Hg	Fischer 344 (4)	2 mg kg^{-1}	saline
PI	Fischer 344 (5)	20 μl kg^{-1}	saline
BEA	Fischer 344 (5)	250 mg kg^{-1}	saline

All treatments were administered by i.p. injection except sodium chromate which was administered by s.c. injection. (BEA, bromoethanamine; HCBD, hexachlorobutadiene; Hg, mercuric chloride; PAP, *p*-aminophenol; PI, propyleneimine; CrO_4, sodium chromate.)

Table 2
Conventional clinical chemistry data for five parameters in urine throughout three time points 0–8, 8–24 and 24–48 h following exposure to six nephrotoxins

	Hg	PAP	CrO_4	HCBD	PI	BEA
GLC8	25.4	206	9.60	8.62	20.6	10.2
GLC24	124	409	32.5	449	35.5	16.1
GCL48	66.2	307	1420	399	14.2	6.74
NAG8	650	5226	323	264	229	263
NAG24	9180	694	2100	3128	1155	453
NAG48	1079	650	6149	552	428	257
UFR8	0.33	0.57	0.18	0.48	0.90	1.54
UFR24	0.33	0.69	0.28	1.64	1.26	1.30
UFR48	0.59	0.62	0.13	0.77	1.21	1.44
GGT8	42.1	25700	11.7	156	29.0	72.9
GGT24	353	994	10.1	1119	43.5	18.9
GGT48	26.0	250	105.8	34.0	39.9	78.4
BUN48	171	88.7	24.0	35.0	24.0	31.1

GLC, glucose [μmol h^{-1} kg^{-1}]; NAG, *N*-acetyl-β-D-glucosaminidase [U h^{-1} kg^{-1}]; UFR, urine flow rate [ml h^{-1}]; GGT, γ-glutamyl transpeptidase [mU h^{-1} kg^{-1}]; BUN, blood urea nitrogen [mg/100 ml]. Numbers represent time after dosing at which the sample was collected, e.g. GLC8 is glucose value for the interval 0–8 h.

transpeptidase (GGT) by the methods of Maruhn [7] and Naftalin *et al.* [8], respectively. Plasma was tested at 48 h for urea nitrogen by the NAD-linked urease method. Table 2 contains data on urine flow rate, NAG, GGT, glucose, throughout three time periods after dosing with the six nephrotoxins, and plasma urea at 48 h.

PMR urine analysis

PMR measurements were made on a Bruker WH400 spectrometer operating at 400.13 MHz and fitted with a 16-bit analog-to-digital converter, Aspect 3000 data system and array processor. A calculated volume of urine [3] was lyophilized and redissolved in an equal volume of ^2H_2O containing sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]-1-propionate (TSP). Sixty-four free-induction decays (FIDs) were collected into 16K points (1.7 s acquisition time) using 28° (3 μs) pulses and a spectral width of 5000 Hz. A further delay of 3.0 s between pulses was added to ensure that the spectra were obtained under full T_1 relaxation conditions. An exponential line-broadening function of 1.0 Hz was applied prior to Fourier transformation.

Scoring of NMR-generated data

NMR spectra of urine samples (containing equal concentrations of TSP) from rats exposed to six nephrotoxins were compared with spectra obtained from untreated control animals at the same time-point plotted on the same vertical scale. The following arbitrary seven-level scoring system was employed for the metabolite signal intensities and the added TSP standard, and taking the modal score for each toxin for computer analysis:

+3 = a major elevation in urinary concentration corresponding to >3 times control level
+2 = an elevation in concentration corresponding to 2 to 3-times control
+1 = a detectable but minor elevation in concentration of up to 2-times control level
0 = not detectably different from control
−1 = minor reduction (20 to 50%) from control concentrations
−2 = moderate reduction (50–90%) from control concentrations
−3 = signals from metabolite not detectable by NMR spectroscopy after toxin treatment, but metabolite is always detectable in controls measured under the same conditions

Table 3 presents the scored information on the changes in 16 metabolite concentrations occurring at three time points after administration of six nephrotoxins, i.e. each toxin is represented by a 48-dimensional metabolic space, with three time-point dimensions per metabolite.

