Pergamon

Biochemical Pharmacology, Vol. 49, No. 8, pp. 1155–1164, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(94)00410-2

HIGH RESOLUTION NMR SPECTROSCOPIC STUDIES ON THE METABOLISM AND FUTILE DEACETYLATION OF 4-HYDROXYACETANILIDE (PARACETAMOL) IN THE RAT

ANDREW W. NICHOLLS,* STEPHEN CADDICK,*† IAN D. WILSON,‡ R. DUNCAN FARRANT,§ JOHN. C. LINDON§ and JEREMY K. NICHOLSON*‖

*Department of Chemistry, Birkbeck College, University of London, Gordon House, 29 Gordon Square, London WC1H 0PP; ‡Department of Safety of Medicines, Zeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire SK10 4TG; §Department of Physical Sciences, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, U.K.

(*Received* 7 *April* 1994; *accepted* 1 *September* 1994)

Abstract—Paracetamol (4-hydroxyacetanilide, acetaminophen) was synthesized with the acetyl group labelled with C^2H_3 (paracetamol-C^2H_3), and dosed to rats i.p. at 25 mg/kg (N = 5) and 40 mg/kg (N = 3) body weight. Paracetamol, with a $^{13}CH_3$ in the acetyl group (paracetamol-$^{13}CH_3$) was also synthesized and dosed to rats i.p. at 40 mg/kg (N = 3). The metabolism and excretion of the ^{2}H-labelled compound was followed in the rat using 600 MHz ^{1}H and 92.1 MHz ^{2}H NMR spectroscopy of urine collected 0–8, 8–24, 24–32 and 32–48 hr post-dosing. The metabolism of paracetamol-$^{13}CH_3$ was also monitored using 600 MHz ^{1}H NMR spectroscopy of urine collected 0–8, 8–24 and 24–48 hr post-dosing. For paracetamol-C^2H_3 the total recovery of the sulphate, glucuronide and *N*-acetyl cysteinyl metabolites via the urine accounted for 61.2 ± 14.1% of the 25 mg/kg dose and 61.4 ± 8.8% of the 40 mg/kg dose. For paracetamol-$^{13}CH_3$ the recovery was 102.7 ± 3.7% indicating that the low % urinary recovery with the C^2H_3-labelled drug is the result of isotope effects on the disposition of paracetamol. In the case of the paracetamol-C^2H_3, quantitative ^{1}H NMR analysis of urine showed that 13.3 ± 0.5 and 10.0 ± 1.2 mole % (25 and 40 mg/kg, respectively) of the urinary paracetamol sulphate recovered following dosing of the deuterium labelled drug had the C^2H_3 acetyl groups replaced by C^1H_3 acetyl groups from endogenous sources. In the case of the paracetamol-$^{13}CH_3$ 8.9 ± 0.7 mole % of the sulphate conjugate had also been transacetylated to paracetamol-$^{12}CH_3$. There was no significant difference between the level of futile deacetylation observed for the deuterated and ^{13}C-labelled drug. Overall these data indicate a high level of deacetylation followed by reacetylation (i.e. futile deacetylation) prior to excretion of paracetamol via the nephrotoxic intermediate 4-aminophenol. The level of deacetylation is much higher than has previously been thought which may cast new light on the role of 4-aminophenol in the development of paracetamol induced nephrotoxicity.

Key words: acetaminophen; deuterium; carbon-13; isotope effects; NMR spectroscopy; transacetylation; urinalysis

Modern high resolution NMR spectroscopy offers a powerful approach to elucidating the structures of biochemicals in complex mixtures with minimal sample preparation [1, 2]. In particular, NMR spectroscopy of body fluids has been shown to be a useful tool for investigating the metabolic fate and excretion of xenobiotics without the need for the radiolabelled compounds required by conventional studies [1–5]. ^{1}H NMR spectroscopy has improved considerably in terms of sensitivity in recent years with detection limits of <1 μM possible at a 600 MHz (14.1 Tesla) ^{1}H NMR observation frequency in favourable cases, and ^{1}H NMR spectroscopy of biofluids is being increasingly applied to problems in drug metabolism and toxicology [1, 2]. Other NMR-active nuclei have also been used as probes in drug metabolism studies. For instance, ^{19}F NMR is now widely used to study the metabolism and disposition of fluorinated compounds because of its high sensitivity (83% of ^{1}H) and negligible background interference from endogenous metabolites [2]. For compounds which do not contain ^{19}F, specificity can be achieved in other ways. Thus, ^{13}C-, and ^{15}N-labelling of drugs with NMR detection of metabolites in biofluids has also proved useful as a probe of xenobiotic metabolism. However, ^{13}C and ^{15}N NMR spectroscopic experiments on unlabelled drugs are much less sensitive and are limited to studies on compounds given in high doses [6, 7]. We have recently shown that high field ^{2}H NMR spectroscopy can provide a good probe of the metabolism and urinary excretion of the experimental anti-tumour drug dimethylformamide-d_7, with all major metabolites being readily detected [8]. The inherent sensitivity of ^{2}H NMR is nearly as good as that of ^{13}C NMR for the same number of nuclei and the short T_1's of the ^{2}H nuclei enable fast acquisition of the NMR data relative to ^{13}C detection [8]. The use of high magnetic fields is an advantage for ^{2}H NMR spectroscopic studies because of the small ^{2}H NMR chemical shift range in Hz, and even at 14.1

† Present address: School of Chemistry and Molecular Science, University of Sussex, Falmer, Brighton BN1 9QJ, U.K.

‖ Corresponding author.

