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Effects of feeding and body weight loss on the ^{1}H-NMR-based urine metabolic profiles of male Wistar Han rats: implications for biomarker discovery

SUSAN C. CONNOR[1], WEN WU[2], BRIAN C. SWEATMAN[1], JODI MANINI[1], JOHN N. HASELDEN[1], DANIEL J. CROWTHER[2] and CATHERINE J. WATERFIELD[1]*

[1] Safety Assessment, GlaxoSmithKline Research and Development Ltd, Ware SG12 ODP, UK
[2] Bioinformatics Division, GlaxoSmithKline Research and Development Ltd, Stevenage SG1 2NY, UK

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For almost two decades, ^{1}H-NMR spectroscopy has been used as an 'open' system to study the temporal changes in the biochemical composition of biofluids, including urine, in response to adverse toxic events. Many of these *in vivo* studies have reported changes in individual metabolites and patterns of metabolites that correlated with toxicological changes. However, many of the proposed novel biomarkers are common to a number of different types of toxicity. These may therefore reflect non-specific effects of toxicity, such as weight loss, rather than a specific pathology. A study was carried out to investigate the non-specific effects on urinary metabolite profiles by administering four hepatotoxic compounds, as a single dose, to rats at two dose levels: hydrazine hydrate (0.06 or 0.08 g kg^{-1}), 1,2-dimethylhydrazine (0.1 or 0.3 g kg^{-1}), α-napthylisothiocyanate (0.1 or 0.15 g kg^{-1}) and carbon tetrachloride (1.58 or 3.16 g kg^{-1}). The study included weight-matched control animals along with those that were dosed, which were then 'pair-fed' with the treated animals so they achieved a similar weight loss. The urinary metabolite profiles were investigated over time using ^{1}H-NMR spectroscopy and compared with the pathology from the same animals. The temporal changes were analysed statistically using multivariate statistical data analysis including principal component analysis, partial least squares, parallel factor analysis and Fisher's criteria. A number of metabolites associated with energy metabolism or which are partially dietary in origin, such as creatine, creatinine, tricarboxylic acid (TCA) cycle intermediates, phenylacetylglycine, fumarate, glucose, taurine, fatty acids and N-methylnicotinamide, showed altered levels in the urine of treated and pair-fed animals. Many of these changes correlated well with weight loss. Interestingly, there was no increase in ketone bodies (acetate and β-hydroxybutyrate), which might be expected if energy metabolism was switched from glycolysis to fatty acid β-oxidation. In some instances, the metabolites that changed were considered to be non-specific markers of toxicity, but were also identified as markers of a specific type of toxicity. For example, taurine was raised significantly in carbon tetrachloride-treated animals but reduced in the pair-fed group. However, raised urinary bile acid levels were only seen after α-napthylisothiocyanate treatment. The methodology, statistical analysis used and the data generated will help improve the identification of specific markers or patterns of urinary markers of specific toxic effects.

Keywords: feeding effects, ^{1}H-NMR, metabonomics, hepatotoxicant, non-specific markers, biomarker, weight loss.

Introduction

For almost two decades, ^{1}H-nuclear magnetic resonance (NMR) spectroscopy has been used as an open system to study the temporal changes in the biochemical

* Corresponding author: Catherine J. Waterfield, Health Assessment, Syngenta, CTL, Alderley Park, Macclesfield SK10 4TJ, UK. e-mail: cathy.waterfield@syngenta.com

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composition of urine in response to adverse toxic events (Nicholson *et al.* 1989, Anthony *et al.* 1994, Beckwith-Hall *et al.* 1998). More recently, the study of endogenous metabolite changes in response to toxic insult was defined as 'the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification' and called 'metabonomics' (Nicholson *et al.* 1999).

Most metabonomic studies have centred on identifying major sources of variation in urinary metabolite excretion that arise following the administration of biochemicals or drugs to animals, usually rats. The aim has been to use this information to identify individual biomarkers or patterns of biomarkers that could be used to predict or monitor for adverse effects. This has resulted in a number of endogenous molecules being proposed as individual biomarkers or as components of a pattern of biomarkers for renal, hepatic and testicular injury, for example (table 1). However, many of the proposed markers highlighted have been similar for a wide range of toxicological effects and disease models (unpublished observations) and are therefore not unique for any particular toxicity, but probably reflect non-specific effects related to toxicity/disease.

Table 1. Examples of metabolites referenced as markers of target organ toxicity.

Target organ	Chemical agent	Urinary biomarkers	Reference
Renal cortical nephrotoxicity *(pars recta)*	mercury (24 μM kg^{-1})	↑acetate, ↑alanine, ↑glucose, ↓citrate, ↓creatinine, ↑2-oxoglutarate, ↑succinate and ↑lactate, ↑2-oxoglutarate	Nicholson *et al.* (1985), Gartland *et al.* (1989)
Papilla nephrotoxicity	bromoethanamine (BEA) (0.25 g kg^{-1})	↑TMAO (Anthony *et al.* 1994) and ↑DMA, ↑acetate, ↑succinate, ↑creatine, ↓TMAO (Robertson *et al.*), ↓taurine and ↓citrate, ↓succinate	Anthony *et al.* (1994), Robertson *et al.* (2000)
Testicular toxicity	cadmium (24 μM kg^{-1})	↑creatine, ↓citrate, ↓hippurate, ↓2-oxoglutarate	Nicholson *et al.* (1989)
Intrahepatic cholestasis with necrosis	α-napthylisothiocynate (ANIT) (0.2 g kg^{-1})	↑creatine, ↓2-oxoglutarate, ↓citrate, ↓hippurate, ↓creatinine	Beckwith-Hall *et al.* (1998), Robertson *et al.* (2000), Azmi *et al.* (2002)
Pan lobular hepatic necrosis	galactosamine (0.6 g kg^{-1})	↑lactate, ↑acetate, ↑taurine, ↑alanine, ↓citrate, ↓hippurate, ↓2-oxoglutarate	Beckwith-Hall *et al.* (1998)
Centrilobular liver necrosis	carbon tetracloride (0.5 ml kg^{-1})	↑taurine, ↑creatine, ↓2-oxoglutarate, ↓citrate ↓hippurate, ↓creatinine	Robertson *et al.* (2000)
Vasculitis	phosphodiesterase inhibitor	↑β-hydroxybuterate, ↓2-oxoglutarate, ↓citrate, ↓succinate, ↓hippurate, ↓taurine	Slim *et al.* (2002)
Phospholipidosis	cationic amphilic drugs	phenylacetyl glycine↑	Nichols *et al.* (2000)

Most of the publications in table 1 have used doses of toxins calculated to cause marked toxicity rather than subtle changes. The NMR results have been validated using both histological and clinical chemistry (Waters *et al.* 2001, Holmes and Antti, 2002). However, 'high-dose toxicity' will also result in reduced food intake and appreciable weight loss (Waterfield *et al.* 1993a, b), a factor which is not generally taken into account or reported when analysing the NMR spectral profiles from toxin-treated animals. The loss of weight and reduced food intake may explain why a number of proposed metabolite biomarkers of target organ toxicity and disease are associated with energy status and gut microflora changes and are, therefore, often the same metabolites, e.g. citrate, succinate, creatinine and 2-oxoglutarate, β-hydroxybutyrate and acetate. The contribution that changes in diet make to urinary profiles has been reported, particularly in relation to the excretion of hippuric acid and other dietary-derived aromatic metabolites in rat (Phipps *et al.* 1998, Gavaghan *et al.* 2001).

The aim of the present investigation was to highlight metabolite changes that were the result of weight loss and reduced food intake following treatment of rats with known hepatotoxicants. When these confounding factors were taken into account, it was possible to identify compound-specific effects more clearly. The study design included the 'pair-feeding' of weight-matched control animals with those that were dosed. The data were analysed statistically using multivariate statistical data analysis (MVDA) including principal component analysis (PCA), partial least squares (PLS; Vandeginste *et al.* 1998), parallel factor analysis (PARAFAC; Bro 1997) and Fisher criteria (Wu *et al.* 1995). Preliminary results have been presented (Sweatman *et al.* 2001).

Materials and methods

Materials

The following materials were supplied by Sigma-Aldrich Co. Ltd (Poole, UK): hydrazine hydrate (HZ), 1,2-dimethylhydrazine (DMH), α-napthylisothiocyanate (ANIT) and carbon tetrachloride (CCl_4) used as hepatotoxic test agents; sterile water for irrigation (SWFI) and corn oil used as the vehicle/control materials. Reagents for ^{1}H-NMR analyses were also supplied by Sigma-Aldrich, including monobasic sodium phosphate monohydrate ($NaH_2PO_4{\cdot}H_2O$), dibasic sodium phosphate heptahydrate ($Na_2HPO_4{\cdot}7H_2O$), sodium 3-trimethyl-silyl-[2,2,3,3-$^2H_4$]-1-propionate (TSP), sodium azide and deuterated water (D_2O).

