Measurement of total phospholipids in urine of patients treated with gentamicin

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Aims The excretion of phospholipids in urine may be a marker of the early renal toxicity of the aminoglycoside antibiotics. Urinary phospholipids are formed in myeloid bodies which develop in the lysosomes of proximal tubules during treatment with the aminoglycosides, and overflow into the urine.

Methods Published assays were modified in order to measure the total phospholipid concentrations in human urine. Phospholipids were extracted from freeze-dried urine samples, digested in concentrated sulphuric acid, and the inorganic phosphorus content determined by complexing with ammonium molybdate and measuring the absorbance at 820 nm. Ten septicemic patients treated with gentamicin for 5–7 days had significantly higher urine phospholipid concentrations than 10 healthy untreated control subjects ($P < 0.0001$). There was a negative linear relationship between phospholipid excretion and creatinine clearance ($r^2 = 0.71$).

Results In 34 patients with acute pyelonephritis, increased phospholipid concentrations were observed prior to treatment compared with healthy controls ($P < 0.001$) and did not alter during treatment with gentamicin. However, the phospholipid concentrations decreased significantly after treatment was completed ($P < 0.03$).

Conclusions These studies suggest that urinary phospholipids may indicate early aminoglycoside toxicity but with poor specificity, as many of the infections being treated may themselves be associated with phospholipiduria.

Keywords: nephrotoxicity, aminoglycosides, gentamicin, phospholipids, creatinine

Introduction

Nephrotoxicity is a significant side effect of the aminoglycoside antibiotics, with a reported incidence of up to 55% [1]. The incidence, however, depends on the criteria used to define toxicity, the dosage of the aminoglycoside, and possibly on the specific aminoglycoside used. An increase in the serum creatinine concentration is the most commonly used index of aminoglycoside nephrotoxicity, but this change is insensitive and may be a late event [2].

Administration of aminoglycosides to rats results in the development of myeloid bodies (lamellar inclusions) in the lysosomes of the proximal tubular cells of the kidney, with a lesser density in the glomerular and distal epithelium [2–4]. The formation of these myeloid bodies may be associated with the development of the nephrotoxicity [2]. Although phospholipidosis in the cells and subsequent phospholipiduria are well recognised in animals, there is conflicting data in human clinical practice. One research group has presented three reports showing that aminoglycosides increase the amounts of phospholipids in the sediment prepared by high speed centrifugation of urine, in patients with urinary tract infections or pelvic inflammatory disease [5–7]. Another study, however, found that tobramycin and

gentamicin did not alter the total phospholipids in the urine of patients with acute pyelonephritis [8]. In a further study of four patients with various infections, the ratio of phospholipids to protein in the urinary sediment was increased by about 30% by treatment with gentamicin [9]. The sedimented fraction was, however, isolated by low speed centrifugation, and probably included cells and not the lighter myeloid bodies.

In view of these contrasting results, we compared the urinary concentrations of total phospholipids in healthy subjects, in a broad group of septicemic patients being treated with gentamicin, and in patients with acute pyelonephritis before, during and after treatment with gentamicin, and in a patient who was inadvertently overdosed with gentamicin. We also report the details of the assay for the measurement of total urinary phospholipids highlighting difficulties and limitations.

Methods

Materials

For the purpose of validation of the assay, phospholipids were obtained from Sigma Chemical Co., MO63178, USA: L- α -phosphatidic acid [PA] (egg yolk lecithin 98%), (–)- α -phosphatidylcholine dipalmitoyl [PC] (synthetic 99%+), ($\pm$)- α -phosphatidylethanolamine dipalmitoyl [PE] (synthetic

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98%), (–)- α -phosphatidylinositol [PI] (soybean 99%), (–)- α -lysophosphatidylserine [PS] (bovine brain 98%), (–)- α -lysophosphatidylcholine [Lyso-PC] (egg yolk lecithin 99%) and sphingomyelin [SM] (bovine brain 98%). Hydrazine sulphate and butylated hydroxy toluene were also obtained from Sigma. Chloroform [CHCl_3] and methanol [MeOH] were h.p.l.c. grade (Mallinckrodt). Calcium nitrate tetrahydrate, 30% w/v, hydrogen peroxide, potassium chloride, potassium dihydrogen phosphate, sulphuric acid and glass wool were analytical reagent (AR) grade (BDH). Ammonium molybdate and sodium azide [NaN_3] were also AR grade from Fisons and Fisher Scientific, respectively. All phospholipid standards were prepared in CHCl_3 /MeOH/water (1:2:0.8) containing 0.01% butylated hydroxy toluene (to prevent oxidation of unsaturated fatty acids). Standards were stored in the dark at 5° C until used.