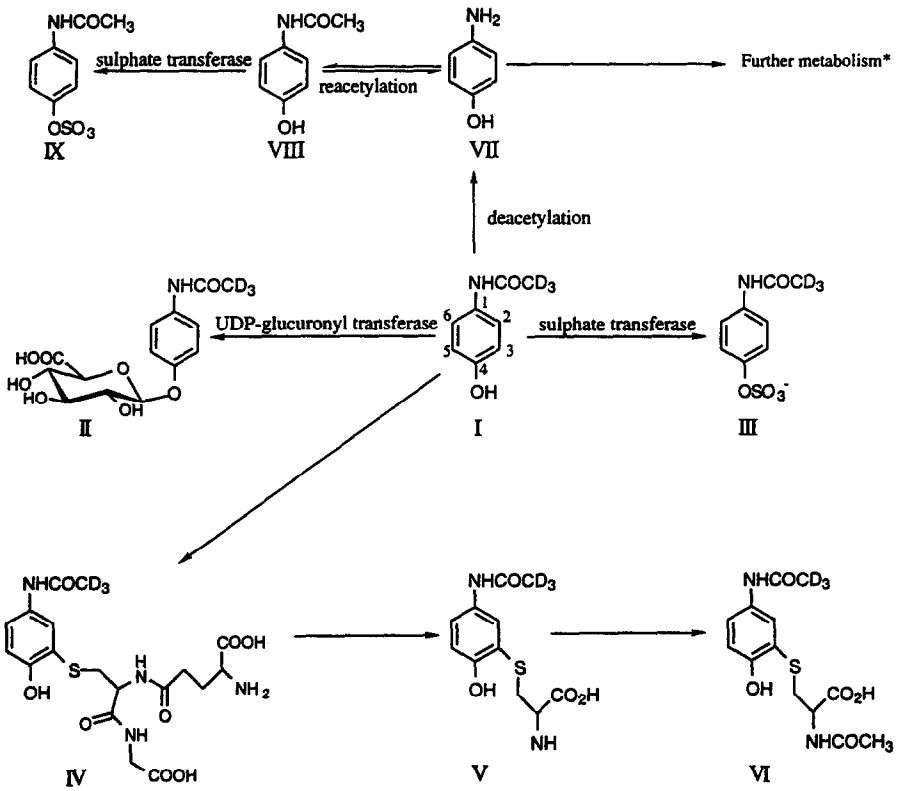

Fig. 1. The structure of paracetamol-C^2H_3(4-hydroxyacetanilide-C^2H_3) and its major metabolites. *Generation of nephrotoxic species' quinone imine formation, GSH conjugation, and polymerization. I = paracetamol-C^2H_3, II = paracetamol-C^2H_3 glucuronide, III = paracetamol-C^2H_3 sulphate, IV = glutathionyl paracetamol-C^2H_3 conjugate, V = L-cysteinyl paracetamol-C^2H_3 conjugate, IV = *N*-acetyl-L-cysteinyl paracetamol-C^2H_3 conjugate, VII = 4-aminophenol, VIII = paracetamol, IX = paracetamol sulphate. (I, II, III, V and VI are excreted in urine.)

Tesla, ^2H nuclei only resonate at 92.1 MHz. An inherent disadvantage of ^2H NMR is that for larger molecules, the ^2H T_2's are very short, resulting in broad lines and loss of spectral resolution. In order to extend the use of ^2H NMR spectroscopy (combined with ^1H NMR) as a probe of drug metabolism, we have investigated the metabolism of paracetamol-C^2H_3 (4-hydroxyacetanilide-C^2H_3, acetaminophen-C^2H_3,I) which was synthesized with the acetyl group fully deuteriated. A primary aim of this study was to use ^1H NMR spectroscopy to investigate the level of metabolic deacetylation of either the ^2H- or ^{13}C-labelled drug followed by reacetylation with endogenous (i.e. protio) acetyl groups. In this way we hoped to gain an insight into the *in vivo* flux of metabolism through 4-aminophenol, a potent nephrotoxin, which may have an important role in the toxicity of paracetamol [9, 10].

Overdosage of paracetamol causes severe centrilobular hepatic necrosis. There is general agreement that for hepatotoxicity to occur, paracetamol undergoes activation via cytochrome P450 mixed function oxidase enzymes to NAPQI¶ [11]. However, despite the extensive research in this area, controversy still surrounds the mechanism of the hepatotoxic lesion. There are two main theories for paracetamol induced hepatotoxicity involving oxidative stress [11], or dysfunction of intracellular Ca^{2+} homeostasis [12, 13]. Large doses of paracetamol also cause renal proximal tubular necrosis in rats [14], the mechanism of which is still unclear [15]. Renal cortical microsomes have been shown to activate paracetamol metabolically via an NADPH dependent cytochrome P450-mediated process [14, 16]. The extent of renal and hepatic damage *in vivo* is almost identical [14], but the renal cortical microsomal content of cytochrome P450 is far lower than that found in the liver [16]. This suggests that other biochemical mechanisms must also be involved in renal necrosis [17]. Therefore, it has been suggested that paracetamol may also undergo deacetylation to 4-aminophenol, a nephrotoxin which causes severe and acute necrosis of the kidney proximal tubules [18]. Both paracetamol and 4-aminophenol cause identical renal lesions and lead to a reduction in renal glutathione concentrations [19]. In previous studies in the rat, 4-aminophenol or its metabolites have been observed in the urine at a level of 1–3% of the paracetamol dose [20] representing a relatively low level of net 4-aminophenol production. However, it is possible

¶ Abbreviations: NAPQI, *N*-acetyl-*p*-benzoquinone imine; SD, Sprague–Dawley; TSP, sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]-1-propionate, FT, Fourier transformation, FID, free induction decay.

that any 4-aminophenol produced by deacetylation may be subject to reacetylation of the amino group *in vivo* leading to an underestimate of the extent of deacetylation. In the present study ^1H and ^2H NMR spectroscopy has been used to study the metabolism and urinary excretion of paracetamol-C^2H_3 and paracetamol-$^{13}CH_3$ in the rat, and in particular to determine the extent of combined deacetylation and reacetylation of paracetamol which might cast new light on the role of metabolically silent 4-aminophenol in paracetamol nephrotoxicity. The structures of the major paracetamol-C^2H_3 metabolites excreted in the urine and their metabolic relationship are shown in Fig. 1.

MATERIALS AND METHODS

All chemicals were obtained from Aldrich Chemical Company Ltd (Dorset, U.K.).

