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# Urinary lipid changes during the development of chemically-induced renal papillary necrosis: a study using mefenamic acid and *N*-phenylanthranilic acid

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Acute renal papillary necrosis (RPN) in animals is characterized by increased renal lipid accumulation. The excretion of renal lipids into urine has been determined to evaluate their possible use as sensitive early biomarkers for the diagnosis of RPN. This study investigates injury induced by two model nephrotoxins, mefenamic acid (MFA), a non-steroidal anti-inflammatory drug (NSAID), and its analogue *N*-phenylanthranilic acid (NPAA). Oral NPAA was given repeatedly at doses of 100, 250 and 500 mg kg$^{-1}$ daily for 5 days, followed by a 2 day respite over the weekend, and then four further daily doses. The same dosing procedure was used with MFA, but at doses of 75, 150 and 300 mg kg$^{-1}$. The control groups were given vehicle orally using the same volume given to the test groups. Urinary phospholipids (PLs), notably sphingomyelin (SPM), phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were measured and compared with other urinary parameters. Histopathological investigations were also performed to confirm the presence or absence of RPN. Following MFA treatment, PC, PI and PE were raised significantly ($p < 0.001$) on days 1 and 3 and for the remaining part of the experiment. After NPAA treatment, PI showed a transient elevation, and PC and PE levels were significantly increased from day 2 onwards. Both drugs caused a dose-related increase in PLs. There was no significant increase in the level of other urinary parameters. However, histopathological examination of the kidney on day 11 revealed lesions in the medulla and papilla following treatment with the two papillotoxins. These findings demonstrate the potential of urinary PLs as diagnostic non-invasive biomarkers for early renal injury associated with RPN, which may provide an important improvement in the approach to the therapeutic management of analgesic nephropathy.

*Keywords*: early markers, renal papillary necrosis, urinary phospholipids, *N*-phenylanthranilic acid, mefenamic acid



## Introduction

Renal papillary necrosis (RPN) in humans develops mainly as a consequence of chronic consumption or long-term abuse of single or mixed analgesics or non-steroidal aniti-inflammatory drugs (NSAIDs) (Segasothy *et al*. 1984, 1987, 1995, Sandler *et al*. 1989, 1991, Rahman *et al*. 1993, De Broe *et al*. 1996). It may then

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progress to end-stage renal disease. The problem of excessive use of analgesics or NSAIDs continues today, and has remained a significant and costly health problem in the developed world (Pommer *et al.* 1989, Griffin *et al.* 1995, Elseviers and De Broe 1995, De Broe *et al.* 1996). There are a large number of pathophysiological similarities between the acute model of RPN in animals and the chronic disease in humans (Bach and Bridges 1984). Clinical and animal studies have shown that a wide range of compounds can induce RPN. Two such compounds are mefenamic acid (MFA; 2-[(2,3-dimethylphenyl)amino]benzoic acid), a common analgesic, and *N*-phenylanthranilic acid (NPAA; 2-(phenylamino)benzoic acid), a chemical agent that is a close structural analogue to MFA. They have both been shown to cause subacute RPN in animals (Hardy 1970, Hewitson *et al.* 1991), with MFA being the 'leading' single substance (38%) causing acute renal failure (Thiel *et al.* 1991).

The earliest manifestations of RPN are silent; currently, it is only once these have progressed to secondary cortical degeneration and loss of renal function that the lesion is clinically apparent (Bach and Bridges 1985, Schwarz *et al.* 1989, Nanra 1993). At present, early diagnosis of RPN is very difficult. Diagnostic advances have been made by the combined use of ultrasound and computed tomography (Braden *et al.* 1991, Elseviers *et al.* 1992), but the changes in renal size and shape only occur after complete papillary necrosis. Attempts to diagnose RPN non-invasively have been based on several different approaches, but to date there is still a requirement for painstaking histopathology via preparation of the kidneys and careful assessment of sections, especially if a focal lesion is present, and in the case of animals only one examination at a given time can be made on a single animal. Often assessment by routine pathology is not sufficiently exacting to section the papilla in such a way that focal lesions are identified, so it is likely that in many cases a diagnosis of RPN is missed.

