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NMR and HPLC-NMR spectroscopic studies of futile deacetylation in paracetamol metabolites in rat and man

Andrew W. Nicholls [a], R. Duncan Farrant [b], John P. Shockcor [c], Steve E. Unger [c], Ian D. Wilson [d], John C. Lindon [a,*], Jeremy K. Nicholson [a]

[a] *Department of Chemistry, Birkbeck College, University of London, Gordon House, 29 Gordon Square, London WC1H OPP, UK*
[b] *Physical Sciences Research Unit, GlaxoWellcome Medicines Research Centre, Gunnels Wood Road, Stevenage SG1 2NY, UK*
[c] *Bioanalysis and Drug Metabolism, GlaxoWellcome Inc., 5 Moore Drive, Research Triangle Park, NC 27709, USA*
[d] *Department of Safety of Medicines, Zeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK*

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Abstract

HPLC-NMR spectroscopy has been used to investigate the level of deacetylation followed by reacetylation (futile deacetylation) of metabolites of paracetamol detected in human and rat urine. This has been achieved through the synthesis and administration of paracetamol isotopically labeled at the acetyl group with C^2H_3, $^{13}CH_3$ and $^{13}CO-^{13}CH_3$. Using paracetamol-C^2H_3 it had been shown that in the rat the sulphate metabolite present in the urine shows 10–13% futile deacetylation depending on the dose, whereas for paracetamol-$^{13}CO-^{13}CH_3$ the corresponding value was about 8%. After solid phase extraction, it was also possible to determine the level of futile deacetylation in the glucuronide metabolite using directly-coupled HPLC-NMR. This approach was facilitated by the use of acetonitrile-d_3 as an HPLC eluent and the HPLC-NMR analyses showed that the level of futile deacetylation in the sulphate and glucuronide metabolites were equal at about 9%. The glucuronide of paracetamol-C^2H_3 was the predominant metabolite in man and following separation using HPLC-NMR, the level of futile deacetylation was shown to be 1% for the glucuronide and 2% for the sulphate, these values being equal within experimental error. This work demonstrates the utility of NMR and HPLC-NMR spectroscopy for isotope exchange studies. © 1997 Elsevier Science B.V.

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1. Introduction

Paracetamol (4-acetamidophenol, acetaminophen) is one of the most widely used anti-pyretic, analgesic compounds currently available. The major metabolites of paracetamol at the therapeutic dose level are paracetamol sulphate and glucuronide and L-cysteinyl and *N*-acetyl-L-cysteinyl conjugates (Fig. 1). Of these paracetamol sulphate and paracetamol glucuronide form the largest proportion and other minor metabolites

* Corresponding author. Tel.: +44 171 3807527; fax: +44 171 3807464.

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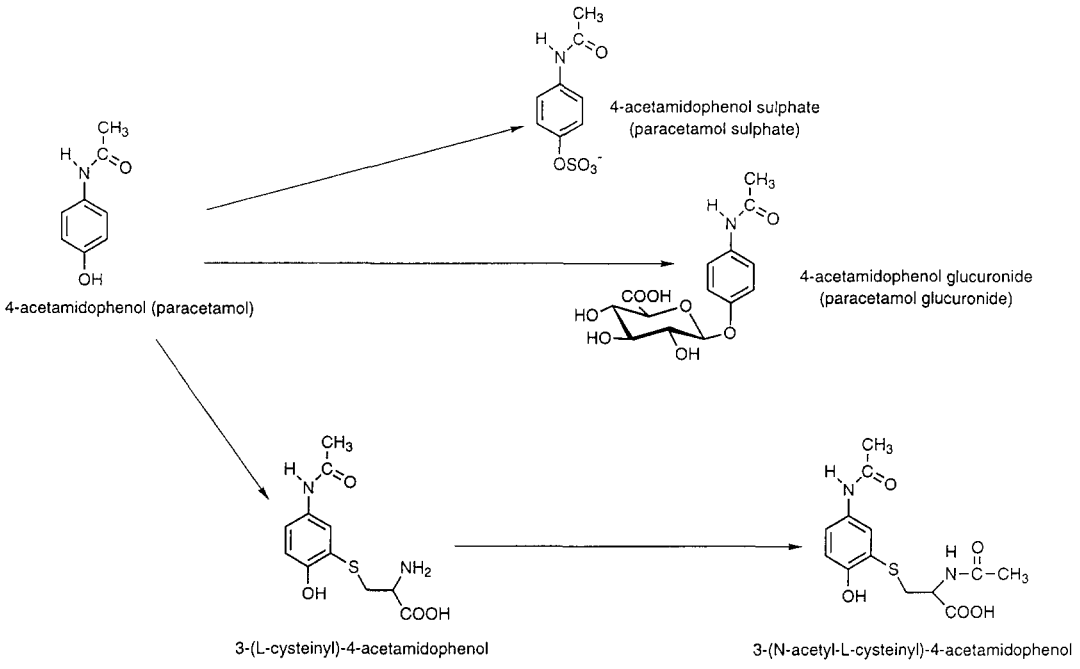

Fig. 1. The structure of paracetamol and its principal metabolites.

may also be observed, but usually only following drug overdose [1].

