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^{1}H-Nuclear magnetic resonance pattern recognition studies with *N*-phenylanthranilic acid in the rat: time- and dose-related metabolic effects

REBECCA E. WILLIAMS[1]*, LISA COTTRELL[1], MATTHEW JACOBSEN[1], LASANTHA R. BANDARA[2], MIKE D. KELLY[2], SANDY KENNEDY[2] and EDWARD A. LOCK[1]

[1] Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK
[2] Oxford GlycoSciences (UK) Ltd, The Forum, 86 Milton Park, Abingdon, Oxon, OX14 4RY, UK

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N-Phenylanthranilic acid (NPAA) causes renal papillary necrosis (RPN) in the rat following repeated oral dosing. Non-invasive early detection of RPN is difficult, but a number of potential biomarkers have been investigated, including phospholipid and uronic acid excretion. This study used ^{1}H-nuclear magnetic resonance (NMR) spectroscopic analysis of urine to investigate urinary metabolic perturbations occurring in the rat following exposure to NPAA. Male Alderley Park rats received NPAA (300, 500 or 700 mg kg^{-1} day^{-1} orally) for 7 days, and urine was collected on days 7–8, 14–15, 21–22 and 28–29. In a separate study, urine was collected on days 1–2, 3–4, 5–6 and 7–8 from rats receiving 500 mg kg^{-1} day^{-1}. Samples were analysed by ^{1}H NMR spectroscopy combined with multivariate data analysis and clinical chemistry. Histopathology and clinical chemistry analysis of terminal blood samples was carried out following termination on days 4, 6, 8 and 29 (4 week time course) and days 2, 4, 6 and 8 (8 day study). Urine analysis revealed a marked, though variable, excretion of β-hydroxybutyrate, acetoacetate and acetone (ketone bodies) seen on days 3–4, 5–6 and 7–8 of the study. It is postulated that the ketonuria might be secondary to an alteration in fatty acid metabolism due to inhibition of prostaglandin synthesis. In addition, an elevation in urinary ascorbate was observed during the first 8 days of the study. Ascorbate is considered to be a biomarker of hepatic response, probably reflecting an increased hepatic activity due to glucuronidation of NPAA.

Keywords: renal papillary necrosis, ketonuria, *N*-phenylanthranilic acid.

Abbreviations: BEA, 2-bromoethanamine, BUN, blood urea nitrogen, DMG, dimethylglycine, MFA, mefenamic acid, NMR, nuclear magnetic resonance, NPAA, *N*-phenylanthranilic acid, NSAID, non-steroidal anti-inflammatory drug, PCA, principal component analysis, PG, prostaglandin, PI, propyleneimine, RPN, renal papillary necrosis, TMAO, trimethylamine-*N*-oxide, TSP, sodium 3(trimethylsilyl)propionate-2,2,3,3-d_4.

Introduction

A major cause of renal papillary necrosis (RPN) in humans is long-term exposure to analgesics and non-steroidal anti-inflammatory drugs (NSAIDs), including amidopyrine, phenacetin, indomethacin, ibuprofen, diclofenac, and mefenamic acid (MFA) (Bach and Bridges 1985, Bach and Hardy 1985, Bach and Thanh 1998, Brix 2002). RPN is difficult to detect in the early stages of the

* Corresponding author: Rebecca E. Williams, Department of Drug Metabolism and Pharmacokinetics, AstraZeneca, Alderley Park, Macclesfield, Cheshire, SK10 4TG, UK. Tel: (+44) 1625 514653/1625 510341; fax: (+44) 1625 516962; e-mail: becky.williams@astrazeneca.com

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disease and usually the lesion only becomes clinically apparent once secondary cortical changes are present (Brix 2002). Decreased glomerular filtration rate, polyuria, increased blood urea nitrogen (BUN) and renal tubular acidosis have been reported; however, these are not specific to RPN and occur after pathology is detected (Hardy and Bach 1984, Bach and Thanh 1998).

Several chemicals have been reported to be papillotoxins in animals, allowing RPN to be investigated experimentally, including ethyleneimine, propyleneimine (PI), 2-bromoethanamine (BEA), MFA and *N*-phenylanthranilic acid (NPAA) (Hardy 1970, Hardy and Bach 1984, Bach and Bridges 1985, Gartland *et al*. 1989, Wolf *et al.* 1992). NPAA, a biphenyl analogue of fenamic acid, causes RPN when given to rats (0.1–5 mmol kg^{-1} orally for 14 days), affecting up to 30% of the papilla and leading to associated secondary cortical changes (Hardy 1970, Hardy and Bach 1984). Degeneration of medullary interstitial cells occurs initially, followed by damage to the endothelial cells, the loops of Henle and the collecting ducts (Gregg *et al.* 1990, Thanh *et al.* 2001a). Renal accumulation of lipids and proteoglycans has also been reported (Gregg *et al.* 1990, Bach *et al*. 1991).

A number of potential biomarkers have been investigated in animals for the early detection of RPN. Increased excretion of sphingomyelin, phosphatidylcholine and phosphatidylethanolamine was observed in rats following exposure to NPAA and MFA (500 or 300 mg kg^{-1} day^{-1}, respectively, for 5 days followed by 2 days off then a further four daily doses) (Thanh *et al.* 2001a). Lipid excretion increased 48 h post-dosing; thus perturbed lipid excretion was detected at an early stage in the development of the lesion. Increased urinary phospholipids have been reported in rats exposed to BEA (Thanh *et al.* 2001b), further suggesting that lipid excretion may be a useful biomarker for RPN. Additionally, increased uronic acid has been reported, occurring prior to the detection of RPN by histopathology, suggesting its use as a biomarker (Nguyen *et al.*, 2001). Increased uronic acid may reflect renal proteoglycan accumulation.

Several groups have reported the use of ^{1}H-nuclear magnetic resonance (NMR) spectroscopy and pattern recognition studies of urine to identify biomarkers characteristic for organ-specific toxicity in laboratory animals. For example, elevated urinary taurine has been identified as a specific marker for hepatotoxicity (Nicholls *et al.* 2001) and creatine as a marker for testicular toxicity (Nicholson *et al.* 1989). Furthermore, in the kidney it has been possible to distinguish urine samples based on site-specific injury (Gartland *et al.* 1989, Holmes *et al.* 1992). Papillotoxicity, induced by BEA and PI, has been characterized by an early increase in urinary trimethylamine-*N*-oxide (TMAO), dimethylamine, methylamine and betaine accompanied by a decline in dimethylglycine (DMG), citrate and α-ketoglutarate (Gartland *et al.* 1989, Holmes *et al.* 1992, Holmes *et al.* 1995a, b, 1997). Additionally, increased urinary excretion of glutaric and adipic acid has been reported following a single dose of BEA in rats (Holmes *et al.* 1997).

In this study we examined the urinary metabolic perturbations that occur following exposure to NPAA using ^{1}H NMR spectroscopy in combination with pattern recognition in order to define metabolic changes associated with RPN. Initially, a 4 week time course was studied, with urine samples collected every 7 days. Following the identification of metabolic perturbations on day 7–8 of this

study, a subsequent 8 day study was performed, with urine samples collected every 2 days. The second study aimed to focus on the onset of these metabolic perturbations and any correlation there may be with the development of the lesion.

Materials and methods

Animals

Male (210–230 g) Alderley Park (Wistar-derived) rats were obtained from the animal breeding unit, Alderley Park, Cheshire, UK, and acclimatized for 6 days prior to the start of each study. The animals were housed five per cage except for the duration of urine collection, when rats were housed individually in metabolism cages. Controlled humidity (30–70%), temperature ($22 \pm 3^{\circ}$C) and a 12 h light/dark cycle were maintained. The animals' bodyweight and clinical observations were recorded at the start of the study, on each day of dosing, and immediately prior to termination. All animals had free access to food (RM-1 diet, Special Diet Services Essex, UK) and water throughout the study, except for the withdrawal of food whilst the animals were in the metabolism cages. Animal procedures were performed in accordance with licenses issued under the animals (Scientific Procedures) Act, 1986.

Treatments

Two separate studies were conducted. Day 1 is defined as the first day of dosing.

Four week time course. Eighty animals were dosed with either NPAA (300, 500 or 700 mg kg^{-1} day^{-1}, 10 ml kg^{-1} orally; $n = 20$ per dose group) or vehicle (corn oil, 10 ml kg^{-1} orally; $n = 20$) for 7 days. Five animals per dose group were killed on days 4, 6, 8 and 29 of the study, with urine samples being collected over dry ice from 15:00 h to 09:00 h on days 7–8, 14–15, 21–22 and 28–29 from the five animals per experimental group that were killed on day 29. The rats were killed by exposure to halothane followed by exsanguination. Terminal blood samples were taken from the heart into heparinized tubes and the plasma separated by centrifugation (3000 *g* at 4°C for 10 min). Plasma was submitted for clinical chemistry analysis and the right kidney was removed for histopathological examination.