Animal treatment and husbandry

Male Wistar Han rats [Crl:WI(Glx/BRL/Han)BR] aged 56–63 days were supplied by OLAC. They were housed five animals per cage in solid-bottom TP4 cages (Techniplast UK Ltd, Kettering, Northants, UK) lined with standard Beekay animal bedding (Beekay Universal Ltd, Aldborough, UK) and allowed to acclimatize for 9 days and identified with a unique serial number. Environmental conditions were regulated to maintain a constant temperature of 20–22°C and a relative humidity of 45–60% with positive-pressure ventilation (approximately 12 complete air changes per hour). The rooms were illuminated with fluorescent lighting on a 12-hour light/dark cycle (light 07.00–19.00 h daily). During the course of the study, each animal had access to standard diet (Rat & Mouse No. 1 Diet, Special Diet Services Ltd, Cambridge, UK) either *ad libitum* or by an amount determined by the 'pair-feeding' regimen and domestic tap water *ad libitum*.

During the urine collection periods, the rats were individually housed in MET 1 metabolism cages (Arrowmight-Biosciences, Hereford, UK) including an additional 3-day acclimatization period before dosing.

Study design

Pair-feeding. Ten rats were randomly allocated to each drug treatment group (Stage B) and a further 10 animals to a group of animals that were fed *ad libitum* throughout the study (true controls) (Stage A). Each individual rat in the treatment groups had a weight-matched partner placed into appropriate groups, which were designated the 'pair-fed' group for that treatment (Stage C). The 24-h food consumption was measured daily for each individual drug-treated rat. The same amount of food was given to each rat's pair-fed control at the end of each 24 h. Thus, the feeding, dosing and necropsy were staggered by 24 h in all the pair-fed groups when compared with the toxin-treated animals (table 2).

Dosing and necropsy. Each animal received a single oral dose (10 ml kg^{-1} body weight) of either control material (vehicle) or test compound: CCl_4, ANIT (in corn oil), DMH or HZ (in SWFI) (table 3). Pair-fed animals were given the appropriate vehicle 24 h after their treated partner (table 2). All dosing solutions were administered as a single dose by gavage at a volume of 10 ml kg^{-1} body weight. The test materials (ANIT/CCl_4/DMH/HZ) and control materials (corn oil/SWFI) were administered to the treatment and true control groups on day 0 (Stages A and B) and the control materials (SWFI/corn oil) were given to the groups designated as pair-fed groups on day 1 (Stage C). Five animals in each group were necropsied 3 days after dosing and the remaining five animals were necropsied 24 h later to enable any recovery in the treated groups to be assessed.

Clinical observations and body weight

Clinical observations were recorded twice daily from days −5 to 4 for Stage A and B animals and until day 5 for Stage C animals. All animals were weight matched into pairs on day −6 for the purpose of the pair feeding aspect of the study. Animals from Stages A and B had their body weight recorded daily from days −6 to 4 inclusive. Animals in Stage C (pair-fed controls) had their weights recorded daily from days −6 to 5 inclusive.

Urine collection

Rats were housed individually in metabolism cages for the duration of the study and urine was collected over 7 h (08.00–15.00) predose and continuously post-dose for 7 and 17 h (08.00–15.00 and 15.00–08.00) (table 2). Before urine collection, approximately 1 ml 1% (w/v) sodium azide solution (aqueous) was added to each collection pot to serve as a preservative and bacteriostatic agent.

Table 2. Study time table for dosing, necropsy and urine collection.

Day−3	Day−2	Day−1	Day 0 (dose)	Day 1 (dose)	Day 2	Day 3	Day 4	Day 5
True controls (*ad libitum* feeding) (stage A)			⇓			⇓		
Metabolism cages	PDU	PDU	PDU dose vehicle	Urine ×2	Urine × 2	Urine ×2 necropsy (five rats)	Urine ×2 necropsy (five rats)	
Experimental group (stage B)			⇓			⇓		
Metabolism cages	PDU	PDU	PDU dose test com-pound	Urine ×2	Urine × 2	Urine ×2 necropsy (five rats)	Urine ×2 necropsy (five rats)	
Experimental group (stage C)							⇓	
	Metabolism cages	PDU	PDU	PDU dose vehicle	Urine × 2	Urine ×2	Urine ×2 necropsy (five rats)	Urine ×2 necropsy (five rats)

Arrows highlight key times in the study and the staggering of these events in the pair-fed group. Metabolism cages, rats introduced into meabolism cages; dosing day; PDU, pre-dose 7-h urine collection from 08.00 to 15.00; urine ×2, 24-h urine collection divided into two collections 08.00–15.00 and 15.00–08.00.

Table 3. Dosing and treatment groups.

Group number	Test material	Dose (g kg^{-1})	Dose solution (mg ml^{-1})	Reference
1	Vehicle — sterile water	0	–	–
2	Vehicle — corn oil	0	–	–
3	Carbon tetrachloride	1.58	158	Waterfield *et al.*
4	Carbon tetrachloride	3.16	316	(1993a)
5	α-Napthylisothiocyanate	0.1	10	Waterfield *et al.*
6	α-Napthylisothiocyanate	0.15	15	(1993a)
7	Dimethylhydrazine	0.03	3	Kuratko *et al.*
8	Dimethylhydrazine	0.1	10	(1994)
9	Hydrazine	0.06	6	Sanins *et al.* (1992)
10	Hydrazine	0.08	8	
11–14	Corn oil (pair-fed controls for groups 3–6)	0	–	–
15–18	Sterile water (pair-fed controls for groups 7–10)	0	–	–

Clinical chemistry

Animals were killed by exsanguination from the abdominal aorta whilst under anaesthesia and necropsied 3 or 4 days after dosing. Blood (2.0 ml) was collected into heparin tubes for the preparation of plasma and a number of clinical chemistry parameters were measured. These included alkaline phosphatase (ALK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamate dehydrogenase (GLDH), bile acids, glucose, urea, total protein, albumin, creatinine, cholesterol, phospholipids and triglycerides using a Hitachi 917 auto-analyser and the appropriate kits (Roche Diagnostics, Lewes, UK).

Pathology

The liver and kidneys were weighed and sections taken into 10% (v/v) neutral buffered formalin. Tissue sections were prepared by embedding in paraffin wax, sectioning and then staining with haematoxylin and eosin. Lipid accumulation was confirmed in frozen liver sections (10 μm) using oil Red 'O' staining.

^{1}H-NMR spectroscopic analysis of urine

Aliquots of urine (400 μl) were mixed with phosphate buffer (200 μl, 0.2 M, pH 7.4) and left to stand at room temperature for 10 min. Any resulting precipitate was removed by centrifugation at 5000 rpm for 15 min at 4°C. An aliquot (500 μl) of the supernatant was put into a 5-mm NMR tube (Wilmad 507PP), to which an aliquot (50 μl) of a TSP/D_2O/sodium azide solution (0.1% w/v TSP in D_2O and 1% w/v sodium azide) had been added.

The TSP and D_2O provided a chemical shift reference ($\delta_H = 0.00$ ppm) and field lock signal, respectively, for the NMR spectrometer. Samples were analysed at 600.13 MHz on a Bruker DRX-600 spectrometer at ambient probe temperature (300 K) using a 5-mm TXI ATMA probe. Spectra were acquired using a standard water presaturation pulse sequence:

$$\mathrm{RD} - 90^\circ - t_1 - 90^\circ - t_m - 90^\circ - \text{collect free induction decay},$$

where RD and t_m were relaxation delays of 2 s and 100 ms, during which the water was selectively irradiated and t_1 represented the delay for the first increment in a two-dimensional nuclear overhauser enhancement spectroscopy (NOESY) pulse sequence and was set to 3 μs. Sixty-four scans and four dummy scans were collected into 64 k data points, with a spectral width of 12019.23 Hz, an acquisition time of 2.73 s and a total recycle delay of 4.73 s. An exponential line-broadening function of 0.30 Hz was used to multiply the free induction decays before Fourier transformation.

NMR spectral data

All spectra were phased and base-line corrected using XWINNMR software (Bruker GmbH, Karlsruhe, Germany). Data were reduced to 245 integrated regions (buckets) of 0.04 ppm corresponding to the region $\delta_H = 10.0$ to 0.2 ppm using AMIX (Bruker GmbH). Seventeen buckets corresponding to the regions $\delta_H = 6.0 - 5.5$ and 4.9 −4.7 ppm were excluded because of the high variability in intensity

of the water and urea resonances. NMR data were then normalized by dividing the sum value of the remaining 228 buckets for each spectrum and multiplying by 1000.

Chemometric feature selection methods

A typical urine NMR dataset for toxicity studies is usually treated as two-way table. In this study, the two ways related to 180 animals (observations) and 228 NMR buckets (features). However, NMR spectra are often recorded from rats over a series of time points and rats are treated in groups, as in this case. The onset and recovery from toxicity has previously been followed over time using PCA. The toxicity trajectory (time trajectory for each individual/group NMR data) would be followed in the two/three-dimensional scores plots. It is usually difficult to find clear time and group effects due to the large number of points and animal variation in the score plot. However, time and group information can also be regarded as a third and a fourth way in the dataset and the time evolution and group effects can be more easily visualized using alternative methods to PCA.

