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NMR spectroscopic studies on the metabolism and futile deacetylation of phenacetin in the rat

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1. ^{1}H-NMR spectroscopy of urine was used to determine the % deacetylation and re-acetylation of ^{2}H-labelled (in the acetyl) phenacetin metabolites in the rat.

2. Male Sprague-Dawley rats were each dosed with either phenacetin or phenacetin-C^2H_3 at 50 mg kg^{-1}. The total urinary recoveries for phenacetin and phenacetin-C^2H_3 were 47·6 ± 16·7 and 50·1 ± 16·2% respectively (not significantly different, $p > 0·05$). Paracetamol sulphate and glucuronide are the major urinary metabolites of both protio and deuteriophenacetin.

3. The futile deacetylation given by the urinary recovery of protio-acetyl metabolites of phenacetin-C^2H_3 was 29·6 ± 0·9% for paracetamol sulphate and 36·6 ± 3·1% for paracetamol glucuronide. These observations demonstrate a high level of futile deacetylation in the paracetamol conjugates formed by metabolism of phenacetin-C^2H_3 and this may indicate a high metabolic flux through the nephrotoxic intermediate 4-aminophenol.

4. The level of futile deacetylation for phenacetin was significantly higher than that found previously in studies of labelled paracetamol in rat or man, and may be important in understanding the higher nephrotoxicity of phenacetin as compared with paracetamol.

Introduction

The metabolism and toxicity of phenacetin (*N*-(4-ethoxyphenyl)acetamide) have been the subject of much research and controversy for the last 40 years. Although in widespread use as an analgesic from the end of the nineteenth century, it was not until the 1950s that phenacetin consumption (as a component of certain proprietary combination analgesics) was first implicated as a cause of chronic interstitial nephritis (Spühler and Zollinger 1953, Gloor 1993). Subsequent studies classified phenacetin in combination analgesics as a cause of analgesic nephropathy (Sandler *et al*. 1989, Buckalew Jr 1996), a condition involving renal papillary necrosis with interstitial nephritis and a reduction in renal size (Duggin 1980). Although phenacetin has not been proven to cause nephropathy in man, the clinically perceived association with toxicity led to its eventual withdrawal from the market (Buckalew Jr 1996). Other toxicologically important phenomena related to phenacetin ingestion are methaemoglobinæmia (Smith 1958) and an increased risk of bladder and/or colon cancer (Hinson 1983).

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Phenacetin is extensively metabolised in the liver by first-pass metabolism such that small quantities appear in the circulation only following very large doses (Duggin 1980, 1993). The metabolites of phenacetin are as shown in figure 1. The major metabolite of the drug has been identified as paracetamol (acetaminophen, 4-acetamidophenol) formed from cytochrome P450 1A2-catalysed de-ethylation (Guengerich *et al*. 1993). The paracetamol so formed from phenacetin is further metabolised via phase II conjugating enzymes to the *O*-sulphate, the *O*-glucuronide and to *N*-acetyl-L-cysteinyl metabolites derived from glutathione conjugations (Thomas 1993). Since first-pass metabolism is so extensive it has been suggested that phenacetin is actually a pro-drug with the true pharmacologically active agent being paracetamol (Brodie and Axelrod 1949). A second metabolite of phenacetin has been shown to be the deacetylation product 4-ethoxyaniline (*p*-phenetidine; figure 1) which can also undergo further metabolism (Brodie and Axelrod 1949, Duggin 1993). Other minor metabolites that have been observed are 2-hydroxyphenacetin (Hinson 1983) and N-hydroxyphenacetin (Nelson 1982), but paracetamol and its conjugates constitute the major proportion of phenacetin metabolites.

We have recently investigated the futile deacetylation of paracetamol in which the acetyl moiety of the drug is lost and then subsequently replaced by an acetyl group from endogenous sources (Nicholls *et al*. 1995). To investigate this 'silent' metabolic pathway isotopically labelled paracetamol was administered to rats and a human volunteer (Nicholls *et al*. 1995, 1997). The labelling used consisted of either full deuteriation of the acetyl group or replacement of the methyl ^{12}C with ^{13}C. ^{1}H-NMR spectroscopy was then used to analyse the urine to assess the level of protio-acetyl content within the molecule. This work showed an extensive degree of futile deacetylation whereby the isotopically labelled (^{13}C and ^{2}H) acetyl moiety of the drug was removed and subsequently replaced by a protio group from endogenous sources. Quantitative NMR measurements indicated a level of approximately 10% futile deacetylation for the sulphate and glucuronide conjugates in the rat and approximately 1–2% futile deacetylation in a single subject study in man (Nicholls *et al*. 1997). These results suggested that there was a significant transient flux through the metabolic intermediate, and potent nephrotoxin, 4-aminophenol. The nephrotoxicity of 4-aminophenol has long been established, with the compound causing severe and acute necrosis of the kidney proximal tubules (Green *et al*. 1969). However, the exact mechanism of 4-aminophenol nephrotoxicity still remains unclear (Goldstein and Schnellmann 1996).

