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A metabonomic approach to the investigation of drug-induced phospholipidosis: an NMR spectroscopy and pattern recognition study

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^{1}H NMR spectroscopy of urine and pattern recognition analysis have been used to study the metabolic perturbations caused following dosing of five novel drug candidates, two of which (GWA, GWB) caused mild lung and liver phospholipidosis, whilst the rest (GWC–GWE) did not cause any detectable toxicity. Urine samples were collected pre-dose, 0–8 h, 8–16 h, 16–24 h and 24–32 h after single, oral dosing with each compound to Han Wistar rats ($n = 3$ per group), and liver and lung samples for were taken at 48 h for histology. ^{1}H NMR spectra of whole urine were acquired, processed and subsequently analysed using principal component analysis. All animals administered the drug candidates showed a significant reduction in serum triglycerides and those animals administered either GWA or GWB were observed to have foamy alveolar macrophages and the presence of multilamellar bodies in hepatocytes by electron microscopy. In the plot of the first two principal components, urinary spectra of those animals dosed with GWA or GWB mapped separately to controls, all pre-dose samples and animals dosed with GWC-GWE. Inspection of the principal components loadings indicated an increase in urinary phenylacetylglycine with a concomitant decrease in urinary citrate and 2-oxoglutarate, possibly constituting a novel urinary biomarker set for phospholipidosis. This work exemplifies the use of NMR spectroscopy and pattern recognition methods for the detection of novel biomarker combinations for poorly understood toxicity types and the potential in screening novel drugs for toxicity.

Keywords: phospholipidosis, ^{1}H NMR, urinalysis, metabonomics, phenylacetylglycine, cationic amphiphilics, phenylacetate.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CADs, cationic amphiphilic drugs; FIDs, free induction decays; GDH, glutamate dehydrogenase; GST, glutathione-S-transferase; HPMC, hydroxypropylmethylcellulose; PAG, phenylacetylglycine; TSP, sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)-1-propionate.

Introduction

Phospholipidosis is a term used to describe histological changes related to the accumulation of phospholipid within animal cells (Kodavanti and Mehendale 1990, Halliwell 1997). A predominant characteristic of phospholipidosis is the presence of multilamellar bodies within the lysosomes of alveolar macrophages, hence the common description foam cells or 'foamy' macrophages (Reasor *et al*. 1988).

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Phospholipidosis has been observed as a recurrent pathological feature in both the clinical (Lullmann *et al*. 1975) and in non-clinical toxicity studies (Kodavanti *et al*. 1990). A common characteristic of compounds reported to induce phospholipidosis is that they are cationic amphiphiles (Halliwell 1997). Examples of such cationic amphiphilic drugs (CADs) include Clorpromazine, Chloroquine and Amiodarone. It has been shown that, following dosing, high concentrations of CADs accumulate in lysosomes, which has been attributed to pH-dependent ion trapping of the CADs due to protonation in the acidic lysosomal environment (De Duve *et al*. 1974). The charged CAD complexes with the charged phosphate group of phospholipids rendering them unsuitable for degradation by phospholipase enzymes (Hollinger 1993). The rise in intra-lysosomal pH may also result in the decline of phospholipase A and A_2 activity. The free pool of both drug and phospholipid will then increase. CADs are also known to inhibit phosphatidate phosphohydrolase, leading to the overproduction of cytidine diphosphate-diacylglycerol and the phospholipids, phosphatidylinositol and phosphatidylglycerol (Brindley and Bowley 1975).

To date, determination of phospholipidosis has relied upon the use of electron microscopy for confirmation of the presence of multilamellar bodies (Hook 1991), examination of peripheral blood lymphocytes (Lullmann *et al*. 1975) and direct chemical analysis of phospholipids (Reasor *et al*. 1988); hence agents that cause phospholipidosis may only be recognized at a late stage in the drug development process. It had been shown previously that two of a series of new drug candidates caused widespread phospholipidosis in the rat following 7 days repeat dosing. Identification of phospholipidosis at an earlier stage would thus have provided a valuable method for screening of potential candidates for toxicological problems.

^{1}H NMR spectroscopy of biofluids such as urine and plasma offers an alternative method of monitoring the biochemical changes elicited by novel drug candidates (Nicholson and Wilson 1987, 1989, Lindon *et al*. 1999). Recent studies have used biofluid ^{1}H NMR spectroscopy to assess target organ toxicity in rats. This has provided a database of spectral profiles and biochemical changes associated with various toxicity types, which can be related to assessment by more conventional toxicological methods (Waterfield *et al*. 1993a, b). ^{1}H NMR spectroscopy has been used to identify biomarkers indicative of organ damage, and including taurine as a specific marker of hepatotoxicity (Sanins *et al*. 1990), and creatine as a marker of testicular toxicity (Gray *et al*. 1986, 1990, Nicholson *et al*. 1989). Furthermore, NMR spectroscopic analysis also allows the visualization of previously identified markers as characteristic metabolic spectral profiles, e.g. the spectral profile of glucose, amino acids and organic acids as markers of nephrotoxicity (Holmes *et al*. 1990, Anthony *et al*. 1992).

High-frequency ^{1}H NMR spectra of biofluids (acquired at >500 MHz) typically contain several thousand resonances providing structural and quantitative information on potentially hundreds of endogenous metabolites. In order to effectively examine ^{1}H NMR spectra containing such large quantities of information, statistical data reduction and multivariate analysis techniques, such as principal component analysis, have been applied (Holmes *et al*. 1998b). Principal components analysis (PCA) is used to calculate a new smaller set of orthogonal variables from a linear combination of a large set of correlated variables, whilst still maintaining the maximum level of variability from the original data. This permits the simple visualization of separation or clustering between the samples caused by

compound-induced metabolic perturbations (Beckwith-Hall *et al*. 1998, Holmes *et al*. 1998b) using 2-dimensional plots of the principal components (scores plots). The weightings given to each variable in calculating the principal components (PCs) allow for the identification of the variables important to the separation/clustering and hence, the deduction of biomarkers of toxicity or disease states (Holmes *et al*. 1998a).