In humans, high doses of paracetamol can result in hepatotoxicity, with occasional observations of nephrotoxicity [2]. One of the proposed mechanisms of the kidney damage involves the formation of 4-aminophenol, a known nephrotoxin that causes severe and acute necrosis of the kidney proximal tubules [3]. For this to occur, paracetamol is required to undergo deacetylation, but this process has generally been considered to be only of minor importance due to the low fraction of the paracetamol dose excreted as 4-aminophenol [4]. However, it has previously been observed that 4-aminophenol may become reacetylated back to paracetamol prior to excretion [5]. This transacetylation or, more correctly, 'futile deacetylation' process has been shown for paracetamol (in rats and man) using GC-MS [5] and inferred by the reduced deacetylation of 4-acetaminohippurate in the presence of paracetamol and the reduced acetylation of 4-aminohippurate in the presence of 4-aminophenol [6]. Studies using ^{14}C-radiolabelling have also been used in an effort to quantify the extent of this metabolic route for paracetamol [7]. However, until recently, no measurement of the futile deacetylation of the individual paracetamol metabolites has been made, nor has NMR been utilised in the investigation of such metabolic processes. Moreover, deficiencies in the recovery of the drug from these earlier studies has not led to an accurate measure of the level of futile deacetylation of paracetamol.

Recently it has been shown that, in the rat, there is a significant level of futile deacetylation which can be detected in the sulphate metabolite [8] and here an extension to the study is reported in which levels of futile acetylation have been determined in both rat and man for isotopically-labelled paracetamol sulphate and glucuronide metabolites. This has been achieved through the use of directly-coupled HPLC-NMR spectroscopy to separate and characterise the metabolites and to quantify the proportions of the different isotopically labelled metabolites in the chromatographic peaks. Directly-coupled HPLC-NMR spectroscopy has been demonstrated to have widespread potential for the identification of drug

Table 1
The percentage futile deacetylation (FD) for paracetamol sulphate and glucuronide conjugates and the percentage total recovery of all paracetamol metabolites in rat and human urine

Compound	Dose (mg kg^{-1})/species	% FD sulphate	% FD glucuronide	% Total recovery in urine
Paracetamol-C^2H_3	25/rat	13.2 ± 0.2	ND	55.6 ± 12.3
Paracetamol-C^2H_3	40/rat	9.98 ± 0.86	ND	60.6 ± 8.8
Paracetamol-$^{13}CH_3$	40/rat	8.87 ± 0.51	ND	97.8 ± 3.5
Paracetamol-$^{13}CO^{13}CH_3$	40/rat	9.80 ± 0.35	ND	75.0 ± 3.7
Paracetamol-$^{13}CO^{13}CH_3$	100/rat	7.9 ± 1.8	6.61 ± 0.1	47.4 ± 11.3
Paracetamol-$^{13}CO^{13}CH_3$	100/rat	9.1[a,b]	5.0[a,b]	47.4 ± 11.3
Paracetamol-$^{13}CO^{13}CH_3$	100/rat	9.2[a,c]	9.1[a,c]	47.4 ± 11.3
Paracetamol-C^2H_3	4.3/human	1.0[a]	2.0[a]	45.6[a]
Paracetamol	4.3/human	—	—	22.6[a]

[a] Single measurement.
[b] After 600 MHz HPLC-NMR separation using methanol/D_2O elution.
[c] After 500 MHz HPLC-NMR separation using acetonitrile-d_3/D_2O elution.
ND, not detected.

metabolites [9,10] and can be carried out either in continuous-flow detection or in stop-flow mode. Here stop-flow HPLC-NMR spectroscopy has been used to study the level of futile deacetylation in both sulphate and glucuronide metabolites of paracetamol.

2. Experimental

2.1. Synthesis of paracetamol-C^2H_3 and paracetamol-^{13}CO–$^{13}CH_3$ and methods for in vivo rat studies

These compounds were synthesised and solid phase extraction chromatography (SPEC) and metabolism studies in the rat were carried out as described previously [8]. The dose levels for the rat studies are given in Table 1.

