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Directly-coupled HPLC-NMR spectroscopic studies of metabolism and futile deacetylation of phenacetin in the rat

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Abstract

The metabolism and futile deacetylation of phenacetin has been investigated in the rat via ^{1}H NMR spectroscopic analysis of urine. Animals were dosed with either phenacetin or phenacetin–C^2H_3 and urine samples were collected for −24–0 (pre-dosing), 0–8, 8–24, and 24–48 h post-dosing. Drug metabolites of the two compounds were concentrated from the urine using solid-phase extraction prior to the use of directly-coupled HPLC-^{1}H NMR spectroscopy for separation and identification. Following dosing of phenacetin, the metabolites identified were paracetamol glucuronide, paracetamol and *N*-hydroxyparacetamol, whilst paracetamol and *N*-hydroxyparacetamol sulphate were identified following dosing of phenacetin–C^2H_3. Quantitatively the percentage futile deacetylation of phenacetin–C^2H_3 metabolites was found to be 32% in both paracetamol and *N*-hydroxyparacetamol sulphate. This study further indicated the importance of futile deacetylation in simple analgesics and the value of directly-coupled HPLC-NMR spectroscopy for the study of this process. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Futile deacetylation; Paracetamol; 4-Acetamidophenol; Phenacetin; Metabolites; Rat; HPLC-NMR; ^{2}H labelling

1. Introduction

The mechanistic basis of the nephrotoxicity of the drug phenacetin has long been a subject of much controversy. The perceived association between phenacetin consumption and the development of nephrotoxic lesions led to the eventual withdrawal of the drug from the market [1], but the cause of phenacetin-induced nephrotoxicity has yet to be fully elucidated. The principal metabolite of phenacetin is paracetamol [2], which then undergoes further metabolism as shown in Fig. 1. The other major metabolite of phenacetin in mammals is *p*-phenetidine (4-ethoxyaniline) [3].

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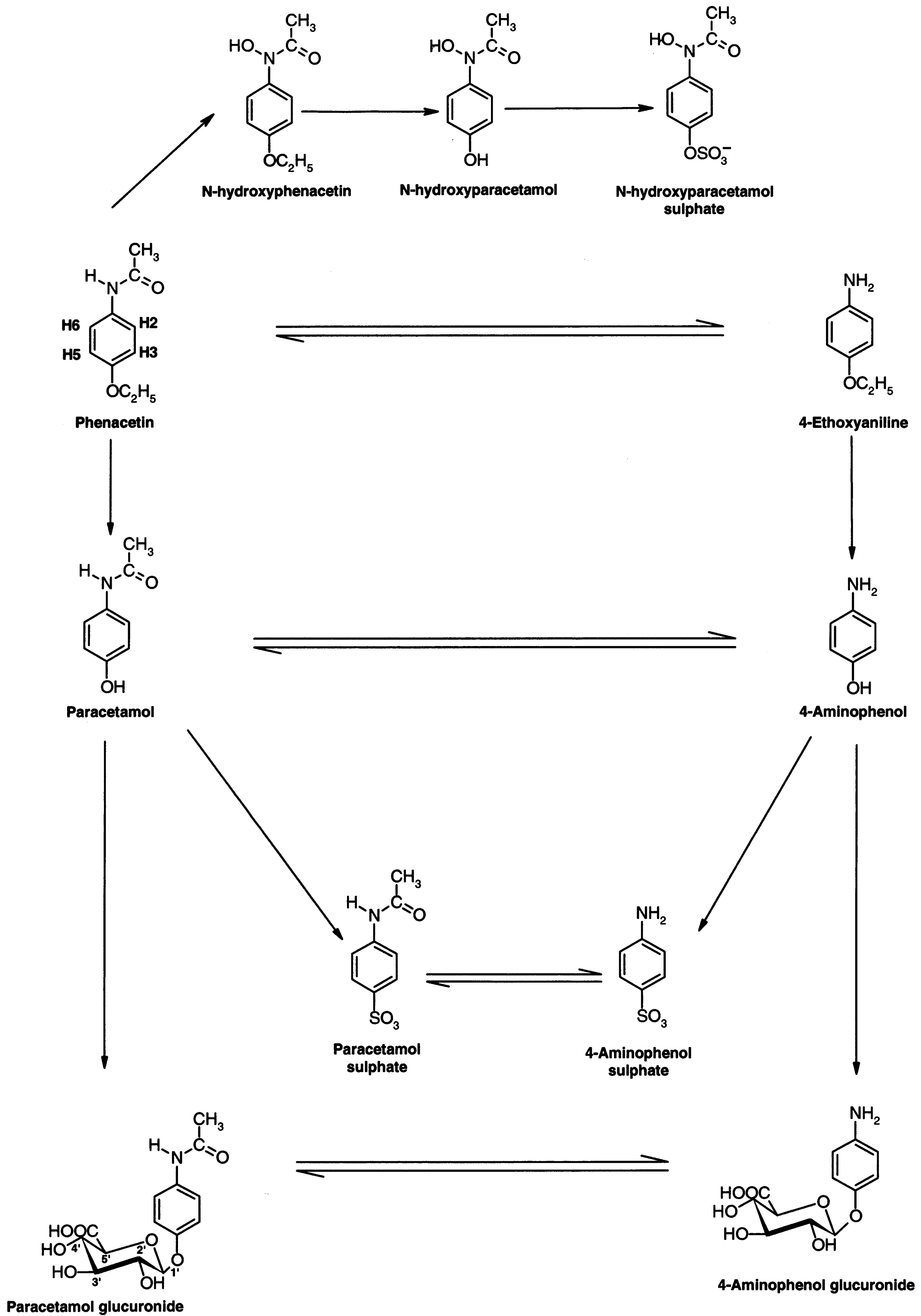

