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β-Hydroxybutyrate: a urinary marker of imipenem induced nephrotoxicity in the cynomolgus monkey detected by high field ^{1}H NMR spectroscopy

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High field nuclear magnetic resonance (NMR) spectroscopy is finding increasing application in the analysis of biological fluids obtained during the investigation of drug metabolism or toxicity (reviewed in Refs 1–3). In particular Gartland *et al.* [4] have demonstrated the utility of proton (^{1}H) NMR of urine as a means of obtaining information on the site of toxic insult and the biochemical mode of action of a wide range of compounds exhibiting nephro-, hepato- and testicular toxicity. We have previously used ^{1}H NMR as a means of urinalysis for studies on the effects of the cephalosporin antibiotic cephaloridine [5, *] in order to monitor the onset and progress of nephrotoxicity. As part of further investigations on antibiotic nephrotoxicity we have used ^{1}H NMR for the qualitative analysis of urine samples obtained from cynomolgus monkeys dosed with the carbapenem antibiotic imipenem as described below.

Materials and Methods

Animals and treatments. Experimental work was conducted at Inveresk Research International (Musselburgh, U.K.). In this study three male and three female cynomolgus monkeys (*Macaca fascicularis*, Shamrock

* Murgatroyd LB, Pickford RJ, Smith IK and Wilson ID, submitted.

Farms Ltd, U.K.), weighing 2.5 ± 0.2 kg were dosed intravenously with 180 mg/kg of imipenem (4% w/v solution in physiological saline), once daily for 7 days. A control group (six animals of each sex) each received an intravenous dose of physiological saline (5 mL/kg) for the same period.

All animals were observed daily for signs of ill health or reaction to dosing. Body weight, food and water consumption were monitored during the dosing period. Routine clinical chemistry and urinalysis indices were measured twice pretest and on days 1, 3 and 7 of the study. Urine samples for ^{1}H NMR were obtained on days 1, 3 and 7. After 7 days dosing, a gross pathological examination was performed together with histopathological evaluation of the kidneys.

Clinical chemistry. Standard clinical chemistry methods were used to determine blood urea nitrogen and creatinine, blood glucose, total protein, albumin, albumin/globulin ratios, sodium, potassium, chloride, alkaline phosphate, alanine aminotransferase and aspartate aminotransferase.

Urinary creatinine, alkaline phosphatase (AP), gamma glutamyl transferase (GGT), *N*-acetyl-β-D-glucosaminidase (NAG) colour, pH, specific gravity, volume, sodium, potassium, osmolality glucose and protein were also measured.

^{1}H *NMR.* Urine samples from treated and control animals were stored frozen until analysis. Aliquots (1.6 mL) of urine were taken and mixed with 5 M urea in ^2H_2O (400 μL) and the pH adjusted to 3.5. ^{1}H NMR was then performed on a Bruker AM400 NMR spectrometer operating at 9.4 tesla (400 MHz). All spectra were recorded at ambient probe temperature. Water suppression was achieved using the WATR technique as modified by Connor *et al.* [6]. Spectra were the result of 64 free induction decays (FIDs) which were collected into 32 × 1024 data points. Compounds detected in urine by ^{1}H NMR were identified by comparison with the spectra of authentic standards and by standard addition. Quantities of β-hydroxybutyrate present in the day 7 urine samples of imipenem dosed animals were estimated using NMR by reference to the known concentrations of creatinine.

Results

Clinical pathology and histopathology. Intravenous administration of imipenem at 180 mg/kg/day for 7 days was associated with increases in blood urea nitrogen and creatinine consistent with an effect of the compound on the kidney (Table 1) whilst there was a transient increase in the concentration of potassium observed for day 3. Urinalysis showed increases in a range of enzymes including alkaline phosphatase (AP), gamma glutamyl transferase (GGT) and *N*-acetyl-β-D-glucosaminidase (NAG) and a decrease in urinary pH. These changes in blood chemistry and urinary enzyme levels were accompanied by gross and histopathological changes in the kidneys of five out of the six dosed animals. At necropsy the kidneys of the affected animals appeared pale, with grey or brown foci, speckling and/or streaks.