2.2. Procedures for in vivo human studies

A male volunteer was administered orally with a 300 mg dose of paracetamol-C^2H_3 corresponding to 4.3 mg kg^{-1}. Urine was collected pre-dose, 0–2 and 2–4 h post-dose. For comparison, the same volunteer also received a 300 mg dose of non-deuterated authentic paracetamol and urine was collected for the same time intervals All samples were weighed and the urine frozen at −19°C until NMR urinalysis. Prior to NMR spectroscopic studies, 1.0 ml aliquots of each urine were freeze-dried and were studied at a four-fold concentration.

2.3. HPLC-NMR analysis of SPEC fractions of human and rat urine

Solid-phase extraction chromatography was carried out on a 1 ml C18 Bond-Elut™ column pretreated with methanol and acidified water (pH 2). The acidified sample was loaded onto the column and eluted with 1 ml acidified water and subsequently 1 ml methanol under vacuum. The HPLC system comprised a Bruker LC22C pump and a Bischoff 1000 Lambda variable-wavelength UV detector (operating at 250 nm). The outlet from the UV detector was connected to the HPLC-NMR flow probe via an inert polyether(ether ketone) capillary (0.25 mm i.d.). A column oven was used to maintain a column temperature at 25°C. Data were collected using the Bruker Chromstar HPLC data system. Analysis was performed on a Knauer column (120 × 4.6 mm i.d.) packed with Spherisorb ODS-2, 5 μm. The mobile phase consisted of D_2O-0.05M sodium dihydrogenphosphate (pH 2) (99:1, v/v) for the first 5 min followed by a linear gradient to D_2O-methanol-0.05M sodium dihydrogenphosphate (pH 2) (49:50:1, v/v/v) after 30 min with a flow rate of 1.0 ml min^{-1}.

HPLC-NMR spectra were acquired using a Bruker AMX-600 spectrometer equipped with a ^{1}H flow probe (cell of 3 mm i.d. with a volume of 100 μl). ^{1}H NMR spectra were obtained in stop-flow mode at 600.14 MHz. Spectra were acquired using the NOESYPRESAT (Bruker GmbH, Rheinstetten, Germany) pulse sequence with double presaturation of the water and methanol signals. FIDs were collected into 64K data-points, using a 90° pulse with an acquisition time of 2.69 s, a recycle delay of 4.79 s and a spectral width of 12 195.2 Hz, for 64 scans. An exponential line-broadening function of 0.4 Hz was applied prior to FT.

2.4. HPLC-NMR analysis of whole 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. Data were collected 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-TFA (pH 2)/acetonitrile-d_3 increasing from 100:0 at the beginning of the run to 50:50 after 30 min at 1.0 ml min^{-1}.

HPLC-NMR spectra were acquired using a Bruker DMX-500 equipped with a ^{1}H flow probe (cell of 3 mm i.d. with a volume of 120 μl). ^{1}H NMR spectra were obtained in stop-flow mode at 500.13 MHz. Presaturation of the water signal was achieved by the use of a selective sinc pulse applied for six cycles of 100 ms each. FIDs were collected into 32 K data-points, using a 90° pulse with an acquisition time of 3.28 s, a recycle delay of 0.6 s and a spectral width of 5000 Hz, for 4096 scans. An exponential line-broadening function of 1.0 Hz was applied prior to FT and the data were zero-filled by a factor of 2.

2.5. Analysis of NMR spectra containing paracetamol metabolites

The regions of the ^{1}H NMR spectrum corresponding to the aromatic and acetyl signals 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 paracetamol metabolite concerned.

3. Results

3.1. Direct measurement of paracetamol-C^2H_3 metabolites in rat urine using NMR

Comparison of the ^{1}H NMR spectra of the post-dose 0–8 h rat urine and pre-dose rat urine samples clearly showed the aromatic signals (AA′ XX′ spin system) at δ7.33 and 7.47 of the paracetamol-C^2H_3 sulphate. Expansion of the spectrum in the region δ1.75–2.30 revealed a number of peaks resulting from the appearance of signals from acetyl groups which were not present in the urine of the rat prior to dosing with paracetamol-C^2H_3. New resonances include the cysteine acetyl group of the *N*-acetyl cysteinyl metabolite at δ1.87 and a signal at δ2.18, previously assigned to the acetyl group of paracetamol sulphate based on the chemical shift of authentic material [11]. The presence of the sulphate acetyl signal indicates that in vivo there is a significant degree of deacetylation of the $CO.C^2H_3$ group and reacetylation with $CO.CH_3$. The mean level of futile deacetylation was dose dependent as shown in Table 1 being about 13% at the lower dose and 10% at the higher dose.