Synthesis of paracetamol-C^2H_3. Acetic anhydride-d_6, 99+ atom % D (1 g, 9.24 mmol) was added to a mixture of 4-aminophenol (0.814 g, 7.46 mmol) in deuterium oxide (2.20 mL) under an inert argon atmosphere. The mixture was stirred and heated at 80° for 10 min, then allowed to cool and the solid filtered off. The crude product was purified by recrystallization from hot water to give paracetamol-C^2H_3 (m.p. 160–161°) 0.438 g, 2.84 mmol. The identity of the product was confirmed using ^1H, ^2H and ^{13}C NMR and mass spectrometry. The NMR data on the synthetic paracetamol-C^2H_3 were as follows: δ^1H (DMSO-d_6, JEOL GSX 270 spectrometer operating at 270.1 MHz ^1H NMR frequency); δ^1H values: 9.65, *s*, OH; 9.14, *s*, NH; 7.31, *m*, 2H (H_2, H_6); 6.67, *m*, 2H (H_3, H_5); $\delta^{13}C$ (DMSO-d_6, JEOL GSX 270 spectrometer operating at 67.9 MHz ^{13}C frequency). $\delta^{13}C$ values: 167.5 (CO), 153.0 (C4), 130.9 (C1), 120.7 (C2, C6), 114.9 (C3, C5); [21]. Deuteration of the acetyl group causes a loss of signal intensity (and hence non-observability) of the ^{13}C NMR signal for the acetyl methyl carbon. This is due to the lack of the nuclear Overhauser enhancement normally obtained via broadband proton decoupling together with a longer T_1 relaxation time, combined with the complex pattern of the signal caused by the ^{13}C-^2H spin–spin coupling giving a 1:3:6:7:6:3:1 multiplet structure to the ^{13}C signal. δ^2H (H_2O, Bruker AMX 600 spectrometer, 92.1 MHz ^2H frequency). δ^2H values: 2.09, s, 3H. The electron impact mass spectrometry data were as follows: *m/z* 154, 110, 81, 53, 46, 43, 39, 33, 29.

Synthesis of paracetamol-$^{13}CH_3$. Acetic-2-^{13}C acid, sodium salt, 99.2 atom % ^{13}C (1.024 g, 12.33 mmol) was added to acetic-2-^{13}C chloride, 99.2 atom % ^{13}C (0.660 mL, 0.738 g, 9.28 mmol) in an ice-bath and mixed thoroughly. Acetic anhydride-2,2′-$^{13}C_2$ was distilled at 135–140° from the mixture to give 0.453 g, 4.62 mmol, δ^1H ($CDCl_3$, JEOL GSX 270 MHz spectrometer operating at 270.1 MHz ^1H NMR frequency). δ^1H values: 2.22, d, $^{13}CH_3$(J_{CH} = 118 Hz); $\delta^{13}C$ ($CDCl_3$, JEOL GSX 270 spectrometer operating at 67.9 MHz ^{13}C frequency). $\delta^{13}C$ values: 166.8 (CO), 165.9 (CO), 22.2 ($^{13}CH_3$). To the distillate was added 4-aminophenol (0.462 g, 4.23 mmol) and the mixture was heated until the solid had dissolved. After removal from the heat, water was added and the product allowed to crystallize. The solid was filtered under suction and washed with distilled water to give paracetamol-$^{13}CH_3$ (m.p. = 163–165°) 0.325(7) g, 2.14 mmol. δ^1H (DMSO-d_6, JEOL GSX 270 spectrometer operating at 270.1 MHz ^1H NMR frequency). δ^1H values: 9.67, *s*, OH; 9.21, *s*, NH; 7.33, *m*, 2H (H_2, H_6); 6.67, *m*, 2H (H_3, H_5); 1.96, *d*, $^{13}CH_3$ (J_{CH} = 127 Hz); $\delta^{13}C$ (DMSO-d_6, JEOL GSX 270 spectrometer operating at 67.9 MHz ^{13}C frequency). $\delta^{13}C$ values: 168.1 (CO), 167.4 (CO), 153.2 (C4), 131.0 (C1), 121.0 (C2, C6), 115.1 (C3, C5), 23.73 ($^{13}CH_3$). The electron impact mass spectrometry data were as follows: *m/z* 152, 131, 119, 109, 80, 78, 69, 65, 63, 53.

Administration of paracetamol-C^2H_3 *(25 mg/kg).* Eight male SD rats (weight approximately 200 g) were housed individually in metabolism cages and allowed free access to food and tap water. The animals were subjected to regular light–dark cycles. Three animals served as controls for the duration of the experiment, receiving only a saline dose. The remaining five animals each received a single intraperitoneal dose of paracetamol-C^2H_3 at 25 mg/kg in 0.9% NaCl. Urine was collected over ice during the following periods: −24–0 hr, 0–8 hr, 8–24 hr, 24–32 hr, 32–48 hr. After collection, urinary volumes were recorded and the urine was centrifuged at 3000 rpm for 20 min at 4° to remove solid debris. All samples were stored at −20° until required for NMR analysis.

Administration of paracetamol-C^2H_3 *and paracetamol-*$^{13}CH_3$ *(40 mg/kg).* Six male SD rats (weight approximately 200 g) were housed individually in metabolism cages and allowed free access to food and tap water. The animals were subjected to regular light–dark cycles. The animals acted as their own controls. The animals were divided into two groups of three and each group received a single intraperitoneal dose of either paracetamol-$^{13}CH_3$ or paracetamol-C^2H_3 at 40 mg/kg (i.p.) in 0.9% NaCl. Urine was collected over ice during the following periods: −24–0 hr, 0–8 hr, 8–24 hr, 24–32 hr, 32–48 hr. After collection, urinary volumes were recorded and the urine was centrifuged at 3000 rpm for 20 min at 4° to remove solid debris. All samples were stored at −20° until required for NMR analysis.

^2H-NMR spectroscopy of rat urine following dosing with paracetamol-C^2H_3 *(25 mg/kg).* Point five millilitres of each of the untreated urine samples was placed in a 5 mm NMR tube and the ^2H-NMR spectrum recorded at 92.13 MHz on a Bruker AMX-600 spectrometer operating at ambient probe temperature (303 K) without a field-frequency lock. Sixty-four FIDs were collected into 16,384 data points using a spectral width of 2777.8 Hz, an acquisition time of 2.95 sec and a further relaxation delay of 2.95 sec, giving a total pulse recycle delay of 5.9 sec. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.5 Hz prior to FT.

^1H-NMR spectroscopy of rat urine. Point five millilitres of each of the untreated urine samples was placed in a 5 mm NMR tube, 0.05 mL of 1 mM TSP in ^2H_2O was added to each to act as a chemical shift

and quantitation reference (δ 0.0), and ^{1}H NMR spectra were measured at 600.14 MHz on a Bruker AMX 600 spectrometer. Sixty-four FIDs were collected into 131,072 data points using a spectral width of 18,518.5 Hz, an acquisition time of 3.54 sec and a total pulse recycle delay of 6.5 sec. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz prior to FT. All data processing was subsequently performed on a Bruker Aspect 1 workstation using Bruker UXNMR software.

