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Short Communication

Proton nuclear magnetic resonance of urine and bile from paracetamol dosed rats*

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Introduction

High resolution nuclear magnetic resonance (NMR) spectroscopy, particularly ^{1}H-NMR, is now an established method for the direct analysis of biological fluids (e.g. urine, plasma, etc.; reviewed in refs 1–4). The technique also has considerable utility in the study of drug metabolism [1–4], particularly for compounds dosed at >1–2 mg kg^{-1}, where the major route of excretion is the urine. Clearly where the elimination of the drug is primarily via the faeces, NMR techniques are less readily applicable. Solvent extraction of faecal samples followed by NMR analysis can provide information on a compound's metabolic fate in suitable cases (Roberts and Wilson, unpublished) but a major difficulty with this approach is that often variable and incomplete recoveries are obtained. An alternative which eliminates the need for extraction is to collect bile for direct analysis by NMR. Previously we have reported the use of NMR to analyse bile from rats, and have studied the metabolism and biliary excretion of *p*-aminophenol in this species [5]. Here we report further studies on the use of NMR to analyse bile for the presence of drug metabolites based on studies using [^{14}C]-labelled paracetamol (acetaminophen) to enable comparison of ^{1}H-NMR and conventional radiotracer techniques to be made.

Experimental

Dose formulation

Paracetamol and [^{14}C]-ring labelled paracetamol (Amersham International, UK), having a radiochemical purity >97%, were dissolved in ethanol–physiological saline (30:70, v/v) at a concentration of 150 mg ml^{-1}.

Animals and treatments

Eight albino male rats (Alpk:APFSD Wistar derived), of between 200–300 g, were acclimatized in metabolism cages for 3 days prior to dosing. The animals were subdivided into two groups of four. One group of normal animals received a single intraperitoneal injection of 300 mg kg^{-1} [^{14}C]-paracetamol (8 μCi/animal). The second group received a similar dose of paracetamol but were fitted with a biliary cannula enabling a complete collection of bile to be made.

Bile was collected for the following periods; predose, 0–6, 6–12 and 12–24 h post dose. Urine was collected from both groups for the periods predose and 0–24 h post dose. Samples were stored frozen at −20°C until analysed.

Animals in both groups had free access to food and water at all times.

Analytical procedures

Determination of radioactivity. The amount of radiolabel present in individual samples was

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determined by scintillation counting. Aliquots of urine and bile (50 μl), in triplicate, were taken and diluted to 1 ml with water to which 10 ml of Beckman "Ready Value" scintillant was added. Radioactivity was determined on an Intertechnique SL 30 liquid scintillation counter (10 min or until 10^4 counts had accumulated). Counting efficiency was determined using the external standard channels ratio method.

Thin-layer chromatography. Metabolite profiles of the excreted radiolabel were obtained by TLC on 20 × 20 cm glass backed silica gel 60 F254 TLC plates incorporating a fluorescent indicator (E. Merck, purchased from BDH Ltd, UK). Samples were applied as 1-cm streaks using a Camag Linomat IV automatic TLC sample applicator (Camag, Switzerland). Ascending chromatography was performed in glass TLC tanks (20 × 20 × 5 cm) using multiple development with two solvent systems. Solvent system A consisted of chloroform–methanol–acetic acid (85:15:1, v/v) with double development to 15 cm. Chromatography in solvent system A was followed by a single development in solvent system B, which was composed of butan-1-ol–water–acetic acid (4:1:1, v/v), to 6.5 cm. Following chromatography sample components were located using fluorescence quenching at 254 nm, autoradiography and by TLC Linear Analyser (Berthold LB285, Berthold UK Ltd).

Identification of metabolites was by comparison with authentic standards applied to the plates with confirmation of selected samples by TLC with off-line fast atom bombardment-mass spectrometry directly from the adsorbent (see ref. [6] for details). Enzymic hydrolysis with β-glucuronidase and aryl sulphatase was also employed to confirm the presence of conjugated metabolites.