The proton aromatic resonances at δ7.37 and δ7.15 (AA′ XX′ spin system) indicated the presence in the urine of small amounts of the paracetamol glucuronide. The signal at δ7.37 was partially overlapped with a signal from an unidentified endogenous metabolite, so only the signal at δ7.15 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 corresponded to 6.9 ± 1.7% of the dose because of

the high capacity for sulphation in the rat which dominates the metabolic profile.

There was no ^{1}H NMR evidence for the presence of either 4-aminophenol or paracetamol-C^2H_3 itself in the urine. These results confirmed previous observations of futile deacetylation in vivo in the rat. They additionally, via the use of high field NMR, identified the futile deacetylation process in the paracetamol sulphate metabolite. However, observations of a low overall percentage urinary recovery of the metabolites, prompted repetition of the experiment at an elevated paracetamol-C^2H_3 dose level of 40 mg kg^{-1} which showed little change in the observed urinary recovery (see Table 1). In addition paracetamol-$^{13}CH_3$ was also dosed to determine whether or not deuterium kinetic isotope effects [12] had affected either the level of futile deacetylation or the overall urinary recovery of the drug since kinetic isotope effects on metabolism from ^{13}C-labelled compounds are generally small [12]. This experiment showed that whilst the level of futile deacetylation remained essentially unchanged at 8.9%, the recovery of metabolites in the urine increased from 55–60 to 97.8%.

3.2. NMR studies on the metabolism of paracetamol-$^{13}CO-^{13}CH_3$ in rat urine

The 600 MHz ^{1}H NMR spectrum of rat urine taken 0–8 h after dosing with paracetamol-$^{13}CO-^{13}CH_3$ at 100 mg kg^{-1} has also been examined. The aromatic resonances from the paracetamol sulphate were again visible at δ7.45 and δ7.31 with the glucuronide conjugate aromatic resonances identified at δ7.36 and δ7.14 (the former being overlapped by an unidentified endogenous signal). A resonance at δ6.90 was assigned to the parent compound and an acetyl signal at δ1.87 was assigned to the *N*-acetyl-L-cysteinyl metabolite. However, no aromatic signals were observed for this metabolite due to its low concentration. Doublet of doublets signals for the acetyl groups ($^1J_{CH}$ = 129 Hz and $^2J_{CH}$ = 5.9 Hz) of the non-deacetylated metabolites were seen at δ2.24–2.29 and δ2.03–2.08.

The paracetamol sulphate acetyl signal resulting from futile deacetylation was visible as a singlet at δ2.18. Integration of this peak gave a mean percentage futile deacetylation level for the paracetamol sulphate of 7.9 ± 1.8%. An acetyl singlet, overlapped by the sulphate signal, was assigned to the paracetamol glucuronide at δ2.17 and this was resolved via Gaussian resolution enhancement. Integration gave a mean percentage futile deacetylation of 6.6 ± 0.1.

The results obtained at a dose level of 40 mg kg^{-1} for all of the different types of labelled paracetamol do not appear to be significantly different showing that there is no isotope effect on the futile deacetylation process. Moreover, the endogenous process causing the futile deacetylation would also appear to be unaffected by the potential level of substrate available to it. The use of the doubly labelled paracetamol-$^{13}CO-^{13}CH_3$ allowed the unusual and unlikely possibility that futile demethylation as well as deacetylation was taking place to be distinguished. However, since no acetyl NMR resonance showing the loss of the one-bond J_{CH} and retention of the two-bond J_{CH} was observed for futile deacetylated products, the level of any futile demethylation must be at most only a very minor process. As a result of the high dose level used in the study with paracetamol-$^{13}CO-^{13}CH_3$ the glucuronide conjugate was also detected and seen to undergo futile deacetylation. It could not be determined from this study whether or not there is a significant difference in the percentage futile deacetylation between the glucuronide and sulphate metabolites. Since no ^{13}C-coupled NMR acetyl peaks were observed, the process in the glucuronide would also appear to be futile deacetylation.

3.3. HPLC-NMR studies on the metabolism of paracetamol-$^{13}CO-^{13}CH_3$ in rat urine

To provide additional evidence of the futile deacetylation in both major metabolites, a sample of the 0–8 h urine following dosing of paracetamol-$^{13}CO-^{13}CH_3$ at 100 mg kg^{-1} was first purified using SPEC (acidified water and 100% methanol fractions). The paracetamol and its metabolites were observed in the acidified water fraction and this was further analysed using HPLC-NMR at 600 MHz.