Computer data analysis

The scored data were analysed using the unsupervized learning PR method principal components analysis (PCA) which forms part of the software package ARTHUR [9]. PR methods work in multi-dimensional parameter space, where in this case each dimension represents the concentration of one metabolite; these methods can also provide dimension reduction techniques for display purposes. PCA is a well established statistical technique for dimension reduction [10, 11]. Principal components (PCs) are linear combinations of the original variables with appropriate weighting coefficients. All data were autoscaled prior to PR analysis. Correlation coefficients (r) were also calculated based on the coordinate similarity between parameters, and correlation matrices generated. Each matrix is a table with toxins along the top and down the left-hand side in the same order, such that the diagonal elements always have a value of 1.0 and all other values reflect inter-toxin correlations. From this is calculated the table of coefficients of non-determination (CND; $1\text{-}r^2$) which provides the data for use in further PCA studies.

Table 3
Scored data* for 16 metabolites† throughout three time periods 0–8, 8–24 and 24–48 h obtained from PMR spectra of urine following exposure to six nephrotoxins

	Hg	PAP	CrO_4	HCBD	PI	BEA
ace8	0	1	0	0	1	1
ace24	1	1	0	1	2	2
ace48	1	1	1	1	3	2
ala8	0	2	0	0	0	0
ala24	1	2	0	2	1	1
ala48	1	2	1	2	1	1
HB8	0	1	0	0	0	0
HB24	0	1	0	1	0	0
HB48	0	1	0	1	0	0
cn8	−1	−1	−1	0	0	0
cn24	−1	−1	−1	0	0	0
cn48	−1	−1	−1	0	0	0
cit8	−1	−1	0	0	0	0
cit24	−2	−1	0	0	0	−1
cit48	−3	−2	0	0	1	−2
glc8	0	2	0	0	0	0
glc24	2	3	0	3	0	0
glc48	2	3	3	3	1	1
gln8	0	1	0	0	0	0
gln24	1	2	0	2	0	0
gln48	1	2	2	2	0	0
2-OG8	−1	−1	0	−1	0	0
2-OG24	−2	−1	0	0	0	−3
2-OG48	−2	−1	−1	−1	−2	−3
hip8	0	−1	0	−1	0	0
hip24	0	−1	0	−1	0	0
hip48	−1	−1	−2	−3	0	−1
lac8	0	2	0	0	0	0
lac24	2	2	0	2	1	1
lac48	3	3	1	3	1	1
suc8	0	1	0	0	−1	−1
suc24	−1	−1	0	0	2	1
suc48	−3	1	−1	1	3	2
TMAO8	−1	0	0	0	2	2
TMAO24	−1	0	0	0	2	−3
TMAO48	−2	0	0	0	−3	−3
val8	0	1	0	0	0	0
val24	1	2	0	2	0	0
val48	1	2	0	2	0	0
DMA8	0	0	0	0	2	1
DMA24	−1	0	0	0	2	2
DMA48	−1	0	0	0	1	1
lys8	0	1	0	0	0	0
lys24	1	2	0	2	0	0
lys48	1	2	1	2	0	0
DMG8	−1	1	0	−1	0	0
DMG24	−1	0	0	−1	0	0
DMG48	0	0	0	0	3	3

*See Methods section for a description of the scoring system used.

†The urinary metabolites were: ace, acetate; ala, alanine; cit, citrate; cn, creatinine; cr, creatine; DMA, dimethylamine; lys, lysine; DMG, *N*,*N*-dimethylglycine; glc, glucose; gln, glutamine; hip, hippurate; HB, 3-D-hydroxybutyrate; lac, lactate; 2-OG, 2-oxoglutarate; suc, succinate; TMAO, trimethylamine *N*-oxide; val, valine.

Results and Discussion

Shown in Fig. 1 are two examples of perturbed PMR urine analysis profiles following exposure to a proximal tubular toxin (hexachlorobutadiene; HCBD) and an agent which causes acute renal papillary necrosis (propyleneimine; PI). Descriptions of the perturbed