Total phospholipid assay

Concentration of phospholipids prior to solvent extraction.

Two methods of concentrating the urine samples prior to solvent extraction were investigated; lyophilisation and centrifugation. Both methods have been employed by previous researchers. The phospholipid recoveries were compared.

Lyophilization. Urine samples [5 ml] were pipetted into labelled 100 × 16 mm borosilicate culture tubes which were then frozen for 15 min in a dry-ice/methanol bath. Frozen urine samples were transferred to a lyophilizer and dried under vacuum for 48 h. Once removed from the freeze-dryer, samples were either sealed with parafilm and frozen at –35° C or extracted immediately.

Centrifugation. Urine samples [5 ml] were pipetted into 100 × 16 mm polypropylene high speed centrifuge tubes and centrifuged at 4° C using a horizontal swing-out centrifuge (Beckman L8-70 Ultracentrifuge, 100 000 g) for 60 min. Tubes were carefully removed from the centrifuge and the urine was then decanted, to avoid disturbing the pellet formed by the accumulation of the myeloid bodies. The resulting pellet was dispersed into 600 μl of deionised water prior to extraction and digestion.

Extraction. Phospholipids were extracted from urine by a method adapted from Folch *et al.* [10] and Olier *et al.* [8]. Urine lyophilizate was reconstituted and extracted as described by Olier *et al.* [8], with the following changes. The lower CHCl_3 phase was collected and filtered through glass wool packed into a pasteur pipette. The glass wool filter was washed with CHCl_3 (1 ml) and finally flushed clean of solvent by forcing air through the filter. The combined CHCl_3 eluents were washed by vortexing with 1M potassium chloride (250 μl) [10] containing 0.01% NaN_3 as an antibacterial. The mixture was then centrifuged at 3000 g for 20 min and the CHCl_3 layer transferred to a clean 100 × 16 mm tube. The solvent was evaporated on a hot plate at 60° C under a stream of oxygen free nitrogen.

Digestion. Phospholipid standards and urine extracts were digested in sulphuric acid together with calcium nitrate [12] and hydrogen peroxide [13]. The extracts were mixed with

10 M sulphuric acid (100 μl), 0.16 M $\text{Ca}(\text{NO}_3)_2$ (25 μl) and 30% hydrogen peroxide (25 μl), and heated for 20 min on the hot plate at 180° C [14]. Some urine extracts required more than 25 μl of peroxide to give the clear digest. For every addition of peroxide, an extra 20 min was added to the digestion time. This was found to be essential for accuracy and reproducibility as the residual peroxide interferes with the subsequent colorimetric assay. The samples were allowed to cool for 30 min then 1.5 ml deionised water was added followed by 2.4% ammonium molybdate (250 μl), and 0.65% hydrazine sulphate. The tubes were vortexed briefly and transferred to the water bath at a temperature of 35° C for 30 min. The concentration of phosphate was quantitated by measurement of absorbance at $\lambda = 820$ nm using a CARY1E UV-VIS spectrophotometer.

Assay validation. Synthetic dipalmitoyl-PC (4.22% elemental phosphorus) was used to construct a standard curve equivalent to 0.65 to 32.3 $\mu\text{mol l}^{-1}$ (0.02 to 1 mg l^{-1}) of elemental phosphorus [P]. The phosphorus content of several natural phospholipids was determined by comparison with a standard curve prepared from synthetic dipalmitoyl-PC. The percentage recovery of individual phospholipids was determined by comparing the recovery by solvent extraction of phospholipid from spiked urine lyophilizate from healthy volunteers ($n=4$) with the same amount of phospholipid digested and assayed for phosphorus without extraction ($n=3$).

Recoveries were determined for each phospholipid at the equivalent of 6.5, 19.4 and 32.3 $\mu\text{mol l}^{-1}$ (0.2, 0.6 and 1 mg l^{-1}) of elemental phosphorus. The between day variation was determined by repeating the analysis of all patient and healthy volunteer samples and ascertaining the coefficient of variation [CV] of the ratio first determination/second determination.

All urine samples and standards were analysed in duplicate. Urine samples were frozen at –35° C prior to lyophilization and were found to be stable for at least four freeze-thaw cycles.

The concentrations of creatinine in serum and urine were measured by the Jaffe method (Canterbury Health Laboratory Ltd). The creatinine clearance was estimated from the serum creatinine concentrations by the method of Cockcroft & Gault [15] as modified by Pesola *et al.* [16].