Shown in Fig. 2(a) is the 600 MHz ^{1}H NMR spectrum obtained using directly-coupled HPLC-NMR in stop-flow mode corresponding to the UV absorbance peak at a retention time of 13.05 min. Although methanol was used as the organic modifier in the HPLC, a detectable level of acetonitrile also leached from the HPLC column. This caused the appearance of a peak in the acetyl region of the ^{1}H NMR spectrum. This paracetamol metabolite was assigned as paracetamol sulphate by comparison to the NMR spectrum of the whole urine. The aromatic resonances were visible at δ7.45 and 7.31 whereas the doublet of doublets corresponding to the non-futile deacetylated paracetamol sulphate was centred around δ2.17. An acetyl singlet was observed at δ2.17 corresponding to the acetyl group of the futile deacetylated paracetamol sulphate and by integration relative to the aromatic resonances, it was determined that 9.1% futile deacetylation had occurred. No evidence was observed for any futile demethylation in the paracetamol sulphate.

The 600 MHz ^{1}H NMR spectrum corresponding to the UV absorbance peak at a retention time of 14.5 min is shown in Fig. 2(b). Again by comparison with the whole urine spectrum, this was assigned to the glucuronide metabolite. The aromatic resonances were visible at δ7.35 and 7.13, whereas the acetyl resonance doublet of doublets of the non-futile deacetylated metabolite was centred around δ2.15. An acetyl singlet was observed at δ2.15 corresponding to the futile deacetylated paracetamol glucuronide. Integration of this signal relative to that of the aromatic resonances gave a futile deacetylation level of 5.0%. This figure is probably less precise than that for the sulphate because of the lower concentration of the glucuronide and the consequent difficulty of NMR integration.

The 600 MHz ^{1}H NMR spectrum of the UV absorbance peak at a retention time of 18.42 min was assigned to the parent compound and is shown in Fig. 2(c). The aromatic resonances were observed at δ7.26 and δ6.90. The acetyl doublet of doublets was centred around δ2.14. No acetyl peak corresponding to futile deacetylated paracetamol could be observed due to the large resonance caused by the residual acetonitrile on the column and the low level of free paracetamol in the urine.

As an alternative to the use of methanol in the HPLC eluent, HPLC-NMR studies were also carried out on whole concentrated urine from rats dosed at 100 mg kg^{-1} using acetonitrile-d_3 as the organic solvent phase. In the stop-flow mode using 500 MHz ^{1}H NMR spectroscopy, spectra were obtained from both glucuronide and sulphate metabolites which gave acetyl resonances from the transacetylated products, these peaks now being observable through the use of deuterated acetonitrile thereby removing a large solvent resonance in the same spectral region. The levels of futile deacetylation were 9.2% for the sulphate and 9.1% for the glucuronide metabolites.

3.4. NMR studies on the metabolites of paracetamol-C^2H_3 present in human urine

Following the results obtained from experiments in the rat, studies into futile deacetylation in man were undertaken using both paracetamol and paracetamol-C^2H_3. Both the 0–2 and 2–4 h urine following dosing of 300 mg of paracetamol-C^2H_3 contained drug metabolites. The glucuronide conjugate was the predominant metabolite with aromatic proton resonances at δ7.37 and δ7.14, whereas resonances for the sulphate were observed at δ7.46 and δ7.33. The recovery of the metabolites for the total 4 h period as a percentage of the dose was 33.3 and 12.3% for the glucuronide and sulphate respectively. Following administration of 300 mg of paracetamol to the same subject, the corresponding figures were 16.4% for the glucuronide and 6.2% for the sulphate. No acetyl signals for the two metabolites were observed because of overlap from NMR resonances from endogenous compounds. Solid phase extraction followed by NMR spectroscopy (SPEC-NMR) of the 2–4 h urine yielded the paracetamol metabolites in the acidified water fraction. Although some endogenous metabolites had been removed, the spectral clarity of the acetyl region had not been sufficiently improved to accurately identify any signals resulting from futile deacetylation. Therefore, due to the background interference in the acetyl region

