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Proton NMR spectroscopy of bile for monitoring the excretion of endogenous and xenobiotic metabolites: application to *para*-aminophenol

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Introduction

One of the most important functions of the liver is bile formation. Bile is both a secretory and an excretory fluid and, as such, its composition is complex and varies according to the nutritional state of the individual [1]. The secretory functions most prominently include the delivery to the intestinal tract of bile salts and their associated lipids to aid fat digestion and absorption, while the excretory functions include the excretion of liver-derived metabolites of potentially toxic endogenous (e.g. steroid hormones, bilirubin) or exogenous (e.g. drugs, environmental chemicals) materials. A large number of drugs and foreign compounds are excreted in bile [2], and monitoring and identifying biliary excretion products is an essential part of the metabolic profiling procedure that has to be undertaken when novel therapeutic compounds are developed.

The analysis of low molecular weight compounds in whole bile by conventional biochemical techniques is an involved and time-consuming procedure since the fluid has very complex multiphasic physico-chemical characteristics, a micellar substructure with a lipid rich matrix, and detergent properties. We have shown that ^{1}H-NMR spectroscopy can be used effectively to identify and quantify metabolites of both endogenous and xenobiotic origin in other intact biological fluids such as blood, plasma and urine which also have complex biochemical matrices [3–8]. We have, therefore, applied high resolution single pulse and spin-echo ^{1}H-NMR methods to study the composition and molecular mobility of endogenous metabolites in whole bile. In addition we have used ^{1}H-NMR to investigate the metabolism and biliary excretion of a model xenobiotic *p*-

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Abbreviations: APAP, paracetamol; CPMG, Carr Purcell Meiboom Gill; gcdc, glycochenodeoxycholate; HB, β-hydroxybutyrate; i.p., intraperitoneal; MW, molecular weight; NMR, nuclear magnetic resonance; PAP, *p*-aminophenol; PC, phosphatidyl choline; ppm, parts per million; TSP, sodium 3-trimethylsilyl-[2,2,3,3,^{2}H$_4$]-1-propionate.

aminophenol (PAP) in the biliary cannulated rat. PAP is a minor metabolite of the drug paracetamol that is nephrotoxic via an unusual mechanism involving primary metabolism in the liver with subsequent biliary excretion of a toxic metabolite which is reabsorbed from the gut and redistributed to the kidney via the blood [8]. The present study forms part of the evaluation of ^{1}H-NMR spectroscopy as a technique for the study of the biliary excretion and compartmentalization of xenobiotic metabolites and endogenous biliary components.

Experimental

Animals and treatments

Twelve male F344 rats (weight, 200–250 g; Charles River) were allocated to two groups of 6 rats, i.e. 6 rats per experiment. These groups of 6 were further divided into two groups of 3 rats: cannulated control and treated groups. The common bile ducts of 6 rats (3 per experiment) were cannulated according to the method of Tomlinson *et al.* [9]. Rats received a single i.p. injection of either saline (control), or a 50 mg ml^{-1} solution of *p*-aminophenolhydrochloride (PAP; Aldrich) in saline equivalent to a dose of 100 mg kg^{-1} body weight. In experiment 1 the toxin was administered 15 min prior to surgery, and the animals maintained in a state of Sagatal^{TM} (pentobarbital; May and Baker)-induced anaesthesia throughout, while in experiment 2 the animals received the same dose of PAP following a 24 h post-operative recovery period. Bile was collected in chilled vessels over two 2-h periods, i.e. 0–2 and 2–4 h (experiment 1), whereas in experiment 2, cannulated animals were placed in glass metabolism cages and bile was collected for a period of 24 h before and at 8 and 24 h after dosing. Food and tap water were provided *ad libitum* following recovery.