Conventional urinalysis or enzyme assay in urine, which is often used to assess renal function, has failed to diagnose RPN at an early and reversible stage. *N*-acetyl-$\beta$-glucosaminidase (NAG) showed a sustained elevation after papillary damage (Stonard *et al.* 1987). However, this was not characteristic for the site of injury, and there was no relationship between the severity of the lesion and the increase in NAG.

In the absence of a definitive diagnostic criterion for the early diagnosis of either the presence or severity of RPN, it has been almost impossible to establish with certainty which single analgesic (or what combinations of analgesic) cause RPN in humans. It has also been difficult to define accurately the total quantity of each compound that has to be consumed in order to produce a papillotoxic effect. Using urinary lipids as markers for RPN could solve this problem since RPN is known to be characterized by increased renal lipid accumulation (Bach *et al.* 1991). It was also suggested from our previous study that phospholipid excretion in urine might be a marker of RPN induced by 2-bromoethanamine in male Wistar rats (Thanh *et al.* 2001a).

This study aimed to assess urinary lipids for their sensitivity as biomarkers for the early non-invasive diagnosis of RPN. Changes in the urinary excretion of three different phospholipids (PLs) – sphingomyelin (SPM), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) – occurring during the development of experimental subacute RPN in rats following the administration of NPAA or MFA were investigated. The doses of NPAA or MFA given were several times greater than

normal therapeutic values. The selected PLs are known to be the major PLs found in renal tissue (Zambrano *et al.* 1975). They were quantified using high performance thin layer chromatography (HPTLC) and scanning densitometry (Thanh *et al.* 2001c). Histopathological investigations were also used to confirm the development of RPN.

## Materials and methods

### *Materials*

NPAA and MFA were purchased from Aldrich Ltd (Poole, UK). All lipid standards were purchased from Sigma Ltd (Poole, UK). Chloroform and methanol (both HiPerSolv for HPLC™), propan-1-ol (n-propanol), and potassium chloride were purchased from BDH Chemicals Ltd, UK.



### *Animal housing and acclimatization*

Males Wistar rats (Dr Karl Thomae GmbH, Biberach/Riss, Germany) weighing 190–200 g were used. The animals were acclimatized to housing conditions in a metabolic cage that separated urine and faeces for 3 days prior to the experiment. The animals were housed under standardized conditions in a temperature (20–25°C) and humidity (45–80%) controlled room. They were kept under specific pathogen-free conditions, with a light/dark cycle of 8/16 h (light period 0800 h–1600 h), at about 100 lux with single housing in Macrolon® cages. A standard fortified rodent diet (Altromin®, Altrogge, Lage/Lippe, Germany) was used, and municipal tap water was offered *ad libitum*. All studies were conducted in accordance with German and US National Institutes of Health guidelines for animal welfare, and all experiments were performed according to good laboratory practice regulations.

### *Experimental protocol*

Following the acclimatization period, control day values (day 0) were obtained. Animals were separated into two main groups, and each was given NPAA or MFA. NPAA was prepared in carboxymethylcellulose as an aqueous suspension and given repeatedly orally to a subgroup of five rats at doses of 100 (low), 250 (medium) or 500 (high) mg $kg^{-1}$ at 10:00 h daily for 5 days, followed by a 2 day respite over the weekend, and then four further daily doses. The daily administration volume was 10 ml $kg^{-1}$. The same dosing procedure was used for MFA given to another subgroup of five rats, but at doses of 75 (low), 150 (medium) and 300 (high) mg $kg^{-1}$. The control groups were given vehicle orally using the same volume as was given to the test groups.