Eight day time course. Forty animals were dosed with either NPAA (500 mg kg^{-1} day^{-1}, 10 ml kg^{-1} orally; $n = 32$) or vehicle (corn oil, 10 ml kg^{-1} orally; $n = 8$) for 7 days. Eight animals per experimental group and two per control group were killed on days 2, 4, 6 and 8, with urine samples being collected over dry ice from each animal immediately prior to their scheduled termination (from 15:00 h to 09:00 h on days 1–2, 3–4, 5–6 and 7–8). The rats were killed as described above.

Clinical chemistry and histopathology

Urine volume was measured and the urine was analysed for specific gravity and total protein content. Creatinine and BUN concentrations were analysed in plasma. Measurements were made using a Konelab 60i instrument (Labmedics Cheshire, UK) using standard assay kits supplied by Labmedics.

The right kidney was weighed and a transverse section, including the papilla tip, was fixed in 10% neutral buffered formalin, embedded in paraffin wax, and 5 μm thick sections were cut and stained with haematoxylin and eosin for histopathological assessment.

^{1}H NMR spectroscopic analysis of urine

Aliquots of urine (500 μl) were mixed with phosphate buffer (250 μl, 0.2 M, pH 7.4, in D_2O) containing sodium 3(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP) (0.5 mg TSP ml^{-1} buffer) (Holmes *et al.* 1998) and centrifuged at 14 000 *g* for 10 min at 4°C. The supernatants (600 μl) were placed into a 5 mm NMR tube (Wilmad).

^{1}H NMR spectra were acquired using a Bruker DPX400 spectrometer operating at 400.13 MHz for ^{1}H. The standard 'noesypr1d' pulse sequence (Bruker Analytik GmbH, Germany) was utilized for data acquisition, which efficiently suppresses the large water signal. A total of 64 free induction decays were accumulated into 64K data points with a spectral width of 8012.82 Hz, an acquisition time of 4.09 s and an interpulse delay of 2 s. A line broadening of 0.3 Hz was applied and all spectra were manually phase- and baseline-corrected.

For the quantification of ascorbate (4.52 p.p.m.), β-hydroxybutyrate (1.22 p.p.m.) and glucose (5.25 p.p.m.), the concentration of each metabolite was calculated with reference to TSP (the internal standard). Each concentration was expressed relative to the creatinine concentration determined by clinical chemistry. Urinary creatinine was not affected by exposure to NPAA.

Automated data reduction and principal component analysis (PCA) of ^{1}H NMR spectra

Using the AMIX software package (version 2.7.5, Bruker Analytische, Germany), each spectrum was segmented into regions of 0.04 p.p.m. (from 0.2 p.p.m. to 10.0 p.p.m.; 256 segments) and the integral value for each segment was calculated (Beckwith-Hall *et al*. 1998, Holmes *et al*. 1998). The regions of the spectrum associated with water and urea (4.5–5.05 p.p.m. and 5.5–6.05 p.p.m., respectively) were removed, as were those arising from NPAA and NPAA metabolites (3.56–3.76 p.p.m., 3.88–3.96 p.p.m., 6.72–7.00 p.p.m., 7.08–7.48 p.p.m. and 8.00–8.16 p.p.m.). The segmented data were exported into Microsoft Excel (version 7.0a), where the integral values were scaled to the total of the summed integrals of each spectrum in order to partially compensate for differences in urinary dilution. The scaled data were then imported into SIMCA (version 10.0.4; Umetrics, Sweden) for PCA. For this analysis the data set was mean-centred.

Statistical analysis

The data were expressed as the mean $\pm$SD. Significant differences were determined using a one-way analysis of variance followed by the Bonferroni test. A corrected p value < 0.05 was considered to be statistically significant.

Results

^{1}H NMR spectroscopic and PCA analysis of urine – 4 week time course

Typical ^{1}H NMR spectra of urine collected following dosing with NPAA are shown in figure 1. NPAA-related metabolites, expected to be NPAA and NPAA glucuronide, were identified in urine samples in both the aromatic region (data not shown) and between 3.0–4.0 p.p.m. (figure 1B, C) in all treated urine samples on days 7–8. These samples were bright yellow, causing interference with the assay used to measure glucose, so the glucose concentration was determined from the ^{1}H NMR spectra. Major perturbations in endogenous metabolite levels were also seen in urine collected on days 7–8, including increased β-hydroxybutyrate, acetoacetate and acetone (figure 1B). These metabolic alterations were not consistent throughout all of the animals (figure 1C), as discussed later in this section.

PCA of the data set, following removal of the spectral regions containing NPAA and NPAA glucuronide, revealed a marked clustering of treated samples away from the control on days 7–8 of the study (figure 2A). The metabolites responsible for the separation were β-hydroxybutyrate, acetoacetate and acetone. The large spread of data demonstrates the variability observed in the excretion level of these metabolites. This is further demonstrated in figure 2C following quantification of β-hydroxybutyrate. Removal of the spectral regions containing β-hydroxybutyrate, acetoacetate and acetone followed by PCA yields the scores plot shown in figure 2B. The data for these metabolites were removed in Excel and the data rescaled prior to PCA. Separation is still evident, including a dose-related effect: the 300 mg kg^{-1} day^{-1} data clusters between the control samples and those exposed to 500 and 700 mg kg^{-1} day^{-1}. The metabolites responsible for the separation were elevated glucose, lactate and ascorbate, and decreased TMAO, DMG, citrate, succinate and α-ketoglutarate (figure 1). The effect on these metabolites was dose-related, as can be seen following the quantification of glucose (figure 2D). PCA using Pareto and unit variance scaling was investigated but no further changes were identified.

NPAA and NPAA glucuronide were not present in urine samples collected on days 14–15, 21–22 and 28–29 after cessation of dosing. PCA of the data set acquired from urine samples collected on days 14–15 (data not shown) and 28–29

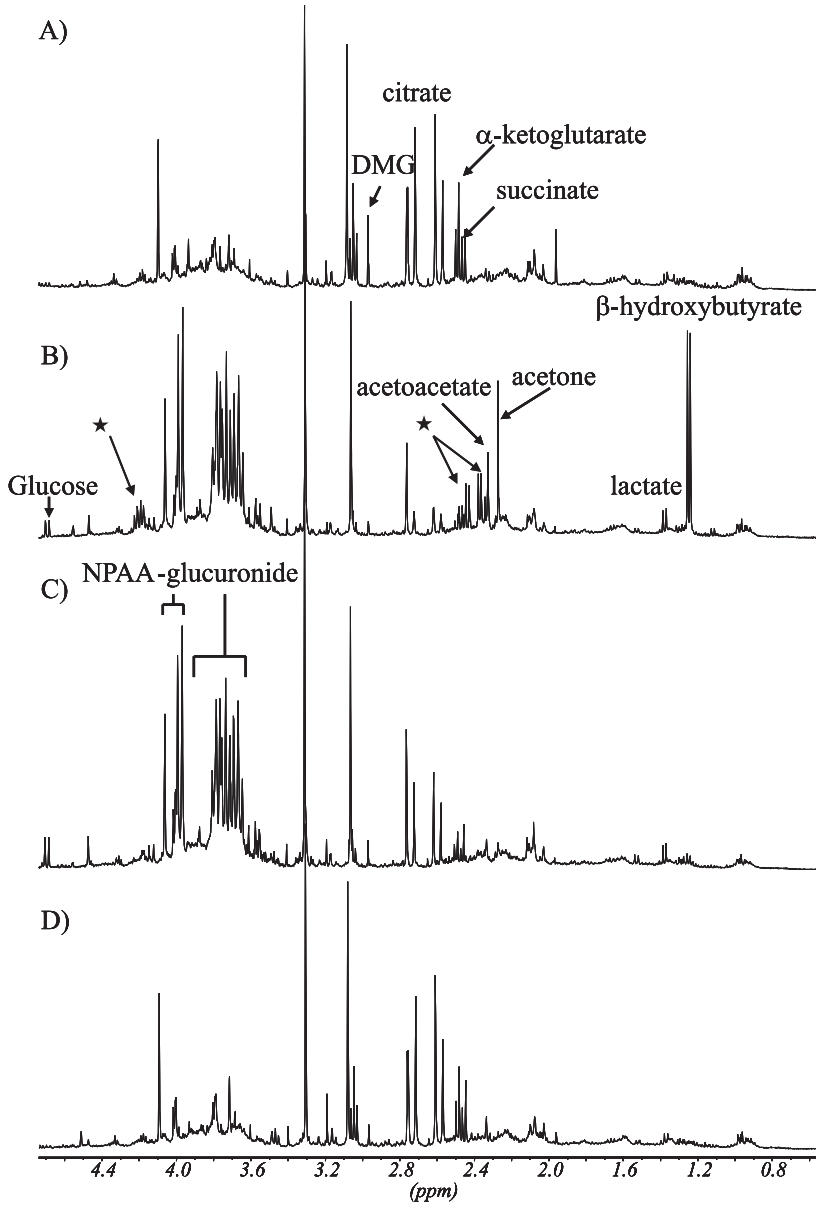

Figure 1. ^{1}H NMR spectroscopy – 4 week time course. ^{1}H NMR spectra (0.5–4.7 p.p.m.) of urine collected from (A) control rat (days 7–8); (B) and (C) rats treated with NPAA 700 mg kg^{-1} day^{-1}, days 7–8; and (D) rat treated with NPAA 700 mg kg^{-1} day^{-1}, days 28–29. *Other resonances attributed to β-hydroxybutyrate at 2.31, 2.41 and 4.16 p.p.m.