Fig. 1. The metabolism of phenacetin.

Directly-coupled HPLC-NMR has been used successfully for the identification of metabolites from complex biological mixtures and the quantification of the percentage futile deacetylation [4–6]. The technique can be used in either continuous-flow or stop-flow modes, with the latter approach used here to study percentage futile deacetylation in phenacetin metabolites.

The extent of 'futile deacetylation' of phenacetin, in which the *N*-acetyl moiety of the drug is lost and then subsequently replaced by an acetyl group from endogenous sources, has been investigated recently since this might have a bearing on the toxicity of the drug [7]. Previously we have investigated this type of futile deacetylation reaction for the closely related drug paracetamol, by synthesising the compound with either ^{2}H or ^{13}C labels in the acetyl group and monitoring, by NMR spectroscopy, the level of non-labelled material excreted in the urine [4,8]. The level of futile deacetylation in individual metabolites of paracetamol was assessed using quantitative ^{1}H nuclear magnetic resonance (NMR) measurements with directly-coupled HPLC, and this indicated a level of approximately 10% futile deacetylation for the sulphate and glucuronide conjugates in the rat [8]. These results suggested that there was a significant transient flux through the metabolic intermediate and potent nephrotoxin 4-aminophenol, which causes severe and acute necrosis of the kidney proximal tubules [9] by ill-defined mechanisms [10].

To investigate the extent of the 'silent' futile deacetylation pathway in individual metabolites of phenacetin, isotopically-labelled material was administered to rats [7] with the label consisting of full deuteriation of the acetyl moiety. ^{1}H NMR spectroscopy was then used to analyse the urine to assess the level of protio-acetyl content within the phenacetin metabolites. Quantitative measurements of the percentage futile deacetylation in the paracetamol sulphate and glucuronide metabolites of phenacetin indicated that a substantial proportion of the drug had undergone the reaction. Further phenacetin-related substances could be observed in the ^{1}H NMR spectra of the whole urine, but their identification was not possible by ^{1}H NMR spectroscopy even using two-dimensional NMR techniques. Identification of these metabolites is important if the total futile deacetylation balance is to be obtained and its possible role in phenacetin toxicity is to be elucidated. Therefore, the known and unknown phenacetin metabolites were isolated from whole urine using solid-phase extraction (SPE) and the extracts analysed using directly-coupled HPLC-NMR spectroscopy to identify the metabolites and to determine the percentage futile deacetylation in each species.