This NMR-PR approach to investigating the time-related metabolic effects of drugs and toxins *in vivo* is described by the term 'metabonomics' (Nicholson *et al*. 1999). Metabonomic data are complementary to those obtained from proteomic and transcriptomic methods that are currently being evaluated for use in toxicological studies. In this paper we describe the use of biofluid NMR-based metabonomics to (i) assess the toxicity of a series of drug candidates, and (ii) to identify urinary changes in endogenous metabolites which might act as specific biomarkers for phospholipidosis.

Materials and methods

Animal experiments

A series of five structurally-related compounds was provided by research chemistry laboratories within Glaxo Wellcome Research and Development. For the purposes of this communication, the compounds have been assigned the identity GWA, GWB, GWC, GWD and GWE.

Male Wistar Han rats (7–8 weeks old at supply) were obtained from Charles River UK Ltd, Manston Road, Kent, UK. Animals were randomly allocated into seven groups ($n = 3$ per group) and acclimatized for 7 days prior to dosing. During the acclimatization period, animals were housed three per cage in Techniplast animal cages (Type 4). For periods of urine collection, animals were housed individually in grid-bottomed urine collection cages (MET 1,20 metabolism cage, Arrowmight-Biosciences, Hereford, UK). Throughout the acclimatization and study periods all animals had access to food (Rat and Mouse 1, SDS, Manea, Cambridgeshire, UK) and water *ad libitum* and were maintained on a 12 h light/dark cycle (21 °C ± 1 °C with 35–65% humidity). Each group of animals was given a single dose of a drug candidate (GW prefix), or vehicle alone to serve as control, by oral gavage at a dose of 500 mg kg^{-1} (using a dose volume of 10 ml kg^{-1} body weight).

Two control groups were used in the study since GWA and GWB were supplied in their base form, whereas GWC, GWD and GWE were provided as co-precipitates of hydroxypropylmethylcellulose phthalate (HPMCP). The dose vehicle for GWA, GWB and their control group (Control 1) consisted of: 0.5% (w/v) hydroxypropylmethylcellulose (HPMC–Methocel K15 Premium HPMC, Colorcon Ltd, Orpington Kent, UK) in Sterile Water for Irrigation (Fresenius Healthcare Group) containing 1% (w/v) polyoxyethylene sorbitan monooleate (Tween 80–Sigma Chemical Co., Poole, Dorset, UK). The dose vehicle for GWC, GWD, GWE and their control (Control 2) was composed of 50 mg HPMCP per g in 0.5% (w/v) HPMC containing 1%(w/v) Tween 80.

Body weights were recorded prior to dosing and then once daily following dosing. All in-life parameters and clinical chemistry parameters were subject to statistical analyses using pairwise comparison to the appropriate control group, using Dunnett's test (Dunnett 1964) for multiple comparisons using a level of significance set at 95% (*) or 99% (**).

Sample collection

Urine samples were collected for an 8 h period on the day prior to dosing, and then 0–8 h, 8–16 h, 16–24 h and 24–32 h post-dose. Urine volumes were recorded and the samples retained at –20 °C until analysis by ^{1}H NMR spectroscopy.

At 24 h post-dose, duplicate blood samples (0.6 ml) were collected into heparinized containers from each animal by tail venepuncture. These samples were analysed by routine methodologies (Hitachi 917 analyser) for assessment of plasma clinical chemistry parameters which included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), bile acids, cholesterol, glutamate dehydrogenase (GDH), glucose, sorbitol dehydrogenase (SDH), total protein, albumin, glutathione-S-transferase(GST), and triglycerides.

Animals were killed by exsanguination whilst under isoflurane anaesthesia, 48 h after dosing. Samples of liver, kidney, adrenal, lungs, heart and mesenteric vessels were retained and fixed in 10% (v/v) neutral buffered formalin for histological processing (3 μm wax sections stained with haematoxylin and eosin) and subsequent microscopic examination. Liver and lung tissues were subsequently prepared for electron microscopic examination from rats treated with GWA, GWB and control rats from material

originally fixed in 10% buffered formalin. Tissues were post-fixed in 1% osmium tetroxide and processed into Agar 100 epon resin. Semi-thin (1 μm) Toluidine Blue stained sections were prepared and examined by light microscopy. Suitable areas for electron microscopy were identified and ultra-thin sections 60–90 nm were then prepared to include alveolar macrophages in the lung, and both centrilobular and periportal zones in the liver. Sections were stained with uranyl acetate and lead citrate using an LKB Ultrastainer and examined in a Philips CM10 transmission electron microscope.

Urine sample preparation for NMR spectroscopy

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 13000 rpm for 10 min. From the supernatant, an aliquot (500 μl) was put in a 5 mm NMR tube (Wilmad 507PP), to which sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$)-1-propionate (TSP) in D_2O was added (50 μl, final concentration of 1mM). The TSP and D_2O provided a chemical shift reference (δ 0.0) and deuterium lock signal for the NMR spectrometer.

Single pulse ^{1}H NMR spectra of all urine samples were obtained at 600.13 MHz on a Bruker DRX-600 NMR spectrometer (Bruker Analytische Messtechnik, Germany). Single dimensional spectra were acquired using a standard presaturation pulse sequence for water suppression (Nicholson *et al*. 1995) with irradiation at the water frequency during the relaxation delay of 3 s and the mixing time of 100 ms. Sixty-four free induction decays (FIDs) were collected into 64 K data points using a spectral width of 7002.8 Hz, an acquisition time of 4.68 s and a total pulse recycle delay of 7.68 s. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz prior to Fourier transformation (FT). Two-dimensional (2D) NMR spectra were acquired to enable identification of biomarkers of toxicity. 2D ^{1}H-^{1}H-total correlation spectroscopy (TOCSY) NMR spectra were acquired on selected samples using 64 scans collected into 4 K data points per increment for 256 increments, with F1 and F2 spectral widths of 7002.8 Hz. The FIDs were multiplied by a shifted sine-bell squared function in both dimensions prior to FT. Heteronuclear multiple quantum coherence (HMQC) ^{1}H–^{13}C spectra were acquired for selected samples using 4 K data points in F2 with 128 increments and 840 scans per experiment, using an inversion recovery delay of 440 ms. The F2 spectral width was 7002.8 Hz and the F1 spectral width was 22504.9 Hz . The FIDs were weighted using a shifted sine-bell squared function in both dimensions prior to FT.