(figure 1D) were indistinguishable from the controls. However, treated urine samples collected on days 21–22 clustered away from the control data set following PCA due to an elevation of taurine (data not shown). Integration of taurine demonstrated a 2.5-fold increase in the urine of animals exposed to NPAA at 700 mg kg^{-1} day^{-1} compared with control animals at this time.

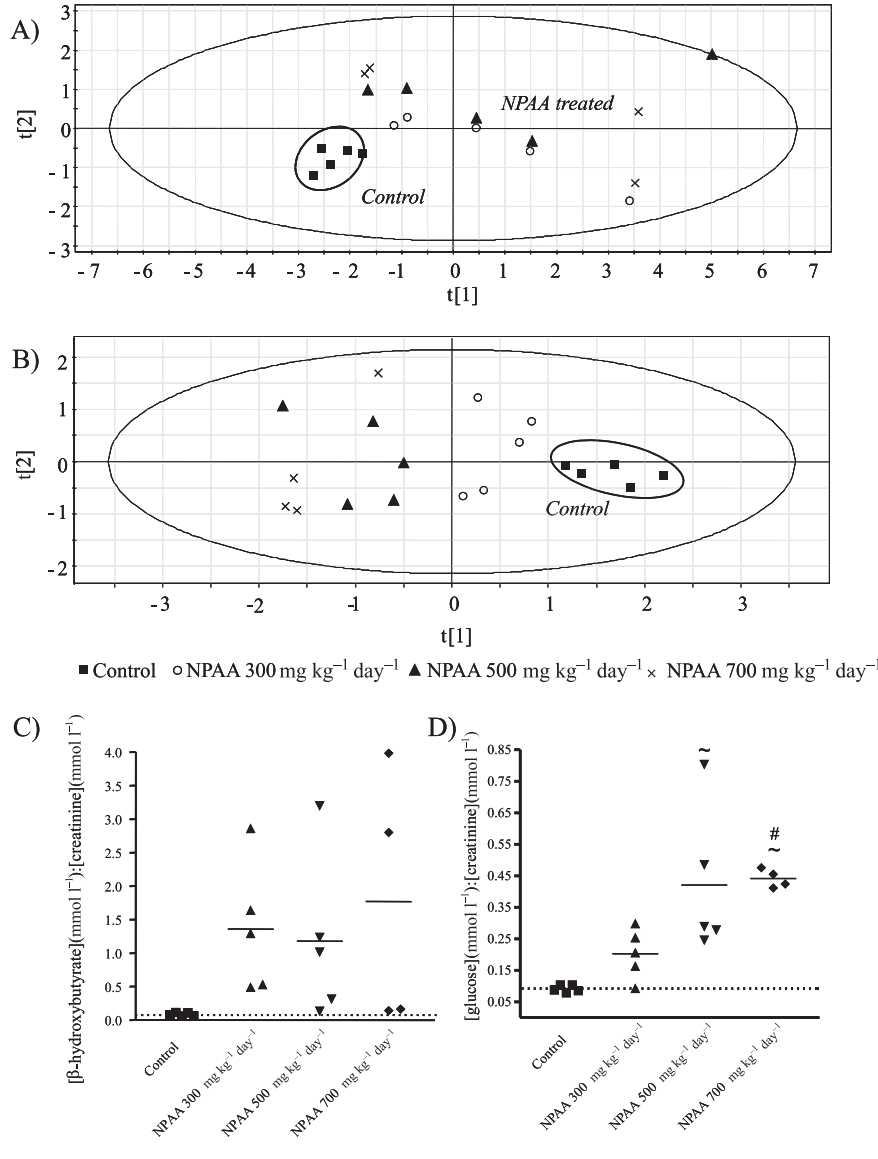

Figure 2. PCA and metabolite quantification – 4 week time course. (A) and (B) Scatter plots of the first two PCA scores (t[1] versus t[2]) obtained from ^{1}H NMR spectra of urine collected overnight on days 7–8 of the study following removal of spectral regions containing NPAA and NPAA glucuronide (A) and with the additional removal of spectral regions containing β-hydroxybutyrate, acetoacetate and acetone (B). (C) and (D) Amount of β-hydroxybutyrate (C) and glucose (D) (expressed as mmol l^{-1} relative to urinary creatinine concentration in mmol l^{-1}) in urine samples collected overnight on days 7–8 of the study. $\sim p < 0.05$ compared with control; # $P < 0.05$ compared with NPAA 300 mg kg^{-1} day^{-1}.

Clinical chemistry, body weight and histopathology – 4 week time course

Animals that received the highest dose of NPAA (700 mg kg^{-1} day^{-1}) showed a significantly lower body weight compared with all other groups on days 2, 4 and 7, which recovered with the cessation of dosing by day 29 (figure 3A).

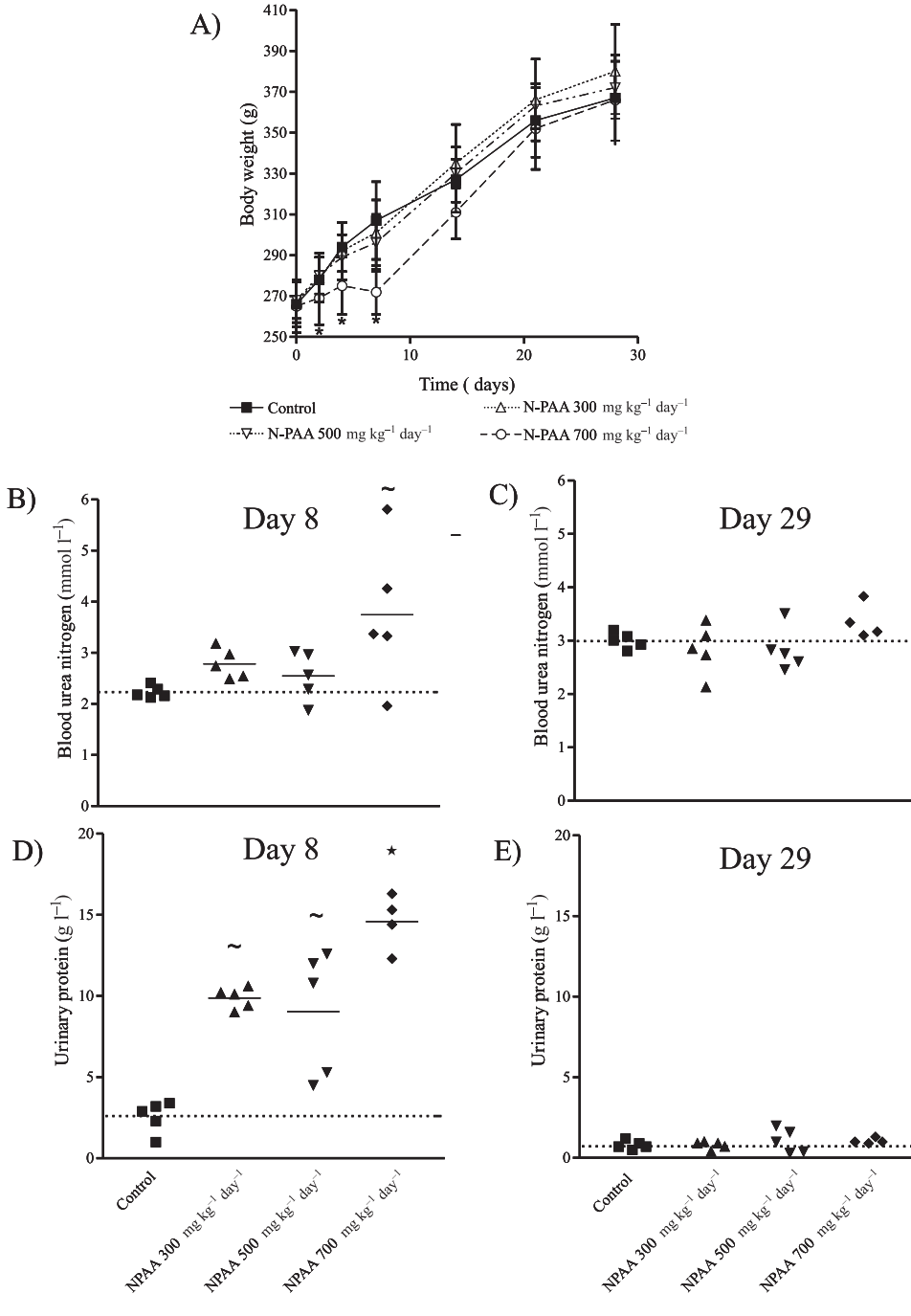

Figure 3. Body weight and clinical chemistry – 4 week time course. (A) Body weight recorded for each animal (number varies as groups reach their scheduled termination). (B) and (C) BUN in terminal blood samples obtained on days 8 and 29. (D) and (E) Urinary protein in samples collected overnight on days 7–8 and 28–29. The mean value of each group is shown as a horizontal line in the graphs for days 7–8, the mean control value is shown as a dashed line in all graphs $^{\star}p < 0.05$ compared with control and NPAA 300 and 500 mg kg^{-1} day^{-1}; $\sim p < 0.05$ compared with control.