Proton NMR spectroscopy of bile

NMR measurements were made on a Bruker WH400 spectrometer (Queen Mary College, ULIRS, London) operating at 400.13 MHz proton resonance frequency, using quadrature detection and fitted with a 16 bit analog to digital converter, Aspect 3000 data system and array processor. The detection limit of ^{1}H-NMR spectroscopy at 9.4 Tesla is approximately 50 μM. Spectra were measured at ambient probe temperature (298 ± 1 K). For non-lyophilized bile a volume of 0.45 ml was diluted with 0.05 ml of ^2H_2O containing the internal chemical shift reference TSP (δ = 0 ppm; Aldrich) in 5-mm tubes.

Lyophilized bile was reconstituted in the same volume of ^2H_2O (field/frequency lock) and 0.45 ml of each sample was diluted with a further 0.05 ml of ^2H_2O containing TSP (2 mM) so that metabolite concentrations would be directly comparable with non-lyophilized samples. All spectra were acquired under identical spectrometer conditions. Sixty-four free induction decays were collected into 16,384 computer points (sweep width: 5000 Hz) employing the Carr Purcell Meiboom Gill (CPMG) spin-echo experiment [10, 11]. The pulse sequence used was

$$[D1\text{-}90_x\text{-}(D2\text{-}180_{\pm y}\text{-}D2\text{-collect FID})_n],$$

where $D1 = T_1$ relaxation delay (2 s), $D2$ = a fixed relaxation delay (2 ms) and n = number of cycles of variable loop, typically 48. This pulse sequence can be used to selectively attenuate broad resonances from protons with short T_2s [12].

As T_2 is related to the molecular mobility of the nucleus via the correlation time T_c, CPMG spectra can be used to probe compartmentalization of molecules in intact biological materials.

Single pulse ^{1}H-NMR experiments were performed on bile samples using the following parameters: 64 free induction decays were collected into 16,384 computer points using 28° (3 μs) pulses over a sweep width of 5000 Hz and an acquisition time of 1.7 s. A further delay of 3.0 s between pulses was added to ensure that the spectra were fully T_1 relaxed. A continuous secondary irradiation field at the resonance frequency of water was applied in order to suppress the intense water signal in non-lyophilized bile, and minimize spectrometer dynamic range problems [3].

Results

Proton NMR spectroscopy of control rat bile

The single pulse ^{1}H-NMR spectrum of lyophilized control bile is shown in Fig. 1 with resonance assignments. The heterogeneous nature of bile in terms of molecular mobility of the different compounds is illustrated with signals having a range of proton resonance linewidths. The single pulse and CPMG ^{1}H-NMR spectra of lyophilized, and non-lyophilized control rat bile samples are shown in Fig. 2. The single pulse spectra of bile are dominated by broad resonances from bile acids which are present in mixed micelles with phospholipids and cholesterol. They are broad as a result of the short spin–spin (T_2) relaxation times reflecting constrained molecular motions within the phospholipid micellar particles. Interestingly, there is a resolved contribution to the single pulse ^{1}H-NMR spectrum of bile from cholesterol and cholesterol derivatives (Fig. 1). In blood plasma, such signals are too broad to be observed due to lipoprotein binding which