### *Collection of urine*

During the urine collection periods, rats had unrestricted access to water. Collection periods lasted for 16 h, after which the animals had access to food for at least 8 h before the start of the next collection period. Samples were collected in plastic universal containers kept at 0–4°C on ice, and total urine volumes were measured and recorded. The frozen urine was sent to the University of East London using a high quality insulated container for analysis of PLs and creatinine. The samples were stored at −20°C and analysed within 1 week. All samples were thawed and centrifuged at 1500 × *g* for 3 min on a bench top centrifuge to sediment the debris and particles before analysis. At the end of the final urine collection period, all five animals in each group were killed by cervical dislocation; the kidneys were removed and used for histopathological examination. Urinary electrolyte quantification, assessment of serum and histopathology were performed at Thomae GmbH, Biberach an der Riss, Germany.

### *Assessment of serum and urinary creatinine and urinary electrolytes*

Serum and urinary creatinine levels were determined by the Jaffé method using the appropriate kits (Sigma Ltd, Poole, UK). Urinary electrolytes ($Na^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$, $PO_4^{2-}$ and $Cl^-$) were also measured, as previously described (Thanh *et al.* 2001b).

### *Quantification of PLs by HPTLC with densitometry*

Lipids were extracted from urine samples using chloroform and methanol in the ratio 2:1 (v/v). Lipid samples were then applied on HPTLC silica gel 60 plates (Merck Ltd, Dorset, UK) with a 200 µl thick layer and separated in a Camag horizontal developing chamber (Camag, Muttenz, Switzerland) using the solvents methanol, chloroform, n-propanol, methyl acetate and 43 mM KCl in the ratios 10:25:25:25:9 (v/v). Integration, calibration and calculation of the results were then performed using a Desaga CD 60 densitometer (Desaga GmbH, Heidelberg, Germany) as described previously (Thanh

*et al.* 2001c). The excretion of lipids into urine was then expressed as ng of lipid excretion per 16 h collection period.

### *Statistical analysis of data*

Comparison between the means of the control and treated groups was performed. The data represent the mean of four or five separate values ± standard error of the mean. Normal ranges were established for all measurements before the various drug regimes; the upper limit of normal was defined as the mean of the control values for all days plus twice the standard deviation. Differences between treated animals and controls were evaluated statistically by analysis of variance (ANOVA), with statistical significance defined as $p < 0.05$.

## Results

### *Histology*

Histological examination of tissues on day 11 showed some evidence of renal lesions in the papilla and the medulla with cortical involvement when rats were treated with MFA at all doses, while only a high dose of NPAA caused severe papillary necrosis (table 1).

### *Effect of NPAA on urinary and serum parameters*

The vehicle of administration of NPAA and MFA was non-toxic to the rats, as all of the parameters measured were within the same range as pretreatment values. NPAA did not produce any significant dose- or time-related changes in urine volume or urinary or serum creatinine during the treatment period.

There was no abnormal change in the excretion rate of electrolytes with low and medium doses of NPAA. At the high dose, however, a significant increase in the excretion of $K^+$, $Cl^-$ ($p < 0.05$) and $Mg^{2+}$ was observed (Thanh *et al.* 2001b).

The accumulation of urinary lipids (SPM, PC and PE) at the medium and high doses of NPAA occurred as early as 48 h after the start of dosing and corresponded to the establishment of RPN on histological examination (which was identified much later in the experiment on day 11).

The effect of repeated oral administration of NPAA at 100, 250 or 500 mg/kg on the urinary excretion of PLs is shown in figure 1. There was a dose- and time-related increase in SPM excretion in the urine of rats treated with NPAA (figure 1a). The low dose produced little change (a small increase on day 8, $p < 0.05$),

Table 1. Histopathological data in rat treated with either NPAA or MFA at the doses specified. Tissues were collected on day 11 following treatment with the different compounds. Data are presented as observations made on five separate determinations.

| | Histological findings | | |
|---|---|---|---|
| Compound (dose) | Papilla | Medulla | Cortex |
| Control | 0 | 0 | 0 |
| NPAA (500 mg/kg) | ++ necrosis<br>++ hyperemia | +++ swelling, vacuolization and single cell necrosis of tubular epithelial cell | 0 |
| MFA (300 mg/kg) | ++ necrosis<br>++ tubular casts (protein) | +++ swelling, vacuolization and single cell necrosis of tubular epithelial cell | +++ cellular debris<br>++ focal necrosis |

0 = no change observed.
+ = slight changes; ++ = moderate changes; +++ = severe changes.