There was a significant increase in BUN on day 8 in animals that received 700 mg kg^{-1} day^{-1} NPAA (figure 3B). At all other time points and doses there was no change in BUN (figure 3C). Plasma creatinine appeared to increase in the same animals; however, the increase was not significant (data not shown).

Clinical chemistry analysis of urine revealed an increase in urinary protein on days 7–8 for all treated samples, with the increase being more pronounced at the highest dose (figure 3D); by days 14–15, 21–22 and 28–29 there was no difference in urinary protein (figure 3E). No difference in urinary volume or specific gravity was observed at any time point (data not shown).

There was no change in kidney weight for any of the animals. The histopathological findings are presented in table 1 and figure 4. RPN was not observed in animals exposed to NPAA at 300 mg kg^{-1} day^{-1}, whilst at doses of 500 and 700 mg kg^{-1} day^{-1} RPN was observed in three out of five animals by day 8. Tubular basophilia of the cortical tubules was observed in four out of five animals given 500 or 700 mg kg^{-1} day^{-1} by day 29, by which time the urinary metabolic profile had returned to normal.

^{1}H NMR spectroscopic and PCA analysis of urine – 8 day time course

Resonances arising from NPAA and NPAA glucuronide were present in spectra obtained from urine samples collected overnight on days 1–2, 3–4, 5–6 and 7–8 from rats dosed with 500 mg kg^{-1} day^{-1} NPAA. Furthermore, perturbations to

Table 1. Outcome of the histopathological examination of the right kidney taken from animals treated with NPAA at 300, 500 or 700 mg kg^{-1} day^{-1}. The number of animals affected and the extent of the injury are shown at each time point for each dose ($n = 5$ per group except 700 mg kg^{-1} on day 29, where $n = 4$).

		300 mg kg^{-1}		500 mg kg^{-1}		700 mg kg^{-1}	
Histopathological finding	Day	Minimal	Moderate	Minimal	Moderate	Minimal	Moderate
Eosinophilia/basophilia of medullary collecting ducts	4	–	–	4	–	1	–
	6	1	–	3	1	3	–
	8	2	–	3	2	3	2
	29	–	–	–	–	–	–
Papillary necrosis	4	–	–	1	–	–	–
	6	–	–	1	–	–	–
	8	–	–	3	–	3	–
	29	–	–	–	–	–	–
Tubular dilation	4	–	–	3	–	–	–
	6	–	–	1	1	3	–
	8	–	–	2	2	2	1
	29	–	–	2	–	1	–
Tubular basophilia of cortical tubules	4	–	–	–	–	2	–
	6	–	–	2	–	2	–
	8	–	–	–	–	–	–
	29	–	–	4	–	3	1

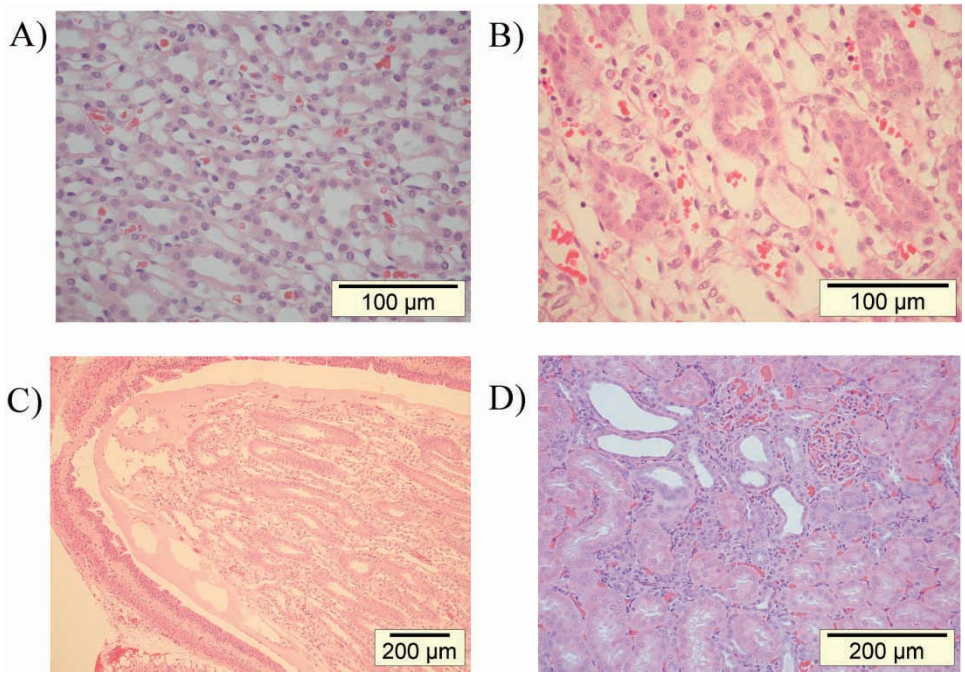

Figure 4. Histopathology – 4 week time course. Sections stained with haematoxylin and eosin obtained from control (A) and NPAA treated (700 mg kg^{-1} day^{-1}) (B)–(D) rat kidney showing the medullary collecting ducts (A), medullary collecting ducts lined by plump cells showing increased cytoplasmic eosinophilia (day 6) (B), RPN (day 8) (C) and tubular basophilia and dilation of the cortical tubules (day 29) (D). Magnification × 40 for (A) and (B), × 10 for (C), and × 20 for (D).

endogenous metabolites similar to those seen on days 7–8 of the 4 week study were observed, including marked increases in β-hydroxybutyrate, acetone and acetoacetate (figure 5). The increases showed a high degree of inter-animal variability, as demonstrated following PCA (figure 6A) and by quantification of β-hydroxybutyrate (figure 7A). Metabolite quantification demonstrated that the alterations in β-hydroxybutyrate first became evident by day 4 (figure 7A).

Examination of the data set following removal of the spectral regions containing β-hydroxybutyrate, acetone and acetoacetate (as for the 4 week time course) demonstrates a clear separation of treated samples from control (figure 6B). The large spread of control data in this analysis was due to metabolite variability related to the day of collection. Increased glucose and lactate, and decreased DMG, TMAO, citrate and α-ketoglutarate were observed. These treatment-related metabolite alterations were not time-related, as shown by the quantification of glucose (figure 7B). DMG was reduced at all time points; however, the decrease was only significant on day 8, when the level of DMG fell to approximately 25% of control values (data not shown).

In addition, by day 2 of the study, ascorbate appeared in the spectra. Ascorbate was also detectable in the spectra of samples collected on days 3–4, 5–6 and 7–8; however, the level of urinary ascorbate declined over time from day 2 (figure 7C).