The apparently high level of futile deacetylation in paracetamol, prompted us to investigate the level of futile deacetylation in the closely-related and more potentially nephrotoxic drug, phenacetin. If the nephrotoxicity of this drug were related to the production of 4-aminophenol, then the level of futile deacetylation in phenacetin might be expected to be higher than for paracetamol. Any large differences in the % futile deacetylation between phenacetin and paracetamol would suggest that metabolites other than those of paracetamol were also contributing to the formation of 4-aminophenol and hence phenacetin toxicity. Hence in the present studies phenacetin-$C^{2}H_{3}$ (fully deuteriated in the acetyl moiety) was administered to rat (i.p.) and the metabolism of the drug followed by ^{1}H-NMR spectroscopy of urine to quantify the level of futile deacetylation.

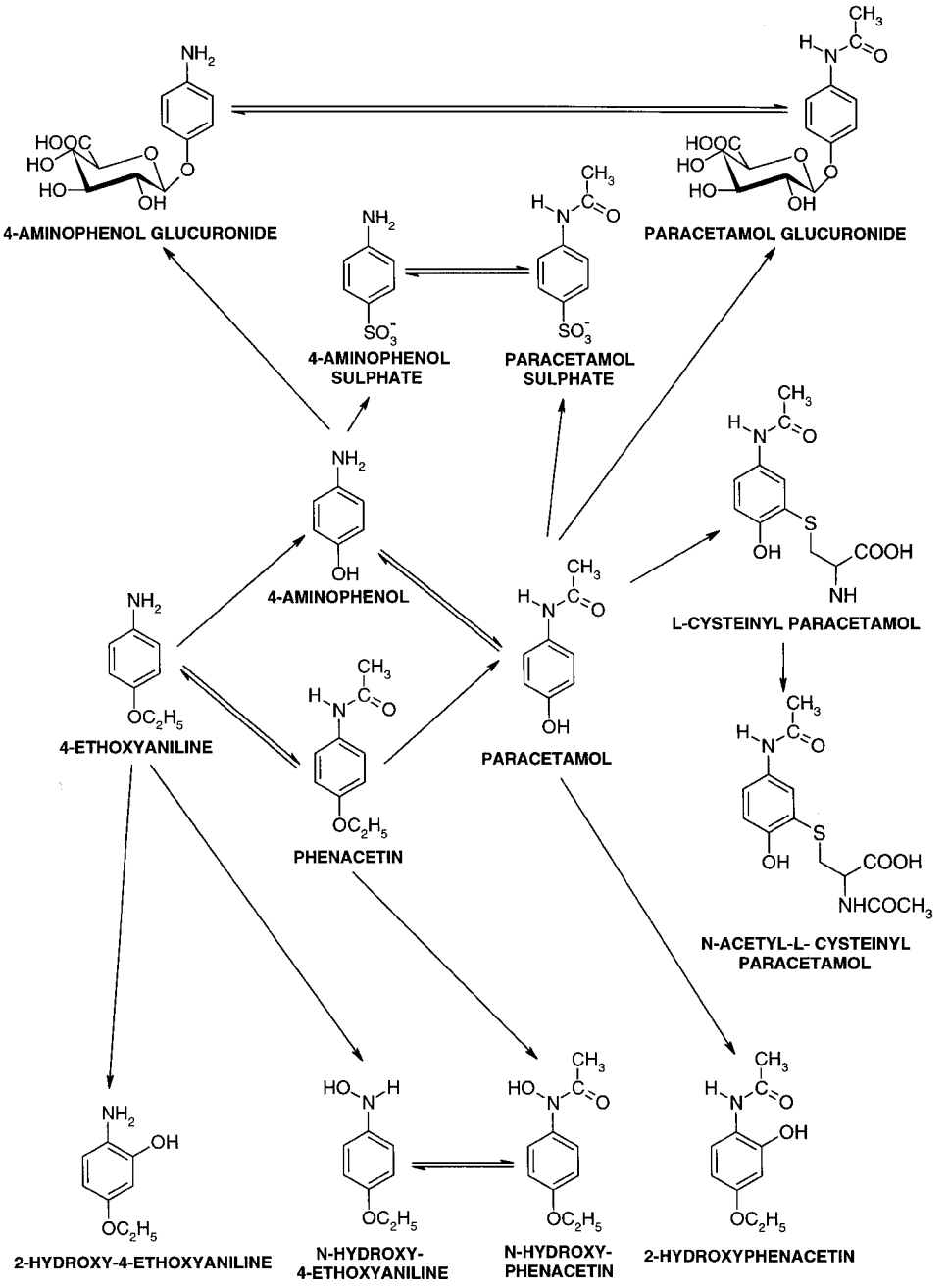

Figure 1. Structures of phenacetin and its principal metabolites. Bi-directional arrows denote the futile deacetylation reactions.

Materials and methods

All compounds were obtained from Aldrich (Poole, UK) except for paracetamol-C^2H_3 (99+ atom % D, purity > 99%), which was synthesized as previously described (Nicholls *et al.* 1995). The synthesis of phenacetin was achieved using the literature method (Vogel 1989).

Synthesis of N-(4-ethoxyphenyl)acetamide (phenacetin)

4-Acetamidophenol (paracetamol) (1·071 g, 6·95 mol) was dissolved in 10 ml ethanol. To this was added 21% w/v sodium ethoxide in ethanol (2·19 ml, 0·460 g, 6·74 mmol). A reflux condenser was added and iodoethane (0·77 ml, 1·5 g, 9·6 mmol) slowly added dropwise down the condenser to the stirring mixture. The mixture was refluxed for 60 min. Water (20 ml) was added and the mixture cooled in an ice-bath. The solid was filtered off to give *N*-(4-ethoxyphenyl)acetamide (phenacetin) (m.p. = 131–2 °C, purity > 99%), 0·233 g, 1·25 mmol.

