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The metabolism of 2-trifluormethylaniline and its acetanilide in the rat by 19F NMR monitored enzyme hydrolysis and 1H/19F HPLC-NMR spectroscopy

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Abstract

The urinary excretion profile and identity of the metabolites of 2-trifluoromethyl aniline (2-TFMA) and 2-trifluoromethyl acetanilide (2-TFMAc), following i.p. administration to the rat at 50 mg kg^{-1}, were determined using a combination of ^{19}F NMR monitored enzyme hydrolysis, SPEC-MS and $^{19}F/^{1}H$ HPLC-NMR. A total recovery of approximately 96.4% of the dose was excreted into the urine as seven metabolites. The major routes of metabolism were N-conjugation (glucuronidation), and ring-hydroxylation followed by sulphation (and to a lesser extent glucuronidation). The major metabolites excreted into the urine for both compounds were a labile N-conjugated metabolite (a postulated *N*-glucuronide) and a sulphated ring-hydroxylated metabolite (a postulated 4-amino-5-trifluoromethylphenyl sulphate) following dosing of 2-TFMA. These accounted for approximately 53.0 and 31.5% of the dose, respectively. This study identifies problems on sample component instability in the preparation and analysis procedures.

Keywords: ^{19}F NMR spectroscopy; Enzyme hydrolysis; HPLC-NMR; Aniline metabolism

1. Introduction

The metabolism of primary aromatic amines is believed to be a major contributing factor leading to toxicity, carcinogenisis and methaemoglobinaemia [1]. Understanding the metabolic fate of these agents/chemicals is, therefore, of key importance, considering their widespread use as intermediates

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in the production of pesticides, industrial chemicals and drugs. Although the risk of environmental exposure to such compounds appears to be significant, formation of aniline (and derivates) can also be a direct result of metabolism of many pharmaceuticals/xenobiotics.

A better knowledge of the metabolic fate of these compounds is likely to be of benefit in understanding their associated toxicity. Despite their structural simplicity, anilines have been shown to undergo complex metabolic transformations [2–11]. A series of studies have been reported in the literature, involving a range of substituted anilines in an attempt to build in vivo metabolic databases. These may serve as tools to (accurately) predict metabolic fate/drug metabolism of anilines in the rat [12–15].

Advances in the hyphenation of HPLC with NMR (and other spectroscopic techniques) have enabled the rapid and efficient structural identification of metabolites [16,17], by eliminating the need for prior isolation and sample clean up, as also shown in this study.

The ^{19}F handle presents a convenient alternative to the use of radiolabelled compounds in metabolism studies. The presence of fluorine in the xenobiotic enables rapid and highly specific detection and quantitation using ^{19}F NMR spectroscopy.

In this study we found ^{19}F NMR spectroscopy provided an efficient means of following the metabolic fate of the trifluoromethyl-substituted anilines investigated. The presence of a CF_3 group not only enabled the simultaneous generation of both excretion balance data and metabolite profiles, but also provided a sensitive and highly specific handle for directly locating and identifying aniline metabolites during the HPLC separation.

Following the same analytical approach as used previously in a related study [11], we employed ^{19}F NMR and HPLC-NMR in combination with enzyme hydrolysis for the quantification, selective detection and identification of the metabolites of 2-trifluoromethyl aniline (2-TFMA) and 2-trifluoromethyl acetanilide (2-TFMAc) in neat urine.

Problems regarding metabolite stability are highlighted and discussed.

2. Experimental methods

2.1. Compounds

2-TFMA and 2-TFMAc were obtained from Fluorochem (97 and 98% chemical purity, respectively; Derby, UK). [^{13}C]-2-TFMAc was a gift from Zeneca Pharmaceuticals.

2.2. Animal dosing

Groups of 3 males Sprague–Dawley rats (200–250 g) each received a single i.p. dose of either 2-trifluoromethylaniline (2-TFMA, 50 mg kg^{-1}) or 2-fluoromethyl-[^{13}C]-acetanilide ([^{13}C]-2-TFMAc, 50 mg kg^{-1}) both dosed at a volume of 1 ml kg^{-1} in corn oil. Prior to dosing the animals were housed individually in metabolism cages designed for the separate collection of urine and faeces. The animals were allowed to acclimatise for 24 h before dosing. Food and water were available ad libitum throughout the experiment. Urine samples were collected over solid carbon dioxide for 24 h predose and for the periods 0–8, 8–24 and 24–48 h post dose (pd). Samples were then stored at −20 °C until analysed. Before analysis the samples were centrifuged at 3000 rpm at 4 °C to remove solid debris (particles of food etc.) and the urinary volume and pH were determined.

2.3. Enzyme hydrolysis of urinary metabolites

Hydrolysis with ß-glucuronidase and aryl sulphatase was performed on selected samples (0–8 hpd) in order to provide an indication of the presence of conjugated metabolites for confirmation by spectroscopic studies.

2.3.1. ß-Glucuronidase

The test and control samples (0.5 ml), buffered to pH 6.8 with 0.4 M potassium phosphate buffer and with the addition of 0.1 ml of D_2O, were incubated overnight (16 h) in glass NMR tubes at 37 °C in a shaking water bath. To the test samples 5 mg of β-glucuronidase (ex. *E. coli*, Sigma) was added.

2.3.2. Aryl sulphatase

The test and control samples (0.5 ml), adjusted to pH 5.3 with 0.4 M acetic acid and with the addition of 0.1 ml of D_2O, were incubated in glass NMR tubes at 37 °C overnight (16 h) in a shaking water bath. To the test samples 5 mg of aryl sulphatase (ex Helix pomatia, Sigma) was added, whilst to both test and control samples 0.05 ml of 100 mM saccarolactone (in 0.4 M sodium acetate buffer, pH 5.3) was added to inhibit β-glucuronidase activity.

The resulting metabolite signal changes were monitored by ^{19}F NMR spectroscopy at 376 MHz (see below).