2. Experimental

2.1. Synthesis of phenacetin and phenacetin–$C^{2}H_{3}$, dosing and urine collection

The compounds were synthesised and metabolism studies in the rat were carried out as described previously [7]. The level of ^{2}H incorporation was $>99.5\%$ and hence the deuterated material did not give rise to an observable acetyl peak in the ^{1}H NMR spectra. Each animal (weight approximately 200 g) received a single intraperitoneal (i.p.) dose of phenacetin or phenacetin–$C^{2}H_{3}$ equivalent to 50 mg/kg.

2.2. ^{1}H NMR spectroscopic analysis of whole rat urine

A 0.5-ml aliquot of each of the untreated urine samples was placed in a 5-mm NMR tube and 0.05 ml of 1 mM sodium 3-trimethylsilyl-[2,2,3,3-$^{2}H_{4}$]-1-propionate (TSP) in $^{2}H_{2}O$ was added to each sample in order to provide a chemical shift and quantification reference (δ 0.0). ^{1}H NMR spectra were measured at 600.14 MHz on a Bruker AMX-600 spectrometer using the NOESYPRESAT [11] pulse sequence for water resonance suppression with irradiation during the relaxation delay of 3 s and the mixing time of 100 ms. A total of 64 free induction decays (FIDs) were collected into 131 072 data points using a spectral width of 18.5 kHz. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz and zero-filled by a factor of two prior to Fourier transformation (FT).

2.3. Solid phase extraction (SPE) of metabolites from urine samples

Urine (1 ml) containing the phenacetin metabolites (0–8 h post-dose, 50 mg/kg i.p.) was acidified to pH 2 with 1 M HCl and extracted on to a C18 bonded cartridge (Bond-Elut™, 100 mg, Jones Chromatography, Hengoed, Wales, UK). The cartridges had previously been activated by washing with methanol (1 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 $^{2}H_2O$ prior to NMR analysis. ^{1}H NMR spectroscopy of the SPE samples showed that the phenacetin metabolites were in the acidified water fraction.

2.4. HPLC-NMR spectroscopy of SPE extracts of rat urine

The HPLC system comprised a Hewlett Packard 1050 Series pump operating at 21°C and a variable-wavelength UV detector operating at 210 nm. The outlet from the UV detector was connected to the HPLC-NMR flow probe via an inert polyether(ether) ketone capillary. The chromatography was controlled using the Bruker Chromstar HPLC data system. Analysis was performed on a Waters Symmetry C18 column (3.9×150 mm i.d.) packed with Spherisorb ODS-2, 3 μm. The mobile phase consisted of D_2O/acetonitrile-d_3 (98:2 v/v) for the first 5 min followed by a linear gradient to D_2O–acetonitrile-d_3 (50:50 v/v) after 55 min with a flow rate of 0.5 ml/min.

HPLC-^{1}H NMR spectra were acquired using a Bruker DMX-500 instrument equipped with a ^{1}H flow probe (cell of 3 mm i.d. with a volume of 120 μl) in the stop-flow mode at 500.13 MHz. Presaturation of the water signal was achieved by the use of a NOESYPRESAT pulse sequence [11] with irradiation during the relaxation delay of 1.8 s and the mixing time of 80 ms. Typically 256 FIDs were accumulated into 32 768 data-points, with an acquisition time of 1.36 s, a recycle delay of 1.8 s and a spectral width of 12 000 Hz. An exponential line-broadening function of 1.0 Hz was applied and the data were zero-filled by a factor of two prior to FT.