Histological changes included moderate to severe tubular necrosis accompanied by tubular epithelial regeneration, the presence of intratubular protein and intracytoplasmic protein droplets. A moderate amount of fat was found in the tubular epithelial cells of four out of five animals showing necrosis whilst a mild amount was observed for the other animal. The remaining animal receiving imipenem showed no changes in the kidney other than a mild amount of fat in the tubular epithelial cells.

^{1}H *NMR spectroscopy.* Typical ^{1}H NMR spectra for one animal are shown in Fig. 1 for days 1, 3 and 7 of the study. In Fig. 1A the day 1 spectrum shows resonances for a variety of endogenous metabolites including acetate, creatinine, creatine, dimethylamine, formate, glycine, hippurate, phenylalanine, succinate, trimethylamine-*N*-oxide and tyrosine; signals for imipenem were not observed. The day 1 spectrum was, in essence, the same as those obtained from control animals. The day 3 spectrum (Fig. 1B) shows many similarities to that obtained for the first day of the study. Differences included the absence of

Table 1. Clinical chemistry blood and urine parameters for control* cynomolgus monkeys and those receiving 180 mg/kg/day of imipenem for 7 days

Parameter + SD	N	Pre-test	Day 1	Day 3	Day 7
Blood creatinine	6	67 ± 6	65 ± 5	93 ± 27§	240 ± 231§
(μmol/L)	(12)	(63 ± 6)	(58 ± 5)	(61 ± 5)	(61 ± 5)§
Blood urea nitrogen	6	9.2 ± 1.2	7.2 ± 1.6	9.4 ± 2.8	20 ± 16.4†
(mmol/L)	(12)	(9.0 ± 1.5)	(7.8 ± 1.5)	(7.7 ± 1.1)	(8.2 ± 1.4)
Urinary creatinine	6	2.6 ± 0.8	1.9 ± 0.6	2.6 ± 0.7	3.9 ± 1.6‡
(mmol/L)	(12)	(1.9 ± 0.6)	(2.6 ± 0.5)	2.6 ± 0.7	(2.1 ± 0.9)
Urinary alkaline	6	6 ± 3	121 ± 251†	50 ± 41‡	94 ± 102§
phosphatase (I.U./L)	(12)	(6 ± 3)	(7 ± 3)	(7 ± 2)	(6 ± 5)
Urinary gamma					
glutamyl transferase	6	29 ± 14	38 ± 22	85 ± 71†	139 ± 140§
(I.U./L)	(12)	(23 ± 9)	(33 ± 11)	(32 ± 9)	(24 ± 9)
N-Acetyl					
glucosaminidase	6	3.0 ± 1.3	6.3 ± 6.3†	8.9 ± 7.4§	22.4 ± 18.7§
(I.U./L)	(12)	(2.7 ± 0.7)	(1.7 ± 0.7)	(2.3 ± 1.1)	(2.2 ± 1.3)
Urinary pH	6	7.4 ± 0.6	7.5 ± 0.4	7.1 ± 0.3	6.2 ± 0.2§
	(12)	(7.3 ± 0.7)	(7.0 ± 0.7)	(7.2 ± 0.5)	(7.3 ± 0.6)

Values are means ± SD.
* Figures for control animals are in parentheses.
† Significantly different from control $P < 0.05$.
‡ Significantly different from control $P < 0.01$.
§ Significantly different from control $P < 0.001$.