Analysis of NMR spectral data

All spectra were phased and base-line corrected in XWINNMR (Bruker GmbH, Karlsruhe, Germany) and data-reduced to 245 integrated regions of 0.04 ppm corresponding to the region δ 10.0 to 0.2 using AMIX (Bruker GmbH, Karlsruhe, Germany). The region δ 6.0–4.5 was set to zero integral for the purposes of pattern recognition analysis value to remove the variability caused in presaturation of the water resonance and cross-relaxation effects on the urea signal. The resulting data table was analysed using principal component analysis (PCA) using Pirouette v2.6 (Infometrix Inc., Woodinville, WA 98072-1528, USA). The region δ 3.32–3.24 was excluded from analysis because of the possibility of metabolite interference from some of the compounds. Each data point was normalized to the sum of its row and all variables were mean centred prior to PCA. Scores plots of the principal components were constructed to visualize any inherent separation of the dose groups, and from the values of the eigenvector loadings (which indicate the weight of each variable contributing to separation) the NMR spectral regions and hence biomarkers were identified. Mean data were calculated for each integrated region for each compound at the same time-period, and analysed using PCA. Plots of the first two principal components (PC1 and PC2) were plotted and mapped to represent a trajectory plot describing the onset of metabolic perturbations with time.

Results

Clinical chemistry and histopathology

Only animals treated with GWA and GWB showed significant weight loss (**$p < 0.01$) following dosing (figure 1(A)). Histologically, rats treated with GWA, GWB or GWC showed reduced liver glycogen. Following treatment with the drug candidates, all animals showed significantly reduced levels of serum triglycerides (**$p < 0.01$) when compared with their respective control group (figure 1(B)). There were no other statistically significant treatment-related changes in any of the clinical chemistry parameters measured.

Only animals treated with GWA were noted to have alterations to their kidney

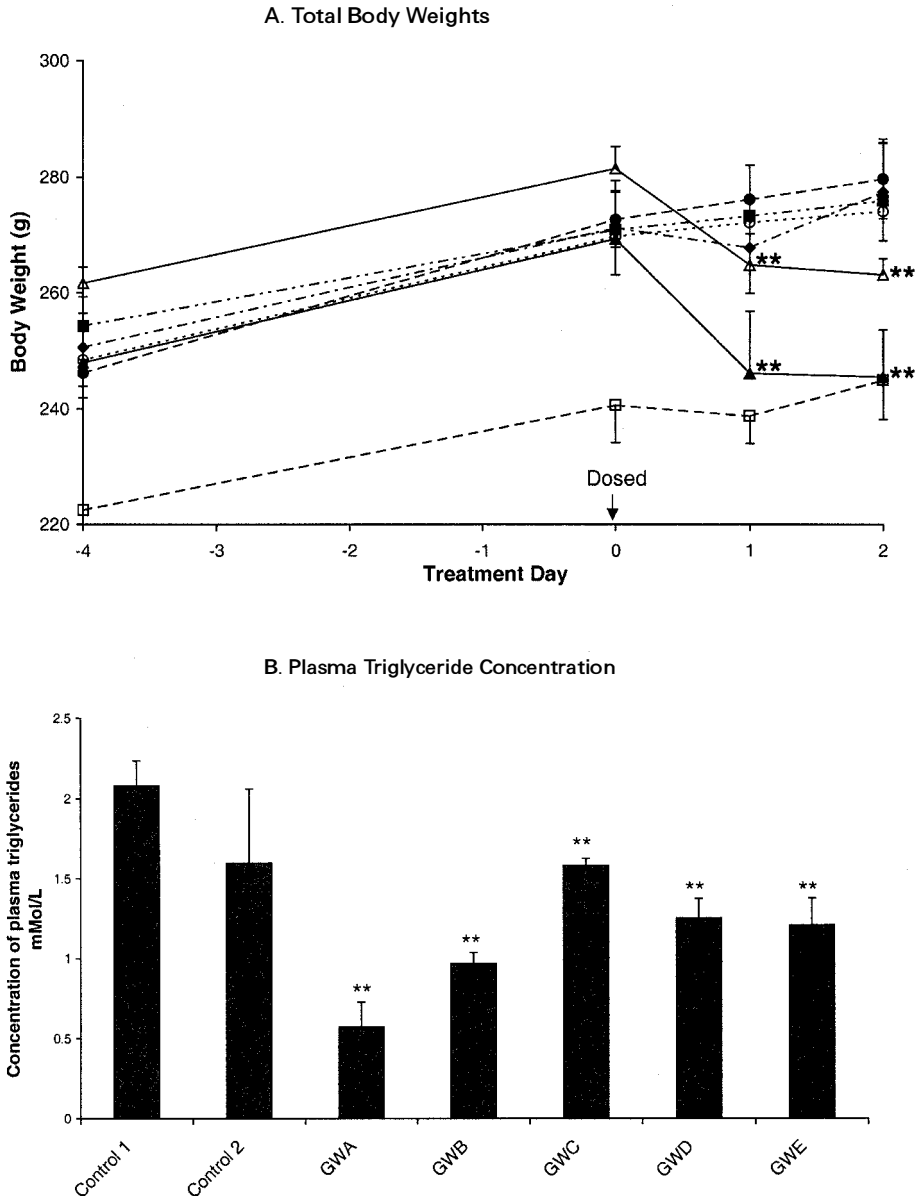

Figure 1. (A) Graph to show animal body weights over the course of the study. Animals dosed with GWA and GWB showed a significant decrease in body weight following dosing (**$p < 0.01$). Key: ■ = Control 1, □ = Control 2, ▲ = GWA, △ = GWB, ● = GWC, ○ = GWD, ◆ = GWE. (B) Bar-chart of the plasma triglyceride concentrations. A significant decrease in triglycerides was noted following dosing of GWA and GWB (**$p < 0.01$).

pathology and this was manifest as a slight cortical tubular dilatation in all three animals. However, the clinical chemistry data did not show any changes in plasma creatinine, blood urea nitrogen or urinary volumes which would have indicated kidney dysfunction.