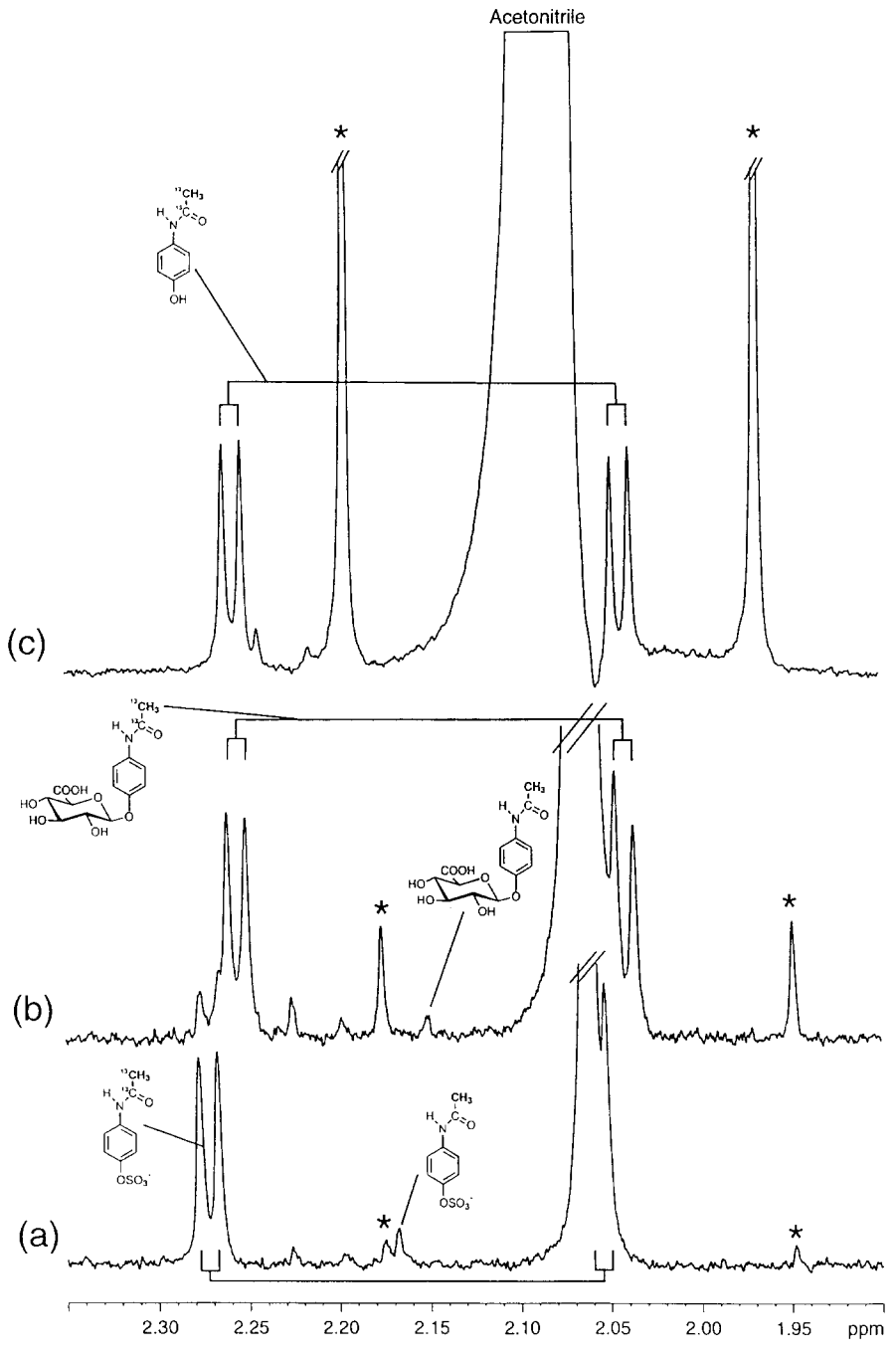

Fig. 2. 600 MHz ^{1}H NMR spectra (δ1.90–2.35) using directly-coupled HPLC-NMR spectroscopy of the acidified water SPEC fraction from rat urine 0–8 h post-dose paracetamol-^{13}CO–$^{13}CH_3$ (a) the $t_r = 13.05$ min peak (paracetamol sulphate); (b) the $t_r = 14.5$ min peak (paracetamol glucuronide); and (c) the $t_r = 18.42$ min peak (paracetamol). * denotes signals from naturally abundant $^{13}CH_3CN$.

and the potential low level of futile deacetylated drug, it was necessary to use HPLC-NMR spectroscopy for quantitative purposes.