δ^1H (DMSO-d_6, JEOL GSX 270 spectrometer operating at 270·1 MHz ^{1}H-NMR frequency); 9·76, s, NH; 7·45, m, 2H (H_2, H_6); 6·83, m, 2H (H_3, H_5); 3·95, q, CH_2; 1·99,s, $COCH_3$; 1·29, t, CH_3.

δ^{13}C (DMSO-d_6, JEOL GSX 270 spectrometer operating at 67·9 MHz ^{13}C frequency); 167·3 (CO), 154·2 (C4), 132·4 (C1), 120·5 (C2,C6), 114·3 (C3,C5), 63·0 (CH_2), 23·7 ($CO\underline{C}H_3$), 14·6 ($CH_2\underline{C}H_3$).

m/z 179 (37%), 137 (35%), 108 (100%), 91 (2%), 80 (14%), 65 (4%), 53 (5%).

Synthesis of N-(4-ethoxyphenyl)acetamide -C^2H_3 (phenacetin C^2H_3)

4-Acetamidophenol-C^2H_3 (paracetamol-C^2H_3) (1·074 g, 6·98 mol) was dissolved in 10 ml ethanol. To this was added 21% w/v sodium ethoxide in ethanol (2·2 ml, 0·462 g, 6·79 mmol). A reflux condenser was added and iodoethane (0·80 ml, 1·56 g, 10·0 mmol) slowly added drop-wise down the condenser to the stirring mixture. The mixture was refluxed for 60 min. Water (20 ml) was added and the mixture cooled in an ice-bath. The solid was filtered off to give *N*-(4-ethoxyphenyl)acetamide-C^2H_3 (phenacetin-C^2H_3), 99+atom % D (m.p. = 130–2 °C, purity > 99%), 0·519 g, 2·85 mmol.

δ^1H (DMSO-d_6, JEOL GSX 270 spectrometer operating at 270·1 MHz ^{1}H-NMR frequency); 9·76, s, N$\underline{H}$; 7·45, m, 2H (H_2, H_6); 6·84, m, 2H (H_3, H_5); 3·94, q, C$\underline{H}_2$; 1·29, t, C$\underline{H}_3$.

δ^{13}C (DMSO-d_6, JEOL GSX 270 spectrometer operating at 67·9 MHz ^{13}C frequency); 167·7 (CO), 154·3 (C4), 132·4 (C1), 120·5 (C2, C6), 114·5 (C3, C5), 63·0 (CH_2), 14·7 (CH_3).

m/z 182 (60%), 138 (45%), 110 (100%), 91 (2%), 81 (21%), 63 (3%), 53 (5%), 46 (17%).

Administration of phenacetin and phenacetin- C^2H_3 to rat

Eight male Sprague-Dawley (SD) rats (weight approximately 200 g) were housed individually in metabolism cages, allowed free access to food (rat maintenance diet TRM9607, Harlan Teklab, Bicester, UK) and tap water and were subjected to regular light cycles. Three of the animals received a single intraperitoneal (i.p.) dose of phenacetin at 50 mg kg^{-1}, whilst three others received an i.p. dose of phenacetin-C^2H_3 at 50 mg kg^{-1}. A DMSO-saline (75:25 v/v) dosing medium was used for both compounds. Two of the animals acted as controls each receiving a single i.p. dose of the dosing medium. Urine was collected over ice during the following periods: −24–0, 0–8, 8–24, 24–48 h. After collection, urinary volumes were recorded and the urine was centrifuged at 1500 *g* for 20 min at 4 °C to remove solid debris. All samples were stored at −20 °C until required for NMR analysis.

NMR-monitored solid phase extraction (SPE) of metabolites from urine samples

The urine containing the phenacetin metabolites (0–8 h post-dose, 50 mg kg^{-1} i.p.) (1 ml) was acidified to pH 2 with 1 M HCl and extracted on to a C18 bonded cartridge (Bond-Elut, 100 mg, supplied by Jones Chromatography, Hengoed, UK). The cartridges had previously been activated by washing with methanol (2 ml) and acidified water (1 ml, pH 2). The fractions were eluted with acidified water and methanol. All samples were reduced to dryness and reconstituted in ^2H_2O prior to NMR analysis.

^{1}H-NMR spectroscopic analysis of urine and SPE fractions

An aliquot (0·5 ml) of each of the untreated urine samples was placed in a 5-mm NMR tube, 0·05 ml 1 mM sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]-1-propionate (TSP) in ^2H_2O was added to each to act as a chemical shift and quantification reference (δ 0·0), and ^{1}H-NMR spectra were measured at 600·14 MHz on a Bruker AMX-600 spectrometer using the first increment of a NOESY 2D experiment, NOESYPR1D (Bruker GmbH, Rheinstetten, Germany), pulse sequence. Sixty-four free induction decays (FIDs) were collected into 131,072 data points using a spectral width of 18,518·5 Hz. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0·3 Hz prior to Fourier transformation. Double-quantum filtered correlated spectroscopy (DQF-COSY) experiments were acquired on a Varian VXR600 spectrometer operating at 600 MHz ^{1}H resonance frequency. The spectra were acquired using a spectral width of 6000 Hz in both the F2 and F1 dimensions. Sixty-four scans were collected per increment for 512 increments into 2K data points. The FIDs were multiplied by a sine-bell-squared function prior to Fourier transformation. The regions of the 1D 600 MHz ^{1}H-NMR spectrum corresponding to the aromatic and acetyl signals of the metabolites were integrated following automatic polynomial baseline correction. The signal intensities of the aromatic ring proton doublets for paracetamol sulphate, glucuronide and parent were integrated relative to TSP and the urinary concentrations of each calculated. The side chain *N*-acetyl singlet of the *N*-acetyl-L-cysteinyl paracetamol