2.3.3. Solid-phase extraction/chromatography (SPEC)

Solid phase extraction, with chromatographic separation provided by stepwise gradient elution, was used to concentrate and partially purify metabolites. An aliquot of a 0–8 h urine sample (2 ml) was acidified to pH 2 with 2 M HCl and loaded onto 3 ml C18 Bond–Elut columns (Jones Chromatography, Hengoed, UK) containing 500 mg of sorbent. Prior to use the column was conditioned with 5 ml of methanol, followed by 5 ml of acidified water (pH 2 with HCl). Following sample application the column was washed with 2 aliquots (1 ml each) of acidified water and then any retained material was eluted using a stepwise gradient of increasing eluotropic strength. This gradient comprised 1 ml aliquots of methanol:acidified water starting at a proportion of 20:80 (v/v) and rising in 20% steps up to 100% methanol. All of the eluates were collected, solvent was removed by a combination of evaporation under a stream of nitrogen and then freeze drying. The eluates were then reconstituted in 1.2 ml of D_2O and taken for ^1H and ^{19}F NMR as described below. Following NMR analysis, the SPEC fractions were freeze-dried again prior to analysis by fast ion bombardment (FIB) mass spectrometry (MS).

2.3.4. Mass spectrometry (MS) (SPEC–MS)

Selected samples obtained by SPEC were analysed on a Quattro triple quadrupole mass spectrometer (Micromass, Wythenshawe, UK) using positive and negative FIB. Samples were dissolved in an ethanol/glycerol matrix prior to FIB with caesium ions.

2.3.5. ^1H NMR spectroscopy (SPEC-^1H NMR)

^1H NMR spectra of the individual SPEC fractions, were obtained on a Jeol GSX500 NMR spectrometer operating at 500 MHz at ambient probe temperature. Typically 64–256 FIDs were collected into 32 K data points using a 45° pulse width with a data acquisition time of 2.73 s. A pulse delay of 2.27 s was used to ensure T_1 relaxation of the sample. Water suppression was achieved using a secondary gated irradiation field at the water resonance frequency. An exponential apodisation function corresponding to a line broadening factor of 0.2 Hz was applied prior to Fourier transformation (FT). Chemical shifts were referenced to the water resonance a $\delta 1_H$ 4.8.

2.3.6. ^{19}F NMR spectroscopy of urine

^{19}F NMR spectroscopy was carried out on neat urine samples (0–8, 8–24, 24–48 hpd) to obtain a metabolic profile, on aliquots of 0–8 h urine following enzyme hydrolysis and on individual fractions obtained by SPEC of a 0–8 h urine sample.

After addition of D_2O to provide a field frequency lock, ^{19}F NMR spectra were recorded on either Varian VXR400 or Bruker AM400 NMR spectrometers operating at a ^{19}F observation frequency of 376 MHz at ambient probe temperature. Typically, 128–512 FIDs were collected into 8 K data points using a 45° pulse width (15 μs), an acquisition time of 0.4 s and a spectral width of 10 000 Hz. A further delay of 6 s (calculated by the inversion recovery method) was added between pulses to ensure complete T_1 relaxation. The time domain spectrum was zero filled to 64 K data points and an exponential weighting corresponding to a line broadening of 0.5 Hz was applied prior to FT. Chemical shifts were referenced to the external secondary reference trifluoroethanol ($\delta 19_F - 77$ from $CFCl_3$).

2.3.7. Quantitation of 2-TFMA rat urinary metabolites

Quantification was performed (solely) using the ^{19}F signals of 2-TFMA (contained in the 0–8, 8–

24, 24–48 hpd urine samples) as a representative for both compounds, based on the similarity of the overall ^{19}F NMR metabolite profiles. Quantification of 2-TFMA related resonances was carried out by ^{19}F NMR spectroscopy, following addition of a known concentration of the internal standard, 2-trifluoromethylbenzoic acid, and subsequent integration of the ^{19}F NMR metabolite signals relative to those of added internal standard. The concentration of the analyte in solution was calculated as follows:

$$Wa = Ws \times \left(\frac{Ia}{Is}\right) \times \left(\frac{MWa}{MWs}\right) \times \left(\frac{Ns}{Na}\right)$$

where, a and s represent the analyte and standard, respectively, W the weight of the substance in the sample, I the integral value of the NMR resonance, MW the molecular weight and N the number of ^{19}F nuclei contributing to the resonance.

2.3.8. HPLC-NMR spectroscopy

^{1}H and ^{19}F HPLC-NMR spectroscopy was performed on the residual samples of 0–8 hpd urine obtained from animals dosed with 2-TFMA. These samples were pooled, freeze dried (total volume of 3 ml) and reconstituted in 0.75 ml of ammonium acetate buffer (0.1 M, pH 5.2) in D_2O:acetonitrile (ACN) (99:1, v/v) prior to chromatography. Typically, 50 μl aliquots of the sample concentrate were analysed. The separation was performed an HPLC system consisting of a Bruker LC22 pump, an LC53 autosampler and LC313 variable wavelength UV detector (operating at 220 nm, data not shown). The 5 μm, Spherisorb ODS 2 C-18 HPLC column (250 × 4.6 mm id) was used with a solvent flow rate of 1 ml min^{-1} at 35 °C. A linear gradient was employed, beginning with an initial 3 min isocratic period with the starting mobile phase composition (ammonium acetate buffer (0.1 M, pH 5.2 in D_2O):ACN; 99:1, v/v) rising over 35 min to 60% ACN.

Solvents were obtained from Fluorochem, Derby, UK (D_2O) and Riedl de-Häen, Seelze, Germany ('Pestanal' grade ACN, Acetonitrile). Ammonium acetate was purchased from Sigma-Aldrich (Gillingham, UK).