Animals treated with GWA and GWB had increased numbers of alveolar foamy macrophages, as seen by light microscopy. These findings were confirmed by

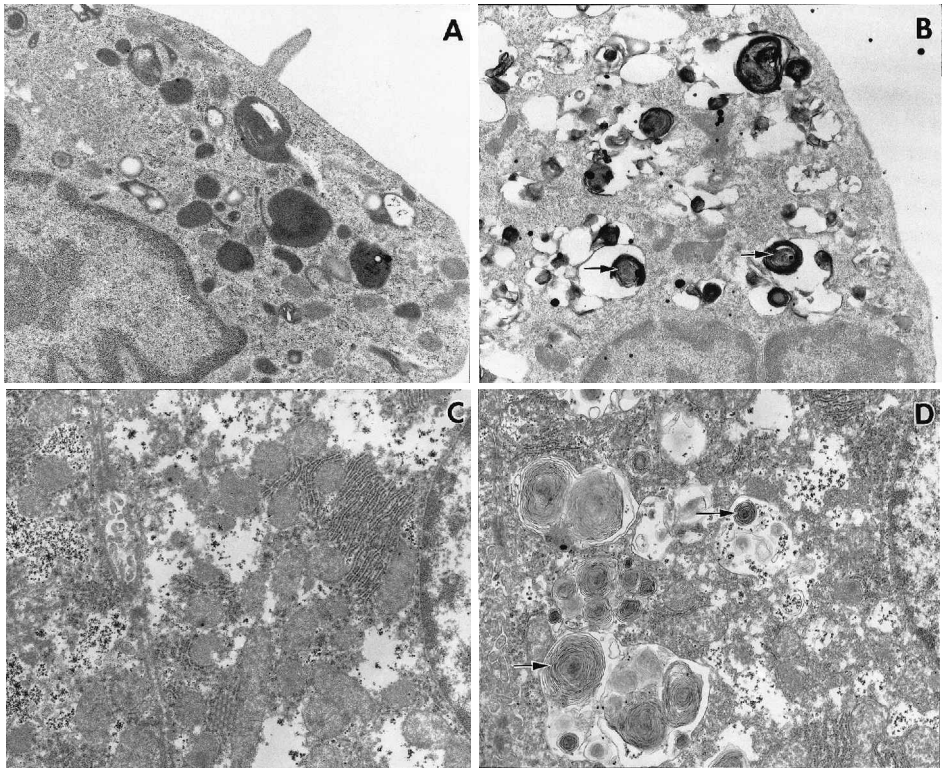

Figure 2. (A) Alveolar macrophage from control animal. (B) Alveolar macrophage showing accumulated multilamellar bodies from treated animal (GWA). (C) Centrilobular hepatocyte from control animal. (D) Centrilobular hepatocyte showing accumulated multilamellar bodies from treated animal (GWA). Arrows denote accumulated multilamellar bodies.

electron microscopy where multilamellar bodies were evident in the alveolar macrophages. There was little evidence of such accumulation in the control animals (figure 2(A and B)) or in animals treated with GWC, GWD or GWE. These effects were not as marked as those seen previously in rats administered GWA or GWB for a 7-day period (unpublished data), where histological lesions, clearly suggestive of widespread phospholipidosis were seen by light microscopy. There was also evidence of multilamellar body accumulation in the livers from animals treated with GWA and GWB when examined by electron microscopy, although this was not identified by light microscopy. The accumulation was apparent in some Kupffer cells from all regions of the liver lobule. Approximately 75% of centrilobular hepatocytes and 10% of periportal hepatocytes in the sections examined from animals treated with GWA showed multilamellar body accumulation. Less than 10% of centrilobular hepatocytes from animals treated with GWB showed multilamellar body accumulation. In the latter livers, no multilamellar bodies were seen in hepatocytes from periportal regions. There was no evidence of phospholipidosis in any cell type in the livers from control animals (figure 2(C and D)) or in animals treated with GWC, GWD or GWE.