NMR spectroscopy

Urine. Samples of urine (800 μl) were made up to 1 ml by the addition of 200 μl of 5 M urea in ^2H_2O, and the pH was adjusted to 3.5 ± 0.5 with 0.1 M hydrochloric acid. Samples were analysed by ^{1}H-NMR, using the modified WATR method for water suppression as described in ref. 7, at 400 MHz (9.4 Tesla) on a Bruker WH 400 NMR spectrometer operating at 400.13 MHz proton resonance frequency. Spectra (typically 64 transients) were measured at ambient probe temperature. Data were collected into 32,768 computer points using 11 (90°) pulses, a spectral width of 5000 Hz, an acquisition time of 3.27 s and a delay between pulses of 1.5 s.

Bile. Samples of bile (750 μl) were freeze-dried and redissolved in an equivalent volume of ^2H_2O containing 3-trimethylsilyl-(2,2,3-$^2H_4$)-1-propionate (TSP) to provide a chemical shift reference (δ = 0 ppm). NMR spectroscopy was performed on a Jeol GSX 500 NMR spectrometer operating at 500 MHz proton resonance frequency (11.75 Tesla) at ambient probe temperature. For each sample 100 transients were collected into 32,768 computer points using 3 μs (40°) pulses, a spectral width of 6000 Hz and an acquisition time of 2.73 s. A delay of 2 s was added to permit full T_1 relaxation. An exponential line broadening function of 1 Hz was applied prior to Fourier transformation (FT). A secondary irradiation field was applied at the water resonance frequency, with the power being gated off during acquisition to minimize radio frequency interference, in order to suppress the signal due to residual water. Paracetamol resonances were assigned as described in ref. 8.

Results and Discussion

The recovery of the radiolabel in urine over the 24 h period following administration was 80% for control and 48% for biliary cannulated animals. The excretion of radioactivity in the bile over this period was 26% with the bulk of the elimination occurring in the 0–6 h period (24.5%).

Metabolite profiling of the urine samples by radio-TLC for both groups of animals revealed the excreted radioactivity to be present as bands which co-chromatographed with paracetamol sulphate and glucuronide, with a small amount of unchanged paracetamol also present. The ratios of the various metabolites, as determined using the linear analyser, varied between the two dosed groups. In the urine of the control group the sulphate conjugate was the major metabolite accounting for some 60% of the total, whilst the glucuronide and unchanged paracetamol accounted for 30 and 10%, respectively. In the case of the bile cannulated animals however the sulphate and glucuronide conjugates were excreted in similar amounts (45 and 45%, respectively) whilst the quantity of paracetamol remained at 10%.

The identification of the urinary metabolites was confirmed by enzymic hydrolysis (β-glucuronidase and aryl sulphates) allied to TLC–FAB–MS on selected samples.

Metabolite profiling of bile showed the major radiolabelled component to be paracetamol glucuronide (80%) with minor quantities of other drug related material (e.g. free paracetamol, paracetamol sulphate, paracetamol glutathione adduct) also present.

The analysis of urine using ^{1}H-NMR spectroscopy also confirmed the presence of paracetamol, paracetamol glucuronide and paracetamol sulphate in samples from both control and bile duct cannulated animals. The differences observed on TLC in the ratios of sulphate and glucuronide conjugates between the two dose groups were immediately apparent, but in addition ^{1}H-NMR also revealed differences in the pattern of endogenous metabolites. Thus, ^{1}H-NMR of urine from the control group showed a normal "fingerprint" of endogenous compounds including hippuric acid, creatinine, citrate, succinate, etc. In the bile duct cannulated animals hippuric acid was absent and concentrations of citrate and succinate were reduced. Such changes, which in other circumstances might have toxicological