metabolite of phenacetin was integrated relative to TSP to determine the concentration of this metabolite. The % total recovery of the metabolites was calculated for the 0–8-h period taking into account the urinary volumes collected over this period. Integration of the CH_3 acetyl signal relative to the aromatic H_3 and H_5 protons of the metabolite concerned allowed calculation of the extent of futile deacetylation.

Results

^{1}H-NMR spectroscopy of urine from rats dosed with phenacetin

The 600 MHz ^{1}H-NMR spectrum of whole rat urine 0–8 h after dosing with phenacetin at 50 mg kg^{-1} is shown in figure 2. A number of the resonances from the endogenous components in urine have been previously assigned and are labelled in the Figure (Nicholls *et al*. 1995, Nicholson and Wilson 1987). Paracetamol resonances were also assigned from previous studies (Bales *et al*. 1984). The singlet resonance observed at δ3·15 was assigned to dimethyl sulphone by addition of authentic material. Figure 3 shows expansions from the 600 MHz ^{1}H-NMR spectra shown in figure 2. As expected the predominant phenacetin metabolites were related to paracetamol, with aromatic resonances (AA′ XX′ spin system) for paracetamol sulphate at δ7·46 and 7·32, and the corresponding protio-acetyl singlet at δ2·18 (Bales *et al*. 1984, Nicholls *et al*. 1995, 1997). Integration of the aromatic resonances relative to an internal standard (TSP) gave a mean % recovery of 37·7 ± 8·6% for the paracetamol sulphate as a % of the administered dose.

Paracetamol glucuronide was also present with the aromatic resonances (AA′XX′ spin system) partially obscured at δ7·36, whilst visible at δ7·15 (Bales *et al*. 1984, Nicholls *et al*. 1995, 1997). The former signal was overlapped by a resonance from an endogenous substance so that only the latter was used for quantification. The glucuronide acetyl (protio) signal was clearly visible as a singlet at δ2·16. The mean % recovery of paracetamol glucuronide was 8·5 ± 3·3% as a % of dose.

One of the aromatic doublets for the *N*-acetyl-L-cysteinyl metabolite of paracetamol was initially assigned to the doublet at δ6·95 and the acetyl singlet of the *N*-acetyl-L-cysteinyl residue to the resonance δ1·86 (Bales *et al*. 1984, Nicholls *et al*. 1995, 1997). However, the relative heights and integrals of these resonances indicated that the signal at δ6·95, which was also phenacetin-related, was not solely due to the paracetamol *N*-acetyl-L-cysteinyl-metabolite. Quantification of the *N*-acetyl-*l*-cysteinyl paracetamol gave a % mean recovery of 1·7 ± 0·6% of the dose. The mean % total recovery for the three paracetamol conjugate metabolites of phenacetin was 50·7 ± 12·4% of the received dose.

To provide further data to enable assignment of the unidentified metabolite at δ6·95 a 600 MHz ^{1}H-^{1}H DQF COSY experiment was obtained of the 0–8-h whole urine (figure 4). The drug metabolite and endogenous metabolite NMR signals were assigned from previous studies (Bales *et al*. 1985). Assignment of the *N*-acetyl-L-cysteinyl conjugate of paracetamol was made to the resonances at δ6·93 and 7·23. The resonances at δ6·99 and 7·16 and those at δ6·89 and 7·25 were assigned to 4-aminophenol sulphate (Gartland 1988) and paracetamol (Bales *et al*. 1985) respectively after comparison with existing data. Due to extensive overlap with other metabolites in the 1-D NMR spectrum, these two metabolites could not be quantified directly. The resonance overlapping with the *N*-acetyl-cysteinyl paracetamol was coupled to another at δ7·31 indicating a *para*-substituted aromatic compound, but complete identification was not possible from these data alone.

To verify whether any further metabolites could be identified solid phase extraction chromatography (SPEC) on a C18 Bond-Elut™ column using acidified