The ^{1}H NMR spectral regions arising from the aromatic and acetyl protons of the paracetamol metabolites were integrated. The extent of futile deacetylation was calculated by integration of the CH_3 acetyl signal relative to those of the aromatic H3 and H5 protons of the phenacetin metabolite concerned. The level of deuteration of the parent compound was such that no residue protio-acetyl methyl peak would be observable in the ^{1}H NMR spectrum.

3. Results

3.1. Extent of futile deacetylation of phenacetin–$C^{2}H_3$ metabolites measured in whole rat urine using ^{1}H NMR spectroscopy

The ^{1}H NMR spectrum of whole rat urine is very complex, with thousands of resonances from a large number of endogenous substances at a wide range of concentrations. However, comparison of expansions of the aromatic and aliphatic regions of the 600 MHz ^{1}H NMR spectra for pre-dose and 0–8 h post-dose whole urine showed the presence of a number of extra resonances in the latter case which arose from phenacetin metabolites. Assignment of these as paracetamol conjugates was made by comparison with previous data [4,12,13]. Based on the known chemical shifts of authentic material, aromatic ^{1}H NMR resonances (AA′ XX′ spin system) at δ 7.46 and 7.32 were assigned to paracetamol sulphate along with a relatively smaller acetyl resonance at δ 2.18. The presence of the acetyl signal indicated that a substantial level of deacetylation of the $COC^{2}H_3$ moiety and reacetylation with $COCH_3$ had occurred. The mean percentage level ($n = 3$) of futile deacetylation measured for paracetamol sulphate was $29.6 \pm 0.9\%$.

Paracetamol glucuronide was also observed in the ^{1}H NMR spectrum of the whole urine with aromatic resonances (AA′ XX′ spin system) at δ 7.36 and 7.14 and an acetyl singlet at δ 2.16. Integration of the acetyl singlet showed a mean percentage ($n = 3$) futile deacetylation of $36.6 \pm 3.1\%$.

^{1}H NMR resonances were also visible at δ 1.86 and 6.95 and these were respectively assigned to the *N*-acetyl of the cysteine residue and H3/H5 of the *N*-acetyl-L-cysteinyl metabolite of paracetamol. The aromatic proton signal was also overlapped with another unassigned phenacetin metabolite. The low concentration of this metabolite, which accounted for approximately $1.9 \pm 0.3\%$ of the dose, meant that quantification of the level of futile deacetylation could not be achieved.

3.2. Identification of phenacetin metabolites using HPLC-NMR spectroscopy of a SPE extract of whole rat urine

An initial study using non-deuteriated compound was made to determine the feasibility of HPLC-NMR separation of phenacetin–CH_3 metabolites from a SPE extract of rat urine. NMR data acquisitions were made in stop-flow mode at retention times indicated by UV-detected peaks. Expansions of the regions δ 2.30–1.80 from the 500 MHz ^{1}H NMR spectra from the stop-flow HPLC-NMR analysis of three phenacetin metabolites present in the SPE extract of rat urine, are shown in Fig. 2. The metabolites were assigned by comparison to previous data [4,12,13] and NMR parameters are listed in Table 1. The first metabolite (retention time = 14 min) was assigned to the glucuronide conjugate of paracetamol with the aromatic proton resonances (AA′ XX′ spin system) at δ 7.31 and 7.08 and the acetyl singlet at δ 2.09 (Fig. 2A). Further resonances (not shown in the figure) corresponding to the glucuronide moiety were observed at δ 5.10 (H1′), δ 4.08 (H5′) and δ 3.58 (H2′, H3′, H4′). The second metabolite detected had a retention time of 22 min and was assigned to paracetamol based on the aromatic proton (AA′ XX′ spin system) resonances at δ 7.20 and 6.83 and the acetyl singlet at δ 2.07, shown in Fig. 2B. These shifts were in agreement with literature values [12]. The final metabolite (retention time, 28 min) gave rise to AA′ XX′ spin system aromatic proton resonances at δ 7.32 and 6.95 and an acetyl singlet at δ 2.09 (Fig. 2C). These chemical shifts are not consistent with paracetamol sulphate, but show a high frequency shift for H2 and H6 suggesting a modification in the acetyl group. As both the acetyl methyl group and the aromatic proton spin system remain intact, substitution at the nitrogen, probably by a hydroxyl group, is postulated. Thus, the metabolite was tentatively assigned to *N*-hydroxyparacetamol, a known metabolite of phenacetin [14]. No further metabolites of phenacetin or paracetamol were isolated in this HPLC-NMR study.