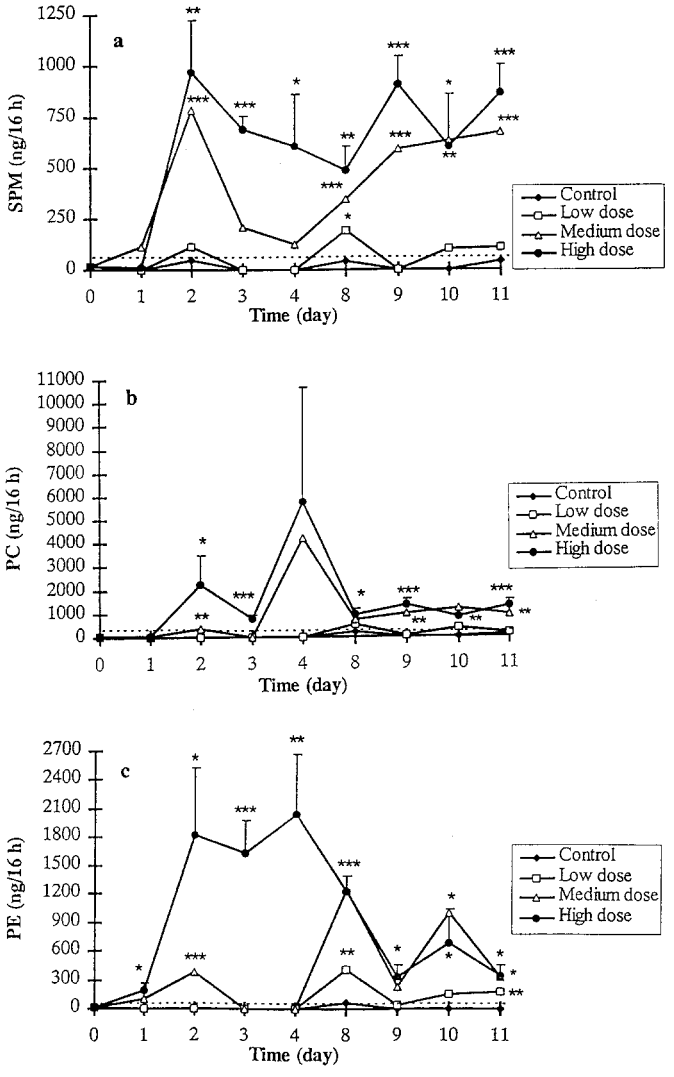


Figure 1. The excretion of (a) SPM, (b) PC and (c) PE in the urine of rats following repeated doses (100, 250 or 500 mg $kg^{-1}$) of NPAA. Values were determined from four to five rats and are presented as the mean ± SEM. The dotted line represents the upper limit of normal. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with controls.

while the medium dose produced a marked though transient increase in SPM excretion on day 2 ($p < 0.001$) and a progressive increase toward the end of the treatment period (days 4–11, $p < 0.001$). At the high dose, NPAA produced nearly a 50-fold increase in SPM excretion, which was maintained throughout the treatment (days 2–11, $p < 0.001$).

NPAA also caused a dose-related increase in the excretion of urinary PC (figure 1b). There was very little excretion of PC in the control or low dose groups. The medium dose produced a small elevation in PC excretion on day 2 ($p < 0.01$), which was enhanced on days 8–11 (generally $p < 0.01$). The high dose caused a

similar pattern of PC excretion, with more than a 200-fold increase as early as day 2 ($p < 0.05$). This effect was maintained throughout the treatment period ($p < 0.05$ to $p < 0.001$).