Analysis of NMR spectra from rats dosed with paracetamol-C^2H_3. The regions of the 600 MHz ^{1}H NMR spectrum corresponding to the aromatic and acetyl signals of paracetamol sulphate were integrated using the standard Bruker UXNMR software following automatic polynomial baseline correction. The signal intensities of the paracetamol sulphate (δ 7.33) and glucuronide (δ 7.15) aromatic ring proton doublets and the side chain *N*-acetyl singlet (δ 1.87) of the *N*-acetyl cysteinyl metabolite were integrated relative to TSP and the urinary concentrations of each calculated. The % total recovery of the paracetamol metabolites was calculated for the 0–8 hr period taking into account the urinary volumes collected over this period. No paracetamol metabolites were detected in any of the urine collection periods after 0–8 hr. The molar ratio of the CH_3 acetyl sulphate signal was calculated relative to that of the aromatic protons H_3 and H_5 of paracetamol sulphate (these represent total protio- and deuterioacetyl paracetamol as the chemical shifts are not affected by acetyl deuteriation). Facile chemical $^1H/^2H$ exchange of the acetyl hydrogens is not possible in aqueous solutions.

Analysis of NMR spectra from rats dosed with paracetamol-$^{13}CH_3$. Quantitative analysis of the 600 MHz ^{1}H NMR spectra was carried out in an identical manner as to that for paracetamol-C^2H_3. The resonances of the paracetamol metabolites derived from dosing with paracetamol-$^{13}CH_3$ used for quantitation were the aromatic doublets at δ 7.31 (paracetamol sulphate) δ 7.14 (glucuronide conjugate), and an acetyl singlet at δ 1.87 corresponding to the *N*-acetyl-L-cysteinyl conjugate. The paracetamol sulphate acetyl corresponding to the metabolite that had undergone deacetylation and reacetylation from endogenous sources was observed at δ 2.18.

RESULTS

Metabolism of paracetamol-C^2H_3 (25 mg/kg)

The 600 MHz ^{1}H NMR spectrum of a typical pre-dose rat urine is shown in Fig. 2a. The complex mixture of endogenous metabolites present in urine gives rise to the multitude of peaks which appear in this spectrum and although all the major peaks have been assigned in previous studies (1) there are still many as yet unassigned. The major endogenous compounds in Fig. 2a are labelled with their signal assignments. Figure 2b shows the 600 MHz ^{1}H NMR spectrum of urine from a rat collected 0–8 hr after dosing with paracetamol-C^2H_3 at 25 mg/kg. The principal metabolite of the drug at this dose was the sulphate conjugate as clearly indicated by the aromatic proton resonances at δ 7.33 and δ 7.47 which have been assigned previously as have those of other metabolites using synthetic standards [3]. As the drug is deuterated at the acetyl methyl a sharp singlet at the acetyl chemical shift of δ 2.18 for the protio-species would not be expected unless some degree of deacetylation and reacetylation had occurred. The ^{2}H NMR spectrum of the same rat urine is clearly shown in Fig. 2c. The deuterated acetyl group of paracetamol-d_3 sulphate is seen at a chemical shift of δ 2.14. The resonance is slightly shifted to a lower frequency compared to the protio acetyl signal as a result of a deuterium isotope effect on the chemical shift.

Expansion of the 600 MHz ^{1}H NMR spectrum of a urine taken 0–8 hours after dosing with paracetamol-C^2H_3 is shown in Fig. 3b. By comparison with Fig. 3a showing the same spectral region before dosing, the aromatic signals (AA′ XX′ spin system) of the paracetamol-C^2H_3 sulphate are clearly visible. In addition expansion of the spectrum in the region δ 1.75–2.30 reveals a number of peaks resulting from the appearance of signals from acetyl groups which were not present in the urine of the rat before dosing with paracetamol-C^2H_3 (Fig. 3a). New resonances include the cysteine acetyl group of the *N*-acetyl cysteinyl metabolite (VI) at δ 1.87, which is present to a minor extent (7 mole % of the dose by integration), and a signal at δ 2.18 previously assigned by Bales *et al.* [3] to the acetyl group of paracetamol sulphate (based on the chemical shift of authentic material). The presence of the signal clearly indicates that *in vivo* there is a significant degree of deacetylation of the $COCD_3$ group and reacetylation with $COCH_3$. Other peaks in this region of the spectrum include that of acetate found normally in the rat as a urinary metabolite and a group of resonances centred at δ 2.05 which arise from *N*-acetyl groups of carbohydrate moieties on glycoproteins [22].

The area of the paracetamol sulphate acetyl signal in the ^{1}H spectra was measured by integration relative to the aromatic peaks and the mole % of protio containing acetyl groups obtained. This gave a mean level of reacetylation in five rats of $13.3 \pm 0.5\%$ of the total sulphate metabolite recovered in the urine ($6.53 \pm 0.06\%$ of received dose, $10.7 \pm 0.1\%$ of mean total recovery). The mean recovery of paracetamol sulphate was $49.1 \pm 11.1\%$ of the dose. The mean total recovery of the observed paracetamol metabolites in the urine was $61.2 \pm 14.1\%$ of the paracetamol-C^2H_3 dose. The proton aromatic resonances at δ 7.37 and δ 7.15 (AA′ XX′ spin system) indicated the presence in the urine of small amounts of the paracetamol glucuronide (II) metabolite (Fig. 3b). The signal at δ 7.37 was partially overlapped with a signal from an endogenous metabolite, so only the signal at δ 7.15 was used for quantification. No ^{1}H resonances from a paracetamol glucuronide acetyl group were observed possibly because the signal was too small to be measured under the NMR acquisition conditions used. The relatively small quantity of glucuronide metabolite observed, corresponding to $7.6 \pm 1.9\%$ of the dose (about four times the therapeutic dose in man), was due to the high