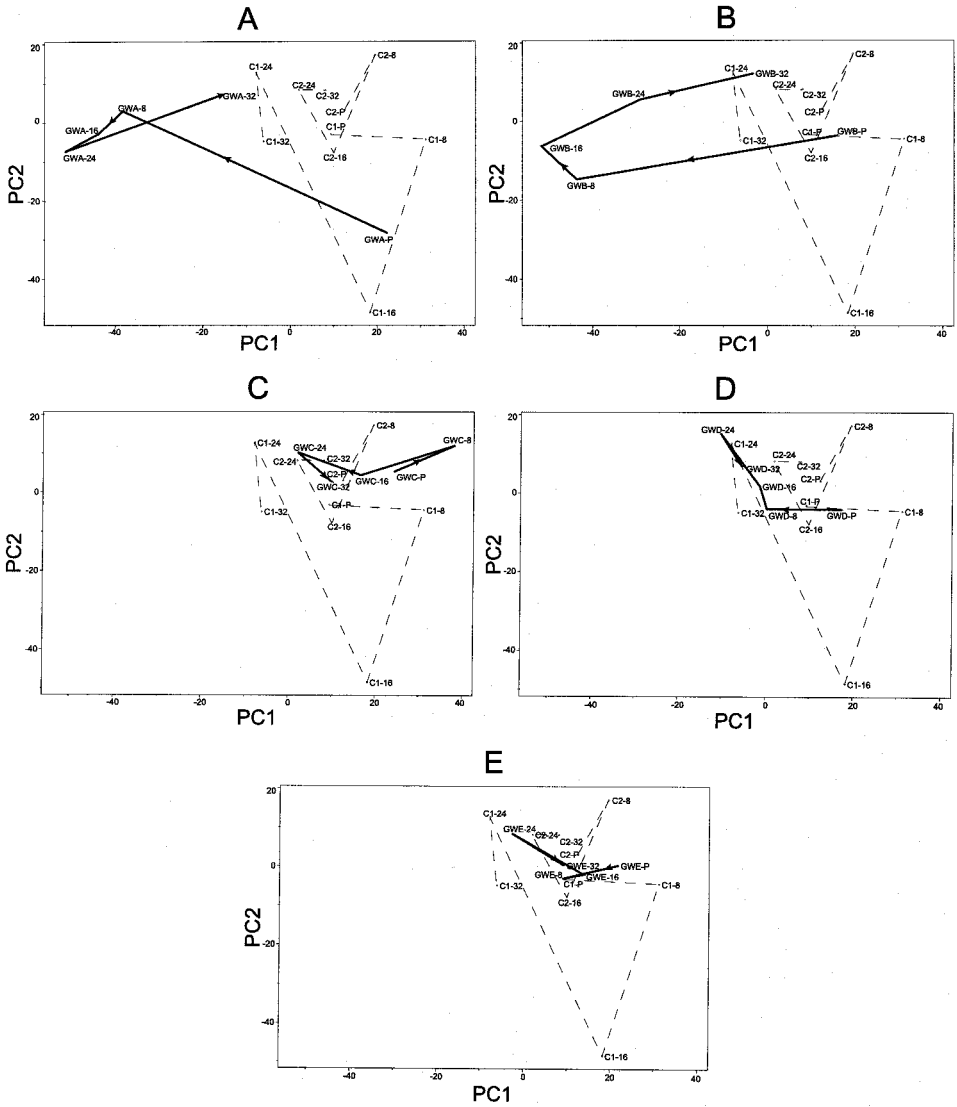

Figure 3. Trajectory plots of the PC1 versus PC2 for the five compounds used in this study versus Control 1 and Control 2. (A) GWA, (B) GWB, (C) GWC, (D) GWD, (E) GWE. P = predose, 8 = 0–8 h, 16 = 8–16 h, 24 = 16–24 h, 32 = 24–32 h. Both GWA and GWB showed a distinct shift in metabolic space after dosing, whereas GWC, GWD and GWE remained in the control region of the plots.

Pattern recognition analysis of urinary NMR spectra

Initially all of the NMR data were analysed using PCA to identify separation between the treatment groups. The scores plot of PC1 vs PC2 (not shown) indicated that the samples from animals dosed with either GWA or GWB were separate from all the other groups. Although these two groups were clearly separate, there was a large data distribution for all groups and, therefore, metabolic trajectory plots were calculated for each compound versus the control groups (Beckwith-Hall *et al.* 1998). The means of each dose group for each time-period