The urinary phospholipid concentrations were quoted as their ratio compared with the urine creatinine concentration to correct for varying degrees of dilution.

Urine specimen collection.

i) Ten healthy volunteers (six male, four female) aged 23 to 47 years (mean 34.3 years) provided a sample of their urine from a single complete emptying of their bladders. None of the healthy volunteers had a urinary tract infection (clinically and by urine culture) or were being treated concurrently with any drug.

ii) Ten patients (all male) aged 15 to 91 years (mean 58.3 years) who were hospitalised and required parenteral antibiotic therapy for the treatment of septicaemia, and who had a calculated creatinine clearance of greater than 20 ml min^{-1} were studied. All patients were given a single daily dose of 5–7 mg kg^{-1} gentamicin based on either lean

body weight (LBW) or actual body weight [16], whichever was lower, by a 30 min intravenous infusion. Doses were adjusted using the once daily gentamicin dosing method described previously by Begg *et al.* [20]. On the fifth to seventh day of gentamicin treatment a urine sample was collected from a complete emptying of the bladder or urethral catheter drainage bag. None of the patients had a urinary tract infection (clinically and by urine culture) or were being treated concurrently with any other potentially nephrotoxic drug.

iii) Thirty-four patients (four male, 30 female) aged 18 to 68 years (mean 32.2 years) requiring hospitalisation and parenteral antibiotic therapy for the treatment of suspected acute pyelonephritis were included in this study. Acute pyelonephritis was diagnosed if the patient had fever $>37.8^{\circ}\text{C}$ together with loin pain or tenderness and infected urine. Patients were allocated to receive one of two treatment regimens based on a combination of gentamicin and ciprofloxacin as described in Bailey *et al.* [21]. One group ($n=15$) received a single large dose of gentamicin (10 mg kg^{-1}) followed by oral ciprofloxacin, while the other group ($n=19$) received conventional gentamicin therapy aiming for peaks of 8 and troughs of 1.5 mg l^{-1} , followed by oral ciprofloxacin. The urinary phospholipids were measured predose, at 24 h, at 3 days while receiving antibiotics, and at 1–2 weeks after the completion of the course of antibiotics.

iv) The concentration of total urinary phospholipids was assayed in the urine of a female patient who was inadvertently overdosed with 240 mg gentamicin every 4 h (instead of once daily) for 16 h.

All study protocols were approved by the Southern Regional Health Authority (Canterbury) ethics committee. All subjects gave written consent prior to urine samples being taken.

Statistical analysis

The Mann-Whitney U-test was used to test the following in relation to phospholipid excretion: the difference between healthy controls and the 10 septicemic patients; the difference between healthy controls and the patients with acute pyelonephritis; the difference between the two treatment groups at the various time points measured. The patients with pyelonephritis were also analysed within patients, before, during (at 1 and 3 days) and after treatment using Wilcoxon's signed rank test. A level of significance for all tests was set at $P<0.05$.

A Deming analysis was used to determine the correlation coefficient (r^2) between the calculated creatinine clearance and urinary phospholipid excretion. A Deming analysis is a linear least squares analysis that accounts for errors in both X and Y data, as opposed to only considering error in the Y data.

Results

Assay

The standard curve was linear over the range 0.65 to $32.3\text{ }\mu\text{mol l}^{-1}$ (0.02 to 1 mg l^{-1}). The coefficient of variation of the standard curve varied from 3.3 to 9.4%, with coefficient of determination (r^2) ranging from 0.9975 to 0.9992. Complete digestion of PC standard was confirmed

by comparison with a standard curve prepared using potassium dihydrogen phosphate which required no digestion. The difference between the absorbances of the PC standard and phosphate was not significant. There was good recovery of all the phospholipid classes at the three concentrations tested; 6.5, 19.4 and $32.3\text{ }\mu\text{mol l}^{-1}$ (0.2, 0.6 and 1 mg l^{-1}). The limit of quantitation for this assay, defined as the smallest concentration with an inter-day coefficient of variation (CV) of $\leq 20\%$ ($n=5$), was $1.94\text{ }\mu\text{mol l}^{-1}$ (0.06 mg l^{-1}). The average ratio of samples assayed on two days (first determination/second determination) was 0.979 with a CV of 10.7%.

The concentrations of phospholipids found by solvent extraction of the pellet after centrifugation of the urine of the septicemic patients was considerably lower than the concentrations found by the solvent extraction of the lyophilisate. The concentration found by centrifugation compared to lyophilisation was $64 \pm 11\%$.