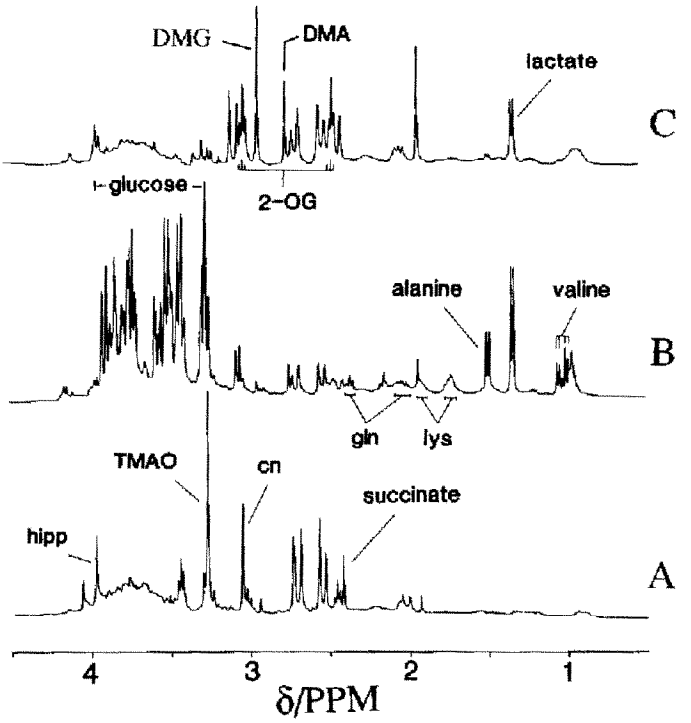

Figure 1
400 MHz PMR spectra of urine samples obtained from a control rat (A), and 24–48 h after dosing with 200 mg kg^{-1} hexachlorobutadiene (B) (a proximal tubular toxin), 24–48 h after 20 μl kg^{-1} propyleneimine (C) (a renal papillary toxin). See text for experimental conditions. Key: cn, creatinine; DMA, dimethylamine; DMG, *N,N*-dimethylglycine; gln, glutamine; hipp, hippurate; lys, lysine; 2-OG, 2-oxoglutarate; TMAO, trimethylamine *N*-oxide.

PMR spectral patterns following exposure to HCBD and PI have been reported previously [3]. Proximal tubular toxins commonly cause manifold changes in the urine profile, in particular, aminoaciduria, glycosuria and L-lactic aciduria, with minor elevations in urinary acetate and succinate [3]. Decreased urinary citrate is also a feature of proximal tubular nephrotoxicity, a particularly good example of which is mercuric chloride, which precipitates a renal tubular acidosis as a result of inhibition of renal carbonic anhydrase activity [2]. Similarly, HCBD causes urine citrate levels to fall (Fig. 1) but to a lesser degree than seen following $HgCl_2$.

Agents causing renal papillary necrosis produce a quite different profile of perturbations in urine to those observed following proximal tubular injury. Early elevations in dimethylamine and trimethylamine *N*-oxide (TMAO) were seen together with later increases in *N,N*-dimethylglycine, acetate δ = 1.95 ppm and succinate and loss of TMAO following exposure to PI (Fig. 1) and BEA [3].

A battery of clinical chemistry parameters is routinely determined in the assessment of both experimental and clinical nephrotoxicity or other form of renal functional impairment. In the present study a good cross-section of these parameters has been studied, including urinary enzymes (GGT and NAG), urinary glucose and urine flow rate, together with plasma urea. Data from these parameters (collected throughout three time periods, and urea at 48 h) were input to the ARTHUR package and principal components analysis carried out. Only the first four principal components were examined, since these contain up to 90% of the variance or information content of the datasets. Although plots of the first two principal components are demonstrated (PC1 and PC2), often a significant proportion of the variance may reside in the third principal component (PC3) and this is being investigated further.

Principal components maps for the clinical chemistry dataset are shown in Fig. 2(A and B). Figure 2A was generated from the 13-dimensional dataset shown in Table 2, while map B was generated using a 6 × 6 correlation matrix. Neither map provides adequate discrimination between proximal tubular toxins and renal papillary toxins, or between toxins