The HPLC separation was monitored by continuous-flow ^{19}F NMR spectroscopy to identify the retention times of the 2-TFMA metabolites, utilising a Bruker AMX 500 NMR spectrometer (470.6 MHz fluorine detection frequency) equipped with a flow through $^{19}F/^{1}H$ dual probe (3 mm i.d; cell volume of 60 μl). ^{19}F-continuous flow experiments were acquired over a spectral width of 25 000 Hz with 24 scans per experiment including four dummy scans. FIDs were collected into 8 k data points with an acquisition time of 0.16 s and a relaxation delay of 1 s between successive scans. FID apodisation was achieved using a sine-bell function prior to FT. Chemical shifts were referenced to external trifluoroacetic acid ($\delta 19_F - 76.8$).

For metabolite identification using ^{1}H NMR at 500 MHz, the separation was repeated, stopping the flow at the retention times corresponding to the ^{19}F-detected peaks. Double solvent suppression was achieved using a standard solvent suppression sequence, irradiating both the residual H_2O signal and the ACN resonance (NOESY-PRESAT, Bruker Biospin, Coventry, UK). Spectra were acquired over a spectral width of 10 000 Hz with between 96 and 4000 transients per experiment. A pulse repetition time of 3.56 s was used with 1 Hz line broadening and zero filling to 32 K data points prior to FT. Chemical shifts were referenced to internal ACN ($\delta 1_H$ 2.0).

3. Results and discussion

3.1. ^{19}F NMR (metabolite) profiling of urine samples

The ^{19}F NMR metabolic profiles were very similar between dosing of the free amine and the acetanilide (data not shown). Based on the similarity of the ^{19}F metabolite profiles of the 0–8 h urines from 2-TFMA and 2-TFMAc treated rats it appears that after *N*-deacetylation, 2-TFMAc followed the same biotransformation pathways as 2-TFMA in the rat, resulting in the production of identical metabolites.

Typical ^{19}F NMR spectra of urines obtained for the periods 0–8, 8–24 and 24–48 h following the administration of 2-TFMA to rat are shown in Fig. 1. The bulk of the excretion of 2-TFMA-related material was seen in the 0–8 h samples where a total of 8 signals for 2-TFMA and its metabolites were observed. Metabolism was extensive, with 2-TFMA itself detected as a minor component (ca. 1.1% of dose), giving rise to a signal at $\delta 19_F - 62.75$ (as confirmed by standard addition; data not shown). The bulk of the signals for compound-related material in these samples were associated with major metabolites at $\delta 19_F -$ 61.62 and -61.65 (ca. 43.4% of dose), and $\delta 19_F -$ 63.23 (ca. 26.9% of dose).

3.2. Enzyme hydrolysis studies

To gain additional information on the presence of sulphate and glucuronide conjugates, enzyme hydrolysis experiments were performed on aliquots of urine samples collected 0–8 h after dosing

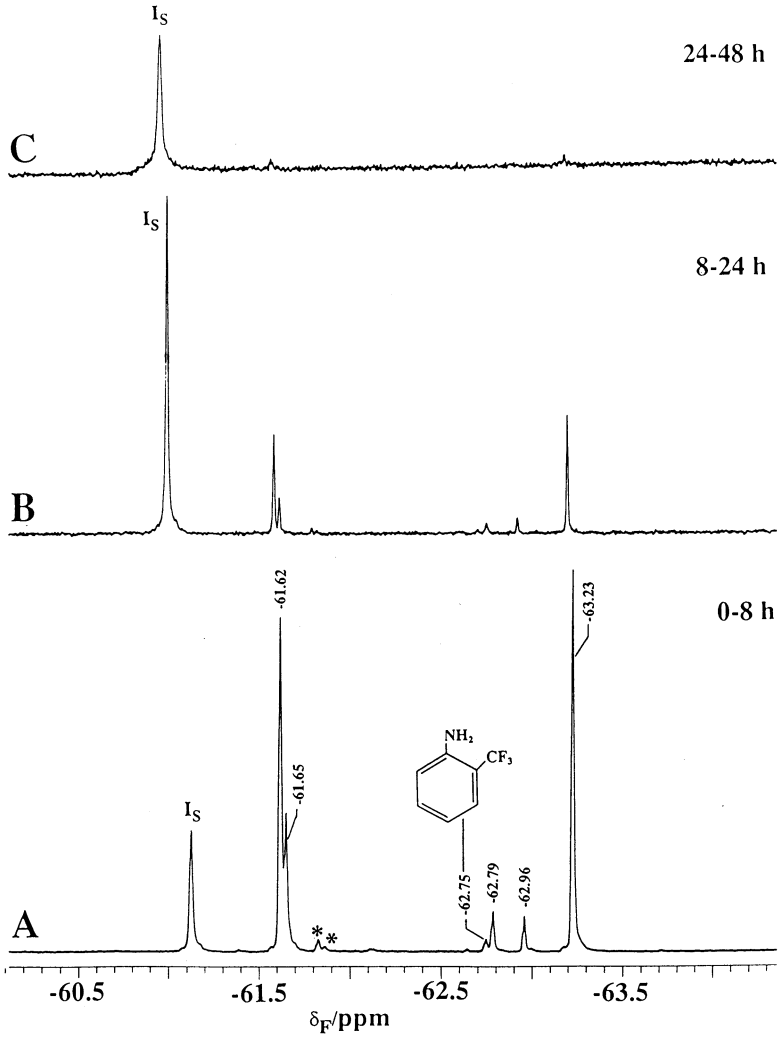

Fig. 1. 376 MHz ^{19}F NMR spectra of urine collected (A) 0–8 h, (B) 24–48 h and (C) 24–48 h after i.p. administration of 50 mg kg^{-1} 2-TFMA to rats. I_S indicates internal standard used for quantification, *, indicates the minor metabolites observed.

with 2-TFMA and analysed by ^{19}F NMR spectroscopy (Fig. 2). The urine samples were deemed to be representative of both, 2-TFMA and 2-TFMAc dosed animals.