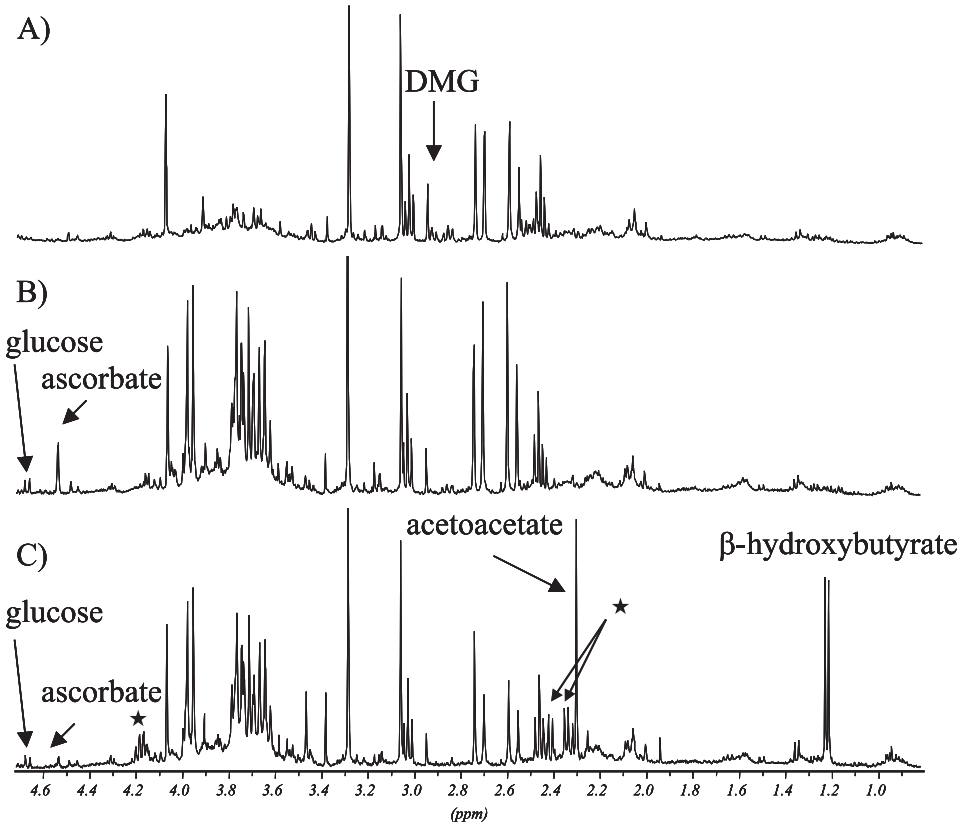

Figure 5. ^{1}H NMR spectroscopy – 8 day time course. ^{1}H NMR spectra (0.5–4.7 p.p.m.) of urine collected from (A) control rat (days 1–2); (B) rat treated with NPAA 500 mg kg^{-1} day^{-1}, days 1–2; and (C) rat treated with NPAA 500 mg kg^{-1} day^{-1}, days 5–6. ⋆Other resonances attributed to β-hydroxybutyrate at 2.31, 2.41 and 4.16 p.p.m.

Clinical chemistry, body weight and histopathology – 8 day time course

There was no difference in body weight gain for treated animals (500 mg kg^{-1} day^{-1} NPAA) compared with control animals at any time point studied (data not shown). All animals lost weight during their final 24 h due to the withdrawal of food during the overnight urine collection; however, treated and control animals lost comparable amounts of weight (days 7–8: controls, 9.1 and 8.6% body weight loss; NPAA treated, 10.9 ± 2.1% bodyweight loss).

There was a significant increase in BUN by day 8 (figure 8A). No changes were seen in plasma creatinine (data not shown). Clinical chemistry analysis of urine revealed an increase in urinary protein on days 1–2, 3–4, 5–6 and 7–8 for treated samples (figure 8B). There was no correlation between urinary protein and β-hydroxybutyrate levels (correlation coefficient = 0.0370 for samples collected on days 7–8). No difference in specific gravity was observed at any time point; however, there was an increase in urine volume, by approximately 1.5-fold, in samples collected on days 1–2, 3–4 and 5–6 (data not shown).

A slight increase in kidney weight was observed in animals terminated on day 4 of the study (controls, 4.03 and 4.43 g kidney weight × 1000 per g body weight;

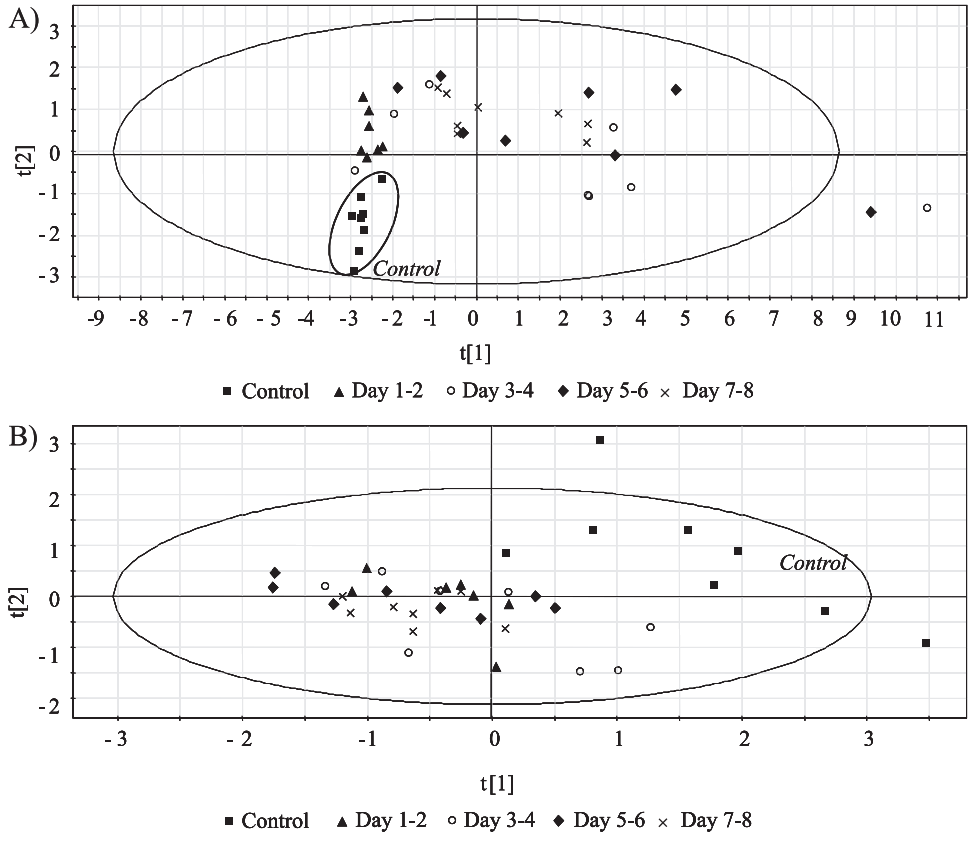

Figure 6. PCA – 8 day time course. (A) and (B) Scatter plots of the first two PCA scores (t[1] versus t[2]) obtained from ^{1}H NMR spectra of urine collected overnight on days 1–2, 3–4, 5–6 and 7–8 of the study following removal of the spectral regions containing NPAA and NPAA glucuronide (A) and with the additional removal of the spectral regions containing β-hydroxybutyrate, acetoacetate and acetone (B).

NPAA-treated, 6.23 ± 1.01 g kidney weight $\times$ 1000 per g body weight). The histopathological findings are presented in Table 2. RPN was observed in 25% of the animals on days 4 and 6, and 50% of the animals on day 8, whilst tubular dilation and eosinophilia/basophilia of the medullary collecting ducts were more consistent. Due to the inter-animal variability in response, we examined the relationship between the pathological response and excretion of β-hydroxybutyrate, acetone and acetoacetate on an individual animal basis. No correlation was observed for either the occurrence of RPN or for the other histopathological findings detailed in Table 2 and the extent of ketonuria. Correlation of the urinary β-hydroxybutyrate concentration with the severity of RPN gave correlation coefficients of 0.145, 0.327 and 0.013 for samples collected on days 4, 6 and 8, respectively.

Discussion

Daily exposure of rats to NPAA for 1 week was shown to cause RPN at a dose of 500 mg kg^{-1} day^{-1} and above, the lesion first appearing about 72 h after the start

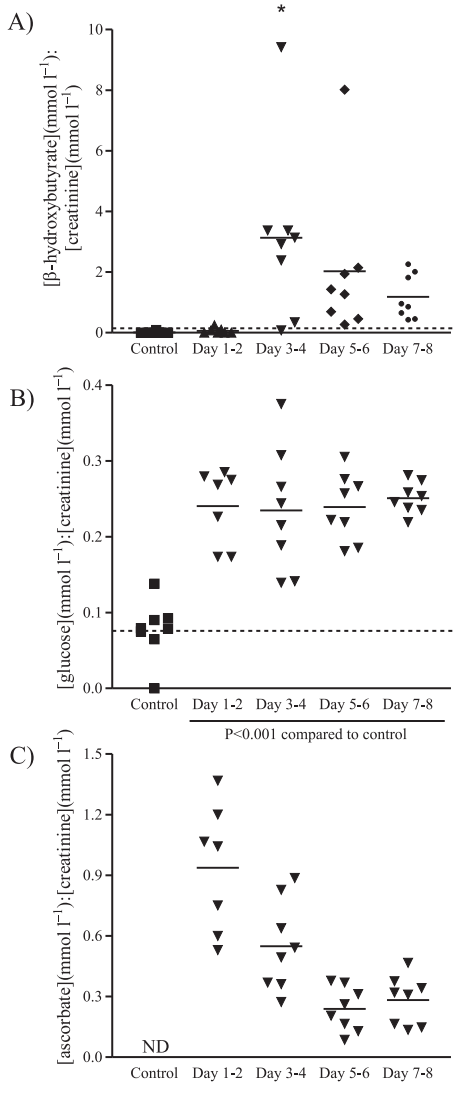

Figure 7. Metabolite quantification – 8 day time course. Amount of β-hydroxybutyrate (A), glucose (B) and ascorbate (expressed as mmol l^{-1} relative to urinary creatinine concentration in mmol l^{-1}) (C) in urine samples collected overnight on days 1–2, 3–4, 5–6 and 7–8 of the study. * $p < 0.05$ compared with control and day 1–2.

of dosing. Rats left to recover for 3 weeks after the cessation of dosing had renal function parameters within the normal range. However, histological examination showed the presence of cortical injury, presumably secondary to papillary damage. These findings with NPAA are consistent with those reported by others (Hardy 1970, Hardy and Bach 1984).