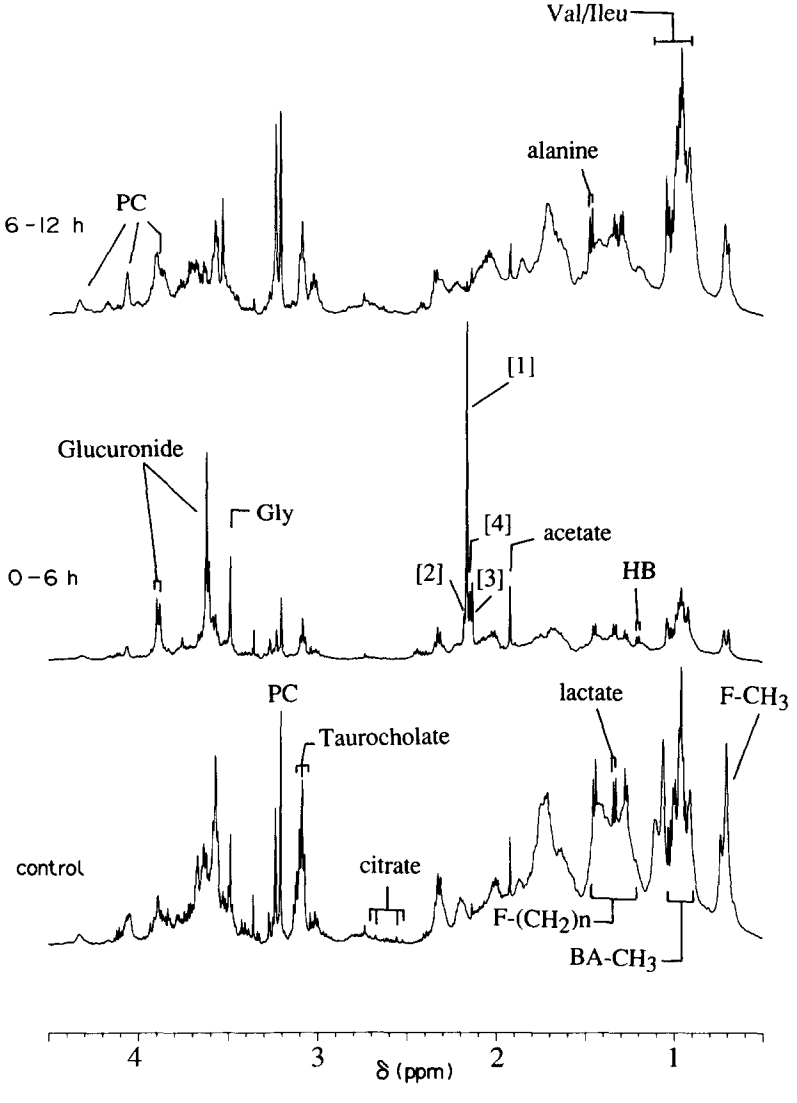

Figure 1
^{1}H-NMR spectra (500 MHz) of rat bile (high field region) showing predose (a) 0–6 h, (b) and 6–12 h, and (c) samples following a single IP dose of 300 mg kg^{-1} [^{14}C]paracetamol. Key: HB, 3-β-hydroxybutyrate; PC, phosphatidyl choline; BA—CH_3, CH_3 groups of bile acids; F—CH_3, CH_3 groups of fatty acids; F—(CH_2), CH_2 groups of fatty acids. Paracetamol metabolites: [1] paracetamol glucuronide; [2] paracetamol sulphate: [3] paracetamol; [4] glutathione adduct of paracetamol.

significance would have gone unnoticed if the sample analysis had been limited to TLC. The changes seen here probably reflect the effect of bile cannulation and not toxicity.

Typical part spectra for the NMR of control, 0–6 and 6–12 h post-dose bile are shown in Figs 1 (aliphatic region) and 2 (aromatic region).

The spectrum of control bile (Figs 1a and 2a) shows the presence of a wide range of endogenous compounds and clearly illustrates the heterogeneous nature of this biofluid in terms of the molecular mobility of the various components as revealed by the range of detectable line widths. As we have discussed elsewhere [5] the spectrum is dominated by broad resonances from bile acids present in micelles together with cholesterol and phospholipids. The compounds present in the sample include 3-β-hydroxybutyrate, phosphatidyl choline, taurocholate, citrate, lactate, glycine, alanine, isoleucine, tyrosine and phenylalanine. When the 0–6 h post-dose sample was analysed in this way signals for paracetamol glucuronide were also observed in both aliphatic (Fig. 1b) and aromatic (Fig. 2b) portions of the spectrum. Additional drug related resonances could also be observed corresponding to unchanged paracetamol, paracetamol sulphate and the glutathione adduct of paracetamol. Examination of the 6–12 h bile sample by ^{1}H-NMR failed to reveal the presence of paracetamol related material, but by this time after dosing bile contained <1% of the dose.