Owing to the differences in pH values between the bulk (untreated post-dose samples) and the incubated samples, slight changes in the ^{19}F chemical shifts of the peaks have been observed. However, for simplicity reasons, the chemical shift values of the 0–8 h urine as quoted in Fig. 1 are referred to throughout the manuscript. It should also be noted, that the ^{19}F NMR signal intensities of the urine samples of individual animals are subject to variability.

Incubation of aliquots of a 0–8 h urine sample with ß-glucuronidase at pH 6.8 for 16 h resulted in the disappearance of the signals at $\delta 19_F - 61.62$, -61.65 and $\delta 19_F - 62.96$ with a concomitant increase in the intensities of the signals for 2-TFMA ($\delta 19_F - 62.75$) and for a probable phase I hydroxylated metabolite observed at $\delta 19_F - 62.69$.

The metabolite at $\delta 19_F - 62.96$ remained stable in the control urine incubation, suggesting it may be an ether-glucuronide conjugate. The signals at $\delta 19_F - 61.62$ and -61.65 appeared slightly attenuated in the control incubation with a corresponding increase in that of 2-TFMA itself,

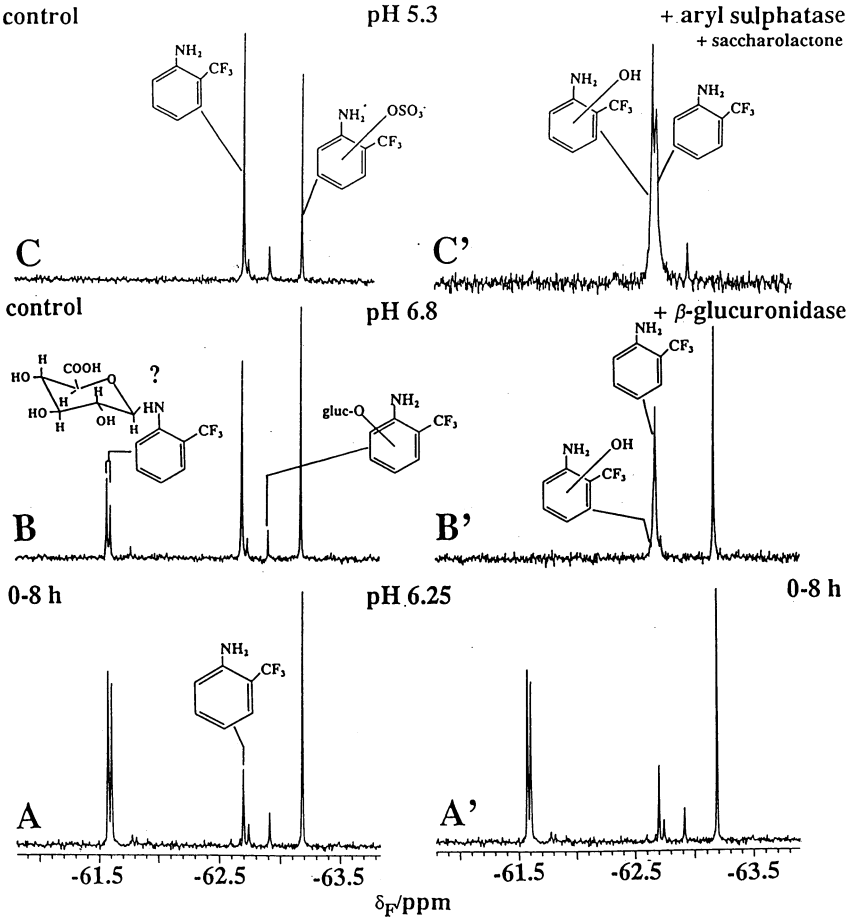

Fig. 2. 376 MHz ^{19}F NMR spectra of 0–8 h urine collected from 2-TFMeA dosed rats before (A = A′) and after incubation with β-glucuronidase (B′) and aryl sulphatase (saccharolactone inhibitor added) (C′). Spectra B and C show the corresponding control urine incubations with phosphate (pH 6.8) and acetate (pH 5.3) buffers, respectively.

indicating the presence of labile metabolites. The signal at $\delta 19_F -62.79$ appeared unaffected.

Incubation of aliquots of the authentic 0–8 h urine sample with aryl sulphatase at pH 5.3 for 16 h caused the major signal at $\delta 19_F -63.23$ to disappear and that of the putative phase I hydroxylated metabolite observed at $\delta 19_F -62.69$ to increase in intensity. The signals at $\delta 19_F -61.62$ and -61.65 were fully attenuated reverting to 2-TFMA as shown in the control incubation. The signal at $\delta 19_F -62.96$, corresponding to the glucuronide conjugate of hydroxy-2-TFMA, and the signal at $\delta 19_F -62.79$ remained unaffected. Attenuation of the signals at $\delta 19_F -61.62$ and -61.65 both in the enzyme and control incubations supported the likelihood of pH labile *N*-substituted metabolites.

The attenuation of the signals at $\delta 19_F -61.62$ and -61.65 with β-glucuronidase, but only slight attenuation in the control incubations at pH 6.8 suggests the presence of slightly labile *N*-glucuronide conjugates. The signal ratios may reflect different ionisation states of the conjugate. The total disappearance of these signals in the control (aryl sulphatase) incubations at pH 5.3 indicates the acid lability of these glucuronide conjugates.

The signal at $\delta 1_H -62.79$ remained unaffected by treatment with β-glucuronidase and aryl sulphatase, suggesting it is a hydroxylated metabolite.