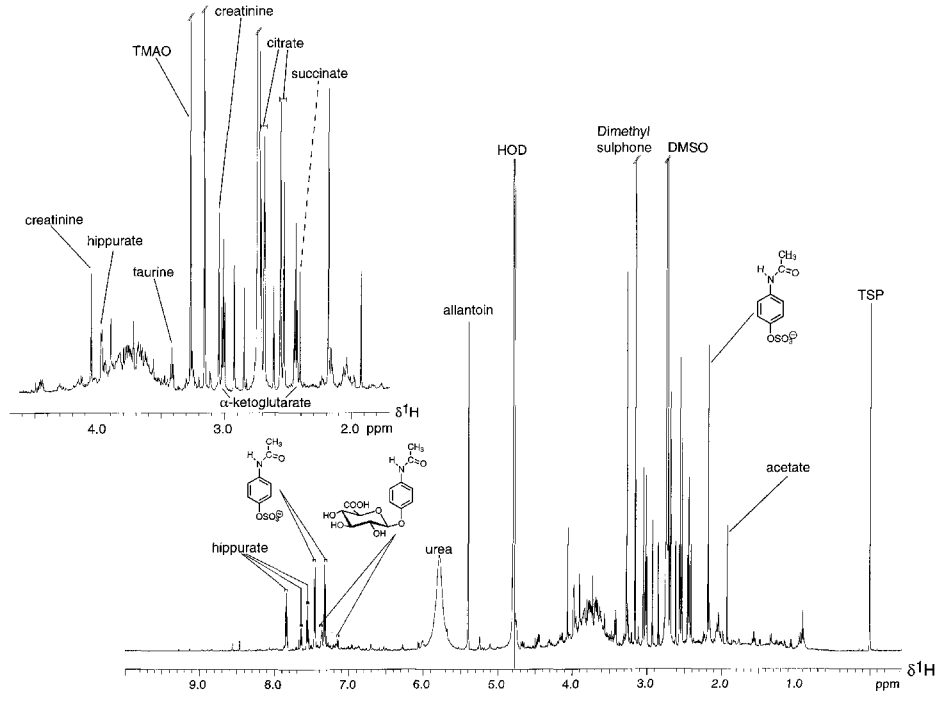

Figure 2. 600 MHz ^{1}H-NMR spectrum of whole rat urine following dosing of phenacetin (50 mg kg^{-1}). The expansion of the aliphatic region shows the major endogenous components. The endogenous and phenacetin metabolites are as labelled. TSP, sodium 3-trimethylsilyl-[2,2,3,3-$^2H_4$]-1-propionate; DMSO, dimethyl sulphoxide; TMAO, trimethylamine-*N*-oxide.

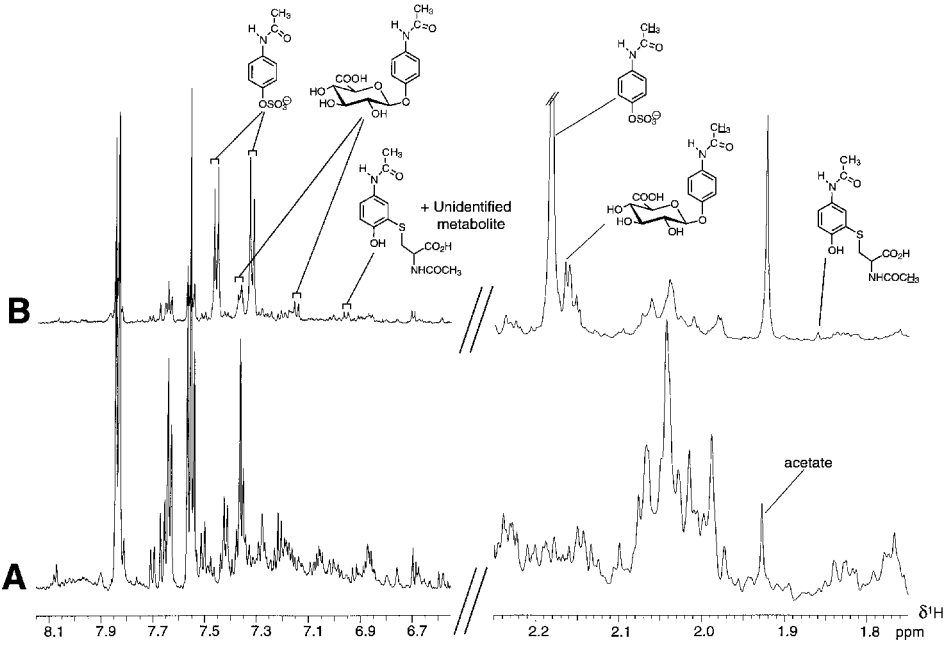

Figure 3. Expansions from the 600 MHz ^{1}H-NMR spectra of whole rat urine pre- (A) and post-dose (B) with phenacetin (50 mg kg^{-1}). The endogenous and phenacetin metabolites are as labelled.