The excretion of PE also followed a dose-related pattern (figure 1c). The low dose did not alter the excretion of PE for the first 4 days of treatment, but between days 8 and 11, urinary PE was higher than the upper limit of normal. The medium dose increased the excretion of PE as early as day 2 ($p < 0.001$), and this effect was maintained on days 8–11 ($p < 0.05$). The high dose caused an increase in the excretion of PE as early as day 1 ($p < 0.05$), a marked increase (200-fold) on days 2–4 ($p < 0.05$ to $p < 0.001$), with some recovery towards the end of the treatment ($p < 0.05$). When the data from all the animals were plotted as urinary lipid (total phospholipid, SPM, PC and PE) against increasing repeated doses of NPAA (figure 2), significant correlations were found for SPM and PE ($p < 0.001$).



### *Effect of MFA on urinary parameters*

While MFA clearly caused RPN, urinary volume appeared normal. However, MFA caused some increases ($p < 0.05$, data not shown) in creatinine excretion, but these were very much less in range than the increase in the urinary lipids.

There was a significant ($p < 0.05$) increase in the $Na^+$ $K^+$ and $Cl^-$ excretion rates with the low dose of MFA, and a significant decrease in $Na^+$ and $Cl^-$ ($p < 0.05$) with the high dose. No changes were observed at the medium dose (Thanh *et al.* 2001b).

The effect of repeated oral administration of MFA for 11 days on the excretion of PLs in the urine is shown in figure 3. Changes in the SPM content of urine after each treatment with MFA are shown in figure 2a. There were small increases in SPM excretion at the low dose throughout the treatment period, but no change at the medium dose. On day 4, however, MFA produced a marked dose-dependent increase in excretion (nearly 5000-fold at the high dose), which had returned to previous levels by day 8.

PC excretion (figure 2b) showed significant increases ($p < 0.05$ to $p < 0.01$) throughout the treatment period at low and medium doses. The high dose of MFA produced a clear linear increase in PC excretion with time up to day 4, followed by variable excretion rates between days 8 and 11, with a peak on day 9. There was considerable variability between individual animals, however.

The excretion of PE (figure 2c) showed a similar pattern to that of SPM. These data were much less variable than those of SPM or PC. MFA increased PE excretion on days 1–4 at the low and medium doses ($p < 0.05$ to $p < 0.001$) and throughout the treatment period at the high dose ($p < 0.05$ to $p < 0.001$). A marked peak was observed on day 4.

## Discussion

Chronic interstitial nephritis and RPN characterize classical analgesic nephropathy. RPN in humans develops over a long period of exposure to single or mixed analgesics or NSAIDs. However, it is possible to induce RPN acutely in animal models, as we have shown in this study with MFA using doses several times higher than the therapeutic dose. Previous studies have shown that a single oral dose of MFA at 1200 mg $kg^{-1}$ produced RPN in 63% of treated animals (Elliott *et al.*