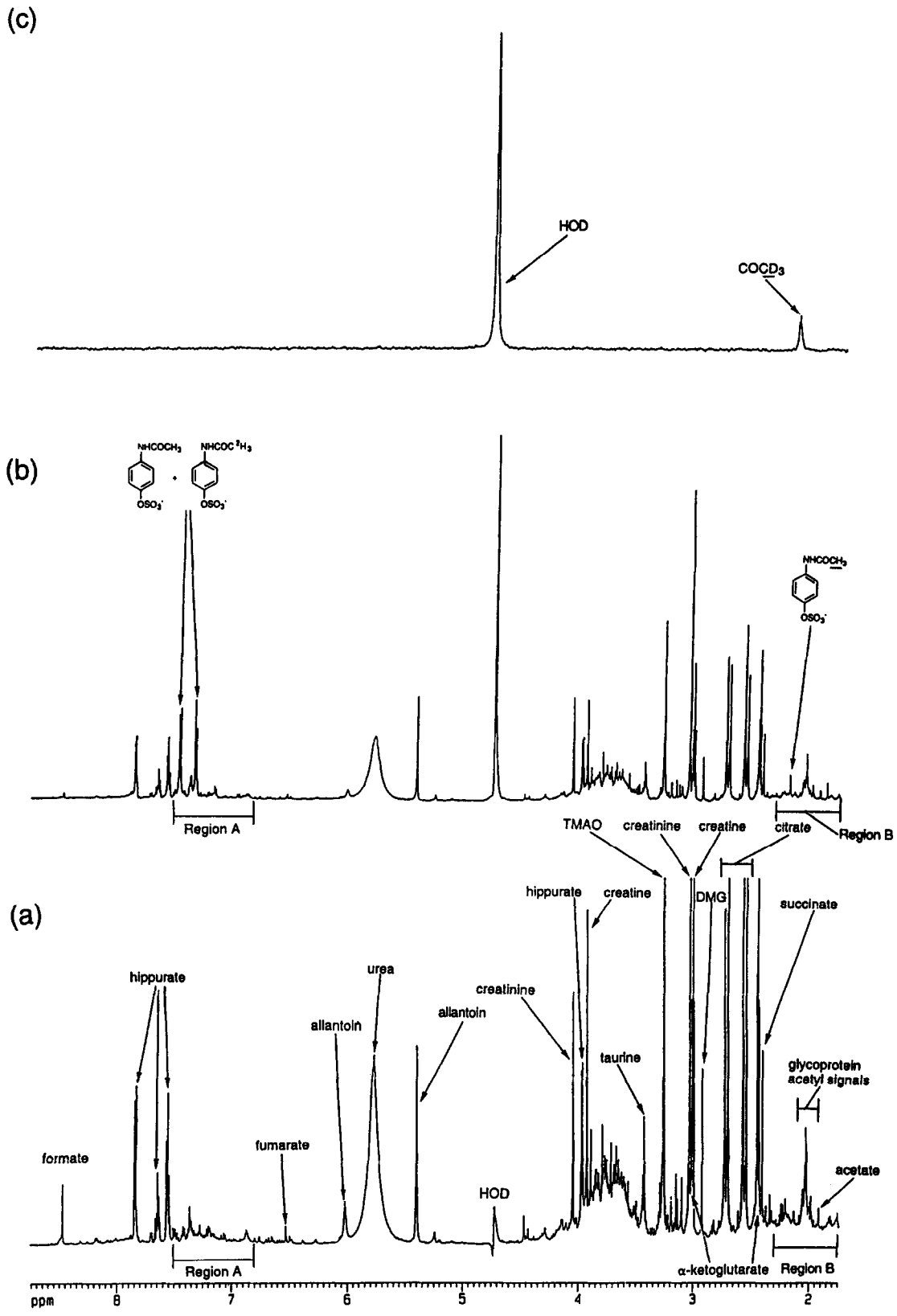

Fig. 2. (a) 600 MHz ^1H NMR spectrum of rat urine before dosing with paracetamol-C^2H_3, (b) 600 MHz ^1H NMR spectrum of rat urine collected 0–8 hr after dosing with paracetamol-C^2H_3 at 25 mg/kg and (c) 92.1 MHz ^2H NMR spectrum of rat urine used for (b). TMAO, trimethylamine-*N*-oxide; DMG, dimethylglycine; HOD, natural abundance of deuterium in water. The regions labelled A and B are shown as expansions in Figs 3 and 4.