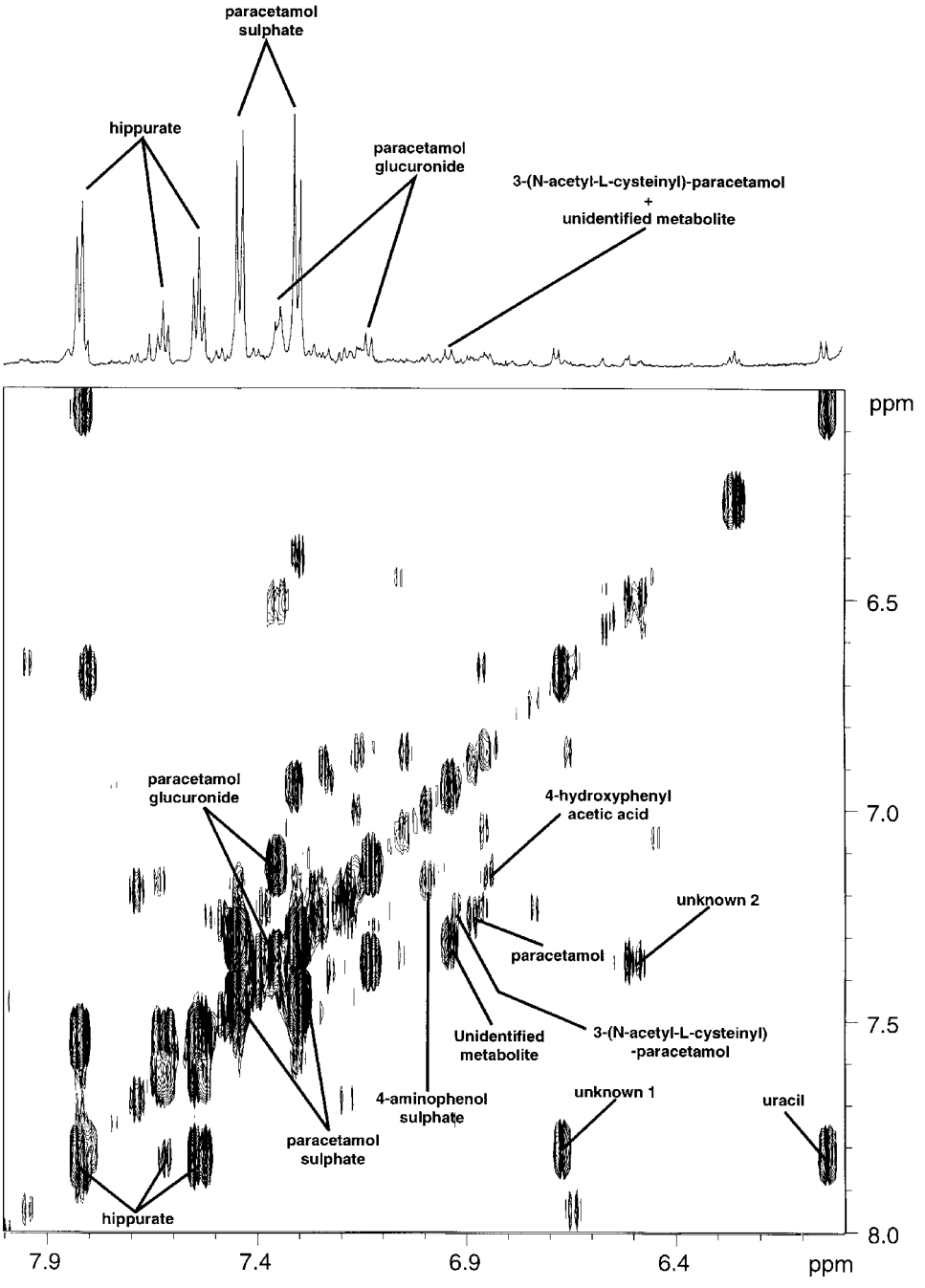

Figure 4. Partial (δ6·0–8·0) 600 MHz ^{1}H-^{1}H DQF-COSY NMR spectrum of whole rat urine following dosing of phenacetin (50 mg kg^{-1}). The endogenous and phenacetin metabolites are as labelled.

water and methanol was used to purify the whole urine. Shown in figure 5 is a stackplot of expansions from the 600 MHz ^{1}H-NMR spectra of the column eluent, acidified water (pH 2) and methanol fractions of the 0–8-h post-dose whole urine. The phenacetin metabolites and endogenous resonances are as labelled. The

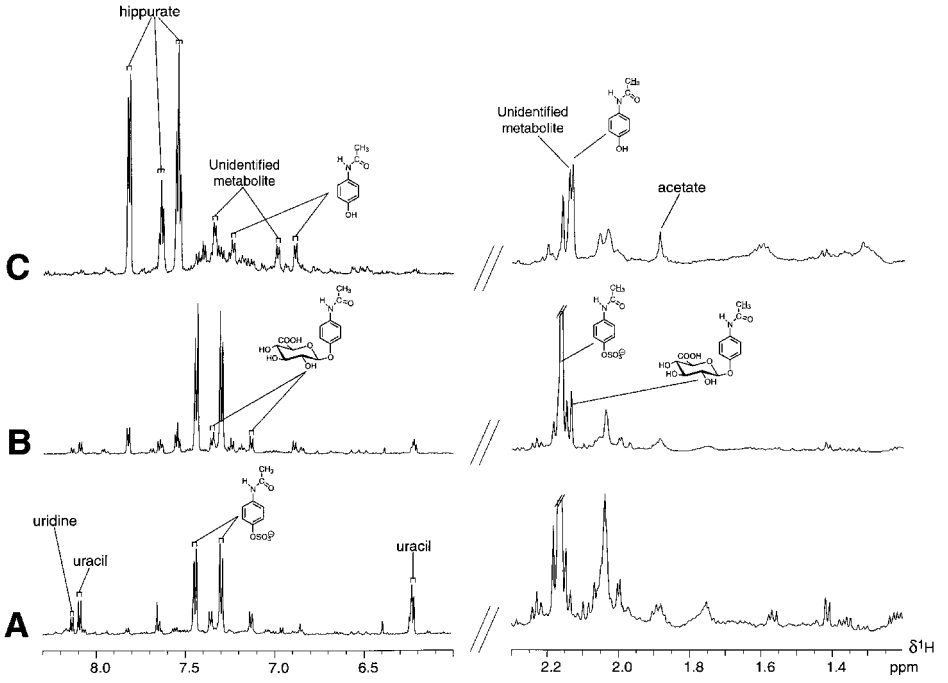

Figure 5. 600 MHz ^{1}H-NMR spectra of SPE fractions of whole rat urine following dosing of phenacetin (50 mg kg^{-1}). (A) Column eluent, (B) acidified water (pH 2), and (C) 100% methanol fraction. The endogenous and phenacetin metabolites are as labelled.