3.5. HPLC-NMR spectroscopic analysis of the metabolites of paracetamol-C^2H_3 in human urine

The directly-coupled HPLC-NMR spectra of the acidified-water SPEC fraction of the whole 2–4 h human urine following dosing with paracetamol-C^2H_3 showed separation of the glucuronide and the sulphate metabolites into two peaks with retention times of $t = 9.33$ min and $t = 19.06$ min. The 600 MHz ^1H NMR spectrum of the first peak is shown in Fig. 3(a). The spectrum was assigned to paracetamol glucuronide by comparison with the HPLC-NMR data obtained above. Aromatic resonances were observed at $\delta 7.32$ and $\delta 7.11$ and the signal for the anomeric proton was observed at $\delta 5.09$. The other glucuronide protons were observed at $\delta 3.89$ and $\delta 3.65-3.55$. The acetyl singlet at $\delta 2.14$ was assigned to the glucuronide and indicated that futile deacetylation had occurred for the metabolite. Integration of this resonance with respect to the aromatic signals gave a futile deacetylation level of 1.0%. The NMR spectrum of the second fraction is shown in Fig. 3(b). The aromatic doublet resonances at $\delta 7.42$ and $\delta 7.27$ were assigned to paracetamol sulphate by comparison with earlier HPLC-NMR. The acetyl singlet at $\delta 2.13$ was assigned to the sulphate by comparison with the acetyl region from the spectrum of paracetamol. Integration of the acetyl signal relative to that of the aromatic protons gave a percentage futile deacetylation level of 2.0%. This figure is probably an over-estimate due to the partial overlap of the signal from the futile deacetylated paracetamol sulphate with the ^{13}C satellite signal from acetonitrile. The results are summarised in Table 1.

4. Discussion

The results given here demonstrate that paracetamol-C^2H_3 in the rat undergoes significant futile deacetylation as seen in the sulphate metabolite. This result is of potential significance in helping to understand the metabolic basis of nephrotoxicity resulting from paracetamol ingestion. Clearly there is a substantial and normally 'silent' metabolic flux for paracetamol in the rat at this dose level.

We have shown earlier [8] that the level of futile deacetylation of paracetamol appeared to be unaffected by the use of either ^2H- or ^{13}C-labelling at equivalent doses, remaining at approximately 9%. Since there was no significant difference in futile deacetylation between paracetamol-C^2H_3 and paracetamol-^{13}CO-$^{13}CH_3$ at the 40 mg kg^{-1} dose level, it might be assumed that the acetyl region of the molecule plays little part in governing the extent of the futile deacetylation process. For both paracetamol-C^2H_3 and paracetamol-$^{13}CO-^{13}CH_3$ futile deacetylation in only the sulphate metabolite was observed due to the high levels of sulphation at the dose level used. At the 100 mg kg^{-1} dose level used in the paracetamol-$^{13}CO.^{13}CH_3$ study, the paracetamol glucuronide metabolite was also observed and seen to have undergone futile deacetylation. The level of futile deacetylation for the glucuronide metabolite was not significantly different to that for paracetamol sulphate.

The metabolism of paracetamol differs quantitatively between man and the rat with in general the glucuronide conjugate being the major metabolite [1]. Due to the greatly increased volume of urine and the complex mixture of metabolites excreted in man, it was not possible to measure any futile deacetylation in the non-purified urine using NMR spectroscopy. Simple SPEC of the urine sample still did not yield sufficiently pure samples to identify any futile deacetylation. Therefore, more selective separation of the urinary components was achieved using HPLC, from which fractions were directly analysed using NMR.

The extent of futile deacetylation for both paracetamol sulphate and glucuronide metabolites in man would appear to be only minor at the dose levels used here showing approximately 2 and 1% levels for the sulphate and glucuronide metabolites respectively. From the results obtained here, the futile deacetylation process seems to be only a