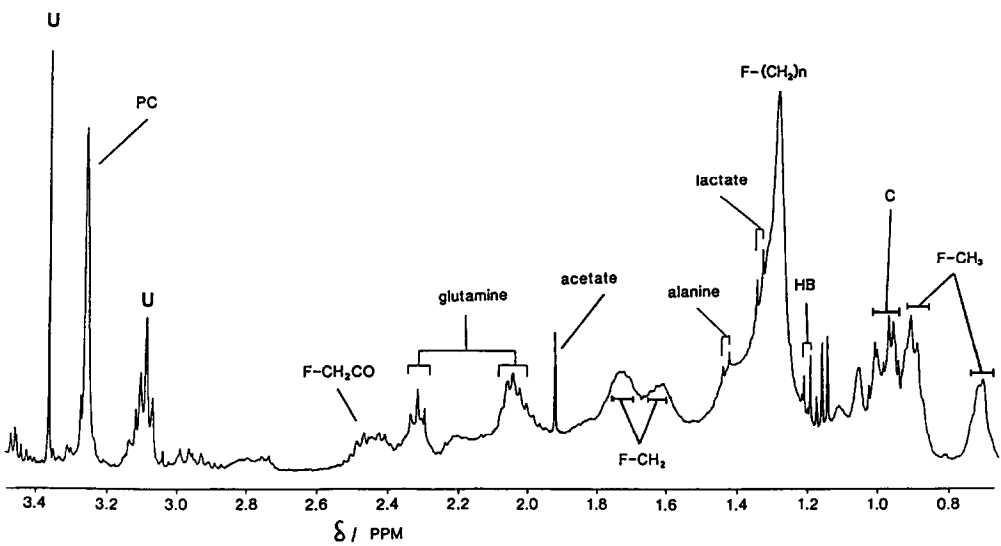

Figure 1
Single pulse 400 MHz ^{1}H-NMR spectrum of lyophilized control rat bile (region to low frequency of water). C, cholesterol; F-CH_2, F-CH_3, F-CH_2CO and F-$(CH_2)_n$ refer to resonances for fatty acids esterified to phosphatidyl choline; U, unknown.

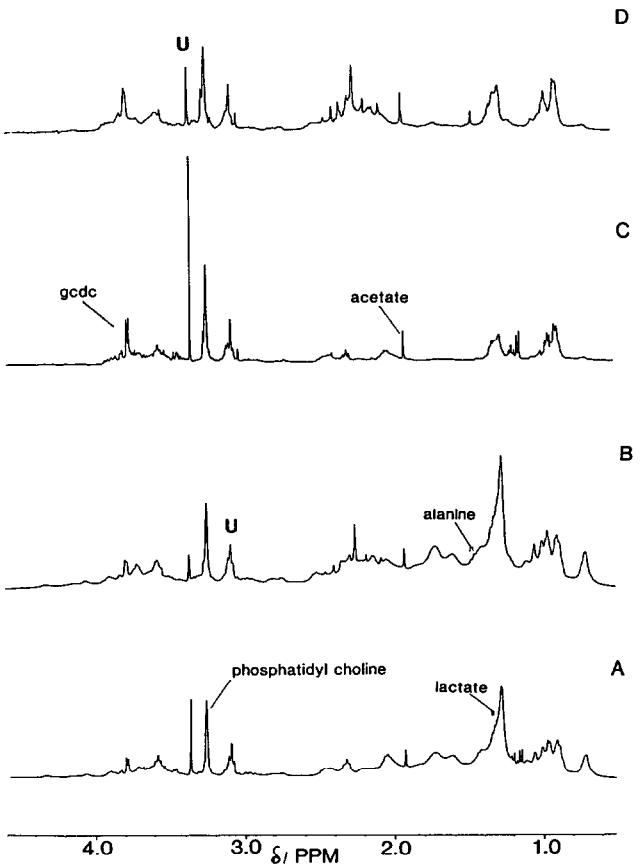

Figure 2
Single pulse (A and B) and CPMG spin-echo (C and D) 400 MHz ^{1}H-NMR spectra of lyophilized (A and C) and non-lyophilized (B and D) control rat bile (region to low frequency of water). U, unknown.

severely constrains their molecular motions. Signals from cholesterol and its esters present in plasma can only be detected in ^{1}H-NMR spectroscopy after lyophilization and extraction with methanol [3]. The CPMG pulse sequence eliminates broad components (short T_2 relaxation times) enabling solutes with high molecular mobility (longer T_2 values) to be studied (Fig. 1). The 400 MHz ^{1}H-NMR spectrum of lyophilized bile differs from that of non-lyophilized in that the signals from gcdc at δ = 3.80 ppm sharpen. In addition, sharp doublets appear at δ = 1.0–1.5 ppm after lyophilization (Fig. 2). The T_2 relaxation times of the aliphatic side-chains of lipid moieties are increased in lyophilized bile, suggesting greater mobility of these molecules. Reduction in signal intensity or disappearance of signals, reflects the loss of volatile (e.g. acetone) or unstable components (e.g. acetoacetate) that occurs during lyophilization. Increases in signal intensities occurring on lyophilization reflect changes in molecular mobility or compartmentation of molecules which is mainly related to the disruption/reorganization of the biliary micellar compartments. Interestingly, signals from β-hydroxybutyrate, valine and other branched chain amino acids do not contribute significantly to the ^{1}H-NMR spectra of non-lyophilized bile, but resonances from these components are clearly