One approach to decomposition of the time evolution is n-way (also known as multimode or multi-order) analysis (Bro 1997). The present work used PARAFAC because of its uniqueness and simplicity (Bro 1996, 1997, Bro *et al.* 1997, Czarnik 1998), although other decomposition methods exist such as Tucker methods.

PARAFAC is a natural higher-order extension of PCA. In PARAFAC, multiway data are decomposed into sets of scores and loadings with the same number of columns (factors). The number of the factors or components is much lower than the number of original variables in each way, so that the data can be visualized in a reduced dimensional space.

Other recent method developments for monitoring the time dependence of metabonomics data analysis have incorporate the use of batch-processing methods to monitor changes in NMR spectra over time (Azmi *et al.* 2002). This is an adaptation of unfolding PCA and usually involves a hierarchical model with a PLS analysis at the basic level using time as a y variable followed by a PCA analysis of the PLS scores at the top level. A typical approach would be to model time evolution in the control group and to look for deviations from this profile in dosed animals in one-dimensional scores plots.

In the modelling of NMR data, the number of variables is usually very large, often with a substantial amount of redundant information in the data set. For interpretation purposes, it is often very useful to make a model of the original data, compressing the data variance into a few components/factors, which are easily interpreted. Uni- or multivariate methods can be used to find individual significant changes or multiple, interacting significant changes, respectively. In these investigations, the multivariate method used was the multiway analysis, PARAFAC. Guo *et al.* (2000, 2002) proposed some new feature selection methods in PCA and sequential projection pursuit (SPP). Wu *et al.* (2003) further extended the feature selection methods from two- to three- or n-way analysis methods of PARAFAC. Here the feature selection method, based on the loadings of the PARAFAC, was applied to find chemical shift buckets that represented toxin-specific and non-specific variables.

PLS analysis was also carried out on the full NMR data and a reduced dataset containing non-specific markers selected using the selection criterion (f1 −f2). The univariate method for feature selection based on Fisher criterion has been proposed previously (Anthony *et al.* 1994, Wu *et al.* 1995, Vandeginste *et al.* 1998). The PLS models used per cent weight loss from the start of the experiment until day 2 as the y variable to which the NMR data were correlated. Validation of the PLS model was carried out using external validation, whereby half of the original dataset was selected by a Kennard & Stone design (Kennard and Stone 1969) as a calibration set and the remaining half was used as the validation set. The calibration set was then used to generate the PLS models using both the full dataset (all bucketed NMR data excluding the water and urea peaks) and a reduced dataset containing only non-specific variables in Table 6. Body weight losses were then predicted for the validation set and the performance of the model expressed in terms of root-mean-square error of prediction (RMSEP):

$$\mathrm{RESEP} = \sqrt{\frac{\sum(y_{\mathrm{predict}} - y_{\mathrm{observed}})^2}{n}},$$

where n is the number of samples.

The univariate method for feature selection was used to select the toxic-specific markers, which were defined as the variables (chemical shift buckets) that were significantly different between toxicant-treated and paired-fed control groups with a minimal significant difference between paired-fed control and true control groups. A non-specific marker was defined as a variable (chemical shift bucket) that was not significantly different between toxicant, treated and paired-fed control groups, but was significantly different between paired-fed and true control groups. Thus, the non-specific marker changes were considered to be related to reducing food intake and/or body weight loss. To quantify this definition, two following criteria were first estimated:

$$f1 = \frac{\text{Variance between treated and paired control groups}}{\text{Variance within treated and paired control groups}}$$

$$f2 = \frac{\text{Variance between true control and paired control groups}}{\text{Variance within true control and paired control groups}},$$

where f1 was used to estimate the separation between toxicant-treated and paired control groups, and f2 was used to estimate the separation between true and paired control groups. Here f1 and f2 are similar to the statistic in the *F*-test, so they are called Fisher criteria. Then, f1 − f2 was used as a selection criterion. When the selection criterion was higher than a threshold, e.g. 1, the variable was designated as a potential toxicity-specific marker. When the selection criterion was lower than a threshold, e.g. −1, the variable was considered as a potential non-toxicity-specific marker. Here 1 and −1 were selected as thresholds by considering the distribution of the selection criterion and examining the selected variables. The markers falling between these two criteria would need to be examined more closely on a study-by-study basis to determine whether they could be considered as specific or non-specific markers, although from a practical point it would be difficult for these to be clearly defined.

Results

Body weights

Dosing with both high and low doses of ANIT, HZ and CCl_4 produced marked body weight loss immediately post-dose, with the most marked effect being on day 2 (figure 1). Weight loss continued until the end of the study following ANIT, and to a lesser extent following CCl_4 treatment, whereas the weight lost trend was reversed for HZ-treated animals by day 4. There was a negligible affect of DMH on body weight change over the time course of the experiment.

Histopathology

To understand the degree of injury caused by the different hepatotoxins and the extent to which the lesions were reversed by day 4 following dosing, sections from the livers and kidneys were examined from all animals killed on days 3 and 4. No abnormalities were seen in the true control animals at either time point. Pair-fed animals showed varying degrees of vacuolation following glycogen depletion from the liver on days 3 and 4, which reflected the reduced food consumption in individual animals. Histopathological findings for all treatment groups are summarized in table 4. Hepatoxicity was observed following both high and low doses of CCl_4, HZ and ANIT, but only minimal effects were observed following dosing with both doses of DMH. Following both CCl_4 and HZ treatment, the maximum effects observed histologically were seen on day 3, with less severe effects on day 4. However, animals treated with both high and low doses of ANIT showed similar effects on day 4 as on day 3, indicating the lack of reversibility of ANIT toxicity over the experimental time course.

Clinical chemistry

The clinical chemistry parameters measured in the terminal plasma samples are presented in table 5. Only those parameters where treatment-related changes were identified are reported. Generally, the data support the liver pathology observed. Both dose levels of ANIT resulted in the most severe pathology and did not regress significantly by day 4. The raised levels of ALK and bile acids were consistent with

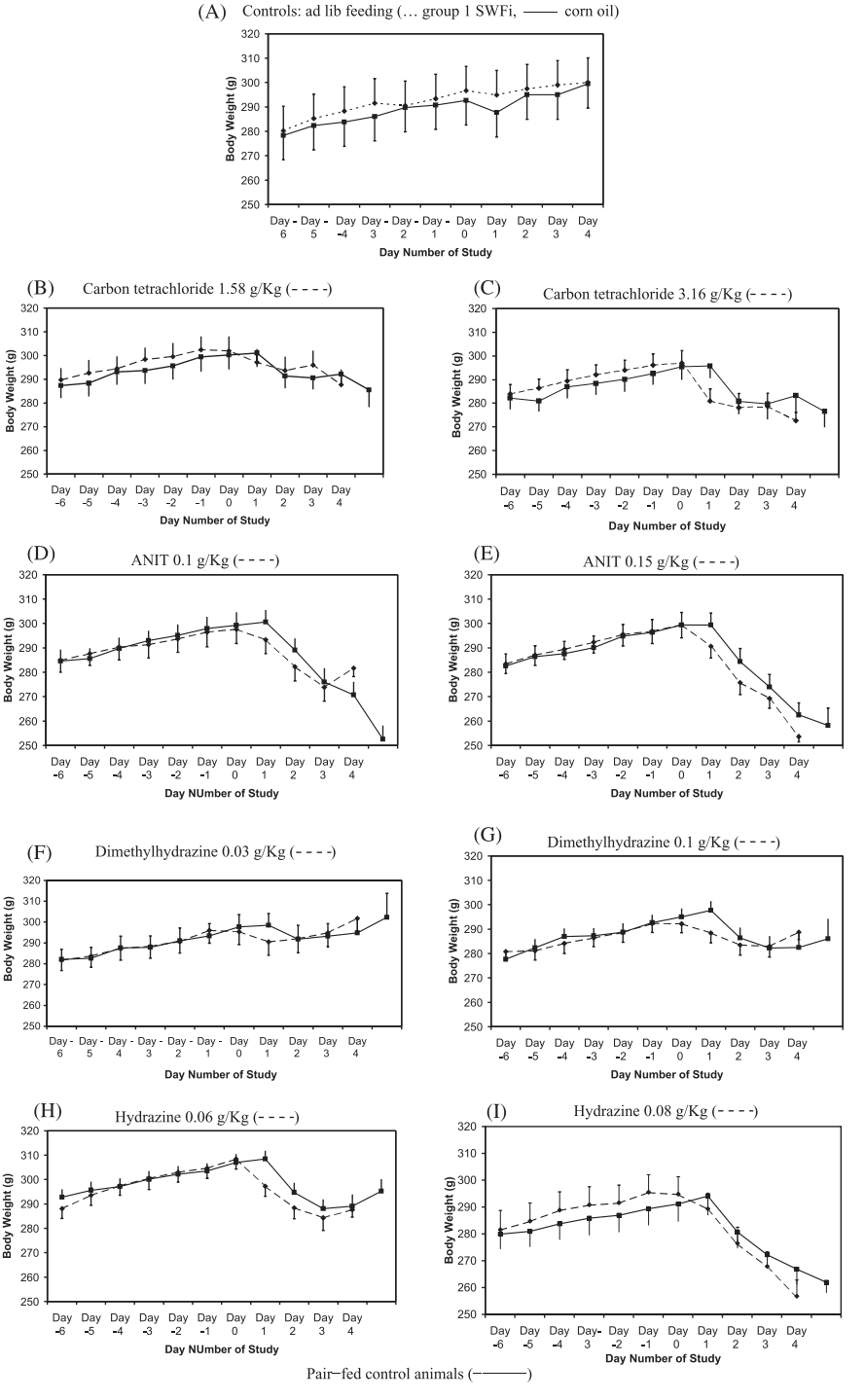

Figure 1. Group average body weights ± standard deviation from day −6 to day 4 post-dosing first dose. *Ad libitum* fed animals (A) and treated plotted with weight matched-paired controls (B–I).