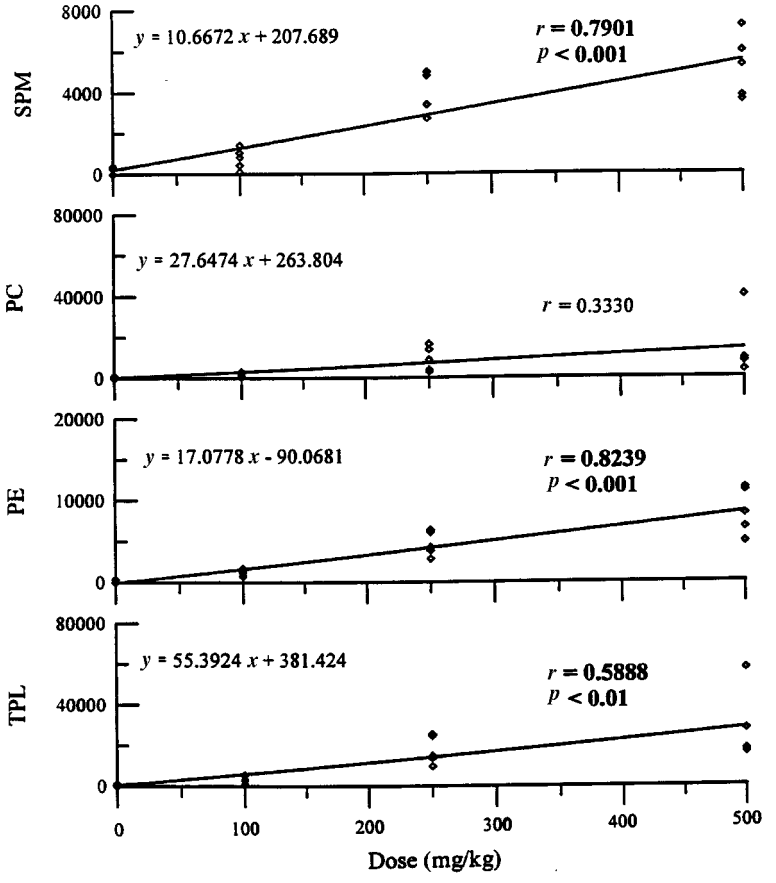


Figure 2. Correlation between the total excretion of urinary lipids (ng) in rats on days 1–4 and 8–11 and the repeated dose of NPAA administered (five animals at each dose). TPL = total PLs.



1986), while the oral administration of a suspension of NPAA, over the range 100 to 400 mg $kg^{-1}$ for 14 consecutive days caused a dose-related subacute RPN after 7–14 days (Hardy and Bach 1984). In the present study, we used lower doses of MFA, which we found to be effective in causing RPN over a short period of treatment.

The histopathology findings in this study have shown that repeated doses of NPAA and MFA at high doses caused RPN, with the MFA effect extending to the renal cortex. While both NPAA and MFA caused RPN at high doses, urinary volume appeared normal; they did not appear to induce diuresis as was previously observed in animals treated with 2-bromoethanamine, a typical papillotoxin (Thanh *et al.* 2001b). The action of 2-bromoethanamine is accompanied by diuresis since it has an effect on the distal tubule and probably increases the tubular reabsorption of $Na^+$, and thus the urine becomes effectively dilute with high urinary flow. However, the actions of NPAA and MFA are not accompanied by an increase in urinary flow. It is difficult to know why these agents act differently from 2-bromoethanamine, and this would warrant further investiga-