Urinary phospholipids

Healthy volunteers vs septicemic patients. The concentrations of phospholipids in the urine of the septicemic patients were highly variable but the mean value was considerably higher than that of healthy subjects ($P<0.0001$, Table 1). The contrast was maintained whether the concentrations of phospholipids were considered as absolute, or relative to urinary creatinine concentration. The two patients with the highest serum concentrations of creatinine (and the lowest creatinine clearances) also had the highest concentrations of phospholipids in urine, although one patient with a very high phospholipid concentration ratio ($4.59\text{ mmol mol}^{-1}$ creatinine) had a normal serum creatinine concentration (Table 1). There was a linear negative relationship between the calculated creatinine clearance and the extent of phospholipid excretion in the 10 patients with septicemia (Figure 1) ($r^2=0.71$).

Patients with acute pyelonephritis. The baseline concentrations of phospholipids were markedly elevated compared with the healthy controls ($P<0.0001$). There were no differences between the two pyelonephritis groups at baseline, at day 1 or 2–4 weeks post-dosing. However at 3 days the group treated with the single large dose had significantly lower phospholipid excretion than the conventionally treated group ($P<0.05$) (Figure 2).

Within group comparisons were made difficult by the non-collection of urine at some time periods, making the sample sizes small. The two groups were therefore combined at baseline, day 1, and 1–2 weeks after treatment, (at which times they were not different from each other). Paired comparisons revealed no difference between baseline and day 1 phospholipid excretion, but there was a significant decrease in phospholipid excretion 1–2 weeks after completion of gentamicin therapy compared with baseline ($P<0.0001$) and 24 h ($P=0.03$).

Accidental overdose

A single urine sample obtained immediately after the last dose of aminoglycoside had a phospholipid concentration of

Table 1 Total concentrations of phospholipids in urine of healthy subjects and septicaemic patients treated with gentamicin.

	Healthy volunteers		Septicaemic patients treated with gentamicin			
	Urinary phospholipid ($\mu\text{mol l}^{-1}$)	Urinary phospholipid:urine creatinine (mmol: mol Cr*)	Urinary phospholipid ($\mu\text{mol l}^{-1}$)	Urinary phospholipid:urine creatinine (mmol: mol Cr*)	Serum creatinine (mmol l ⁻¹)	Creatinine clearance (ml min ⁻¹)
	4.1	0.30	29.6	5.28	0.15	37.2
	2.7	0.53	48.3	5.08	0.08	66.0
	3.0	0.76	11.6	1.53	0.05	173
	3.9	0.28	6.3	1.58	0.07	132
	4.0	0.32	35.7	2.42	0.07	79.8
	2.6	0.25	30.6	5.77	0.14	26.4
	2.9	0.29	5.2	0.83	0.05	200.0
	2.0	0.72	47.7	4.59	0.06	115.0
	0.3	0.53	3.0	3.33	0.09	50.4
	2.7	0.30	24.9	2.14	0.06	139.8
Mean	2.82	0.43	24.3**	3.25**	0.057	102
s.e. mean	0.35	0.06	5.4	0.57	0.011	18.71
s.d.	1.12	1.19	17.08	1.8	0.036	59.17

*Creatinine.

** $P < 0.0001$ compared with concentrations in healthy subjects. **Figure 1** Calculated creatinine clearance (ml min⁻¹) vs phospholipid:creatinine ratio (mmol: mol Cr) in 10 septicaemic patients receiving gentamicin (5–7 mg kg⁻¹ 24 h⁻¹). ($r^2 = 0.71$). **Figure 2** Profile of the urinary phospholipid excretion (mmol: mol Cr) in pylonephritis patients receiving either a single large dose ($\square$) of gentamicin or conventional treatment ($\blacktriangle$) (mean $\pm$ s.e. mean) (* $P < 0.05$ compared with conventional dosing on day 3)).

9.07 mmol mol⁻¹ of creatinine, which was considerably higher than the ratio obtained for any other patient. Over the following 2 days, her serum creatinine concentrations increased from 0.05 to 0.10 mmol l⁻¹. The patient then developed oliguric renal failure, although this may have been associated with her underlying disease, necrotising fasciitis, which ultimately proved fatal.

Discussion

Assay

In the present studies, a method adapted from Folch *et al.* [10], and Olier *et al.* [8] was used to extract the phospholipid from urine, and our recoveries (95%) were consistent with theirs. For accurate results, precautions were necessary at two stages in the assay. Firstly, when separating the CHCl₃ rich phase, it was important to minimise contamination by the interfacial film which contains large amounts of inorganic phosphates. Filtration of the chloroform rich phase is required to remove insoluble phosphates but careful separation of the CHCl₃ rich phase also minimises contamination of the extracts.