3.3. HPLC-NMR analysis of a SPE extract of rat urine from an animal dosed with phenacetin–C^2H_3

Following the above study, HPLC-NMR spectroscopy was then used to separate metabolites from a SPE extract of whole rat urine following dosing of phenacetin–C^2H_3. The δ 2.30–1.80 region of the 500 MHz ^{1}H NMR spectra of two metabolites of phenacetin–C^2H_3 in the rat obtained from stop-flow HPLC-NMR spectroscopy of the solid-phase extracted post-dose urine, shown in Fig. 3. Two metabolites of phenacetin could be detected by this approach and their chemical shifts are listed in Table 1. The first (retention time, 14 min) possessed aromatic proton (AA′ XX′ spin system) NMR resonances at δ 7.20 and 6.83 (Fig. 3A) and was assigned to paracetamol–CH_3 and paracetamol–C^2H_3 by comparison to previous data [4,12,13]. The acetyl singlet from this molecule was observed at δ 2.07 and quantification of the signals indicated a level of futile deacetylation of 32%. The second metabolite of phenacetin–C^2H_3 (retention time, 23 min) possessed aromatic proton NMR resonances consistent with an AA′ XX′ spin system at δ 7.41 and 7.23 (data not shown). Since no other ^{1}H NMR resonances were observed (other than an acetyl singlet at δ 2.09, Fig. 3B) the molecule was not paracetamol glucuronide. However, the ^{1}H NMR chemical shifts and AA′ XX′ splitting pattern were consistent with the substance being a substituted 4-acetamidophenol. Sulphation was the most likely form of conjugation to occur at the *para* position in such species, but comparison of the chemical shifts and the coupling constants between the AA′ XX′ resonances indicated that this metabolite was not paracetamol sulphate.

Thus, further substitution must have occurred at the nitrogen, but the precise nature of this could not be determined from the NMR spectra alone. Following the tentative identification of *N*-hydroxyparacetamol after phenacetin dosing and the high likelihood of sulphation, the metabolite was tentatively assigned as *N*-hydroxyparacetamol sulphate, the level of futile deacetylation of this metabolite being measured at 32%.

Previous studies of phenacetin metabolism have noted the presence of paracetamol sulphate as the major metabolite, but in this study it was not observed. Since the HPLC-NMR was undertaken on a single SPE fraction of the whole urine, the