Metabolism of NPAA

Resonances associated with NPAA were observed in the NMR spectra of urine obtained from NPAA-treated rats during the daily dosing regime. NPAA is rapidly

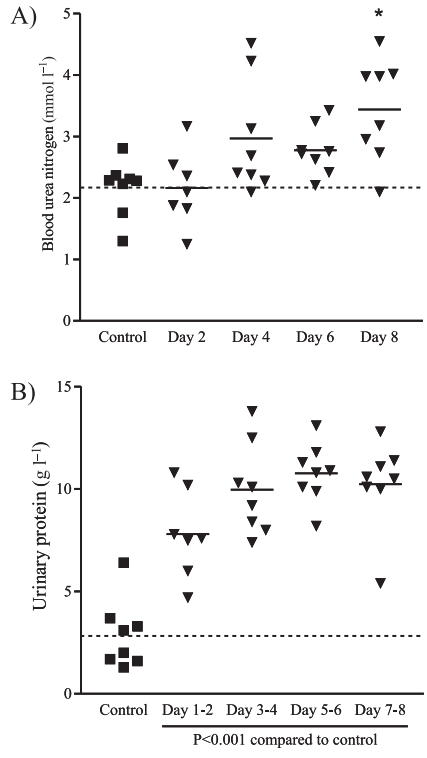

Figure 8. Clinical chemistry – 8 day time course. (A) BUN in terminal blood samples obtained on days 2, 4, 6 and 8. (B) Urinary protein in urine samples collected overnight on days 1–2, 3–4, 5–6 and 7–8. The mean value of each group is shown as a horizontal line. * $p < 0.05$ compared with control.

absorbed, widely distributed and excreted in both urine and bile as NPAA and NPAA metabolites (Whittingham *et al.* 1989). Following repeated dosing of NPAA, urinary excretion predominates. Possible metabolism, based on anthranilic acid metabolism, includes ring hydroxylation and glycine or glutathione conjugation (Naito *et al.* 1984); however, the major metabolite of anthranilic acid in rats, dogs and rabbits is the glucuronide (Williams 1959). Comparison of the resonances observed at 3.58–3.78, 3.95 and 5.82 p.p.m. with literature values for related glucuronides (Sidelmann *et al.* 1996a,b) suggested that NPAA-glucuronide was the major urinary metabolite in this study.

Ketonuria

Ketonuria, characterized by elevated β-hydroxybutyrate, acetoacetate and acetone (ketone bodies), was detected in some of the urine samples collected overnight on days 7–8 of the 4 week study, the extent and presence of which was highly variable at all three dose levels. Furthermore, in urine samples collected during the 8 day study, a variable ketonuria was observed by day 4 that was maintained during the ensuing days of the study, although it appeared to peak at day 6. No correlation was observed between the extent of ketonuria and urinary protein levels or pathological findings. Ketogenesis occurs predominantly in the

Table 2. Outcome of the histopathological examination of the right kidney taken from animals treated with NPAA at 500 mg kg^{-1} day^{-1}. The number of animals affected and the extent of the injury are shown at each time point for each dose ($n = 8$ per group except on day 2, where $n = 7$).

Histopathological finding	Day	Minimal	Slight	Moderate
Eosinophilia/basophilia of medullary collecting ducts	4	–	–	–
	6	2	2	1
	8	3	–	3
	29	3	2	2
Papillary necrosis	4	2	–	–
	6	2	–	–
	8	3	1	–
	29	–	–	–
Tubular dilation	4	6	1	–
	6	2	4	2
	8	4	4	–
	29	1	7	–
Tubular basophilia of cortical tubules	4	–	–	–
	6	4	–	–
	8	4	–	–
	29	4	3	–

liver, with ketone bodies being formed from acetyl coenzyme A formed as a result of fatty acid oxidation (Beylot 1996). Starvation results in the production of large amounts of ketone bodies, which are excreted in significant amounts during the second day of fasting (Bales *et al.* 1986). Although the animals exposed to NPAA at 700 mg kg^{-1} day^{-1} lost weight compared with controls, all other animals maintained the same weight gain. Food consumption was not measured, but the absence of a marked difference in body weight between the controls and the treated animals shows that the observed ketonuria was not the result of reduced food intake. The animals were starved overnight during urine collection, however, and it has been demonstrated that the handling of ketone bodies by the kidney is different in starved rats compared with fed rats (Elhamri *et al.* 1993). It may be that rats are more susceptible to alterations in energy metabolism in a situation where the normal function of the kidney has been compromised. Investigation of the effect of NPAA without the withdrawal of food would be of interest to determine whether it was a confounding factor in the development of ketonuria in the treated animals.

^{1}H NMR spectroscopy and ketonuria. Several studies utilizing ^{1}H NMR spectroscopy of urine have reported elevated ketone bodies. Chemical-induced vasculitis has been reported to cause significant ketonuria that is correlated with the presence and severity of the vascular lesion (Robertson *et al.* 2001). Marked body weight loss was observed in some of the ketotic animals, suggesting that reduced food intake might explain the ketonuria. It was also suggested that it could in part be the result of the vascular lesion, as excessive vasodilation, caused by the chemical, decreases local nutrient delivery and thus alters the focus of energy metabolism (Slim *et al.*

2002). The proximal tubule toxins uranyl nitrate, cephaloridine and imipenem also cause elevated β-hydroxybutyrate. However, in the case of uranyl nitrate this was in the absence of increased acetoacetate or acetone (Harrison *et al.* 1991, Anthony *et al*. 1992Anthony *et al*. 1994a, b). The alteration in urinary ketone body excretion was suggested to occur as a combined result of disturbed energy metabolism and a reduced capacity to reabsorb β-hydroxybutyrate due to damage to the proximal tubules (Harrison *et al.* 1991, Anthony *et al*. 1992). Ketonuria has not been observed in the rat following exposure to the papillotoxins BEA and PI (Gartland *et al.* 1989, Holmes *et al.* 1992, 1995a). Increased β-hydroxybutyrate was reported in the multimammate mouse following exposure to PI (Holmes *et al.* 1997); however, the reason for the increase was unclear.

Mechanistic relevance of ketonuria. The presence of ketone bodies in urine has been reported to occur as a result of altered energy metabolism. Proposed mechanisms for the papillotoxicity caused by analgesics and NSAIDs include direct toxic injury to the interstitial cells and inhibition of prostaglandin (PG) synthesis (Brix 2002). The interstitial cells synthesize PGs, which have a vasodilatory role, and hence damage to these cells or reduced PG synthesis could lead to alterations in the vasculature resulting in decreased vascular perfusion and ischaemic necrosis of the papilla. Furthermore, some NSAIDs have been shown to be non-selective inhibitors of cyclo-oxygenase, thus preventing cyclo-oxygenase-mediated PG production (Elliot *et al.* 1986, Akhund *et al.* 2003). Diclofenac sodium and indomethacin cause a dose-dependent reduction in the level of PGE_2 and $PGF_{2\alpha}$ in the rabbit renal medulla (Oliw *et al.* 1978). It has also been reported that treatment with 16,16-dimethyl PGE_2 reduces the incidence of MFA-induced RPN from 63% to 27% (1200 mg kg^{-1}, single oral dose) (Elliot *et al.* 1986). In the rat, renal inner medulla phospholipids are an important source of the fatty acids required for the high level of PG synthesis (Limas and Limas 1983); hence accumulation of phospholipids in the kidney and urine during the development of RPN may reflect their reduced role as precursors for PG synthesis (Bach *et al.* 1991, Thanh *et al.* 2001a). As mentioned previously, ketone bodies are formed following metabolism of lipids and fatty acids. An accumulation of phospholipids due to the inhibition of PG synthesis might also lead to a local accumulation of fatty acids. Ketogenesis, which can also occur in the kidney (Nakatani *et al.* 1996), might, therefore, be stimulated in order to metabolize the accumulated lipids and fatty acids. Interestingly, the appearance of β-hydroxybutyrate in urine following exposure to imipenem was associated with tubular fat accumulation and reduced mitochondrial respiration (Harrison *et al.* 1991), suggesting an alteration in the metabolism of lipids. The perturbations that we have detected with NPAA have not been reported to occur with other papillotoxins, although BEA and PI have been investigated using the same technique (Holmes *et al.* 1992, Holmes *et al.* 1995a, b, Holmes *et al.* 1997, Gartland *et al.* 1989). Gartland *et al.* (1989) did, however, suggest that BEA might have an effect on fatty acid oxidation due to the elevation of urinary succinate and acetate, which are also products of fatty acid metabolism.