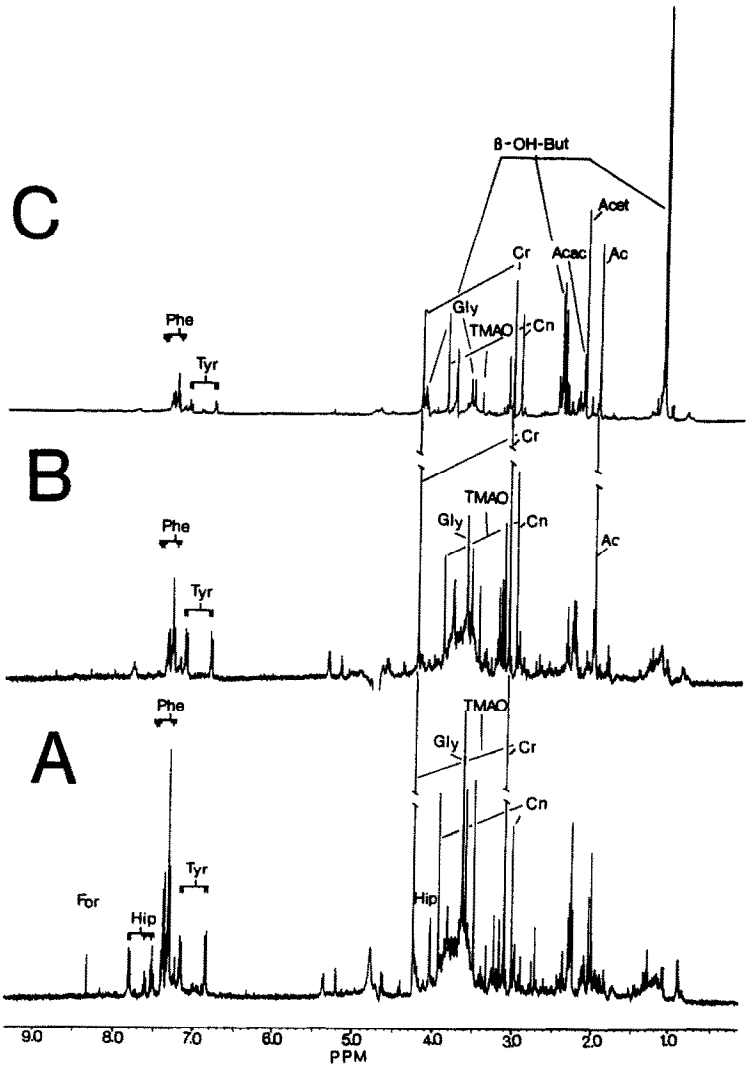

Fig. 1. Typical ^{1}H NMR spectra of urine obtained from the same cynomolgus monkey on days 1, 3 and 7 (A, B and C, respectively) following administration of 180 mg/kg/day of imipenem. Key: Ac, acetate; Acac, acetoacetate; Acet, acetone; β-OH-But, β-hydroxybutyrate; Cr, creatinine; For, formate; gly, glycine; hip, hippurate; phe, phenylalanine; TMAO, trimethylamine-*N*-oxide; Tyr, tyrosine.

hippurate from the spectra of all animals receiving imipenem and the elevation of acetate concentrations in two animals. We have previously observed in rats dosed with antibiotics, or in bile cannulated animals, that hippurate is often absent. This observation may not, therefore reflect an effect of imipenem directly on the kidney but may be associated with the antibiotic effect of the drug and the suppression of gut microflora. In the spectrum of one animal we also observed signals for β-hydroxybutyrate. The spectra for samples obtained for day 7 of the study were, in comparison, grossly distorted for four of the six imipenem dosed animals.

The spectra of two of these animals (1 and 3, Table 2) were dominated by the presence of high concentrations of β-hydroxybutyrate (Fig. 1C) together with signals consistent with the presence of acetate, acetone and acetoacetate. The urine of two further animals (2 and 5, Table 2) also contained large amounts of β-hydroxybutyrate, acetone and acetoacetate. The urine of the remaining animals (4 and 6, Table 2) also contained β-hydroxybutyrate but at lower concentrations than seen with the other four monkeys. Animal 6 exhibited almost normal clinical chemistry and kidney histopathology (apart from the accumulation of tubular fat). The approximate quantities of β-hydroxybutyrate present in the samples were determined from the day 7 NMR spectra by calculating the amount present relative to creatinine. These data, together with the individual animal clinical chemistry data and qualitative histopathological assessments are given in Table 2. From these data it is clear that those animals showing the highest concentrations of β-hydroxybutyrate in the urine (animals 1 and 3) also show the highest levels of urinary enzymes. In contrast animals 4 and 6 had the lowest concentration of β-hydroxybutyrate and the lowest levels