Table 4. Summary of pathology changes observed in treated groups on days 3 and 4.[1]

Compound, dose ($g\ kg^{-1}$)	Day 3	Day 4	Reversibility
CCl_4, 1.58 and 3.16	Slight ballooning of trilobular/centrilobular hepatocytes Slight/marked steatosis centrilobular and mid-zonal hepatocytes Depletion of glycogen	Hepatocytes normal Slight steatosis in centrilobular and mid-zonal hepatocytes Some glycogen depletion	yes
High dose only, 3.16	Slight/marked inflammatory response Slight/marked increase in mitotic index	Slight ballooning cenrilobular hepatocytes (1/5) Slight centrilobular inflammatory response (4/5)	yes
ANIT, 0.1 and 0.15	Slight/moderate bile duct hyperplasia Slight periportal inflammation and fibroplasia Few necrotic cells Glycogen depletion in two rats per dose group	Slight/moderate bile duct hyperplasia Slight periportal inflammation and fibroplasia Few necrotic cells Glycogen depletion in two rats per dose group	no
High dose only, 0.15	Bile duct degeneration/ necrosis	Bile duct degeneration/ necrosis Sperm retention (Waters *et al.* 2002)	no
DMH, 0.03 and 0.1	Two to five rats with a slight mid-zonal mitotic increase	Two of five rats slight mid-zonal mitotic increase	possibly
High dose only, 0.1	Slight single cell necrosis One of five centrilobular necrosis and inflammation Wide variation between rats glycogen depletion	Slight single cell necrosis One of five centrilobular necrosis and inflammation No glycogen depletion	possibly
Hydrazine, 0.06 and 0.08	Slight/moderate mid-zonal steatosis Slight/moderate increase in mid-zonal mitosis (5/5 LD, 3/5 HD) Glycogen depletion (2/5 LD, 5/5HD) Tubules in medullary rays had cytoplasmic 'foaminess'	No liver changes Tubules in medullary rays had cytoplasmic 'foaminess' (2/5 LD, 5/5 HD)	yes
High dose only, 0.08		Slight/moderate increase in mid-zonal mitosis Very slight mid-zonal steatosis and marked periportal steatosis (1/5) Glycogen depletion	yes

[1] Unless stated otherwise, the pathology applies to all animals in the group.

the cholestatic effects of this compound. The resulting hepatocellular necrosis is reflected in the high levels of ALT, AST and glutamate dehydrogenase (GDH). CCl_4 showed marked toxicity at the highest dose, which was resolving by day 4. This was reflected in the raised levels of ALT, AST and GDH, which were also present following treatment with the lower dose 3 days after dosing. The main effect of HZ was the reduction in ALT, which has been reported before (Waterfield *et al.* 1993a). Bile acid levels were also raised and there was a reduction in total protein and albumin. There was a slight increase in phospholipid consistent with

Table 5. Summary of plasma clinical chemistry parameters (means) that were significantly changed following treatment and pair-feeding on days 3 and 4.

Days after dosing	Dose group (g kg^{-1})	ALK	ALT	AST	URN	CRE	BAC	GDH	TPR	ALB	CHL	TRG	GLU	PLI
3	Corn oil	402.0	45.6	68.4	7.4	49.2	7.6	3.6	62.0	31.9	1.5	1.8	10.2	1.8
	CCl_4 1.58	309.6	72.8	100.3	6.9	55.1	15.3	16.1***	64.9	32.1	1.1*	1.7	9.2	1.1**
	CCl_4 3.16	397.1	211.8***	293.2***	7.1	55.1	46.9***	69.7***	62.5	31.2	1.4	2.2	8.2**	1.5
	ANIT 0.1	1413.9***	838.5***	804.1***	5.5**	71.0***	805.8***	171.4***	66.4	29.5*	6.7***	1.6	6.3***	5.0***
	ANIT 0.15	1714.2***	674.5***	676.7***	6.1	66.3***	689.9***	249.5***	66.8	28.0***	7.8***	1.5	8.2*	5.7***
	Pair-fed low	275.0*	33.6	74.6	7.1	50.7	13.8	4.3	61.2	32.3	1.2	1.3	11.8	1.3*
	Pair-fed high	237.6**	22.5**	64.7	7.9	52.4	11.9	3.5	59.8	32.7	1.1	2.0	11.8	1.0***
4	Corn oil	243.8	37.5	58.9	6.7	48.4	7.4	3.4	60.8	30.8	1.3	1.1	11.4	1.5
	CCl_4 1.58	308.3	37.1	58.1	7.0	52.1	13.1	5.3	60.6	30.9	1.5	1.2	11.5	1.5
	CCl_4 3.16	279.1	86.8*	76.9	7.6	48.5	17.0	32.6***	63.0	32.2	1.5	1.4	10.2	1.6
	ANIT 0.1	1159.0***	235.1***	236.4***	5.8	56.7*	97.1***	41.2***	64.6	28.7	4.2***	2.3**	7.8***	2.8***
	ANIT 0.15	1420.0***	421.3***	628.9***	5.0**	59.6**	376.8***	205.9***	68.5**	28.3*	8.8***	1.9*	7.9***	7.1***
	Pair-fed low	267.7	33.0	67.1	7.3	48.2	16.3	3.4	59.9	31.6	1.2	1.1	10.4	1.3
	Pair-fed high	270.2	30.8	74.2	8.2*	50.0	11.6	4.9	57.9	32.0	1.1*	1.8**	10.8	1.0**
3	Water	303.7	47.6	67.4	6.9	51.8	7.6	3.5	61.6	32.0	1.3	1.3	9.5	1.6
	DMH 0.03	266.8	53.1	76.4	7.6	51.4	11.2	4.7	61.5	31.5	1.3	1.5	9.6	1.6
	DMH 0.1	254.0	58.2	104.2***	6.9	49.0	40.6*	10.8***	56.3*	29.0**	1.1	1.4	9.8	1.6
	HZ 0.06	280.7	13.2***	74.7	7.2	48.7	87.8***	7.4*	55.6**	29.8*	1.5	2.7***	9.5	1.7
	HZ 0.08	245.4	10.8***	78.1	7.6	49.4	14.8	6.7	53.3***	28.8**	1.1	2.9***	9.9	1.4
	Pair-fed low	264.2	29.3**	63.7	7.8	47.8	11.8	3.7	59.6	31.3	1.3	1.1	10.0	1.3
	Pair-fed high	258.9	24.1***	64.9	7.8	51.2	9.8	3.7	60.6	32.1	1.1	1.9***	12.0**	1.1***
4	Water	295.4	42.5	59.1	7.7	50.4	9.3	3.4	61.0	31.3	1.2	1.4	10.3	1.4
	DMH 0.03	236.0	40.1	67.4	7.6	47.6	10.3	4.0	60.1	30.8	1.3	2.4	11.4	1.6
	DMH 0.1	319.3	52.6	77.4	7.5	47.0	20.1	5.6	59.9	30.8	1.3	1.9	12.3	1.7
	HZ 0.06	244.6	24.3	56.1	7.6	45.8	31.9*	3.1	61.7	32.6	1.9**	2.1***	11.8	1.6
	HZ 0.08	238.1	14.6***	77.2	6.1*	49.0	26.4	8.2*	55.8*	29.4	2.2***	2.6***	10.4	2.0***
	Pair-fed low	276.4	30.3	66.6	7.9	47.0	17.2	3.6	60.3	31.0	1.2	1.1	10.0	1.3
	Pair-fed high	250.1	23.5*	62.6	9.0	47.2	12.6	3.5	59.7	31.8	1.2	1.6***	11.1	1.2

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ treatment groups or pair-fed animals compared with respective water or corn oil controls (ANOVA and Dunnett's test). $N = 5$/treatment group or $N = 10$/pair-fed group (both low and both high pair-fed groups have been combined).