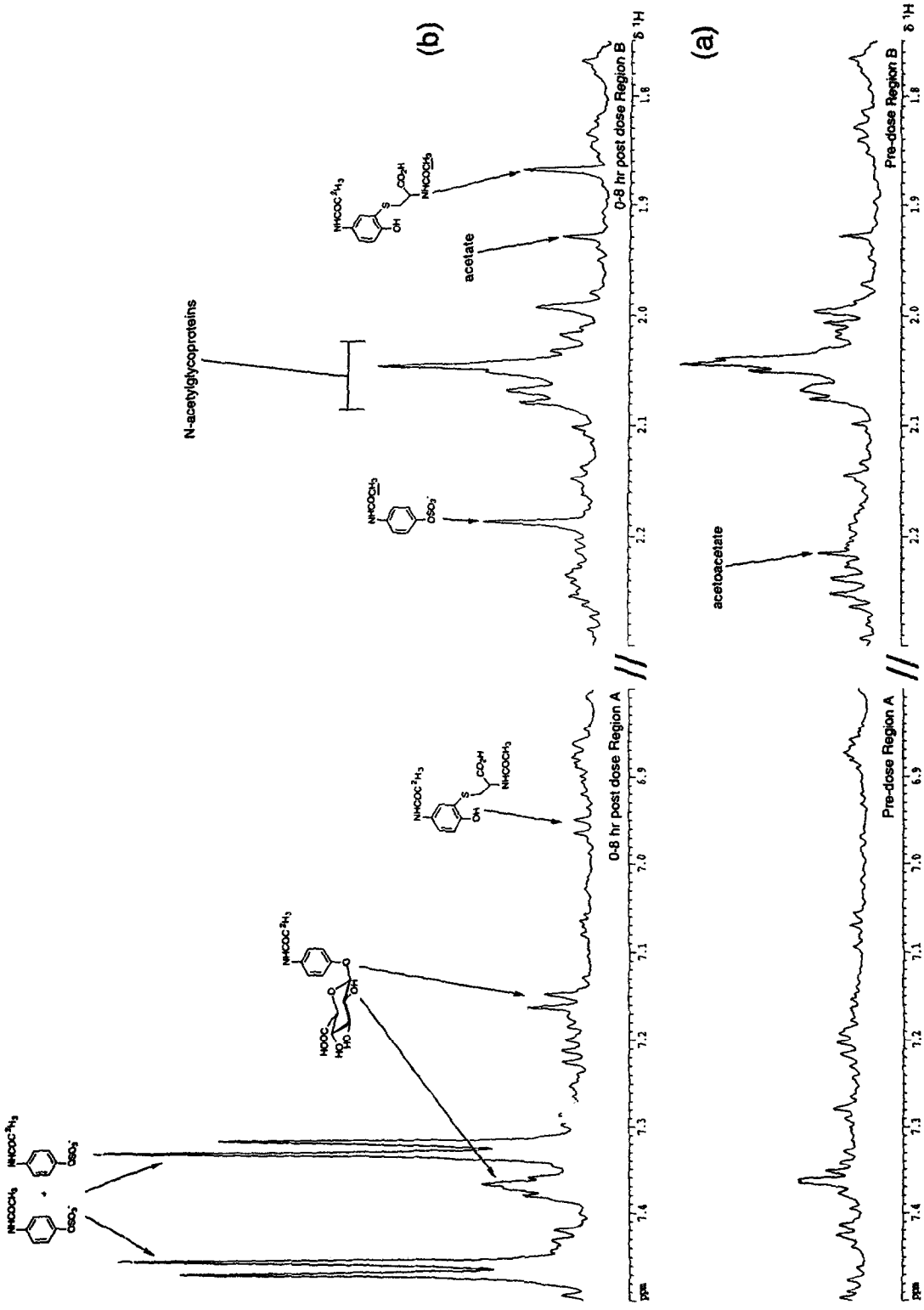

Fig. 3. Expansion of regions labelled A and B in the 600 MHz ^1H NMR spectra of rat urine shown in Fig. 2, (a) pre-dose and (b) 0–8 hr after dosing with paracetamol-C^2H_3 showing the level of reacetylated paracetamol sulphate-C^2H_3.

capacity for sulphation in the rat which dominates the metabolic profile. The mean recovery of the *N*-acetyl-L-cysteinyl metabolite was 4.1 ± 1.1 of the administered dose. There was no ^{1}H NMR evidence for the presence of either 4-aminophenol or the parent drug in the urine. The low overall % urinary recovery of the metabolites prompted us to repeat the experiment at a higher dose level which would also serve to improve the chance of observing futile deacetylation in the glucuronide and *N*-acetylcysteine metabolites (see below).

Metabolism of paracetamol-C^2H_3 (40 mg/kg)

Similar paracetamol metabolite resonances were observed in the 600 MHz ^{1}H NMR spectra of the 0–8 hr urines obtained following dosing with paracetamol-C^2H_3 at 40 mg/kg compared to those seen dosing at 25 mg/kg. The aromatic proton resonances of the paracetamol sulphate were observed at δ 7.46 and δ 7.31, with the corresponding acetyl singlet resulting from futile deacetylation at δ 2.18. Glucuronide aromatic resonances were again observed at δ 7.36 and δ 7.14, with only the δ 7.14 resonance being used for quantification due to overlap of the δ 7.36 signal with an endogenous metabolite. An acetyl singlet corresponding to the *N*-acetyl-L-cysteinyl metabolite of paracetamol was observed at δ 1.87. Measurement of the extent of reacetylation of the paracetamol sulphate gave a mean level of 10.0 ± 1.2% (4.7 ± 0.6% of initial dose, 7.6 ± 0.9% of the mean total recovery) with a mean sulphate recovery of 46.6 ± 5.0% of the dose. The mean total recovery of the glucuronide metabolite, as measured from the resonance at δ 7.14, was 10.6 ± 1.3% of the initial dose whilst the mean recovery of the *N*-acetyl-L-cysteinyl metabolite was 4.19 ± 1.1% of the dose. Again no acetyl signal was observed for the paracetamol glucuronide. None of the parent drug or 4-aminophenol was observed in these samples. The mean recovery of the observed paracetamol metabolites in the urine was 61.4 ± 8.8% (N = 3) of the dose administered, almost exactly that observed at 25 mg/kg.

Metabolism of paracetamol-$^{13}CH_3$ (40 mg/kg)

Due to the possibility of significant deuterium isotope effects affecting the observed futile deacetylation process a further study was performed using a ^{13}C-labelled form of paracetamol as isotope effects for ^{13}C-labelled compounds are generally small. Expansions of the regions δ 7.50–6.80 and δ 2.30–1.75 from the 600 MHz ^{1}H NMR spectra for pre-dose and 0–8 hr urine following dosing with paracetamol-$^{13}CH_3$ at 40 mg/kg (Fig. 4) showed the presence of metabolite resonances similar to those from paracetamol-C^2H_3. The aromatic resonances for paracetamol sulphate were clearly visible (δ 7.45 and δ 7.31), as was one of the paracetamol glucuronide doublets at δ 7.13, the second glucuronide metabolite resonance being overlapped by endogenous metabolites at δ 7.36. The acetyl singlet of the *N*-acetyl-L-cysteinyl conjugate was also observed at δ 1.87. The acetyl resonances for the paracetamol metabolites still containing a $CO^{13}CH_3$ group were observed as ^{13}C-coupled doublets (J = 129 Hz) between δ 2.30–δ 2.25, and δ 2.08–δ 2.03. A single acetyl resonance resulting from $COCH_3$ was observed at δ 2.18 again indicating that futile deacetylation had occurred only for the paracetamol sulphate metabolite. Integration of this resonance to the paracetamol sulphate aromatics gave a mean level of reacetylation of 8.9 ± 0.7% (6.5 ± 0.5% of the initial dose, 6.6 ± 0.6% of mean total recovery) for the paracetamol sulphate conjugate with a mean recovery of 73.3 ± 2.4% (N = 3) of the dose. The mean recoveries of the paracetamol glucuronide and *N*-acetyl-L-cysteinyl metabolites were 15.3 ± 4.0% and 9.8 ± 1.9% of the dose, respectively. Again no acetyl signal was observed for glucuronide undergoing futile deacetylation. The aromatic ^{1}H NMR signals for the parent drug were observed at δ 6.90 and gave a mean recovery of 4.17 ± 0.7%. The mean total recovery of all of the observed paracetamol metabolites was 102.7 ± 3.7% of the $^{13}CH_3$-paracetamol dose.

The quantitative data on the recovery and % of futile deacetylation of paracetamol-C^2H_3 (at two dose levels) and paracetamol-$^{13}CH_3$ are summarized in Table 1.