resolved after lyophilization, indicating a dramatic lengthening of their T_2s and consequently their molecular mobility in the intact biofluid (Fig. 2). This suggests that they may be partly immobilized in micellar compartments in untreated bile.

Proton NMR spectroscopy of rat bile after dosing with PAP

Metabolites of PAP give rise to sharp lines in both single pulse (data not shown) and CPMG ^{1}H-NMR spectra suggesting that they are free in solution in bile and do not interact with the lipophilic micellar compartment on the NMR timescale or (less likely) are in fast exchange between different environments.

Lyophilization had no effect on the intensities of the PAP metabolites, further suggesting a lack of interaction with the micellar compartment. Metabolites of PAP can only be seen by ^{1}H-NMR in bile collected for the first 2 h after dosing (Fig. 3), or throughout the first 8 h (0–8 h) after dosing (Figs 4 and 5). Three sets of aromatic resonances are present (Fig. 3). The A_2B_2 splitting pattern suggests that these metabolites still contain *para*-substituted benzene rings. Two of these metabolites (**1** and **2**) appear to be acetylated (see below) and have chemical shifts identical with those of paracetamol-*O*-glucuronide (δ = 7.15 and 7.37 ppm; ^3JH = 8.8 Hz) and paracetamol-*O*-sulphate (δ = 7.32 and 7.46 ppm; ^3JH = 8.8 Hz), respectively (Fig. 3). The *N*-acetyl signals for these metabolites are at δ = 2.16 ppm and at δ = 2.17 ppm for the glucuronic acid and sulphate conjugates, respectively (Figs 3 and 4). The glucuronic acid conjugate also has a characteristic β-anomeric proton resonance from the sugar ring at δ = 5.13 ppm, and composite signals from the remaining sugar ring protons at δ = 3.6–3.94 ppm. The β-anomeric proton signals are partially suppressed due to irradiation of the water signal at δ = 4.80 ppm. A third metabolite of PAP is also present giving an A_2B_2 splitting