3.3. SPEC–NMR and SPEC–MS

SPEC–NMR was performed on an aliquot of a 0–8 h urine sample as a means of isolating and concentrating some of the metabolites from the urine matrix. The resultant SPEC-fractions were analysed by ^{19}F and ^{1}H NMR spectroscopy prior to submission to MS. The acid wash to 60% MeOH fractions contained a major ^{19}F signal (in different proportions) at $\delta 19_F$ -61.93 (at pH 2). This metabolite was identified as the sulphate conjugate of 2-TFMA by ^{1}H NMR and MS. The ^{1}H NMR spectrum revealed the characteristic coupling and substitution pattern for a 1,3,4- or 1,4,5 substituted aromatic ring system (as seen in Fig. 3). Analysis by negative FAB–MS revealed fragments of *m/z* 256 (corresponding to 3-amino-4-trifluoromethyl- or 4-amino-5-trifluoromethylphenyl sulphate), and *m/z* 176 and 80 (derived from the di-substitited phenol moiety and SO_3^-, respectively).

The major metabolite(s) at $\delta 19_F -61.62$ and -61.65, which were identified as labile under enzyme hydrolysis conditions, could not be isolated by SPEC and detected by ^{19}F NMR spectroscopy and, hence, failed identification attempts by NMR and MS.

3.4. Metabolite identification using HPLC-NMR

3.4.1. ^{1}H and ^{19}F NMR spectroscopy of urine from 2-TFMA-dosed rats

The pseudo two-dimensional (2D) contour plot of the continuous flow ^{19}F NMR-detected HPLC separation obtained for the concentrated 0–8 h urine sample is shown in Fig. 4. The horizontal axis represents the ^{19}F NMR chemical shift scale and the vertical axis gives the chromatographic retention times for the various fluorine-containing metabolites of 2-TFMA. The 1D ^{19}F NMR spectrum from a previously acquired 0–8 h urine sample is shown as a skyline projection. In the 2D contour plot it is possible to distinguish 6 fluorine-containing peaks (A–F), ranging in retention time (t_R) from approximately 7.7 to 27 min. The sample was reanalysed using ^{1}H-NMR in stop-flow mode on each of these peaks. The resulting ^{1}H NMR spectra are shown in Fig. 5.

For peak A, eluting at $t_R = 7.74$ min the spectrum is consistent with that of an ether glucuronide metabolite of 2-TFMA. Thus, the spectrum reveals the distinctive resonances for the anomeric proton of ß-D-glucuronic acid, appearing as a doublet at $\delta 1_H$ 4.9 with the remaining sugar ring protons detected between $\delta 1_H$ 3.4 and 3.9 (not shown). The substitution pattern of the aromatic ring, evident from the signals at $\delta 1_H$ 6.9 (doublet, $^{3}J_{HH} = 9.7$ Hz), $\delta 1_H$ 7.17 (doublet, $^{3}J_{HH} = 9.7$ Hz and $^{4}J_{HH} = 3$ Hz) and $\delta 1_H$ 7.23 (singlet, $^{4}J_{HH} = 3$ Hz) indicates that the structure of this conjugate is either 3-amino-4-trifluormethyl- or 4-amino-5-trifluoromethylphenyl glucuronide. Based on considerations of the chemical shifts of the ring protons, the most probable structure is 4-amino-5-trifluoromethylphenyl glucuronide (see structure inset to figure).

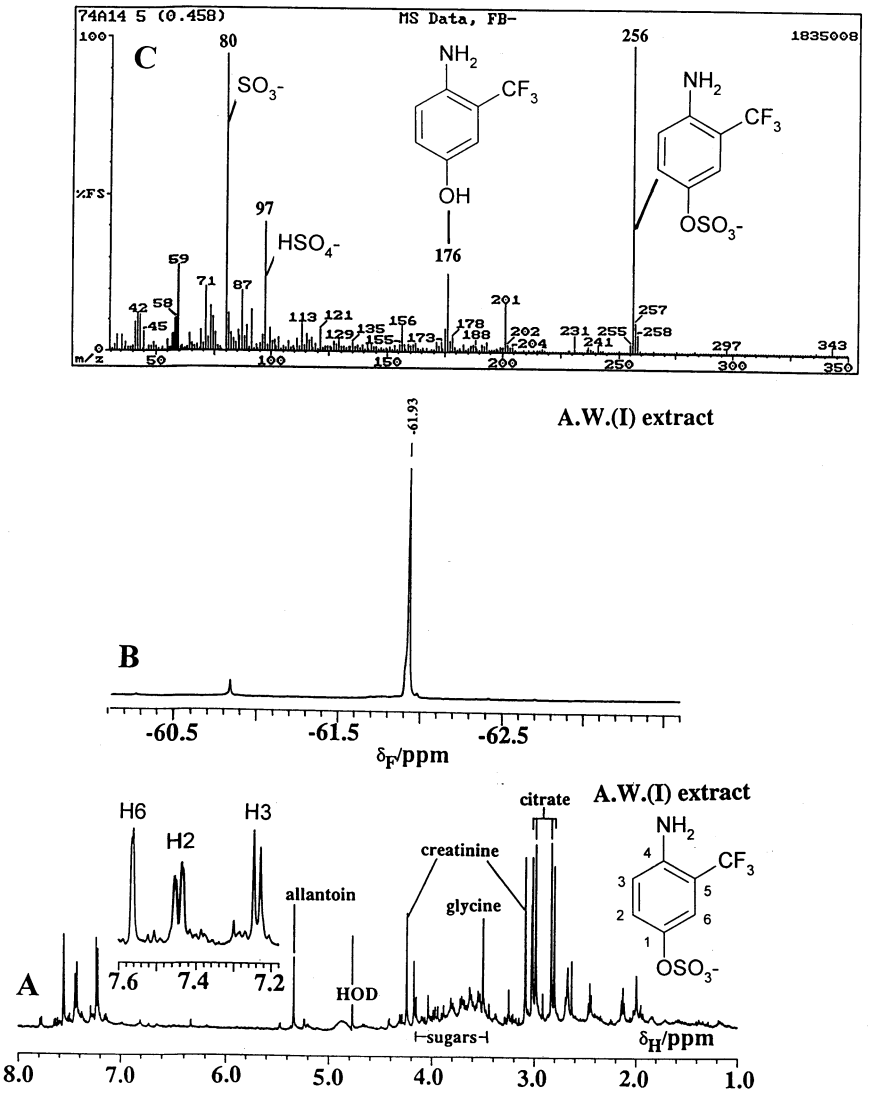

Fig. 3. SPEC as an aid for sample clean up and metabolite isolation: (A) 500 MHz ^{1}H and (B) 376 MHz ^{19}F NMR spectra of the C_{18} Bond-Elut^{TM} solid-phase extracts of the 0–8 h urine collected from 2-TFMA treated rats. (C) displays the corresponding negative FAB-mass spectrum. AW indicates acidified water, HOD, residual water.