DISCUSSION

We have shown that there are significant amounts of protio acetyl paracetamol sulphate (approx. 9–13%) in the urine following paracetamol-C^2H_3 or paracetamol-$^{13}CH_3$ dosing. This deacetylation/reacetylation process, which we have termed futile deacetylation, represents a major metabolic pathway for paracetamol in the rat at the 25–40 mg/kg dose level. It involves a significant flux, which is normally metabolically "silent" in unlabelled drug, through either 4-aminophenol or, perhaps less likely, 4-aminophenol sulphate. In considering these results we were mindful of the possibility that the level of deacetylation found for paracetamol-C^2H_3 might well have been different to that found with paracetamol-C^1H_3 dosing because of kinetic isotope effects on the deacetylation process. Therefore, a comparative study using paracetamol labelled with ^{13}C, which would be expected to give much smaller kinetic isotope effects, was used to trace the metabolism of the acetyl group and thus gauge the level of any deuterium isotope effects on the observed level of futile deacetylation more accurately. This gave a futile deacetylation level of *ca.* ~9% for the ^{13}C-labelled drug indicating that deuterium isotope effects had a minimal effect on the pathway. Therefore, this indicates a major level of futile deacetylation and hence the possibility of a considerable intracellular flux through the nephrotoxic intermediate 4-aminophenol. Such results are likely to be of toxicological significance and may help explain some of the nephrotoxic effects of paracetamol. Indeed, the production of 4-aminophenol has often been invoked as a possible cause of the nephrotoxicity of paracetamol, but in studies on the *in vivo* production of 4-aminophenol following paracetamol dosing have indicated that the extent of its formation is low [19]. Therefore, its importance as the proximate nephrotoxic species may have been underestimated. However, as indicated here, and confirmed by our previous

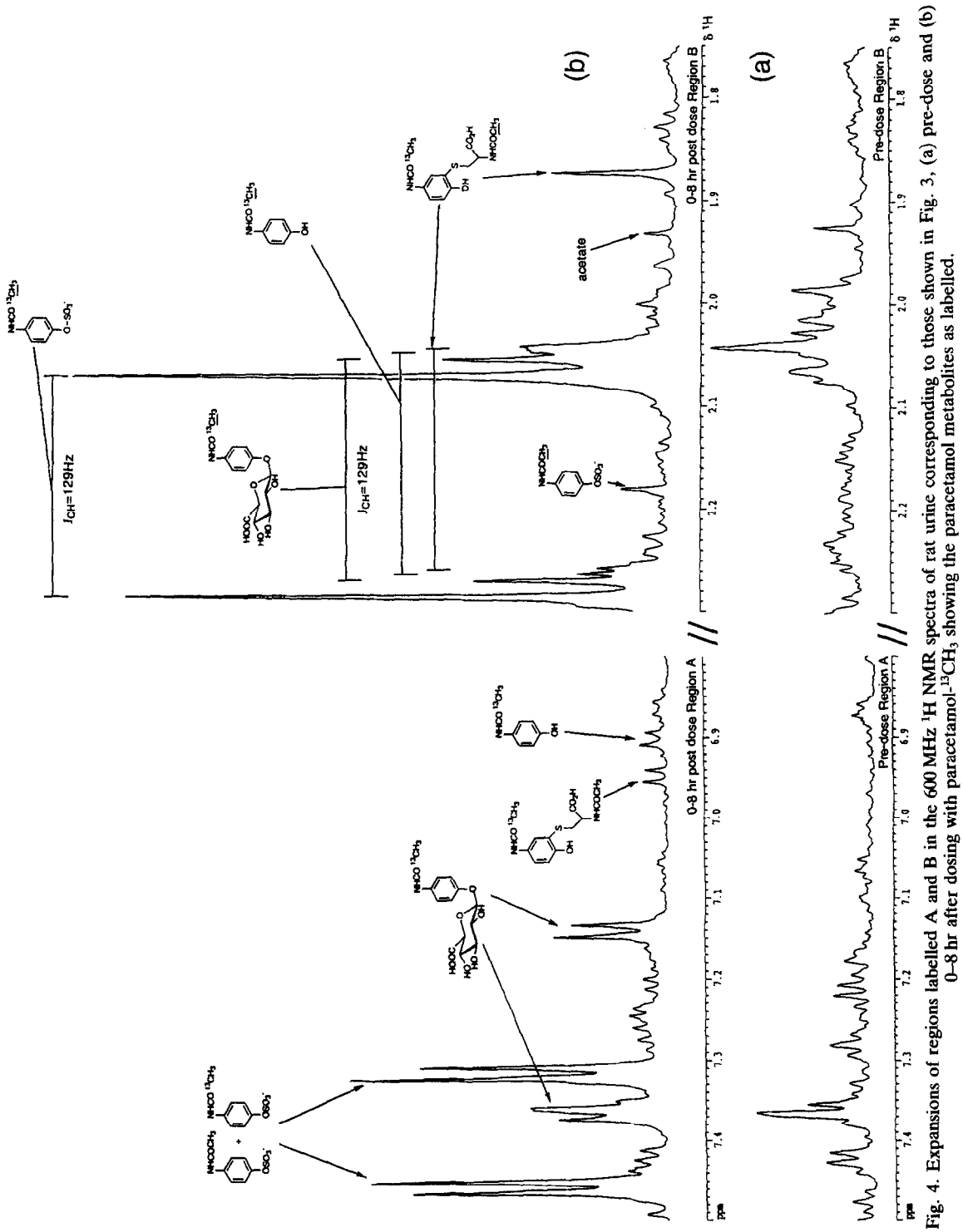

Fig. 4. Expansions of regions labelled A and B in the 600 MHz ^{1}H NMR spectra of rat urine corresponding to those shown in Fig. 3, (a) pre-dose and (b) 0–8 hr after dosing with paracetamol-$^{13}CH_3$ showing the paracetamol metabolites as labelled.

Table 1. Mean % futile deacetylation data and mean % urinary recovery of paracetamol-C^2H_3 (at two dose levels) and paracetamol-$^{13}CH_3$

Compound	Dose/mg/kg	No. of animals	% FD of paracetamol sulphate	% mean recovery of paracetamol sulphate	% mean recovery of total metabolites
Paracetamol-C^2H_3	25	5	13.3 ± 0.5	49.1 ± 11.1	61.2 ± 14.1
Paracetamol-C^2H_3	40	3	10.0 ± 1.2	46.6 ± 5.0	61.4 ± 8.8
Paracetamol-$^{13}CH_3$	40	3	8.9 ± 0.7	73.3 ± 2.4	102.7 ± 3.7

experimental work with 4-aminophenol itself [19], paracetamol is a major metabolite of 4-aminophenol *in vivo*. Paracetamol metabolites are normal end products of the metabolism of 4-aminophenol, as shown by previous studies of the biliary excretion in rats which showed the presence of both paracetamol glucuronide and sulphate 0–8 hr after dosing with 100 mg/kg 4-aminophenol [9, 10]. The determination of the amount of 4-aminophenol excreted in the urine unchanged does not provide any indication of the total flux through the futile deacetylation pathway and the previous studies would tend to underestimate 4-aminophenol production [19]. The current study clearly indicates that a significant amount of the dose does pass through an *N*-deacetylated intermediate, most probably 4-aminophenol.