ALK, alkaline phosphatase; ALT, alanine amino transferase; AST, aspartate amino transferase; URN, blood urea nitrogen; CRE, creatinine; BAC, bile acids; GDH, glutamate dehydrogenase; TPR, total protein; ALB, albumin; CHL, cholesterol; TRG, triglycerides; GLU, glucose; PLI, phospholipids.

the foamy changes seen in the kidney that may have been due to phospholipid accumulation. DMH had the least effect on the any of the clinical chemistry parameters confirming that this was the least hepatotoxic of the compounds at the doses administered. Only the higher dose resulted in changes in the clinical chemistry parameters, which were the slightly raised AST levels, bile acids and GDH with a reduction in albumin and total protein. All of the effects of DMH were resolved by the fourth day.

Interestingly, there were also changes in the clinical chemistry parameters in the plasma samples taken from the animals that had been pair fed. Levels of ALT were reduced, particularly on the third day, although not to the same extent as that seen following HZ treatment. Phospholipid levels were reduced on both days 3 and 4. There was also a reduction in ALK on day 3 in the animals dosed with corn oil. The extent of the pathological changes were broadly reflected in the loss of body weight in the treated groups and were mirrored by the respective pair-fed controls. The percentage body weight loss was greatest for ANIT (H) > HZ (H) > CCl_4 (H) > HZ (L) > ANIT (L) > CCl_4 (L) > DMH (H) > DMH (L).

Statistical analysis of urine NMR data

For each group, the mean NMR spectrum was calculated and all the mean spectra were put together as mean data for PCA analysis. The score plot of initial PCA did not show any clustering of samples (data not shown).

PCA of each toxin-treated group versus its pair-fed control and true control showed varying degrees of separation of the pair-fed controls and the true control versus the treated groups. Figure 2 shows a PCA analysis of the effect of feeding in relation to high dose ANIT treatment. PC1 showed three clear clusters of the pair-fed, ANIT-treated and true controls. The major source of variation, described by PC1, was the effect of feeding and body weight loss rather than toxicity. The ANIT-specific effects were observed across PC2. Separation was due to creatine, creatinine, TCA cycle intermediates, taurine, phenylacetylglycine and hippurate (figure 2B).

To probe the feeding and weight loss effects more systematically and comprehensively, PARAFAC and Fisher criteria were used to highlight influential spectral features of both specific toxicity and non-specific effects related to feeding and body weight loss. A four-way dataset (group × animal × time × NMR bucket) was analysed by PARAFAC. The loading plot of group mode (figure 3A) indicated that factors 1 and 2 separated the ANIT-treated, pair-fed and true controls, comparable with the PCA results shown in figure 2A. The separation of ANIT-treated versus true controls was seen in factor 2 and versus pair-fed groups was seen in factor 1. The time evolution of the samples can be seen in the loading plot of time mode (figure 3B). The NMR mode loadings plot in figure 3C shows the variables that caused the most separation between the groups. For example, citrate at 2.7 ppm (circled, lowest factor 2 loading point) is influential in separating control and pair-fed samples across factor 2. Creatinine at 3.06 ppm (also circled, highest factor 2 loading point) contributes to the difference in pair-fed and ANIT-treated animals versus the true controls in factor 2, whilst bucket 3.26 (highest factor 1 loading point) separated ANIT from the pair-fed and true controls across factor 1.

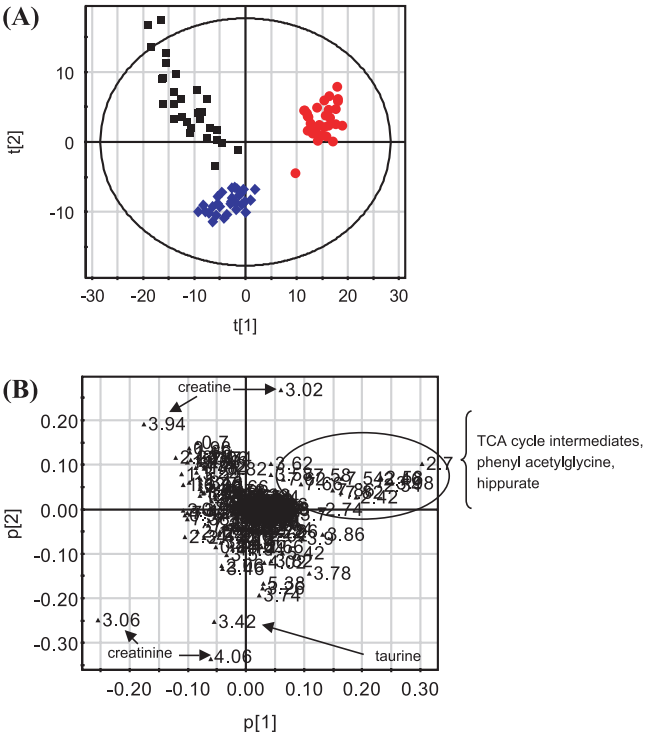

Figure 2. PCA score (A) and loading (B) plots of 48–96-h urine data for high-dose ANIT-treated (◆), pair-fed control (corn oil) (◆) and vehicle-treated freely fed control (corn oil) (●) groups; autoscaling was used.

For the bucket at 2.7 ppm, one three-way dataset (group × animal × time) was constructed and further analysed by PARAFAC. The group mode plot (figure 4B) shows the clustering of treatment groups representing just the citrate variable at 2.7. This plot clearly shows that citrate concentrations are similar in the pair-fed and ANIT-treated animals, which in turn are very different from the citrate levels in true control samples. The time mode plot (figure 4C) shows how the citrate concentration varies throughout the time course of the experiment with a general decrease in concentration at the earlier time points and the largest difference in citrate levels between the groups being observed at the later time points. figure 4A summarizes the mean intensity ± standard deviation information for citrate at 2.7 over the experimental time course, showing that during the acclimatization period citrate excretion was decreased in all groups, but that the true control levels increased back to the starting levels by day 4. All other groups including pair-fed and ANIT-treated groups showed a continued reduction in citrate until the end of the experiment.

In figure 5A, variables with peaks of f1 were the buckets, which could discriminate the ANIT-treated group from the pair-fed control group. Variables corresponding to peaks of f2 were the buckets that separated the pair-fed control from the true control groups. Some of these discriminating variables were overlapping, so it was difficult to decide which one was due to the toxin-specific