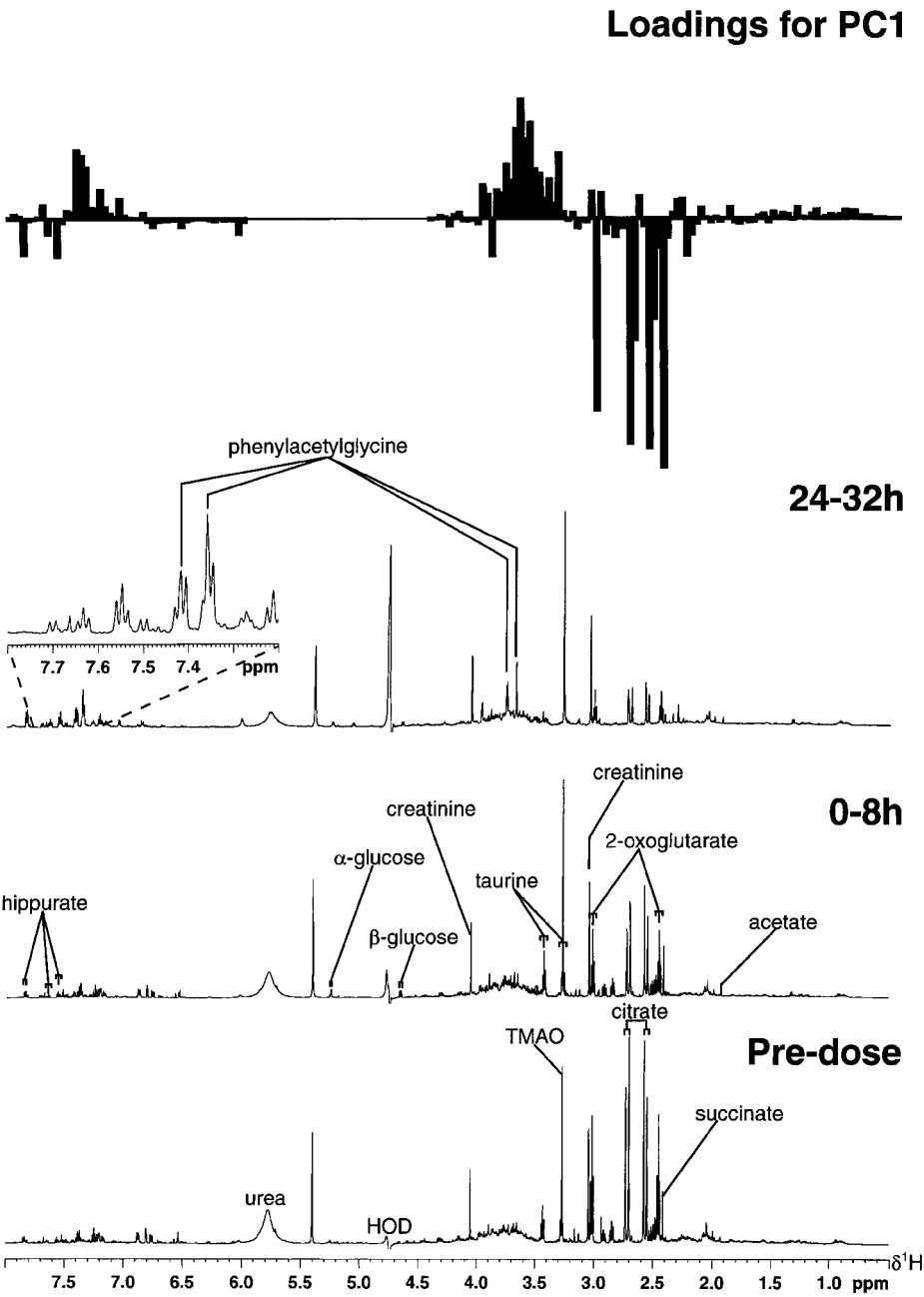

Figure 4. Expansions from the NMR spectra of whole rat urine from an animal administered GWA at predose, 0–8 h and 24–32 h post-dose. Also shown are the loadings for the NMR spectral regions indicating those causing the separation seen in figure 3(A and B) from animals dosed with GWA or GWB. This indicated an elevation in the concentration of phenylacetylglycine and a decrease in the levels of the tricarboxylic acid intermediates citrate and 2-oxoglutarate.

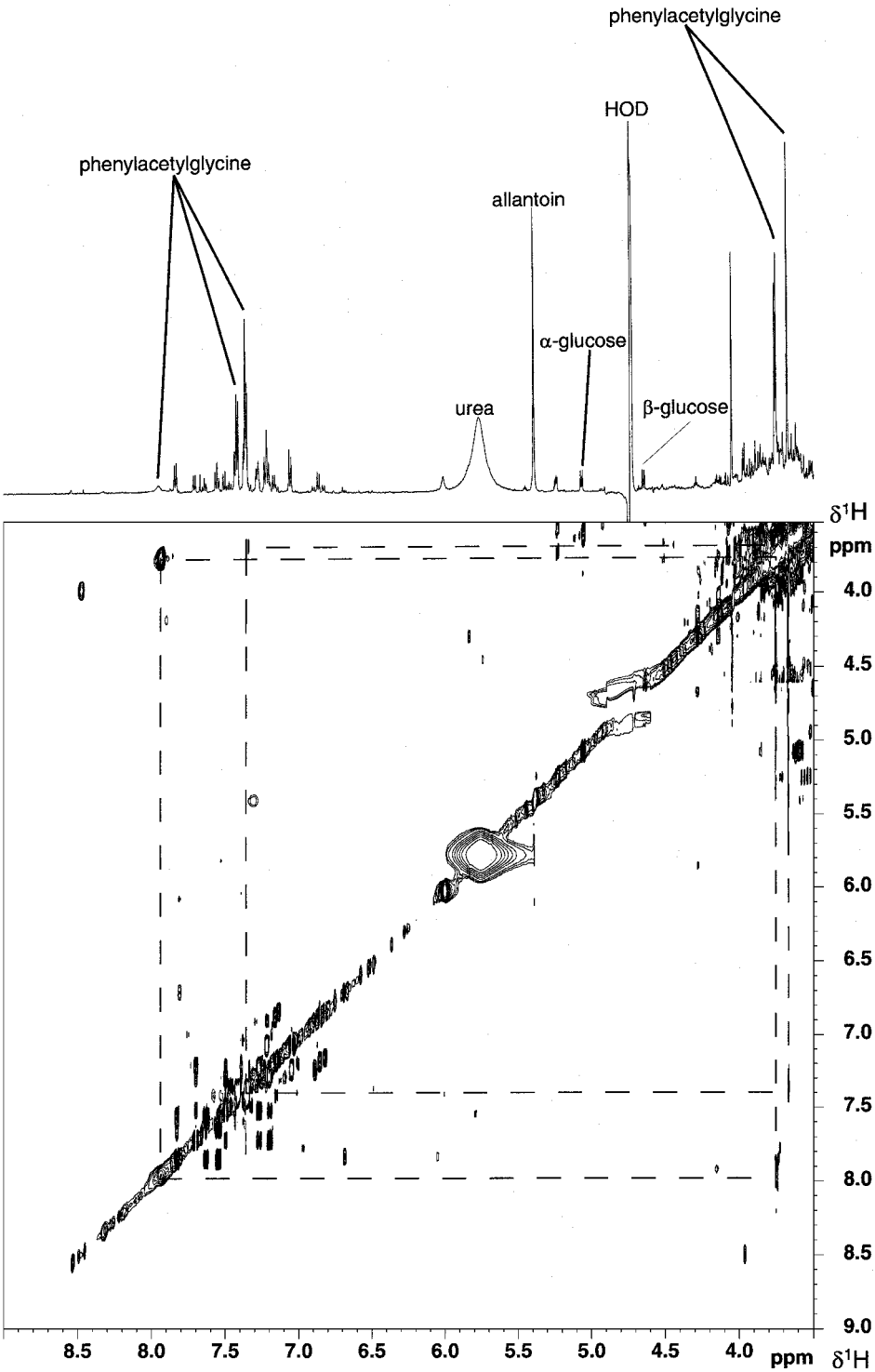

Figure 5. Expansion of the 600 MHz ^{1}H-^{1}H TOCSY NMR spectrum of whole rat urine 24–32 h after dosing of GWB. The resonances for phenylacetylglycine are indicated along with other endogenous urinary metabolites.