The ^{1}H NMR spectrum for the major peak, B, seen in the chromatogram at $t_R = 11.18$ min shows the same splitting pattern as the earlier eluting metabolite. Enzyme hydrolysis experiments (with aryl sulphatase) indicated that this peak is a sulphate conjugate reverting to the same phase I precursor as peak A. This metabolite had been additionally confirmed following isolation by SPEC and subsequent identification by negative FAB–MS. By analogy with the structure of the glucuronide at $t_R = 7.74$ min this metabolite was assigned as 4-amino-5-trifluoromethylphenyl sulphate.

Metabolite C, eluting at $t_R = 12.23$ min, presented difficulty in its assignment. This HPLC-peak appeared to be severely contaminated by the tailing end of peak B.

Metabolite C still revealed high concentrations of 4-amino-5-trifluoromethylphenyl sulphate. In

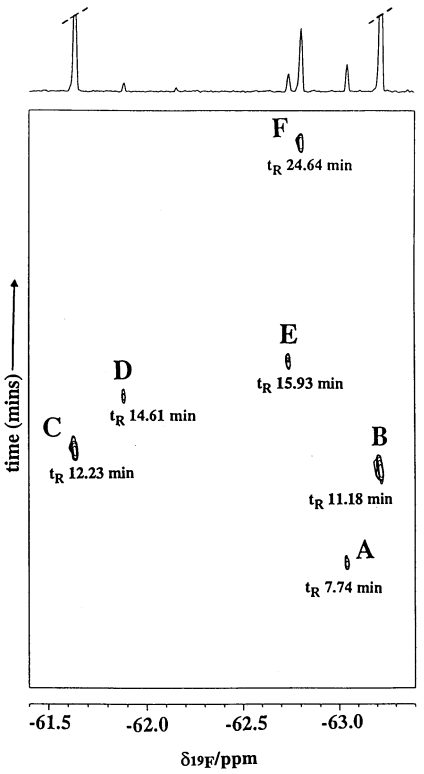

Fig. 4. The pseudo-2D contour plot of a continuous-flow 470 MHz ^{19}F NMR detected HPLC separation of the metabolites contained in the concentrated 0–8 h urine from rats treated with 2-TFMA (24 scans per experiment, trifluoroacetic acid $\delta 19_F - 76.8$ as external chemical shift reference).

addition, minor signals from another ring substituted metabolite were visible, the coupling patterns suggesting substitution para to the amino- or the CF_3-group of 2-TFMA (doublet at $\delta 1_H$ 6.79, singlet at $\delta 1_H$ 6.7 and doublet at $\delta 1_H$ 6.65), possibly corresponding to a hydroxysulphate or hydroxy metabolite. A further minor metabolite was detectable, however, the coupling patterns and the low signal intensities did not allow full characterisation.

Identification of the postulated *N*-glucuronide conjugate metabolites could not be achieved. The expected unsubstituted ring pattern for 2-TFMA (triplet, doublet, triplet, doublet) was not clearly observed, due to signal overlap, lack of resolution and poor signal-to-noise. However, there was evidence of a small unresolved signal at approximately $\delta 1_H$ 5.15, a possible indication of an anomeric proton.

The failure to identify these major metabolites (as seen in the ^{19}F NMR 0–8 h urinary profile and the on-flow ^{19}F NMR experiment, Figs. 1 and 4, respectively) indicated that these postulated glucuronide conjugates may have decomposed during the stop-flow ^{1}H NMR analysis.

Metabolite D, eluting at $t_R = 14.61$ min, appears to be a di-hydroxylated metabolite. The ^{1}H NMR spectrum of ^{19}F contour D revealed a pair of doublets consistent with a dihydroxy metabolite. The presence of a doublet at $\delta 1_H$ 5.05 suggested that one of the hydroxy groups was conjugated to ß-D-glucuronic acid.

Although this metabolite failed characterisation in the enzyme hydrolysis experiment (due to its low concentration, see Fig. 2), concentration of the sample by freeze drying allowed its detection by on-flow ^{19}F NMR.

The peak eluting at $t_R = 15.93$ min. (contour E) corresponds to 2-TFMA itself. The triplet, doublet, triplet, doublet coupling pattern observed for the aromatic ring protons suggests absence of substitution.

In a related study, freeze-drying and reconstitution of an aliquot of a 0–8 h sample has revealed loss of volatile parent. It appears plausible, that the hydrolysis of the postulated *N*-glucuronide conjugate (peak C) has resulted in the increase in concentration of 2-TFMA itself.

The coupling patterns observed for metabolite F ($t_R = 24.64$ min.) corresponds to a ring substituted metabolite. This metabolite is consistent with a mono-hydroxylated phase I metabolite as identified in the enzyme hydrolysis experiment (signal at $\delta 19_F - 62.79$). However, the substitution pattern suggests hydroxylation ortho to the amino or the trifluoromethyl group. Hydroxylation ortho to the amino group appears to be more likely in view of the retention characteristics of this polar metabolite. The late elution of this metabolite must be due to inter- or intramolecular hydrogen bonding.

A metabolic pathway for 2-TFMA and 2-TFMeAc based on the metabolites identified in urine is shown in Fig. 7.