resonances at δ6·95 and 7·25 were assigned to unconjugated paracetamol (Bales *et al*. 1984, Nicholls *et al*. 1995, 1997). The resonance at δ6·99 was attributed to the unidentified phenacetin metabolite with the chemical shift variation caused by the pH of the solution. An acetyl resonance at δ2·13 was found to integrate with a 3:2 ratio to that of the unidentified at δ6·99 indicating that the molecule was probably an *N*-acetylated *para*-substituted aniline. Since resonances of an ethyl group were absent, the unidentified molecule could not be phenacetin itself and based on the chemical shifts the unidentified molecule was tentatively assigned as N-hydroxyparacetamol or a conjugate of this.

The ^{1}H-NMR resonances at δ8·10 and δ6·23 were assigned to the nucleotide base uracil and the resonances at δ8·12 and δ6·24 to uridine (Lynch *et al*. 1994). Increases in the intensity of signals from both substances were observed following dosing of phenacetin.

^{1}*H-NMR spectroscopy of urine from rats dosed with phenacetin-*C^2H_3

Expansions of the 600 MHz ^{1}H-NMR spectra for pre-dose and 0–8 h post-dose whole urine following dosing of phenacetin-C^2H_3 at 50 mg kg^{-1} are shown in figure 6. The endogenous and phenacetin metabolite ^{1}H-NMR resonances were assigned from previous studies (Bales *et al*. 1984, Nicholls *et al*. 1995, 1997). The paracetamol sulphate aromatic resonances (AA′XX′ spin system) were observed at δ7·46 and 7·32 with the corresponding acetyl singlet at δ2·18 (Nicholls *et al*. 1995, 1997). The acetyl singlet was integrated relative to the aromatic signals to give a

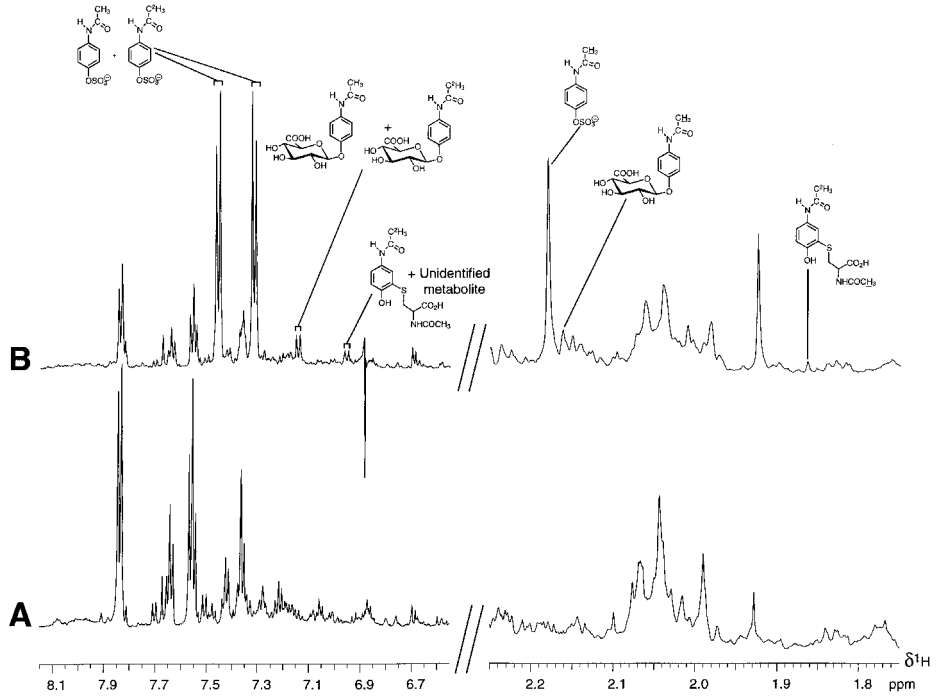

Figure 6. Expansions of the 600 MHz ^{1}H-NMR spectra of whole rat urine, pre- (A) and post-dosing (B) with phenacetin-C^{2}H$_3$ (50 mg kg^{-1}). Both the endogenous and phenacetin metabolites are as labelled.

mean % futile deacetylation of 29·6 ± 0·9% (12·3 ± 0·1% of dose, 23·3 ± 0·9% of the recovery) for the paracetamol sulphate metabolite. The mean % recovery for the paracetamol sulphate was calculated to be 41·6 ± 9·4% of the received dose.

Paracetamol glucuronide was also detected with aromatic resonances (AA′XX′ spin system) at δ7·36 and 7·14 and an acetyl singlet at δ2·16. Integration of the acetyl singlet was used to give a mean % futile deacetylation of 36·6 ± 3·1% (2·5 ± 0·1% of dose, 4·7 ± 0·4% of the recovery). The mean % recovery for this metabolite was 6·8 ± 1·8% of dose.