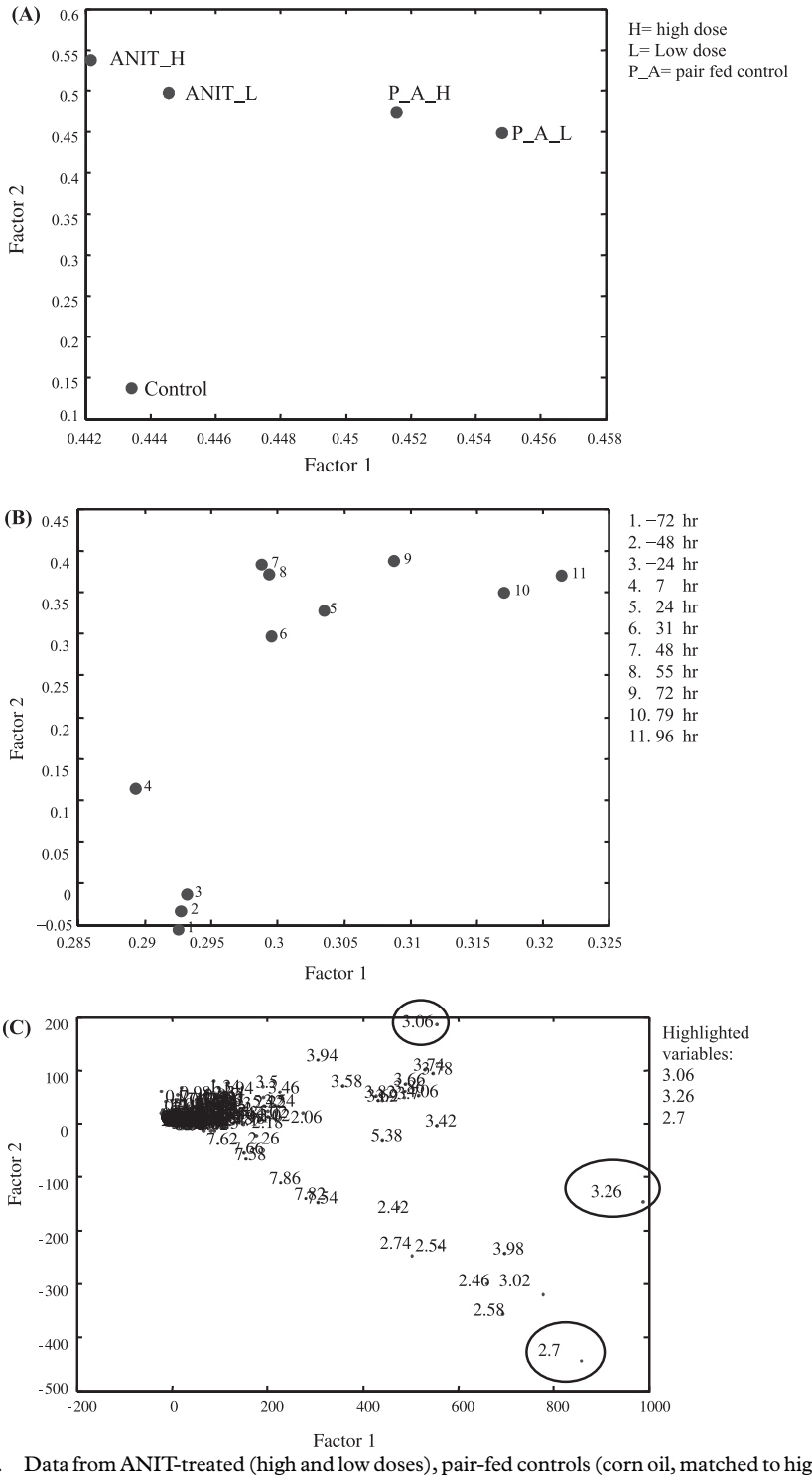

Figure 3. Data from ANIT-treated (high and low doses), pair-fed controls (corn oil, matched to high- and low-dose ANIT-treated animals), and control animals (corn oil). Loading plots of group (A), time (B) and NMR (C) modes obtained by PARAFAC of the four-way data (group × animal × time × NMR); raw data without scaling. Daytime samples, 08.00–15.00 h (−72, −48, −24, 7, 31, 55, 79 h). Late afternoon/night-time samples 15.00–08.00 h (24, 48, 72 and 96 h).

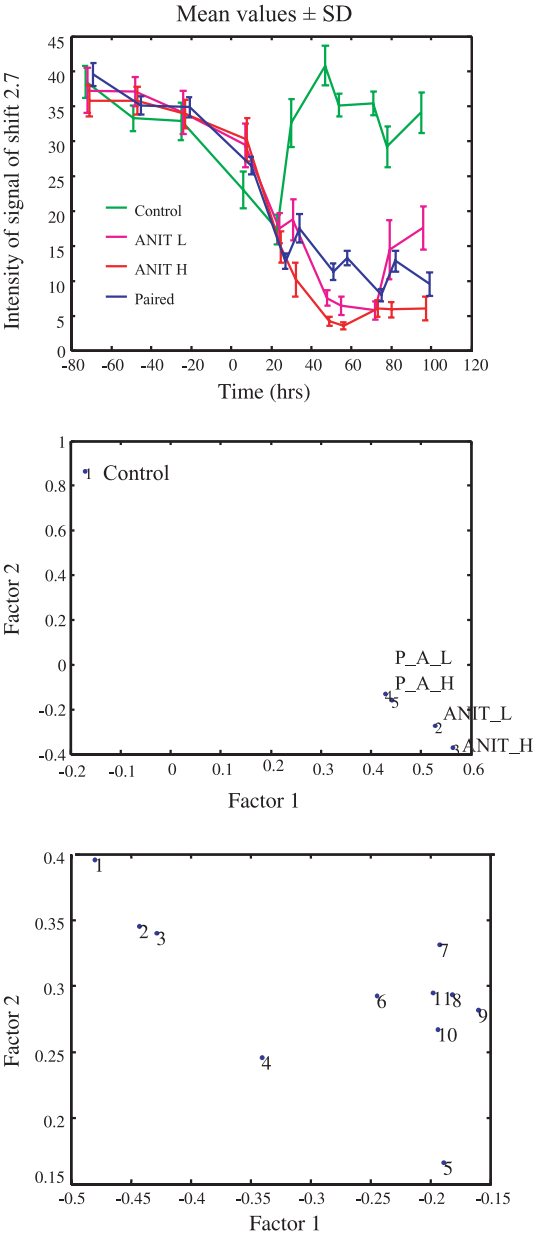

Figure 4. Data from ANIT-treated animals (low and high doses), meaned data from pair-fed controls (corn oil) and true controls (corn oil). (A) Plot of mean signal intensity ± standard deviation at 2.7 ppm versus time; (B) loading plot of group mode; (C) loading plot of time mode; obtained by PARAFAC of the three-way data (group × animal × time) at 2.7 ppm using raw data without scaling. (See Figure 3 for a key to urine collection times.)

effect and which one was due to the non-specific effect related to reducing food intake and body weight loss. The selection criterion (f1 − f2) in figure 5B indicated which effect dominated the separation. Variables with high f1 − f2 were the buckets dominated by the toxin-specific effect, while variables with lower f1 − f2 are the

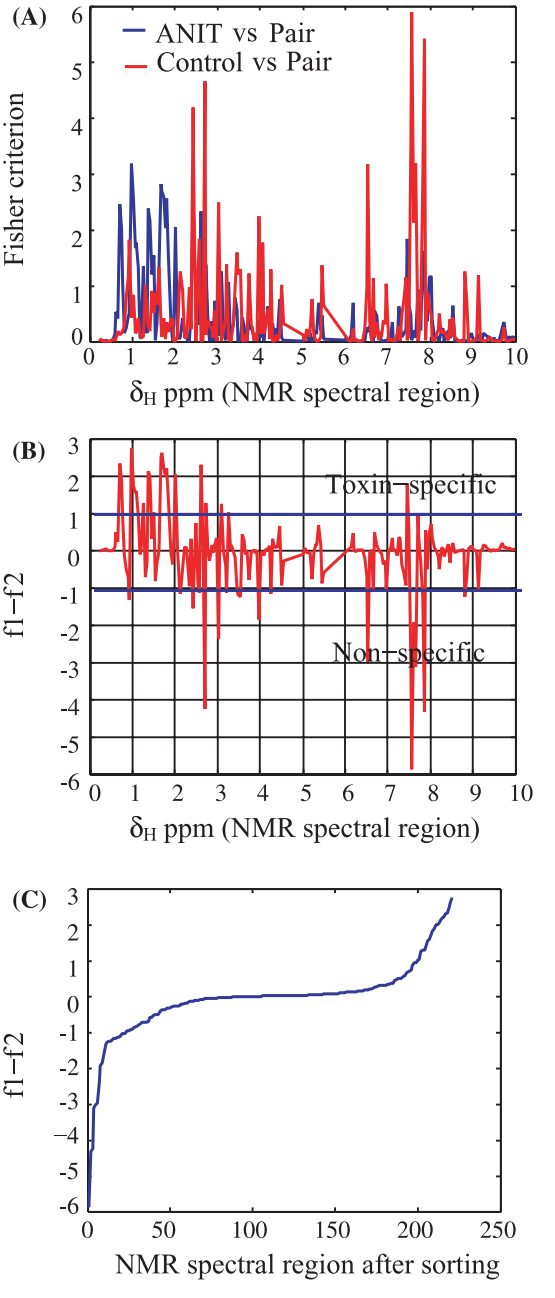

Figure 5. Data from ANIT-treated animals (high dose), pair-fed controls (corn oil) and true controls (corn oil). (A) Representation of Fisher criteria f1 and f2 (see the equation in the Materials and methods), Fisher criteria f1 (blue) versus f2 (red) versus NMR spectral region; (B) result of subtraction (f1 −f2) with thresholds indicated for feature selection related to toxin and non-toxin-specific markers (arbitrary cut-off); (C) selection criterion (f1 −f2) after sorting from small to large.

buckets dominated by the non-specific effect. Figure 5C shows that most of the variables had f1 −f2 values between −1 and 1. About 10% of the variables have f1 −f2 >1 or < −1.

Figure 5B shows that the selection criterion (f1 −f2) reached the maximum when the chemical shift is 0.98 ppm (unassigned). Figure 6 shows the treatment group and time characteristics relating to the 0.98 ppm spectral region. Figure 6A illustrates that there was no group difference in 0.98 during the acclimatization period or between pair-fed and true controls over the time course of the experiment, but there was a large increase at 24 h post-ANIT dosing. The PARAFAC analysis also illustrated this fact, showing that levels of 0.98 in the ANIT groups increased (factor 1, figure 6B) from 31 h, returning to control levels by the end of the experiment (factor 1, figure 6C).

Using the selection criterion f1 −f2 (figure 5B), 48 spectral regions represented non-specific effects related to reduce food intake and body weight loss were found (table 6). Using the same methodologies, several toxin-specific spectral regions were also highlighted for each toxin (table 7). PARAFAC models were then constructed to look at the specific (table 6) and non-specific markers (table 7) separately. Data from tables 6 and 7 have been used in figure 7A and B to produce group mode plots of the PARAFAC for toxin-specific and non-specific spectral regions, respectively. The ANIT-treated groups were clearly separated from the pair-fed and true control groups using the toxin-specific markers whereas the pair-fed and ANIT groups were indistinguishable using the non-specific markers.

PLS was also used to investigate further whether it was possible to model body weight loss by NMR data. The root-mean-square errors for the calibration and validation sets using the 50-variables model were 0.01827 and 0.01931, respectively. The root-mean-square errors for the calibration and validation using all 228 variables were 0.0208 and 0.01995, respectively. Using both the 228 and reduced 50-variable data sets, it was possible to get a good correlation between the NMR data and body weight loss at 48 h (figure 8). By checking the PLS model with all variables, the majority of influential variables corresponded to those selected as non-specific markers using the selection criterion (Table 6).