Ascorbic acid elevation

Increased urinary ascorbate was also detected following treatment with NPAA. Elevated ascorbate excretion has previously been reported following administration of certain compounds including barbitone, chlorbutanol, antipyrine, aminopyrine, phenylbutazone, tetrachlorodibenzodioxin, pentachlorobiphenyl and 3-methylcholanthrene (Poon *et al.* 1994, Burns *et al.* 1960, Marselos *et al.* 1978). Concurrent with this increase, stimulation of certain cytochrome P450s and UDP-glucuronyl transferase has been reported (Poon *et al.* 1994, Marselos *et al.* 1978). Ascorbate, a major metabolite of the glucuronic acid pathway that is synthesized in the liver, has consequently been proposed as a non-invasive marker of hepatic response in rats (Poon *et al.* 1994). Glucuronide formation is not a prerequisite to ascorbate elevation (Marselos *et al.* 1978); however, NPAA is metabolized to the glucuronide and therefore it is expected that the glucuronic acid pathway and UDP-glucuronyl transferase will be stimulated, resulting in increased production of ascorbate.

Additional metabolic perturbations

In addition to the marked ketonuria and ascorbic acid elevation, alterations in urinary TMAO, DMG, citrate, succinate and α-ketoglutarate were observed similar to those reported following exposure to BEA and PI. In rats, BEA and PI have been reported to cause an early elevation in TMAO, methylamine, betaine and dimethylamine, with decreased DMG, citrate and succinate, followed by a later decrease in TMAO (Holmes *et al.* 1992, 1995a, Gartland *et al*. 1989). TMAO, methylamine, betaine and dimethylamine are present as osmolytes in the renal medullary interstitial cells and accumulate in response to an increase in extracellular osmolality (Holmes *et al.* 1992). An increase in the excretion of these compounds is thought to be reflective of damage to the renal papilla (Holmes *et al.* 1992). Glycerophosphocholine and choline also accumulate in the interstitial cells; therefore a decrease in DMG, a degradation product of choline, might reflect reduced degradation of these compounds (Moolenaar *et al.* 1999, Burg 1994). Elevated taurine was also observed in urine samples collected 21–22 days after the start of dosing in the 4 week study. Taurine elevation has previously been attributed to hepatotoxicity (Sanins *et al.* 1990). In this study, clinical chemistry markers of liver injury were not measured, so hepatotoxicity cannot be excluded. Taurine, however, is also involved in the protection of renal medullary cells from osmotic stress (Nakanishi *et al.* 1991) and can reflect a perturbation in protein synthesis (Waterfield *et al.* 1998). Therefore this late elevation in taurine might be related to the recovery of the papilla and the re-establishment of normal osmolyte levels.

Summary

In summary, we have provided evidence that NPAA-glucuronide is probably the major metabolite of NPAA and have shown that NPAA increases the excretion of ascorbate, which is considered to be a biomarker of hepatic response. Furthermore, we have demonstrated that NPAA causes major perturbations to the normal urinary metabolic profile of rats in addition to the markers of papillotoxicity previously described for other papillary toxins. It is suggested that the observed

ketonuria is indicative of increased fatty acid metabolism resulting from an accumulation of fatty acids and lipids due to inhibition of PG synthesis. It would be of interest to determine whether these changes correlate with the changes in urinary phospholipid and uronic acid reported by Thanh *et al.* (2001a).

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References

Akhund, L., Quinet, R. J. and Ishaq, S. 2003, Celecoxib-related renal papillary necrosis. *Archives of Internal Medicine*, **163** (1), 114–115.

Anthony, M. L., Gartland, K. P., Beddell, C. R., Lindon, J. C. and Nicholson, J. K. 1992, Cephaloridine-induced nephrotoxicity in the Fischer 344 rat: proton NMR spectroscopic studies of urine and plasma in relation to conventional clinical chemical and histopathological assessments of nephronal damage. *Archives of Toxicology*, **66** (8), 525–537.

Anthony, M. L., Gartland, K. P., Beddell, C. R., Lindon, J. C. and Nicholson, J. K. 1994a, Studies of the biochemical toxicology of uranyl nitrate in the rat. *Archives of Toxicology*, **68** (1), 43–53.

Anthony, M. L., Sweatman, B. C., Beddell, C. R., Lindon, J. C. and Nicholson, J. K. 1994b, Pattern recognition classification of the site of nephrotoxicity based on metabolic data derived from proton nuclear magnetic resonance spectra of urine. *Molecular Pharmacology*, **46** (1), 199–211.

Bach, P. H. and Bridges, J. W. 1985, Chemically induced renal papillary necrosis and upper urothelial carcinoma. *Critical Reviews in Toxicology*, **15** (3), 217–441.

Bach, P. H. and Hardy, T. L. 1985, Relevance of animal models to analgesic-associated renal papillary necrosis in humans. *Kidney International*, **28** (4), 605–613.

Bach, P. H. and Thanh, N. T. K. 1998, Renal papillary necrosis – 40 years on. *Toxicologic Pathology*, **26** (1), 73–91.

Bach, P. H., Scholey, D. J., Delacruz, L., Moret, M. and Nichol, S. 1991, Renal and urinary lipid changes associated with an acutely induced renal papillary necrosis in rats. *Food and Chemical Toxicology*, **29** (3), 211–219.

Bales, J. R., Bell, J. D., Nicholson, J. K. and Sadler, P. J. 1986, ^{1}H NMR studies of urine during fasting: excretion of ketone bodies and acetylcarnitine. *Magnetic Resonance in Medicine*, **3** (6), 849–856.

Beckwith-Hall, B. M., Nicholson, J. K., Nicholls, A. W., Foxall, P. J., Lindon, J. C., Connor, S. C., Abdi, M., Connelly, J. and Holmes, E. 1998, Nuclear magnetic resonance spectroscopic and principal components analysis investigations into biochemical effects of three model hepatotoxins. *Chemical Research in Toxicology*, **11** (4), 260–272.

Beylot, M. 1996, Regulation of *in vivo* ketogenesis: role of free fatty acids and control by epinephrine, thyroid hormones, insulin and glucagons. *Diabetes and Metabolism*, **5**, 299–304.

Brix, A. E. 2002, Renal papillary necrosis. *Toxicologic Pathology*, **30** (6), 672–674.

Burg, M. B. 1994, Molecular basis for osmoregulation of organic osmolytes in renal medullary cells. *Journal of Experimental Zoology*, **268** (2), 171–175.

Burns, J. J., Conney, A. H., Dayton, P. G., Evans, C., Martin, G. R. and Taller, D. 1960, Observations on the drug-induced synthesis of D-glucuronic, L-gulonic and L-ascorbic acids in rats. *Journal of Pharmacology and Experimental Therapeutics*, **129**, 132–138.

Elhamri, M., Martin, M., Ferrier, B. and Baverel, G. 1993, Substrate uptake and utilization by the kidney of fed and starved rats *in vivo*. *Renal Physiology and Biochemistry*, **16** (6), 311–324.

Elliott, G., Whited, B. A., Purmalis, A., Davis, J. P., Field, S. O., Lancaster, C. and Robert, A. 1986, Effect of 16,16-dimethyl PGE_2 on renal papillary necrosis and gastrointestinal ulcerations (gastric, duodenal, intestinal) produced in rats by mefenamic acid. *Life Sciences*, **39** (5), 423–432.

Gartland, K. P., Bonner, F. W. and Nicholson, J. K. 1989, Investigations into the biochemical effects of region-specific nephrotoxins. *Molecular Pharmacology*, **35** (2), 242–250.

Gregg, N. J., Courtauld, E. A. and Bach, P. H. 1990, High resolution light microscopic morphological and microvascular changes in an acutely induced renal papillary necrosis. *Toxicologic Pathology*, **18**(1), part 1, 47–55.

Hardy, T. L. 1970, *N*-Phenyl anthranilic acid: an agent for inducing and studying renal papillary necrosis in the rat. *British Journal of Experimental Pathology*, **51** (4), 348–355.

HARDY, T. L. and BACH, P. H. 1984, The effect of *N*-phenylanthranilic acid-induced renal papillary necrosis on urinary acidification and renal electrolyte handling. *Toxicology and Applied Pharmacology*, **75** (2), 265–277.