The difference in the phospholipid concentrations found by centrifugation compared with lyophilisation is important when comparing our results with those of previous researchers [6, 8]. When these differences in methodology are accounted for, all researchers appear to have achieved similar results.

Urinary phospholipids

Three features are of special note. Firstly, there was considerable interpatient variability in the concentrations of phospholipids both in healthy control/pre-treatment subjects and patients treated with gentamicin as noted in other studies [5–8]. Secondly, the total concentrations of phospholipids in the urine of healthy subjects was much lower than

in the septicæmic patients and in the patients with acute pyelonephritis. Consistent with this was the negative correlation in phospholipid excretion in relation to creatinine clearance. This implies that phospholipiduria may indeed be a marker of damage to the kidneys, resulting in the decline in renal function. Thirdly, urinary phospholipid excretion did not alter during treatment with gentamicin in patients with acute pyelonephritis but lower concentrations were observed 1 to 2 weeks after treatment had ceased. Importantly, the reduction in phospholipid excretion began earlier in the patients treated with the single large dose of gentamicin compared with those treated conventionally.

It was difficult to compare our results directly with the previously published work because of the differences in the aminoglycosides examined, the doses used, the disease states of the patients and the methods of analysis. It appears, however, that the phospholipid concentrations during treatment with aminoglycosides are similar in all studies when corrected for recoveries related to different methodologies, but the mean concentrations in the control subjects varied considerably. This is likely to be the result of a different choice of control subjects (healthy versus pretreatment sick) in the various studies.

The only previous study in which total phospholipid concentrations were measured was that of Olier *et al.* [8] who examined urinary concentrations of phospholipids in patients with acute pyelonephritis. The concentrations were high before treatment with aminoglycosides and did not change further during treatment, a result which we confirmed. Products of tissue breakdown which are present in urine in this condition may very well increase the urinary levels of phospholipids before treatment. Furthermore, we suggest that a gentamicin-induced enhancement of phospholipid excretion may be countered by a simultaneous fall in the excretion of tissue breakdown materials as the disease is treated successfully. The net result will be no consistent change in the concentration of urinary phospholipids. The lower urinary concentrations of phospholipids in the single large dose group at day 3 and after cessation of treatment in both groups supports this hypothesis.

Ibrahim *et al.* [5–7] found mean phospholipid concentrations of up to $2.0 \text{ mmol mol}^{-1}$ creatinine (adjusted for methodological differences) in the centrifuged urinary sediment of patients treated with netilmicin, although the concentrations in patients treated with amikacin were lower. The major difference between our results and those of Ibrahim *et al.* [6] lies in the concentrations in the controls. Their controls were the urine of patients with pelvic inflammatory disease or urinary tract infections prior to treatment. These patients had pre-treatment values of urinary phospholipids above the values which we found in healthy subjects. The contrast probably reflects the disease processes, as with acute pyelonephritis.

We believe that our results have assisted in understanding the reasons for the conflicting findings in the literature. The results in septicæmic patients support the hypothesis that gentamicin increases phospholipid excretion, but the benefits of using urine phospholipids as an index of nephrotoxicity may be diminished by lack of specificity. It appears that many of the infections treated with aminoglycosides are themselves associated with phospholipiduria. These results

highlight the necessity to measure phospholipids prior to treatment in order to distinguish drug effect from that due to the disease state.

Our results do not assist in recognising which patients are likely to progress clinically to renal failure. Further work should be directed towards determining if there is a level of urinary phospholipid excretion which is predictive of renal toxicity. Studies in rats indicate that the urinary excretion of phospholipids increases before creatinine clearance falls [4, 18], and also 'that a critical threshold in phospholipid accumulation needs to be reached before it leads to cell death, whereas keeping below this critical value will allow the cell to survive, and even to return to its normal state if the drug is withdrawn' [2]. If such a critical threshold can be demonstrated in the urinary concentrations of phospholipids, then measurement of urinary phospholipid concentrations may well be superior to the measurement of plasma concentrations of creatinine and may anticipate and help pre-empt aminoglycoside toxicity. The threshold may however be so high as to make considerations of pre-treatment phospholipid concentrations irrelevant to the prediction of renal failure, but more detailed clinical examination of urinary phospholipid concentrations before, during and after treatment with aminoglycosides is required.

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