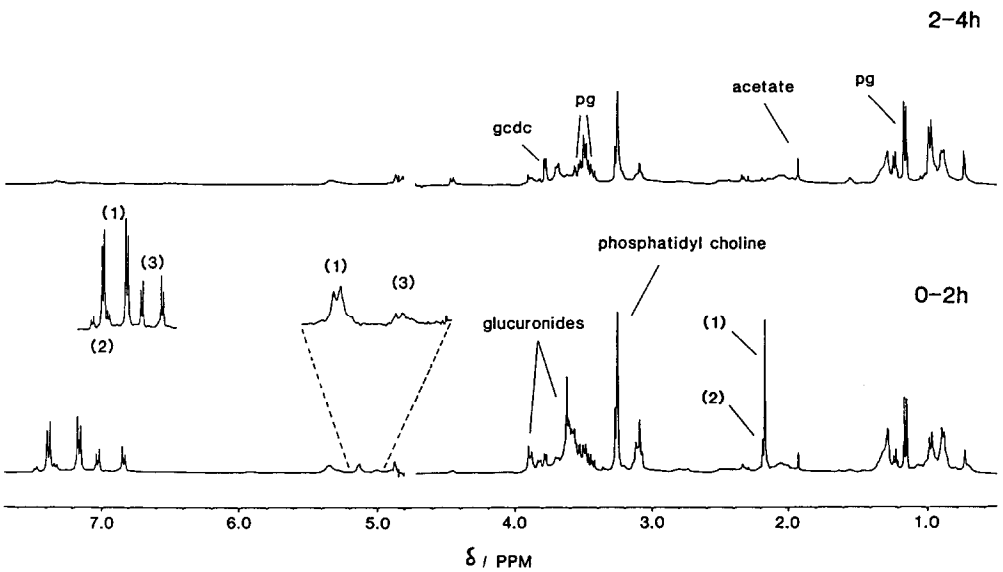

Figure 3
CPMG spin-echo 400 MHz ^{1}H-NMR spectra of rat bile, 2 and 4 h after dosing with 100 mg kg^{-1} PAP (experiment 1). Metabolites: (1) paracetamol-*O*-glucuronide; (2) paracetamol-*O*-sulphate; (3) PAP-*O*-glucuronide. pg, propylene glycol. The intensities of the glucuronide β-anomeric proton signals (δ = 5.01 ppm and δ = 5.13 ppm) are partially suppressed by irradiation of the water signal at δ = 4.8 ppm.

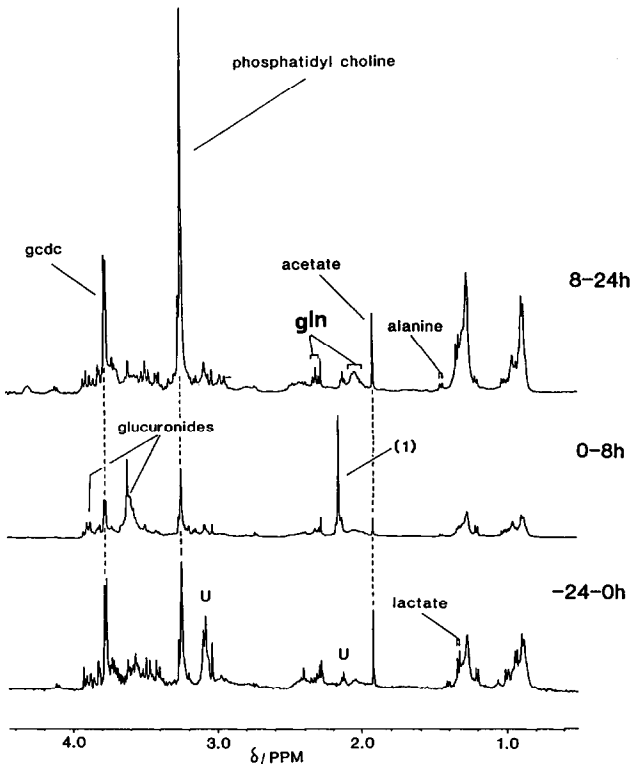

Figure 4
CPMG spin-echo 400 MHz ^{1}H-NMR spectra (region to low frequency of water) of rat bile before (−24–0 h) and after (0–8 and 8–24 h) dosing with 100 mg kg^{-1} PAP (experiment 2). Metabolites: (1) paracetamol-*O*-glucuronide. gln, Glutamine; U, unknown.

pattern (δ = 6.83 and 7.02 ppm; ^3J_H = 8.8 Hz). Due to the presence of a second doublet (with a small coupling constant: J = 3 Hz) at δ = 5.01 ppm (Fig. 3), and the absence of a corresponding *N*-acetyl signal at $\delta \approx$ 2.1–2.2 ppm, we tentatively assign these resonances, and those at δ = 6.83 and 7.02 ppm, to the glucuronic acid conjugate of PAP.