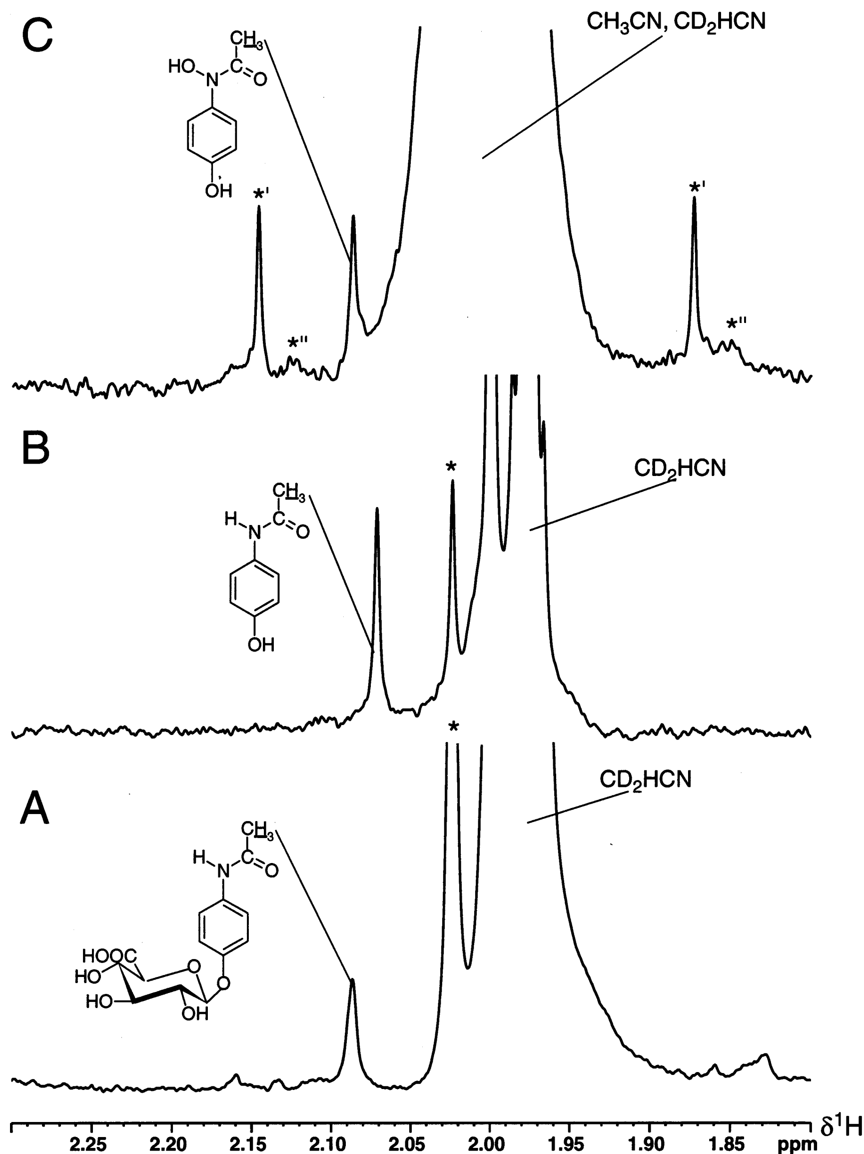

Fig. 2. Partial (δ 2.30–1.80) 500 MHz HPLC-^{1}H NMR spectra of phenacetin metabolites showing the region of the acetyl resonances (A) paracetamol glucuronide (retention time, 14 min), (B) paracetamol (retention time, 22 min), (C) *N*-hydroxyparacetamol (retention time, 28 min); *probable solvent impurity; *′, resonances for $^{13}CH_3CN$; *″, resonances for $^{13}CD_2HCN$.

Table 1
^{1}H NMR chemical shifts for phenacetin metabolites isolated from SPE extract of whole rat urine

Metabolite	Chemical shift (δ)
Paracetamol	7.20 (H2, H6), 6.83 (H3, H5), 2.07 (CH_3)
Paracetamol glucuronide	7.31 (H2, H6), 7.08 (H3, H5), 5.10 (H1′), 4.08 (H5′), 3.58 (H2′, H3′, H4′), 2.09 (CH_3)
N-Hydroxy-paracetamol	7.32 (H2, H6), 6.95 (H3, H5), 2.09 (CH_3)
N-Hydroxy-paracetamol sulphate	7.41 (H2, H6), 7.23 (H3, H5), 2.09 (CH_3)

most likely reason for the lack of this metabolite was that it eluted in one of the other fractions.