The much lower urinary recovery of paracetamol-C^2H_3 in comparison to paracetamol-$^{13}CH_3$ is intriguing. This result is most probably due to a deuterium isotope effect on the de-conjugation of paracetamol metabolites by intestinal microflora. Thus, reabsorption of the drugs via the entero-hepatic circulation is reduced causing a higher proportion of the drug to be lost via the faecal route. This possibility is currently under investigation.

The observation of significant amounts of futile deacetylation may serve to reopen the debate concerning the role of 4-aminophenol in paracetamol nephrotoxicity. If similar reactions occur in man, then 4-aminophenol may be produced in significant amounts following paracetamol administration (*ca.* 80 mg following a 600 mg dose assuming rat and man to be identical in this respect). Whilst this level of exposure may be of little consequence to individuals capable of rapid reacetylation of paracetamol, there may be a greater risk of nephrotoxicity for those whose acetylation capacity is reduced. Clearly the existence of a single bimodal gene in the human population for acetylation displaying two alleles, one for fast and one for slow acetylation [23], may be of interest in relation to nephrotoxicity in overdose situations.

Acknowledgements—We thank the SERC and the Wellcome Foundation Ltd for provision of a studentship for AWN and Bruker Analytische Messtechnik GmbH for donation of NMR data processing and computer equipment.

REFERENCES

1. Nicholson JK and Wilson ID, High resolution proton magnetic resonance spectroscopy of biological fluids. *Prog NMR Spectrosc* **21**: 449–501, 1989.
2. Malet-Martino MC and Martino R, The application of nuclear magnetic resonance spectroscopy to drug metabolism studies. *Xenobiotica* **19**: 583–607, 1989.
3. Bales JR, Sadler PJ, Nicholson JK and Timbrell JA, Urinary excretion of acetaminophen and its metabolites as studied by proton NMR spectroscopy. *Clin Chem* **30**: 1631–1636, 1984.
4. Bales JR, Nicholson JK and Sadler PJ, Two dimensional proton NMR "maps" of acetaminophen metabolites in human urine. *Clin Chem* **31**: 757–762, 1985.
5. Wilson ID, Ismail IM, Fromson J and Nicholson JK, Proton magnetic resonance spectroscopy of human urine: excretion of 1-(3′-carboxypropyl)-3,7-dimethylxanthine by man after dosing with oxpentifylline. *J Pharm Biomed Anal* **5(2)**: 157–164, 1987.
6. Nicholson JK and Wilson ID, High resolution nuclear magnetic resonance spectroscopy of biological samples as an aid to drug development. *Prog Drug Res* **31**: 427–479, 1987.
7. Preece NE, Nicholson JK and Timbrell JA, Identification of novel hydrazine metabolites by ^{15}N NMR. *Biochem Pharmacol* **41**: 1319–1324, 1991.
8. Farrant RD, Salman SR, Lindon JC, Cupid BC and Nicholson JK, Deuterium NMR spectroscopy of biofluids for the identification of drug metabolites: application to *N,N*-dimethylformamide. *J Pharm Biomed Anal* **11**: 687–692, 1993.
9. Gartland KPR, Bonner F and Nicholson JK, The effect of biliary cannulation and buthionine sulphoximine pretreatment on the nephrotoxicity of *p*-aminophenol. *Arch Toxicol* **64**: 14–26, 1989.
10. Gartland KPR, Eason CT, Wade KE, Bonner FW and Nicholson JK, Proton NMR spectroscopy of bile monitoring the excretion of endogenous and xenobiotic metabolites: application to *para*-aminophenol. *J Pharm Biomed Anal* **7**: 699–707, 1989.
11. Adamson GM and Harman AW, Oxidative stress in cultured hepatocytes exposed to acetaminophen. *Biochem Pharmacol* **45**: 2289–2294, 1993.
12. Tsokos-Kuhn JO, Hughes H, Smith CV and Mitchell JR, Alkylation of the liver plasma membrane and inhibition of the Ca^{2+} ATPase by acetaminophen. *Biochem Pharmacol* **37**: 2125–2131, 1988.
13. Moore M, Thor H, Moore G, Nelson S, Moldèus P and Orrenius S, The toxicity of acetaminophen and *N*-acetyl-*p*-benzoquinone imine in isolated hepatocytes is associated with thiol depletion and increased cytosolic Ca^{2+}. *J Biol Chem* **260**: 13035–13040, 1985.
14. McMurtry RJ, Snodgrass WR and Mitchell JR, Renal necrosis, glutathione depletion and covalent binding after acetaminophen. *Toxicol Appl Pharmacol* **46**: 87–100, 1978.
15. Mourelle M, Beales D and McLean AEM, Electron transport and protection of liver slices in the late stage of paracetamol injury. *Biochem Pharmacol* **40**: 2023–2028, 1990.
16. Tarloff JB, Goldstein RS and Hook JB, Xenobiotic

biotransformation by the kidney: pharmacological and toxicological aspects. *Prog Drug Metabol* **12**: 1–39, 1990.

17. Newton JF, Bailie MB and Hook JB, Acetaminophen nephrotoxicity in the rat. Renal metabolic activation *in vitro*. *Toxicol Appl Pharmacol* **70**: 433–444, 1983.
18. Green CR, Ham KN and Tange JD, Kidney lesions induced in rats by *p*-aminophenol. *Br Med J* **1**: 162–164, 1969.
19. Newton JF, Yoshimsoto M, Bernstein J, Rush GF and Hook JB, Acetaminophen nephrotoxicity in the rat II: strain differences in nephrotoxicity and metabolism of *p*-aminophenol, a metabolite of acetaminophen. *Toxicol Appl Pharmacol* **69**: 307–318, 1983.
20. Newton JF, Yoshimoto M, Bernstein J, Rush GF and Hook JB, Acetaminophen nephrotoxicity in the rat. I Strain differences in nephrotoxicity and metabolism. *Toxicol Appl Pharmacol* **69**: 291–306, 1983.
21. Pretsch E, Clerc T, Seibl J and Simon W, *Tables of Spectral Data for Structure Determination of Organic Compounds*. 2nd edn, Spring–Verlag, Berlin, 1989.
22. Foxall PJD, Parkinson JA, Sadler IH, Lindon JC and Nicholson JK, Analysis of biological fluids using 600 MHz proton NMR spectroscopy: application of homonuclear two-dimensional J-resolved spectroscopy to urine and blood plasma for spectral simplification and assignment. *J Pharm Biomed Anal* **11(1)**: 21–31, 1993.
23. Timbrell JA, *Principles of Biochemical Toxicology*. 1st Edn, Taylor and Francis Ltd, London, 1982.