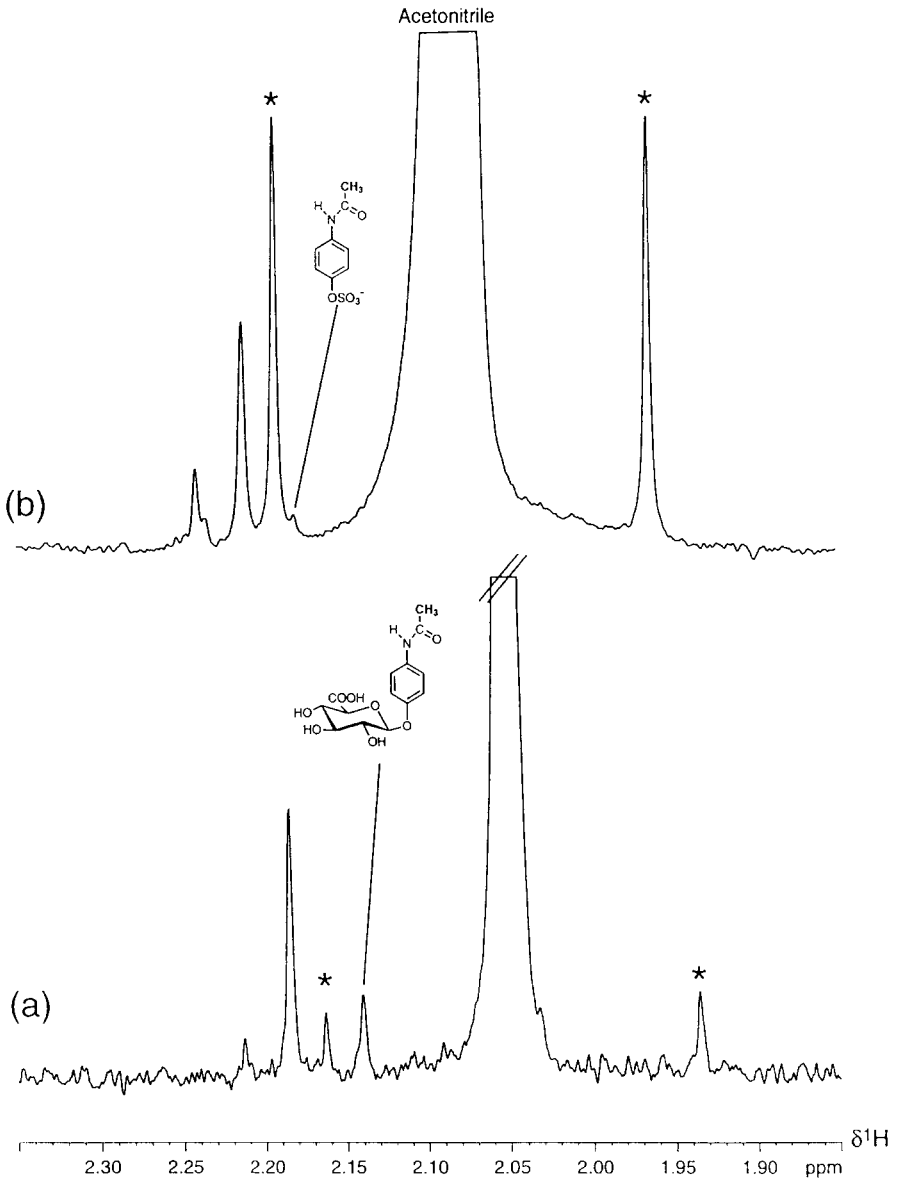

Fig. 3. 600 MHz ^{1}H NMR spectra (δ 1.85–2.35)) using directly-coupled HPLC-NMR spectroscopy of concentrated whole human urine following oral administration of 300 mg of paracetamol-C^2H_3 (a) the $t_r = 9.33$ min fraction (paracetamol glucuronide); and (b) the $t_r = 19.06$ min fraction (paracetamol sulphate). * denotes signals from naturally abundant $^{13}CH_3CN$. Other peaks in this region of the spectrum are probably of endogenous origin as there are no other resonances in the spectrum to indicate that they are related to paracetamol.

minor contributor to paracetamol nephrotoxicity in man, based on this single volunteer study. To provide better insight a larger group study is required so as to determine the inter-individual variation of paracetamol futile deacetylation in man. Studies should also incorporate examination of the dose-dependency of the paracetamol futile deacetylation in man. However, the therapeutic dose of paracetamol can be up to 1000 mg three times a day. If only 1% of this is converted, albeit

possibly transiently, into 4-aminophenol, this represents 30 mg of this very toxic compound. Whilst most people suffer no ill effects from the possible presence of this process, there may be classes of patients where for genetic reasons, the process of futile deacetylation produces toxic effects.

This study demonstrates the use of directly-coupled HPLC-NMR spectroscopy to study futile deacetylation of paracetamol metabolites. Even the use of very high field NMR spectroscopy on whole urine samples is limited by the degree of overlap of resonances from the paracetamol metabolites and endogenous species. Nevertheless, NMR spectroscopy on whole urine can allow quantification of the futile deacetylation in paracetamol metabolites. However, additional confirmation becomes possible by the use of directly-coupled HPLC-NMR spectroscopy. This has the dual advantage of confirming that the NMR resonances from the futile deacetylated glucuronide and sulphate metabolites do indeed arise from these substances since they co-elute with the isotopically labelled corresponding metabolites. Quantification of the level of futile deacetylation is simpler in the HPLC-NMR spectra because of the lack of resonances from endogenous species. In addition, this work demonstrates that ^{1}H HPLC-NMR spectroscopy can be used to detect and quantify metabolites even for the low levels observed for humans.

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