Table 2. Individual clinical chemistry blood and urine parameters and histopathology for day 7 for cynomolgus monkeys receiving 180 mg/kg/day of imipenem for 7 days

Animal		1	2	3	4	5	6
Parameter	Sex	♀	♂	♂	♂	♀	♀
β-Hydroxybutyrate (mmol/L)		78.4	15.2	23.9	2.9	13.2	4.3
Blood creatinine (μmol/L)		103	109	99	611	449	71
Blood urea nitrogen (mmol/L)		15.2	12.9	10.5	51.4	27.4	7.9
Urinary creatinine (mmol/L)		6.9	3.0	4.1	2.5	3.7	3.4
Urinary alkaline phosphatase (I.U./L)		276	65	148	18	30	24
Urinary gamma glutamyl transferase (I.U./L)		338	105	214	18	43	63
N-Acetyl glucosaminidase (I.U./L)		56.4	24.5	25.5	9.7	14.8	3.5
Urinary pH		6.5	6.1	6.0	6.5	6.0	6.3
Acute tubular necrosis*		++	++	++	+++	+++	—
Tubular fat accumulation†		++	++	++	+	++	+

* Acute tubular necrosis: ++ approximately one half of each cortex involved; +++ nearly all cortical tubules involved.

† Tubular fat accumulation: + droplets in one quarter to one half of cortical tubules; ++ droplets in over half of cortical tubules.

of urinary enzymes. The remaining two animals (2 and 5) had intermediate concentrations of β-hydroxybutyrate and urinary enzymes. It is perhaps noteworthy that monkeys 4 and 5 exhibited somewhat higher blood urea nitrogen and creatinine concentrations than the rest and that this may be related to the greater degree of tubular necrosis observed in these animals.

A trend is thus apparent between β-hydroxybutyrate concentrations and urinary markers of toxicity. However, it is not possible, based on the limited data obtained by monitoring days 1, 3 and 7 of the study, to fully describe the onset and progression of this toxicity. It may be, therefore, that the observed differences between animals 1 and 3, 2 and 5, 4 and 6 reflect only a different rate of progression of nephrotoxicity between individuals.

Discussion

Various mechanisms have been proposed for β-lactam induced nephrotoxicity. The most favoured of these proposed mechanisms include:

(a) Concentrative uptake of the β-lactam into the tubular cell [7].
(b) Cell membrane lipid peroxidation [8].
(c) Respiratory toxicity due to the acylation and inactivation of mitochondrial transporters for anionic substrate uptake [9–11].

Both the oxidative (b) and mitochondrial toxicity (c) mechanisms have also incorporated concentrative uptake as the first step leading to cell necrosis [11]. This is based on the observation that the nephrotoxicity of compounds such as cephaloridine, cephaloglycan and imipenem can be prevented by the co-administration of probenecid (or cilastatin in the case of imipenem).

Recent studies have suggested that, with imipenem, oxidative stress is only transient and not the primary cause of nephrotoxicity for this compound, whilst mitochondrial respiration was significantly reduced [11]. The presence of high concentrations of β-hydroxybutyrate, together with acetate, acetoacetate and acetone, in the urine of animals receiving imipenem, and exhibiting tubular necrosis, would appear to be consistent with a severe disruption of energy metabolism. This may also be reflected in the observed accumulation of fat within the cells. Whilst the significance of the ketone bodies present in the urine of imipenem treated animals is not yet clear it may well be that they reflect the underlying mechanism of toxicity. Further, the presence of large amounts of β-hydroxybutyrate provides a useful marker for the toxic effects of the compound.

In summary, the use of ^{1}H NMR as a complement to conventional clinical chemistry and histopathology resulted in the detection of hitherto unsuspected changes in urine composition as a result of imipenem induced nephrotoxicity. Large quantities of β-hydroxybutyrate, as well as other ketone bodies were detected, indicating a disruption of energy metabolism. β-Hydroxybutyrate may provide a useful non-invasive marker for imipenem toxicity.

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