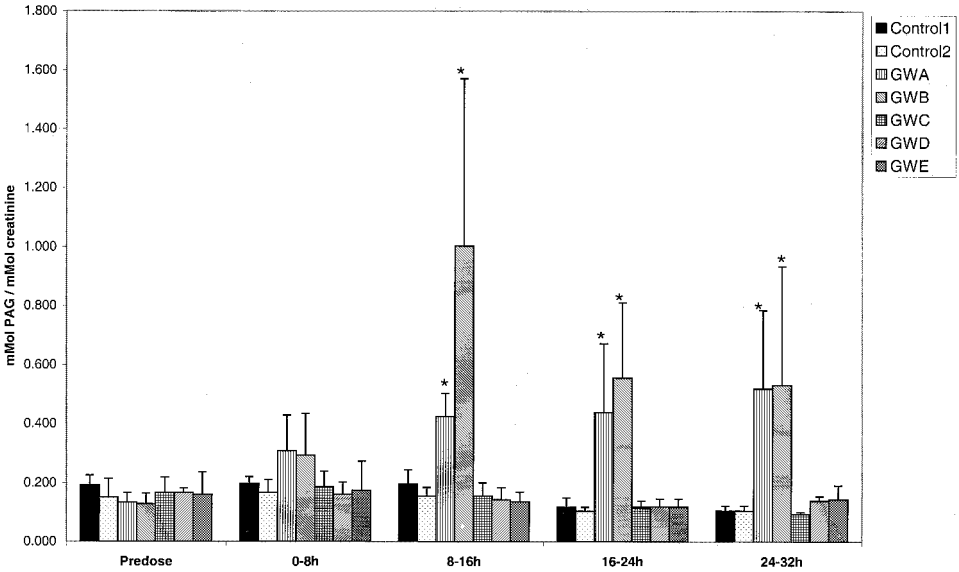

Figure 6. Bar-chart of the mean concentration of phenylacetylglycine (PAG) relative to the concentration of creatinine for all groups at each time-point. *indicates statistically significantly difference from control ($p < 0.05$).

were calculated and analysed using PCA. The first two PCs accounted for 73% of the variation within these original data. The trajectory plots of PC1 vs PC2 (figures 3(A–E)) indicated that those compounds which gave rise to phospholipidosis (i.e. GWA and GWB) showed a distinct shift to the left with time (figure 3(A, B)), whilst the other three compounds remained clustered with the control data (figure 3(C–E)). The effect of dosing with either GWA or GWB was apparent by 8 h with a marked shift in metabolic space from the control region. Maximal separation from control was noted at 16 h for GWB (figure 3(B)) and at 24 h for GWA (figure 3(A)). From 24 h to 32 h post-dose, the trajectories indicated that a metabolic recovery from the effects of GWA and GWB appeared to occur, as the mean data moved back towards the control region of the PCA plot. The samples from Control 2 and GWC–GWE (figure 3(C–E)) showed little movement in metabolic space through the course of the study. However, the mean of the data for group Control 1 showed a distinct shift at 16 h post-dose.

To identify the compounds influencing the observed separation and hence any potential biomarkers of phospholipidosis, the variable eigenvector loadings used to calculate PC1 (figure 4) were analysed to determine those NMR regions influencing the separation. The loadings shown in figure 4 indicated which NMR spectral regions were increasing or decreasing following dosing with GWA or GWB. From analysis of the NMR spectra and comparison with literature data (Lindon *et al.* 1999), the loadings indicated that a decrease in the tricarboxylic acid intermediates citrate (δ 2.70, d; δ 2.55, d) and 2-oxoglutarate (δ 2.45, t; δ 3.02, t) along with an elevation in the previously unassigned urinary metabolite phenylacetylglycine (PAG), were responsible for the separation of the GWA and GWB samples from the remaining data. PAG was assigned to the resonances at δ 7.42, 7.35, 7.35, 3.75 and 3.67 based on analysis of the 2D ^{1}H-^{1}H TOCSY NMR spectrum of whole rat urine after GWA administration (figure 5) and confirmed by comparison with the

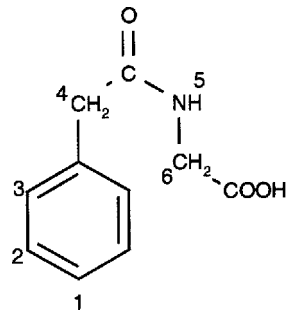

Table 1. The structure and chemical shifts of phenylacetylglycine based on 2D NMR spectroscopic data.

	1	2	3	4	5	6
^{1}H chemical shift/δ ppm	7.35	7.42	7.35	3.67	7.95	3.75
^{13}C chemical shift/δ ppm	127	127	127	41	–	42

NMR spectrum of a synthetic standard (table 1). ^{1}H-^{13}C-HMQC NMR spectra of the urinary sample and standard were also acquired and analysed (spectra not shown), to enable assignment of the ^{13}C resonances for PAG (table 1). Examination of the NMR spectra from all animals dosed with either GWA or GWB indicated an elevation in the urinary concentration of PAG. Although the resonances from PAG were visible in the NMR spectra of the later time points, the variation in the 0–8 h sample could not be easily distinguished. From analysis of the loadings contributing to PC2 (not shown) the separation of the Control 1 group at 16 h was attributed to a pH shift in the citrate resonances.

The concentration of PAG was measured relative to the concentration of creatinine by integration of the triplet at δ 7.42 from PAG to the singlet (δ 4.05) for creatinine. Figure 6 shows the bar-chart of the mean concentration and the standard deviation for PAG in each group at each time-point. There was a substantial increase in the concentration of PAG at all time-points following administration of GWA or GWB which was not observed following administration of GWC, GWD or GWE. The logarithm of each value was calculated and an unpaired *t*-test was used to compare the means of the dosed animals to the appropriate control. This indicated that were was a statistically significant ($p<0.05$) increase in PAG at all time-points after 8 h.