HARRISON, M. P., JONES, D. V., PICKFORD, R. J., WILSON, I. D. 1991, β-Hydroxybutyrate: a urinary marker of imipenem induced nephrotoxicity in the cynomolgus monkey detected by high field ^{1}H NMR spectroscopy. *Biochemical Pharmacology*, **41**(12), 2045–2049.

HOLMES, E., BONNER, F. W., SWEATMAN, B. C., LINDON, J. C., BEDDELL, C. R., RAHR, E. and NICHOLSON, J. K. 1992, Nuclear magnetic resonance spectroscopy and pattern recognition analysis of the biochemical processes associated with the progression of and recovery from nephrotoxic lesions in the rat induced by mercury (II) chloride and 2-bromoethanamine. *Molecular Pharmacology*, **42** (5), 922–930.

HOLMES, E., BONNER, F. W. and NICHOLSON, J. K. 1995a, Comparative studies on the nephrotoxicity of 2-bromoethanamine hydrobromide in the Fischer 344 rat and the multimammate desert mouse (*Mastomys natalensis*). *Archives of Toxicology*, **70** (2), 89–95.

HOLMES, E., CADDICK, S., LINDON, J. C., WILSON, I. D., KRYVAWYCH, S. and NICHOLSON, J. K. 1995b, ^{1}H and ^{2}H NMR spectroscopic studies on the metabolism and biochemical effects of 2-bromoethanamine in the rat. *Biochemical Pharmacology*, **49** (10), 1349–1359.

HOLMES, E., BONNER, F. W. and NICHOLSON, J. K. 1997, ^{1}H NMR spectroscopic and histopathological studies on propyleneimine-induced renal papillary necrosis in the rat and the multimammate desert mouse (*Mastomys natalensis*). *Comparative Biochemistry and Physiology. Part C, Pharmacology. Toxicology and Endocrinology*, **116** (2), 125–134.

HOLMES, E., NICHOLLS, A. W., LINDON, J. C., RAMOS, S., SPRAUL, M., NEIDIG, P., CONNOR, S. C., CONNELLY, J., DAMMENT, S. J. P., HASELDEN, J. and NICHOLSON, J. K. 1998, Development of a model for classification of toxin-induced lesions using 1H NMR spectroscopy of urine combined with pattern recognition. *NMR in Biomedicine*, **11**, 235–244.

LIMAS, C. and LIMAS, C. J. 1983, Phospholipid metabolism in the rat renal inner medulla. *Biochimica Biophysica Acta*, **753** (3), 314–323.

MARSELOS, M., TORRONEN, R. and AITIO, A. 1978, Responses of the d-glucuronic acid pathway in rat tissues to treatment with tetrachlorodibenzodioxin. *Xenobiotica*, **8** (7), 397–402.

MOOLENAAR, S. H., POGGI-BACH, J., ENGELKE, U. F., CORSTIAENSEN, J. M., HEERSCHAP, A., DE JONG, J. G., BINZAK, B. A., VOCKLEY, J. and WEVERS, R. A. 1999, Defect in dimethylglycine dehydrogenase, a new inborn error of metabolism: NMR spectroscopy study. *Clinical Chemistry*, **45** (4), 459–464.

NAITO, J., SASAKI, E., OHTA, Y., SHINOHARA, R. and ISHIGURO, I. 1984, Anthranilic acid metabolism in the isolated perfused rat liver: detection and determination of anthranilic acid and its related substances using high-performance liquid chromatography with electrochemical detection. *Biochemical Pharmacology*, **33** (20), 3195–3200.

NAKANISHI, T., UYAMA, O. and SUGITA, M. 1991, Osmotically regulated taurine content in rat renal inner medulla. *American Journal of Physiology*, **261**(6), part 2, F957–962.

NAKATANI, T., SAKAMOTO, Y., ANDO, H. and KOBAYASHI, K. 1996, Enhanced ketogenesis in the kidney during hepatic inflow occlusion with the administration of Ringer's acetate solution. *Surgery*, **119** (6), 684–689.

NGUYEN, T. K., OBATOMI, D. K. and BACH, P. H. 2001, Increased urinary uronic acid excretion in experimentally-induced renal papillary necrosis in rats. *Renal Failure*, **23** (1), 31–42.

NICHOLLS, A. W., HOLMES, E., LINDON, J. C., SHOCKCOR, J. P., FARRANT, R. D., HASELDEN, J. N., DAMMENT, S. J., WATERFIELD, C. J. and NICHOLSON, J. K. 2001, Metabonomic investigations into hydrazine toxicity in the rat. *Chemical Research in Toxicology*, **14** (8), 975–987.

NICHOLSON, J. K., HIGHAM, D. P., TIMBRELL, J. A. and SADLER, P. J. 1989, Quantitative high resolution ^{1}H NMR urinalysis studies on the biochemical effects of cadmium in the rat. *Molecular Pharmacology*, **36** (3), 398–404.

OLIW, E., LUNDEN, I. and ANGGARD, E. 1978, *In vivo* inhibition of prostaglandin synthesis in rabbit kidney by non-steroidal anti-inflammatory drugs. *Acta Pharmacologica et Toxicologica*, **42** (3), 179–184.

POON, R., CHU, I., LECAVALIER, P., BERGMAN, A. and VILLENEUVE, D. C. 1994, Urinary ascorbic acid — HPLC determination and application as a noninvasive biomarker of hepatic response. *Journal of Biochemical Toxicology*, **9** (6), 297–304.

ROBERTSON, D. G., REILY, M. D., ALBASSAM, M. and DETHLOFF, L. A. 2001, Metabonomic assessment of vasculitis in rats. *Cardiovascular Toxicology*, **1** (1), 7–19.

SANINS, S. M., NICHOLSON, J. K., ELCOMBE, C. and TIMBRELL, J. A. 1990, Hepatotoxin-induced hypertaurinuria: a proton NMR study. *Archives of Toxicology*, **64** (5), 407–411.

SIDELMANN, U. G., NICHOLLS, A. W., MEADOWS, P. E., GILBERY, J. W., LINDON, J. C., WILSON, I. D. and NICHOLSON, J. K. 1996a, High-performance liquid chromatography directly coupled to ^{19}F and ^{1}H NMR for the analysis of mixtures of isomeric ester glucuronide conjugates of trifluoromethylbenzoic acids. *Journal of Chromatography A*, **728**, 377–385.

Sidelmann, U. G., Lenz, E. M., Spraul, M., Hofmann, M., Troke, J., Sanderson, P. N., Lindon, J. C., Wilson, I. D. and Nicholson, J. K. 1996b, 750 MHz HPLC-NMR spectroscopic studies on the separation and characterization of the positional isomers of the glucuronides of 6,11-dihydro-11-oxodibenz[*b,e*]oxepin-2-acetic acid. *Analytical Chemistry*, **68** (1), 106–110.

Slim, R. M., Robertson, D. G., Albassam, M., Reily, M. D., Robosky, L. and Dethloff, L. A. 2002, Effect of dexamethasone on the metabonomics profile associated with phosphodiesterase inhibitor-induced vascular lesions in rats. *Toxicology and Applied Pharmacology*, **183** (2), 108–116.

Thanh, N. T. K., Stevenson, G., Obatomi, D. K., Aicher, B., Baumeister, M. and Bach, P. H. 2001a, Urinary lipid changes during the development of chemically-induced renal papillary necrosis: a study using mefenamic acid and *N*-phenylanthranilic acid. *Biomarkers*, **6** (6), 417–427.

Thanh, N. T. K., Obatomi, D. K. and Bach, P. H. 2001b, Phospholipiduria in 2-bromoethanamine-induced renal papillary necrosis. *Biomarkers*, **6**, 326–334.

Waterfield, C. J., Asker, D. S., Patel, S. and Timbrell, J. A. 1998, Is there a correlation between taurine levels and xenobiotic-induced perturbations in protein synthesis? A study with tetracycline in rats. *Amino Acids*, **15** (1–2), 161–177.

Whittingham, A., Delacruz, L., Gregg, N. J., Albores, A., Ijomah, P. and Bach, P. H. 1989, The kinetics of papillotoxic doses of ^{3}H-*N*-phenylanthranilic acid in rats. *Renal Physiology and Biochemistry*, **12** (5–6), 406–412.

Williams, R. T. (editor), 1959, *Detoxification Mechanisms*: *The Metabolism and Detoxicification of Drugs, Toxic Substances and other Organic Compounds, 2nd edition* (London: Chapman and Hall), pp. 447–448.

Wolf, D. C., Carlton, W. W. and Turek, J. J. 1992, Experimental renal papillary necrosis in the Mongolian gerbil (*Meriones unguiculatus*). *Toxicologic Pathology*, **20**(3), part 1, 341–349.