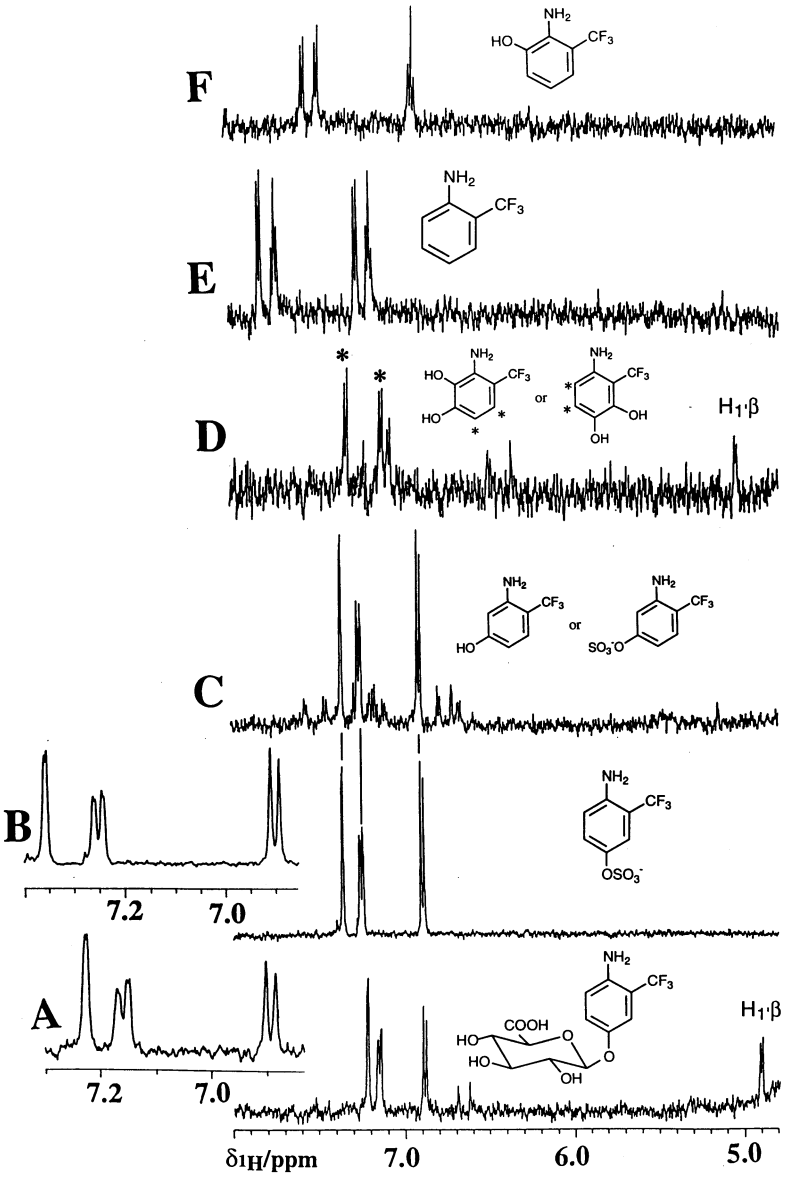

Fig. 5. The corresponding 500 MHz ^{1}H NMR spectra acquired in the stop-flow mode. H_1·β-represents the β-anomeric proton of glucuronic acid (96–4000 scans, exponential linebroadening of 1 Hz, ACN $\delta 1_H$ 2.0 as internal chemical shift reference).

3.5. Investigations of stability of the postulated N-glucuronide conjugates (^{19}F NMR signals $\delta 19_F$ −61.62 and −61.65)

Further experiments were carried out in order to characterise the postulated *N*-glucuronide conjugates of 2-TFMA and to explain their lack of detection and identification in the SPEC- and HPLC-NMR experiments.

3.5.1. Effects of pH and freeze-drying on 2-TFMA metabolite chemical shifts and stability

Acidification of an aliquot of the bulk 0–8 h urine sample to pH 2 (using 2 M HCl, as used for