4. Discussion

We have demonstrated here that phenacetin–C^2H_3 undergoes substantial in vivo futile deacetylation in the rat. Previous studies in the rat using ^{1}H NMR spectroscopy have quantified the extent of futile deacetylation at 30 and 36% in the paracetamol sulphate and glucuronide metabolites respectively, with a total urinary recovery of $50.1 \pm 16.2\%$ of the dose [7]. However, it was not possible to measure the level of deacetylation for minor phenacetin metabolites (Table 2). We have now shown that the level of futile deacetylation of paracetamol and *N*-hydroxyparacetamol sulphate metabolically derived from phenacetin was 32% (Table 2). We have previously shown that following administration of paracetamol itself approximately 6% of the total dose undergoes futile deacetylation in the rat, whilst the percentage futile deacetylation in the individual metabolites was ca. 10% [4,8]. These earlier studies used ^{2}H and ^{13}C labelling of paracetamol and both NMR spectroscopy [4] and HPLC-NMR spectroscopy [8] analyses to determine the level of futile deacetylation. Therefore, it is most likely that the deacetylation/reacetylation reactions of phenacetin occur predominantly on the parent compound itself and not its metabolites.

The level of futile deacetylation observed may aid the understanding of the significance of so-called 'silent' metabolism in relation to the potential nephrotoxicity of phenacetin. The nephrotoxic effects of phenacetin have been reported extensively [1,2,15,16] and paracetamol has also been reported to give rise to kidney damage, but at a much lower incidence and at higher doses [17]. It has been postulated that the level of futile deacetylation of phenacetin or paracetamol may be characteristic of the severity of nephrotoxic damage by indicating the degree of production of the potent nephrotoxin 4-aminophenol [4]. Since paracetamol is the major metabolite of phenacetin, a number of metabolic routes could lead to its formation (Fig. 1). One possibility is that phenacetin first undergoes futile deacetylation and is then de-ethylated to paracetamol. If so, this indicates that the presence of the ethoxy-group confers greater reactivity towards the deacetylation enzyme system. Alternatively phenacetin may be deacetylated to its secondary metabolite *p*-phenetidine (Fig. 1) which could subsequently be de-ethylated to 4-aminophenol. This species can be then acetylated to form paracetamol. This latter route would afford the formation of potentially significant levels of 4-aminophenol thereby providing a reason for the nephrotoxicity observed with phenacetin. Such a mechanism would indicate that 'silent' metabolic reactions, like futile deacetylation, might have been underestimated in their importance with regard to drug toxicity.

Other minor metabolites of phenacetin were also observed which indicate that the formation to paracetamol is not necessarily a direct de-ethylation step. Following dosing the metabolites *N*-hydroxyparacetamol and, tentatively, *N*-hydroxyparacetamol sulphate were noted in the urine. Further studies, perhaps employing HPLC-NMR-MS, are required so as to confirm the structure of latter metabolite. These metabolites have been previously noted for phenacetin [14], but have not been observed for paracetamol and thus the *N*-hydroxylation may require the presence of the ethoxy group. The variation in the metabolites observed between the unlabelled and ^{2}H-labelled phenacetin may have been due to a degradation

of the *N*-hydroxyparacetamol sulphate back to *N*-hydroxyparacetamol.

This study has emphasised the potential importance of the futile deacetylation reaction pathway in drug metabolism of substituted *N*-acetylarylamines. We have shown that although measurements of futile deacetylation can be made from the ^{1}H NMR spectra of the whole biofluid, HPLC-^{1}H NMR spectroscopy affords a more accurate quantification of the percentage futile deacetylation by the removal of interference from overlapping resonances arising from endogenous species. In addition, we have shown that the use of acetonitrile-d_3 as an HPLC mobile phase was more beneficial for futile deacetylation studies due to the lack of an acetonitrile methyl resonance that would obscure the signals of interest. Although suppression techniques could be used to