Conclusion

The results of this study underline the utility of using ^{1}H-NMR methodology as a means of studying the biliary elimination of drugs and

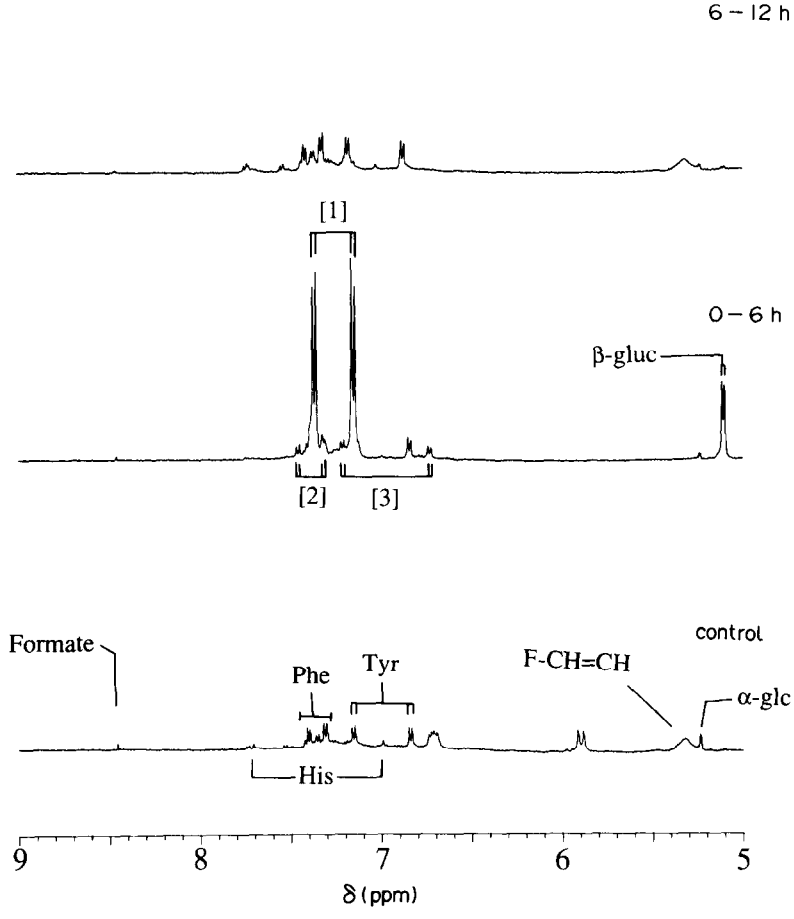

Figure 2
NMR spectra (500 MHz) of bile (low field region) showing predose (a) 0–6 h (b) and 6–12 h, and (c) samples following a single 1P dose of 300 mg kg^{-1} [^{14}C]paracetamol. Key as for Fig. 1 with addition α-glc, α-anomeric proton of glucose; β-gluc, β-anomeric proton of paracetamol glucuronide; F—CH=CH, olefinic protons of fatty acids; His histidine; Phe, phenylalanine; Tyr, tyrosine.

their metabolites. Comparison of the results from ^{1}H-NMR spectroscopy and conventional studies involving the use of radiolabelling showed good agreement in respect of metabolite profiling. Radiotracer techniques are clearly inherently more sensitive than ^{1}H-NMR, but obviously require the availability of a suitably radiolabelled form of the xenobiotic to be studied. In addition, whilst metabolites are easily detected by both techniques, the NMR spectrum may contain sufficient information to enable unequivocal identification to be made in a way which is not possible based on chromatographic data alone. In addition to basic qualitative or quantitative analytical data generated by NMR spectroscopy subtle information on the physicochemical interactions between molecules in whole bile are carried in the line width (T_2 relation time) data provided in the spectrum. This can provide information which relates to micellar compartmentation/immobilization of compounds. Such information is difficult to obtain by conventional methods and may be of value in understanding mechanisms by which drugs and their metabolites are eliminated in the bile.

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