Discussion

The liver changes described in these studies are consistent with those reported in the literature following treatment with the different hepatotoxicants (Waterfield *et al.* 1993a, b, Beckwith-Hall *et al.* 1998, Nicholls *et al.* 2001). Although pathology was not recorded at 48 h, the most marked toxicity-related effects were probably manifested 48 h after dosing as this was the time that the most significant changes were seen in the urine samples and there was the greatest weight loss in most animals. Pathology and clinical chemistry results indicated that there was recovery from these lesions in all treatment groups, although less marked in the ANIT-treated animals where the severity of the lesions was greatest. For this reason, the PLS models (figure 8) were constructed using body weight loss

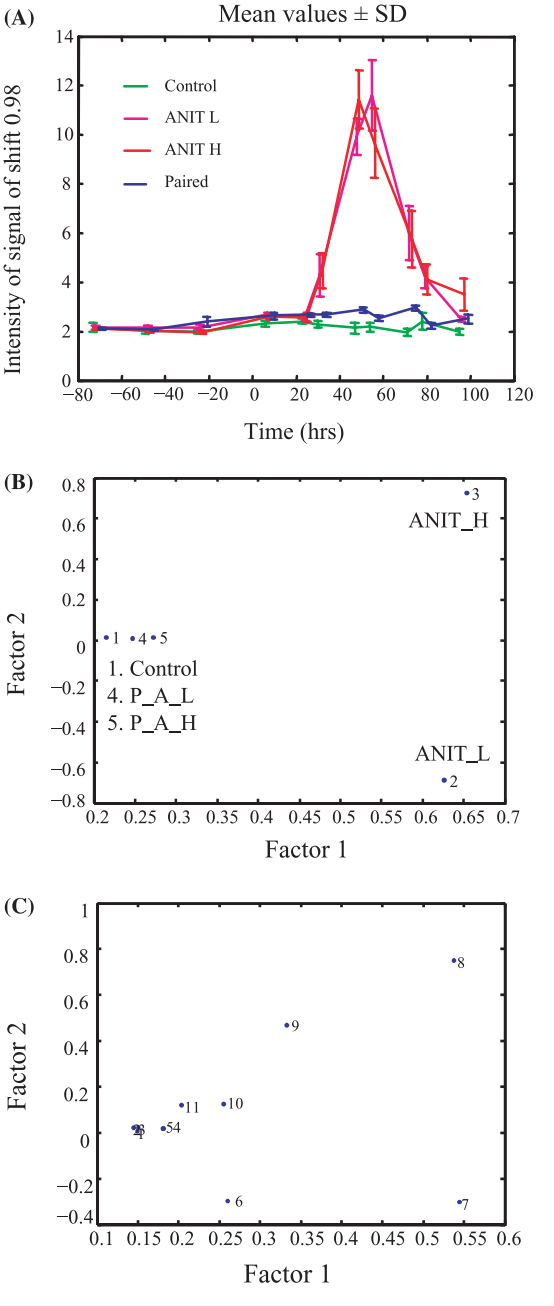

Figure 6. Data from ANIT-treated animals (low and high doses), meaned data from pair-fed controls (corn oil) and true controls (corn oil). (A) Plot of mean signal intensity ± standard deviation at 0.98 ppm versus time; (B) loading plot of group mode; (C) loading plot of time mode; obtained by PARAFAC of the three-way data (group × animal × time) at 0.98 ppm using raw data without scaling. (See Figure 3 for a key to urine collection times.)

in the first 48 h after dosing to provide the most information about specific toxicity-related effects and the non-specific changes due to reduced feeding and weight loss.

Table 6. Non-specific spectral regions highlighted by comparison of the pair-fed control versus true control groups for each treatment group.

Non-specific spectral regions (δ_H ppm)	Annotation	Pair-fed group corresponding to toxin
2.70	citrate	CCl_4 (L, H),
3.50	glucose	CCl_4 (H), ANIT (L, H), hydrazine (L)
3.54		CCl_4 (H), ANIT (L, H), hydrazine (L)
3.22		ANIT (L, H), hydrazine (L)
3.38		ANIT (L)
3.74		CCl_4 (H), ANIT (L), DMH (H), hydrazine (L)
5.22		ANIT (L), hydrazine (L)
5.46		ANIT (L, H)
0.90	short and medium chain fatty acids	ANIT (L)
0.94, 1.54, 2.10, 2.14, 2.34		ANIT (L, H)
2.42	succinate	ANIT (L), DMH (H)
2.46	2-oxoglutarate	ANIT (L)
3.02		ANIT (H), hydrazine (L, H)
2.54	citrate	ANIT (L), hydrazine (L)
2.58		ANIT (L)
2.70		ANIT (L, H), hydrazine (L, H)
6.54	fumarate	ANIT (L, H), hydrazine (L)
7.54, 7.62, 7.82, 3.98	hippurate	ANIT (L, H), hydrazine (L)
7.58, 7.66, 7.86		ANIT (L, H), DMH (H), hydrazine (L, H)
7.78		ANIT (L, H)
8.82, 9.14	N-methyl nicotinic acid	ANIT (L, H)
8.86		ANIT (L)
3.46	taurine/glucose	ANIT (L, H), DMH (H), hydrazine (L)
4.26, 4.50	unassigned	ANIT (L, H)
5.10		DMH (H)
5.50		ANIT (H)
6.46, 6.50, 6.66, 6.98		ANIT (L)
7.06, 7.30		hydrazine (L)
7.34, 7.38		ANIT (H)
7.42		ANIT (L)
7.94		ANIT (H)
8.22		ANIT (H)

Urine samples analysed from treated animals with the greatest reduction in body weight and most severe lesions, also had the highest number of changes in endogenous metabolites, which were considered to be non-specific to the toxicity (high dose ANIT and HZ). Citrate, 2-oxoglutarate, hippurate and creatinine appeared to change even with minimal weight loss. With greater weight loss fumarate, glucose, taurine, fatty acids and N-methylnicotinamide altered in the urine. Most of these metabolites are associated with energy metabolism or are the end products of compounds involved in energy metabolism, e.g. N-methylnicotinamide from nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). The changes in taurine and hippurate are likely to be due to altered food intake, as a percentage of both molecules are known to be of dietary origin. Interestingly, there was no increase in ketone bodies (acetate and β-hydroxybutyrate), which might be expected if energy metabolism was switched from glycolysis to fatty acid β-oxidation. There were, however, increased urinary levels of short- and medium-chain fatty acids, which may have reflected an increase in the mobilization of fatty acids for β-oxidation.

Table 7. Toxin-specific spectral regions highlighted by comparison of the treated group with the pair-fed control for each treatment group.

Toxin	Specific spectral regions (δ_H ppm)	Unconfirmed annotation [1]
CCl_4 (low dose only unless otherwise specified)	0.26 (L & H), 0.30, 0.58, 0.62, 0.74, 0.78	alpha-2-microglobulin?
	3.26, 3.3, 3.46 (L & H)	taurine
	6.46, 6.74, 7.74 (L & H), 7.78, 8.46 (L & H), 8.74, 8.78, 8.82, 8.94, 9.06	unassigned aromatics (plus formate)
	2.62 (H only)	methylamine?
	3.10 (H only)	unassigned
	3.94 (H only)	creatine
	5.42 (H only)	maltose (diose/triose)
ANIT (both doses unless otherwise specified)	0.70, 0.74, 0.78 (L only), 0.98, 1.02, 1.06, 1.10, 1.26, 1.38, 1.42, 1.50, 1.66, 1.70, 1.74, 1.78, 1.82, 1.86, 1.90 (L only), 2.02	bile acids and unassigned alkyl's (probably also from bile acids)
	2.42 (H only)	succinate
	2.46 (H only)	2-oxoglutarate
	2.62	methylamine?
	2.74	dimethylamine
	2.94 (H only)	*N*,*N*-dimethylglycine
	3.26	trimethylamine *N*-oxide
	3.10	unassigned
	4.46 (H only)	NMN?
	5.38 (H only)	allantoin
	7.46	unassigned aromatic
	7.70 (L only), 8.02 (L only)	unassigned aromatics
DMH (both unless otherwise stated)	0.70, 0.74, 0.82 (L only)	cholic acid derivative
	1.78	unassigned
	2.62	methylamine
	5.38	allantoin
	8.70, 8.74, 8.78, 9.02, 9.06	unassigned aromatic
	0.82–1.18	unassigned
	1.22 (H only)	3-D-hydroxybutyrate
	1.26	unassigned fatty acid
	1.30	unassigned fatty acid
	1.38	unassigned fatty acid
	1.46	alanine
	1.62–1.94	citrulline, Nα-acetyl-citrulline, argininosuccinate
	2.02	Nα-acetyl-citrulline
	2.26 (H only), 2.38 (H only),	2-aminoadipate
	2.42 (H only)	succinate
	2.46	2-oxoglutarate
	2.50	balanine? (plus part of citrate)
	2.78–2.86	argininosuccinate
	2.90 (L only)	trimethylamine
	2.98	2-oxoglutarate
	3.14–3.18	citrulline, b-alanine
	3.30	argininosuccinate
	3.94	creatine
	4.02 (H only)	unassigned
	4.06	creatinine
	4.18 (H only)	unassigned
	4.26	argininosuccinate
	4.30 (H only)	unassigned
	4.46 (L only)	N-methylnicotinamide
	5.10, 5.18	unassigned sugars