Resonances arising from propylene glycol can also be seen both at 0–2 and 2–4 h after dosing. This is present as a vehicle in the Sagatal™ formulation employed to maintain anaesthesia. Metabolites of PAP are also identified in bile collected from conscious animals. However, whereas in the above experiment metabolite resonances were only seen 2 h after dosing, in this experiment metabolites were identified in bile collected 8 h after PAP (Figs 4 and 5). Aromatic resonances from four PAP metabolites are shown in Fig. 5, the fourth set of resonances probably arising from PAP-sulphate. The spectral region to high frequency of the water signal of control bile (Fig. 5) is almost entirely devoid of signals except for those arising from the CH protons of unsaturated fatty acids (δ = 5.4 ppm) and the presence of a small doublet at δ = 5.23 ppm which we assign to the α-anomeric proton of glucose. In the region of the spectrum to low frequency (high field) of the water resonance, signals from endogenous metabolites including alanine, lactate and acetate can be observed (Fig. 4).

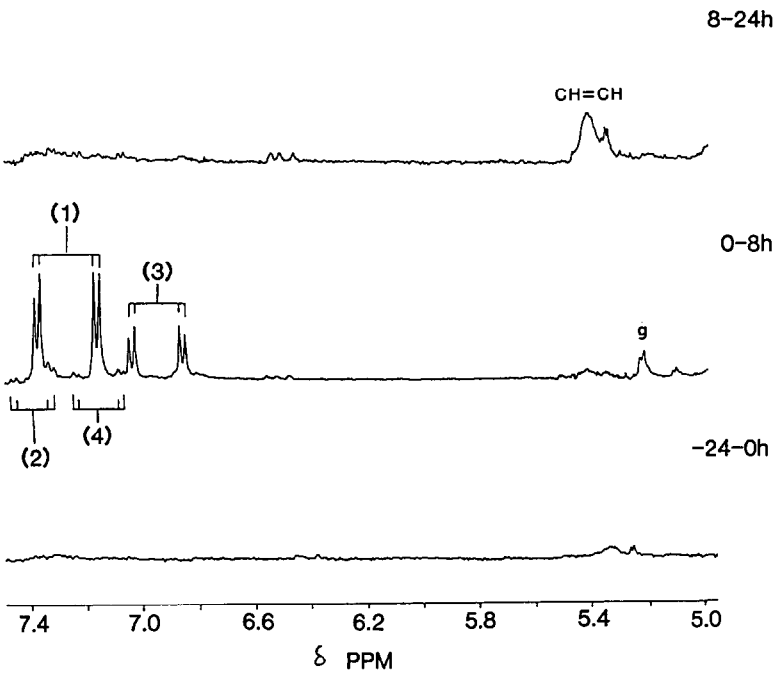

Figure 5
CPMG spin-echo 400 MHz ^{1}H-NMR spectra (region to high frequency of water) of rat bile before (−24–0 h) and after (0–8 and 8–24 h) dosing with 100 mg kg^{-1} PAP (experiment 2). Metabolites: (1) paracetamol-*O*-glucuronide; (2) paracetamol-*O*-sulphate; (3) PAP-*O*-glucuronide; (4) PAP-*O*-sulphate. g, α-Anomeric proton of glucose; U, unknown.

Discussion

Although the metabolism of PAP has been examined previously employing conventional [13] and ^{1}H-NMR urinalysis [8] techniques, the biliary excretion of this nephrotoxin has not been examined previously employing ^{1}H-NMR analysis. There are a number of advantages in using ^{1}H-NMR analysis of bile to study the metabolism of aromatic drugs and other xenobiotics. A high resolution ^{1}H-NMR spectrum can be obtained from bile in a matter of minutes. This rapidity is combined with non-selectivity and specificity in that experimental conditions do not have to be selected in order to detect a range of chemical classes of compound. The technique is non-selective in that all molecules with suitable NMR-detectable protons and present in bile at near millimolar concentrations should give resonances. In addition, ^{1}H-NMR analysis requires only minimum sample pretreatment, and is non-destructive so that the sample may be further analysed using conventional biochemical methods if required. The spectral region from δ = 5–10 ppm of control bile is nearly devoid of resonances from endogenous components, i.e. it is low in chemical noise [14], and this makes studies on the biliary excretion of aromatic drugs and xenobiotics, particularly attractive. Drugs giving signals in the low frequency (high field) region of the ^{1}H-NMR spectrum are less amenable to study because of the high chemical noise conferred by the endogenous components. An exception to this would be drugs giving signals at $\delta < 0.80$ ppm, δ = 2.5–3.0 ppm and δ = 3.9–4.5 ppm, in single pulse experiments. The range of chemical shifts in which