Discussion

This study has shown that the NMR-based metabonomic approach to drug toxicity evaluation (Nicholson *et al.* 1999) provides a powerful tool for the identification of subtle biochemical changes found in drug-induced phospholipidosis. Detection of phospholipidosis appears to be extremely difficult by conventional biochemical procedures and relies on histopathological studies. However, in the present study we have identified a candidate urinary biomarker for phospholipidosis, namely PAG, by statistical analysis of NMR spectral data.

The reduction in serum triglycerides coupled with animal weight loss observed for all compounds was probably due to reduced food intake. However, there may

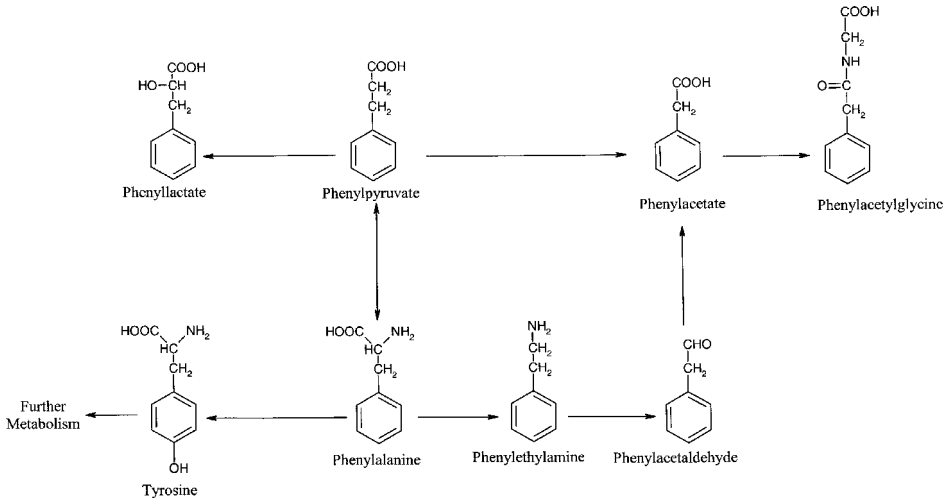

Figure 7. The metabolism of phenylalanine, showing the formation of phenylacetylglycine.

also have been a specific drug-related effect. CADs which cause phospholipidosis are known to affect lipid metabolism by inhibiting phosphatidate phosphohydrolase (EC 3.1.3.4), leading to the over production of cytidine diphosphate-diacyl glycerol. This also results in raised levels of phosphatidylinositol and phosphatidyl glycerol (Brindley and Bowley 1975). The consequent reduction in triglyceride production may be an additional reason for the reduced plasma triglyceride levels in the study. The compounds administered in these studies may have some of the same characteristics.

Electron microscopy confirmed the presence of multilamellar bodies in alveolar macrophages and in the liver of animals treated with GWA and GWB. PAG was observed as a potential major urinary biomarker of phospholipidosis, but the mechanism of formation remains unclear. PAG is formed by the conjugation of phenylacetyl-CoA with glycine (Jones 1982). However, the source of the phenylacetate required for formation of phenylacetyl-CoA remains unclear. Two potential routes exist for formation of phenylacetate *in vivo*, the β-oxidation of phenyl containing fatty acids and the metabolic formation from phenylalanine. Knoop originally showed that β-oxidation was a two-carbon degradation process in which acids with an even number of carbons in the chain were excreted as phenylacetate (Stryer 1988). However, this study relied on dosing of phenylbutyrate in the dog rather than as a measure of the endogenous components of urine. If this route were responsible for the formation of phenylalanine and hence PAG, it would require a source of phenyl containing-fatty acids and to date such a source has not been reported, although they may be dietary derived. Phenylalanine can be degraded to phenylacetate via both phenylethylamine and phenylacetaldehyde or via phenylpyruvate (figure 7). This latter route is observed in sufferers of the inborn error of metabolism, phenylketonuria (Lehninger *et al.* 1993). In this condition, the normal hydroxylation of phenylalanine to tyrosine does not occur due to a defect in the phenylalanine 4-monoxygenase (EC 1.14.16.1). Further work is needed to determine whether this enzyme is being inhibited in cases of phopholipidosis. Verification of the presence of urinary PAG following dosing with previously reported phospholipidosis causing CADs is

required, so as to determine whether the induction of phenylalanine is due to phospholipidosis or to some other effect of the CADs.

Studies in animals have also shown an elevation in urinary phenylacetate in germ-free rats feed with food contaminated with faeces from specific pathogen-free rats (Goodwin *et al*. 1994). This study indicated that the gut flora contributed to the final levels of phenylacetate excreted. Furthermore, studies in man have shown an elevation in plasma phenylacetate and its conjugates following fasting (Davis *et al*. 1982). In the current study, the animals which received GWA or GWB showed a weight loss attributed to a reduction in food consumption. This reduced consumption may have altered the gut environment and hence affected the gut flora, leading to an alteration in the concentration of urinary phenylacetate and its conjugates. However, further work is needed to confirm the concentration of urinary phenylacetate in fed and fasted rats. Phenylacetate has also been shown to be a major plasma and urinary marker of patients suffering depression with a number of studies reporting a reduction in plasma and urinary concentrations (Sabelli *et al*. 1983, Karoum *et al*. 1984, Davis *et al*. 1994). This effect was also observed in sufferers of bulimia nervosa, but was shown not to be related to the patients' eating behaviour (Davis *et al*. 1994). In the current study, we have used NMR spectroscopy to detect and identify PAG in animals suffering a mild phospholipidosis. This work has indicated that an elevation in PAG may be a potential biomarker for phospholipidosis, but further work is needed to conclusively link the marker to the effect. This work has also shown that by exploiting the simple visualization of NMR spectral data using metabonomics techniques it is possible to rapidly identify toxicological effects arising from administration of novel drug candidates.

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