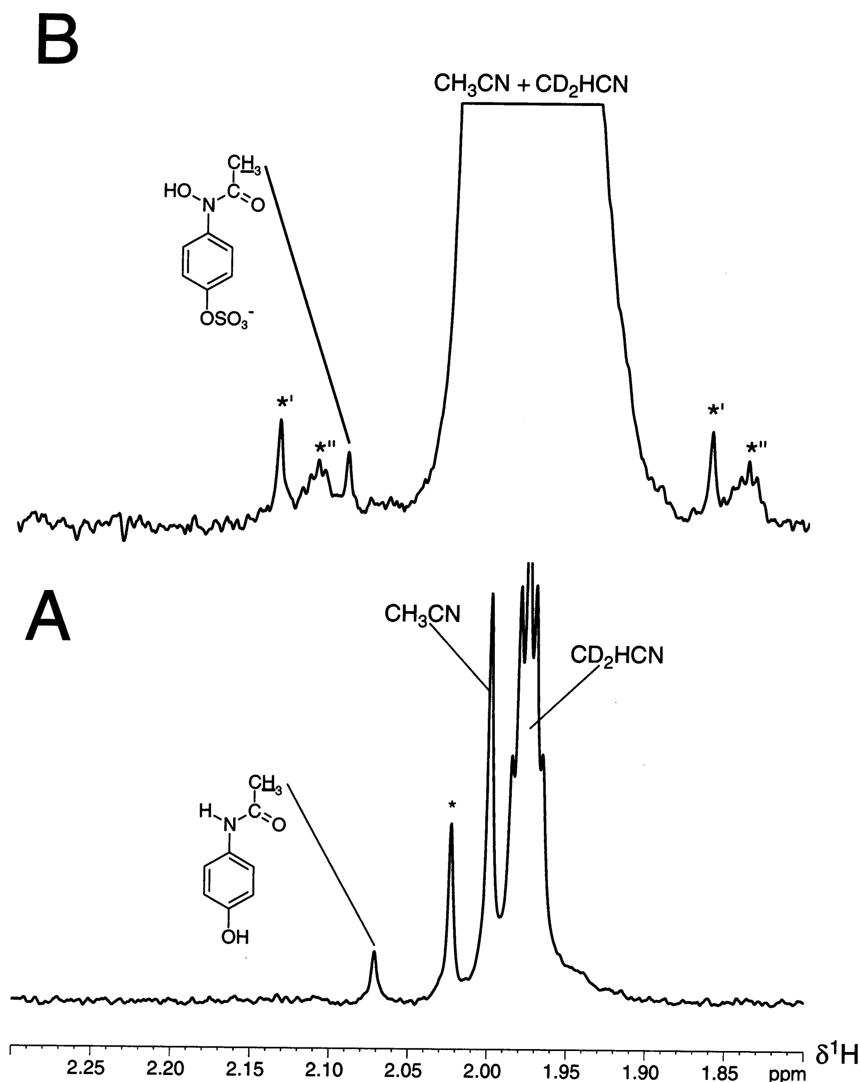

Fig. 3. Partial (δ 2.30–1.80) 500 MHz HPLC-^{1}H NMR spectra of phenacetin–$C^{2}H_3$ metabolites showing the region of the acetyl resonances (A) paracetamol–CH_3 and paracetamol–$C^{2}H_3$ (retention time, 14 min), (B) *N*-hydroxyparacetamol–CH_3 sulphate and *N*-hydroxyparacetamol–$C^{2}H_3$ sulphate (retention time, 23 min); *probable solvent impurity; *′, resonances for $^{13}CH_3CN$; *″, resonances for $^{13}CD_2HCN$.

Table 2
Percentage futile deacetylation in phenacetin metabolites following dosing at 50 mg/kg in the rat

Phenacetin metabolite	Percentage futile deacetylation
Paracetamol	32.0[a]
Paracetamol sulphate	29.6 ± 0.9
Paracetamol glucuronide	36.6 ± 3.1
N-Hydroxyparacetamol sulphate	32.0[a]

[a] Single measurement after 500 MHz HPLC-NMR separation using acetonitrile-d_3/D_2O elution.

remove the acetonitrile resonance and its ^{13}C-satellites, this would result in suppression of the acetyl resonances of interest due to the close proximity of the signals. Therefore, ^1H NMR spectroscopy allied with directly-coupled HPLC and isotopic labelling has provided a simple, readily interpretable analytical method for the measurement of such 'silent' metabolic reactions.

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