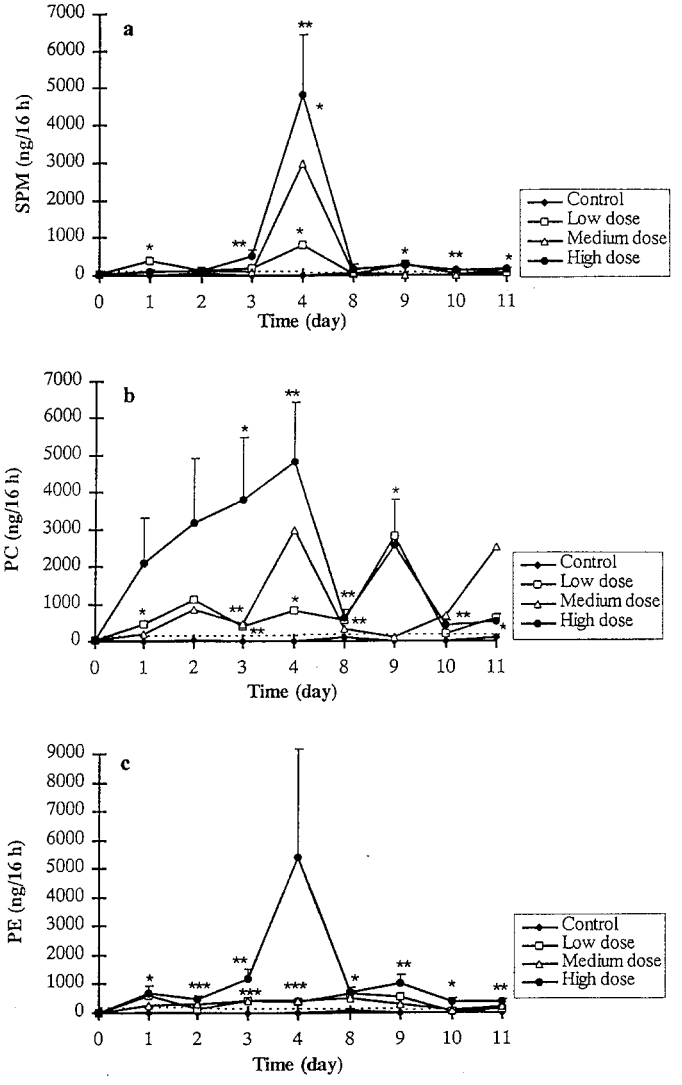


Figure 3. The excretion of (a) SPM, (b) PC and (c) PE in the urine of rats following repeated doses (75, 150 or 300 mg kg$^{-1}$) of MFA. Values were determined from four to five rats and are presented as the mean ± SEM. The dotted line represents the upper limit of normal. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with controls.

tion. It cannot be speculated why diuresis is observed with one and not with the others since the mechanism of diuresis is very complex.

Urinary creatinine and electrolytes appeared to be normal in the dosed groups compared with the controls in the two models, although NPAA and MFA caused some rather variable changes in creatinine excretion. However, these changes were much smaller than those of the urinary lipids (more than 200-fold). The small changes seen in the parameters (urinary volume, electrolytes and serum creatinine) commonly used to assess renal function showed that they are not a sensitive enough indicator for assessing the development of RPN. The lack of any significant elevation in the serum parameters following treatment with either

NPAA or MFA points to the fact that this invasive method of investigation may not be useful in detecting the onset of RPN. This justifies the search for non-invasive and sensitive means of detecting RPN and the results of this study suggest the usefulness of urinary lipids.

It is accepted that the primary lesion of analgesic drug abuse is in the medulla. The primary morphological changes occur in the medullary interstitial cells, followed by damage to the endothelial cells and loops of Henle, and then changes in the collecting duct. The interstitial cells are an important part of the medulla as they occupy 10–20% of the tissue volume in the outer medulla and 40% near the papilla tip (Moffat 1981, Kriz and Bankir, 1988). Also, 2–4% of the total volume of these cells in the rat are cytoplasmic lipid droplets, which are mainly PLs and unsaturated fatty acids (Bojesen 1974). With respect to renal lipid changes, it has been have shown that indomethacin (an NSAID) also causes PL accumulation in the rat kidney and that the papilla is the most sensitive site ((Fernandez-Tome and Sterin-Speziale 1994).

The two model papillotoxins used in this study caused different changes in the urinary PL profiles. The enhanced excretion of lipid in the urine, however, was a common feature of the two lesion models. While the effect of MFA was more immediate (occurring within 24 h), NPAA did not produce increases in urinary lipids until day 2. The present studies have confirmed that the relatively crude biochemical changes in renal tissue as reported by Bach *et al*. (1991) are indeed reflected in changes in biochemically identifiable marker molecules in the urine.

The particularly pronounced biomarkers SPM, PC and PE are therefore good indicators of early RPN. Their excretion in urine might have resulted from the product of complex degenerative processes whose mechanisms are not clearly understood, and careful consideration is required before any major conclusions can be drawn about their value in clinical diagnosis.

On the basis of the results of the present study, it is suggested that urinary lipids from known analgesic drug abusers with confirmed RPN and from individuals who have taken high doses of analgesic drugs should be measured. It is proposed that urinary lipids should be detailed as a first step in establishing the presence of RPN in patients. In cases where there is doubt, biopsy should be performed for histopathological investigations. Urinary PL assessment appeared to be selective and more sensitive than common histology for identifying papillary necrosis and may therefore have considerable clinical value in assessing RPN.



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