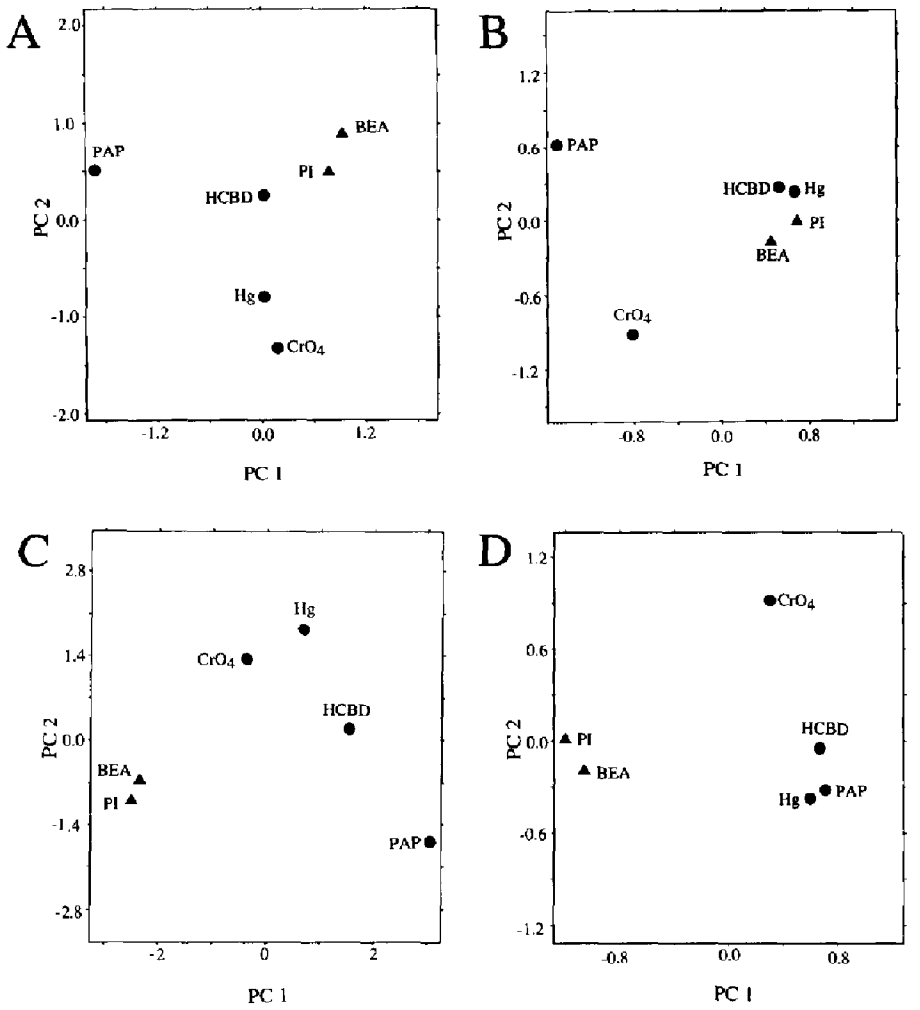

Figure 2
Plots of the first two principal components generated from 13 clinical chemistry time-course parameters (A), 48 proton NMR time-course parameters (C), and the corresponding CND maps for the clinical chemistry (B) and NMR datasets (D). Improved clustering is seen with the NMR dataset. Values of the variance in each of the first three principal components are: PC1 = 40%, PC2 = 29%, PC3 = 19% (part A); and PC1 = 72%, PC2 = 23% PC3 = 4% (part B); PC1 = 51%, PC2 = 20%, PC3 = 14% (part C); PC1 = 66%, PC2 = 19%, PC3 = 7% (part D).

which target the *pars convoluta* (convoluted portion) or the *pars recta* (straight portion) of the proximal tubule. However, the raw data did produce slightly better results (Fig. 2A). Principal components maps for the PMR dataset are shown in Fig. 2 (C and D). Map C was generated using the 48-dimensional dataset shown in Table 3, while map D was generated using a 6 × 6 correlation matrix derived from the 48-dimensional dataset. Although good classification of region-specific nephrotoxicity can be seen in both maps, with proximal tubular toxins clearly discriminated from renal papillary toxins, tighter clustering is apparent in map D with sodium chromate (a chemical which produces necrosis of the *pars convoluta*) clearly discriminated from the remaining three proximal tubular toxins, which all produce necrosis in the *pars recta* segment.

In the present study the authors have clearly demonstrated the value of PMR urine analysis data over clinical chemistry data for the classification of experimental region-specific nephrotoxicity. The use of 48 metabolic dimensions in the case of the PMR dataset, as compared to only 13 metabolic dimensions in the case of clinical chemistry, was undoubtedly a contributory factor in this improved classification. However, in addition to the quantity of data collected, one must also consider data

quality. The discriminatory capacity of the maps generated from the PMR dataset clearly shows that higher order information is being carried in the PMR dataset than in the clinical chemistry dataset. The authors consider that PMR–PR analysis of toxicological data is a novel and exciting means of classifying toxicity states and could therefore be used predictively in order to assess the toxicological profile of novel therapeutic agents.

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