Table 7 (*Continued*)

Toxin	Specific spectral regions (δ_H ppm)	Unconfirmed annotation [(1)]
	5.30 (H only), 5.14 (H only)	unassigned sugars
	5.38	allantoin
	5.46 (L only)	maltose
	5.50	unassigned sugar
	6.34, 6.58, 6.62, 6.66, 6.70, 6.74, 6.78, 6.82, 6.94	includes chlorogenic acid metabolites
	6.98 (H only), 7.10 (H only), 7.26–7.38 (H only)	includes chlorogenic acid metabolites
	7.14 (L only), 7.18 (L only), 8.14 (L only)	tyrosine?
	7.70 (H only), 7.74 (H only), 8.10 (H only), 8.46 (H only), 8.50 (H only), 8.54.	histidine
	8.97, 8.90,	N-methylnicitinamide
	8.42, 8.50, 8.62, 8.66, 8.70, 8.74, 8.78, 8.82, 8.86, 8.94, 9.02, 9.06, 9.10, 9.14, 9.20, 9.34	NAD-trypophan metabolites plus baseline

[1] Assignment unconfirmed, taken from literature values (Lindon *et al.* 1997, Nicholls *et al.* 2001).

These studies have confirmed what has long been suspected. Namely, that a number of metabolites that have been referred to as 'biomarkers' of various types of toxicity, are more likely to be biomarkers of general toxicity due to reduced food intake and a loss of body weight. No doubt, some proportion of these changes may also be due to specific toxicities, but the extent of this change will be difficult to determine. The non-specificity also means these 'markers' have uncertain value as specific biomarkers.

The PCA and PARAFAC plots (figures 2 and 3) illustrate the possible pitfalls of interpreting metabonomics data (and probably other 'omics' data), without putting the results in context of the in-life data such as body weight loss and food intake, in addition to gold standard end points of toxicity. Without the presence of a pair-fed control group, the conclusion from figure 2, for example, would be that all the variability in PC1 describes ANIT toxicity, whereas in reality almost all of the changes would be related to body weight loss and reduced feeding.

Despite the presence of these potentially very substantial confounding factors, it is still possible to eliminate them from the analysis through statistical techniques. Ideally, this would be through using the 'pair-feeding' regimen described here, as the data need to be correlated with the loss of body weight and food intake. This implies that a pair-fed group would be needed for all groups in a study, which would then make it possible to elucidate toxin-specific markers by paired comparison of the toxin-treated group versus its pair-fed control. However, we acknowledge that this would be both impractical and wasteful for routine studies. For practical purposes, once one study has been conducted under conditions in a particular laboratory setting for a particular species, it should be possible to use these data as prior knowledge of the effects of feeding/body weight loss/stress alongside future data. This would be possible providing the animal weights and food intake are recorded for each study and that the strains of rats and the type of diet are consistent. It would also be possible to use these in-life data as part of a

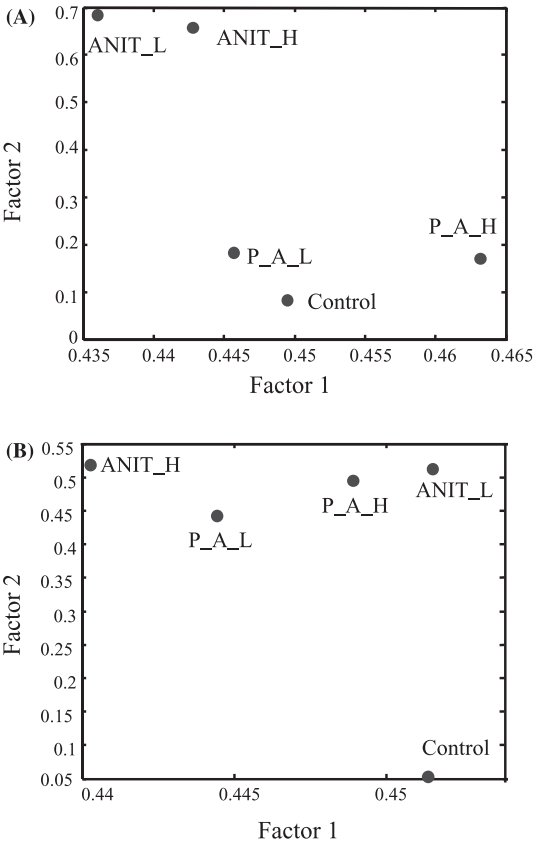

Figure 7. Data from ANIT-treated animals (low and high doses), from pair-fed controls (corn oil, matched to high- and low-dose ANIT-treated animals) and true controls (corn oil). Loading plots of group mode obtained by PARAFAC of the four-way data (group × animal × time × NMR); raw data without scaling; (A) using toxin-specific markers in Table 7 and (B) using non-specific markers in Table 6.

PLS model to look at the effects of body weight loss in relation to toxicity. However, this would not be feasible without prior knowledge of the expected confounding effects if toxicity and body weight loss are tightly correlated, as is often the case.

An analysis of the specific and non-specific effects across the four compounds used in this study highlighted that there might be occasions where a non-specific marker is much more greatly elevated than expected for a weight loss or dietary effect, or has an effect in the opposite direction. One example of this is taurine, which is elevated after treatment with CCl_4 to a much larger extent than the changes seen in response to body weight loss in any of the groups. Taurine is therefore a marker of CCl_4 treatment, which could be used as part of a predictive model of toxicity, but its value as a specific individual marker of effect is questionable. This would also be the case for altered tricarboxylic acid cycle intermediates, as urinary levels are depleted during renal tubular acidosis and the ratios of the different molecules are altered in diabetics). Changes in levels of these metabolites may, therefore, be of interest in cases where they are markers of altered energy metabolism or metabolic acidosis that could be a component of a more

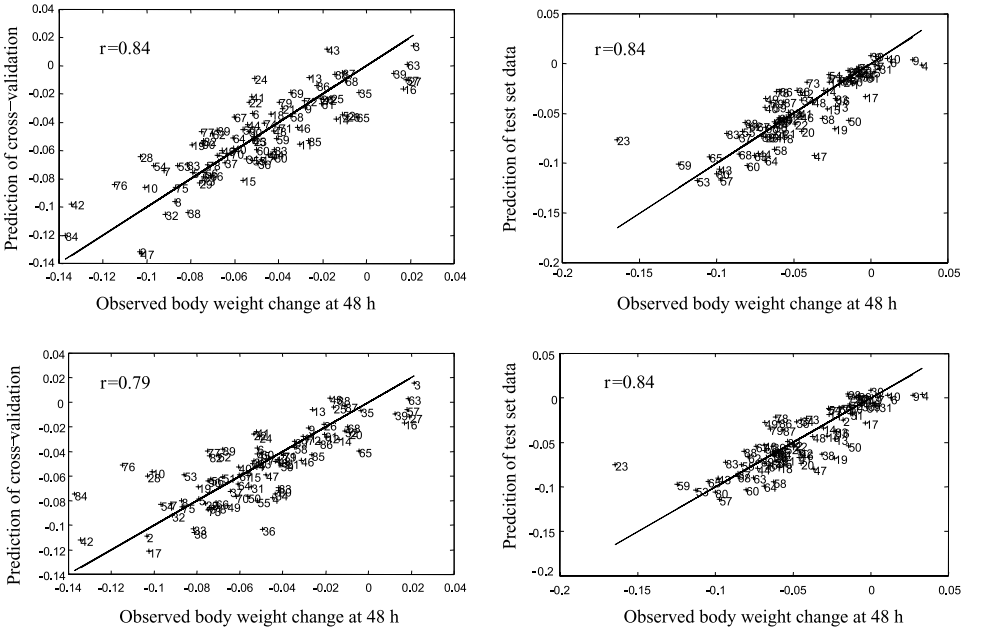

Figure 8. PLS model prediction versus observed body weight change at 48 h; (A, B) calibration set and validation sets using 50 non-specific markers; (C, D) calibration set and validation sets using all 228 NMR buckets.

subtle toxicity/disease effect, or a reflection of altered kidney function (Anthony *et al.* 1994, 1995, Holmes *et al.* 1998). In such cases, it would be necessary to establish the correlation between toxicity and body weight loss by using PLS and running parallel models with and without these non-specific markers. This will help to detect both the contribution and importance of these non-specific markers to a particular toxicity, and make it easier to detect potentially more subtle but also more specific metabolite changes that could give novel biological insight into a particular process.

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