As with phenacetin, the ^{1}H resonance at δ1·86 was assigned to the *N*-acetyl-L-cysteinyl metabolite of paracetamol and the signal at δ6·95 was attributed to *N*-acetyl-L-cysteinyl paracetamol overlapped with a signal from the unassigned phenacetin metabolite. The low concentration of this metabolite, which accounted for only 1·9 ± 0·3% of dose, meant that quantification of the level of futile deacetylation was not possible in the present studies. No other metabolites were identified from the 0–8-h urine. Thus the mean % total recovery of the metabolites of phenacetin-C^{2}H$_3$ was 52·8 ± 11·5% of dose. There was no significant difference (Student's *t*-test, $p > 0{\cdot}05$) between the total % urinary recoveries for the non-labelled and labelled phenacetin.

Discussion

Futile deacetylation is potentially a very common *in vivo* metabolic transformation for many *N*-acetylated arylamines, the extent of the process being dependent on ring electronic and side-chain steric interactions with deacetylases and *N*-acetyltransferases. However, relatively few studies have dealt specifically with

Table 1. Percentage urinary recoveries of phenacetin, phenacetin-C^2H_3, paracetamol-C^2H_3 and paracetamol-$^{13}CH_3$ dosed to rat along with the % futile deacetylation for the isotopically labelled drugs. Data derived from * Nicholls *et al.* (1995) and ** Nicholls *et al.* (1997). —, Not observed.

Compound dosed	No. of animals	Dose level (mg kg^{-1})	Mean % futile deacetylation of paracetamol sulphate	Mean % futile deacetylation of paracetamol glucuronide	Mean % urinary recovery of total metabolites
Phenacetin	3	50	—	—	47·6 ± 16·7
Phenacetin-C^2H_3	3	50	29·6 ± 0·9	36·6 ± 3·1	50·1 ± 16·2
Paracetamol-C^2H_3*	3	40	10·0 ± 1·2	—	61·4 ± 8·8
Paracetamol-$^{13}CH_3$	3	40	8·9 ± 0·7	—	102·7 ± 3·7
Paracetamol-$^{13}CO^{13}CH_3$**	3	100	9·2	9·1	47·4 ± 11·3

these biochemically 'silent' processes. The importance of a futile deacetylation reaction with regard to toxicity of arylamines has been demonstrated previously for paracetamol (Nicholls *et al.* 1995, 1997). The results of the present and previous studies on futile deacetylation in paracetamol are compared in table 1. The earlier studies (Nicholls *et al.* 1995) indicated that a proportion of the paracetamol, approximately 6% of the dose, underwent futile deacetylation probably via 4-aminophenol. This extent of futile deacetylation was considered to be a possible contributing factor to the nephrotoxicity of paracetamol, underlying the importance of metabolic reactions that are 'silent' in non-labelled material (Nicholls *et al.* 1995).

Phenacetin, which is shown herein to undergo extensive futile deacetylation (15% of dose, i.e. twice that observed for paracetamol), has been shown to lead to an increase in the predisposition to kidney damage when taken for long periods, and nephrotoxicity is similarly seen in patients who have experienced overdose (Sandler *et al.* 1989). The extent of futile deacetylation for phenacetin supports previous evidence from ^{14}C-labelling studies, which also showed a total deacetylation of 15% for phenacetin (Smith and Griffiths 1976). Since the level of % futile deacetylation was significantly higher than that found for paracetamol, this indicates that products formed from the deacetylation of phenacetin itself need to be considered as major metabolites of the drug. Thus 4-ethoxyaniline, which has previously only been considered as a minor metabolic product of phenacetin (Brodie and Axelrod 1949, Bernhammer and Krisch 1965, Smith and Griffiths 1976), might be an important metabolite formed by futile deacetylation.

The difference between the % futile deacetylation for paracetamol and phenacetin suggests two possible routes for paracetamol formation from phenacetin. The first route involves the deacetylation of phenacetin to 4-ethoxyaniline followed by re-acetylation back to phenacetin with subsequent de-ethylation to paracetamol (figure 1). Another route involves the deacetylation of phenacetin to 4-ethoxyaniline that in turn is de-ethylated to form 4-aminophenol. This can then be either acetylated to paracetamol followed by conjugation or conjugated with subsequent acetylation (figure 1). The latter of these two routes would allow for a potentially greater quantity of 4-aminophenol to be formed, which might explain the increased nephrotoxicity of phenacetin over paracetamol. Circumstantial evidence for the formation of these compounds in addition to the nephrotoxicity of phenacetin has been the occurrence of methaemoglobinæmia following metabolite ingestion (Smith 1958, Gloor 1993). This toxicological condition has also been shown to occur

following ingestion of 4-ethoxyaniline (Timbrell 1992). If indeed 4-aminophenol and 4-ethyoxyanilineare the main toxic species then this would indicate that such 'silent' metabolic routes represent an, as yet, little appreciated major route of metabolism with regard to drug toxicity for compounds of this class.

In conclusion, this study has emphasised the potential importance of the futile deacetylationreaction pathwayin drug metabolism. In addition, the use of ^{1}H-NMR spectroscopy and isotopic labelling has provided a simple, readily interpretable analytical method for the measurement of such 'silent' metabolic reactions.

Acknowledgements

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