these are windows in the chemical noise can be extended to δ = 1.5–3.0 ppm and δ = 4.0–4.6 ppm, if CPMG spin-echo experiments are performed and the metabolites do not have their motions constrained by micellar compartmentation, as broad line interferences from endogenous components is attenuated (Fig. 2). As shown, CPMG ^{1}H-NMR experiments provide high order information on the molecular mobility of endogenous components and could be extended to studies on drugs which show micellar compartmentation, if lyophilization procedures are also employed.

The biliary metabolites of PAP identified by ^{1}H-NMR in the present study are paracetamol-*O*-glucuronide, paracetamol-*O*-sulphate (using the assignments of Bales *et al.* [7]), and two others which have been only tentatively assigned to PAP-*O*-sulphate and PAP-*O*-glucuronide (Fig. 6) since at present we do not have authentic standard materials. Abou-El-Makarem *et al.* [15] examined the biliary excretion of benzene and 21 of its simple derivatives, including PAP, in the rat. Following challenge with PAP (50 mg kg^{-1}), the glucuronic acid conjugate of PAP was the only metabolite identified in the bile, 7.6% of the dose being excreted by this route in 24 h. It is very likely that this group overlooked the possibility of *N*-acetylation since they employed a diazotization method which requires the presence of a free amino group [16], and consequently underestimated the degree of biliary excretion.

In agreement with the qualitative results described here for PAP, quantitative analysis of the biliary metabolites of paracetamol (a drug structurally related to PAP) in the rat show that the major biliary metabolite is, in fact, the glucuronide conjugate followed by the sulphate and glutathione conjugates [17, 18]. When PAP (100 mg kg^{-1}) was given to male F344 rats, metabolites were seen by proton NMR in bile only throughout the first 2 h (0–2 h) period following treatment, and also in bile collected 8 h (0–8 h) following treatment (Figs 3–5). Furthermore, metabolites can only be identified in urine collected 8 h after treatment (data not shown). This is consistent with the rapid clearance of the drug from the liver into the bile (within 2 h), and later into the urine (within 8 h) [19].

The present study clearly demonstrates the value of high resolution ^{1}H-NMR for studying xenobiotic metabolism and biliary excretion of drugs. In addition, ^{1}H-NMR

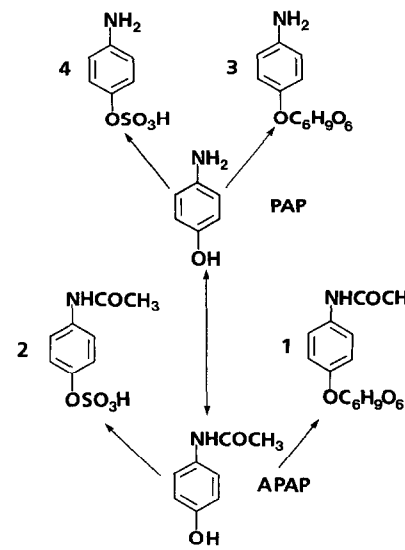

Figure 6
PAP and the major metabolites of PAP in the rat. APAP, paracetamol; **1**, paracetamol-*O*-glucuronide; **2**, paracetamol-*O*-sulphate; **3**, PAP-*O*-glucuronide; **4**, PAP-*O*-sulphate. APAP is a metabolite of PAP in the Fischer 344 rat [13] and, in addition, PAP is a metabolite of APAP in this strain [20].

analysis can at the same time highlight any changes occurring in the endogenous biliary components which could have toxicological significance. The non-selectivity combined with specificity of ^{1}H-NMR makes it a powerful technique in the analysis of biofluids and a valuable tool to the biochemical toxicologist.

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