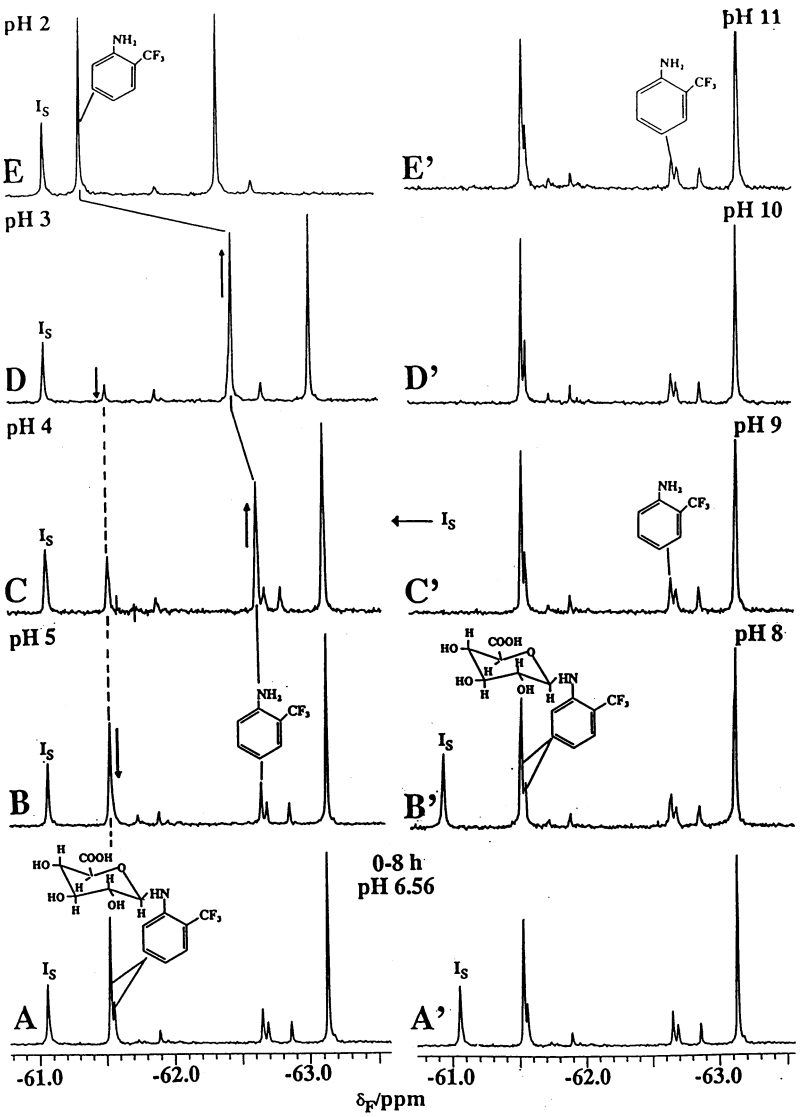

Fig. 6. The effect of pH modulation on the signal intensity of the putative *N*-glucuronide conjugate of 2-TFMA:376 MHz ^{19}F NMR spectra of urine collected 0–8 h after administration of 2-TFMA to rats (A = A′) following acid titration (B–E) and base titration (B′–E′) (I_S indicates internal standard (4-trifluoromethylphenol), the signal is shifted to higher frequency at pH 9 and above).

the SPEC separation) caused all the ^{19}F signals to shift to higher frequency, suggesting the presence of a free amino group, while the signals at $\delta 19_F -$ 61.62 and -61.65 reverted to 2-TFMA ($\delta 19_F -$ 62.75), indicating rapid acid hydrolysis of the amine (data not shown).

Further pH titration experiments (pH 6.56–1 and 6.56–11, in approximately ΔpH 1 units) were performed to assess the stability of the postulated 2-TFMA-N-glucuronide conjugates (Fig. 6). An internal standard of known concentration was added as an internal quantification standard. It appeared that the signals from the putative *N*-glucuronides were stable at alkaline pH. Yet, acidification to pH 5 resulted in the collapse of the two signals into one (the area under the curve,

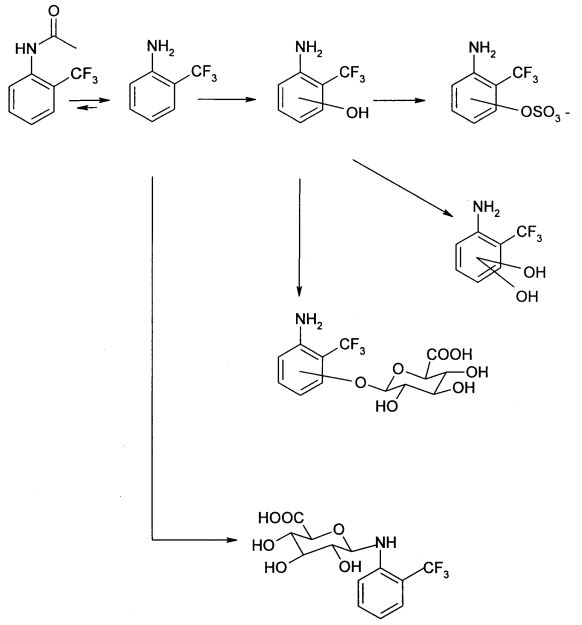

Fig. 7. The putative metabolic pathway for 2-TFMeA and 2-TFMeAc from analysis of urine.

however, remaining the same). A further drop in pH resulted in attenuation of the signal with a concomitant proportional increase in the intensity of the parent signal. At pH 3, only small amounts of the postulated *N*-glucuronide were detectable, while at pH 2 no signal was observed, hence, accounting for the failure to isolate the *N*-glucuronide metabolite by SPEC.

These findings, together with the data from the control enzyme incubation at pH 5.3, appear to support the theory that the pH of the mobile phase used for the HPLC-NMR separation (pH 5.2) must have contributed to the hydrolysis of this labile *N*-glucuronide conjugate. The freshly prepared sample in initial mobile phase buffer allowed detection of the signal(s) by on-flow ^{19}F HPLC-NMR (peak C), however, repeating the chromatographic separation with sequential stop-flow analyses, affording considerable time on peaks A and B, must have caused loss of this acid labile metabolite.

The effects of freeze-drying have also been assessed. An aliquot of a 0–8 h sample containing an internal standard for peak area comparison was freeze-dried and reconstituted in the same volume of D_2O. Comparison of the ^{19}F NMR spectra of the initial and reconstituted sample revealed loss of the volatile parent compound and collapse of the *N*-glucuronide signals ($\delta 19_F - 61.62$ and -61.65) into a single peak (similar to the observation on acidification), yet retaining the original signal integral value (data not shown).

It, therefore, follows, that the combination of freeze-drying (in order to concentrate the sample for HPLC-NMR) and reconstitution in mobile phase at pH 5.2 may have caused hydrolysis of the *N*-glucuronide metabolite during the HPLC-NMR analysis with concomitant increase of the signal for 2-TFMA itself.

3.6. Quantification of the urinary metabolites of 2-TFMA (and 2-TFMAc)

Following identification, the mean percentages of the dose recovered in urine up to 48 hpd were assessed. These data were derived from the ^{19}F NMR spectra of neat urine samples.

Owing to the similarity of the ^{19}F NMR metabolite profiles from 2-TFMA and 2-TFMAc dosed rats, quantification has been carried out on the urines from 2-TFMA as being representative for both compounds. The total amount of 2-TFMA-related material excreted over the 48 h of the experiment was $96.4 \pm 6.6\%$ of the dose, with some $79.7 \pm 5.9\%$ of that eliminated in the first (0–8 h) sample (Table 1). The major urinary metabolites, the postulated *N*-glucuronide and the hydroxysulphate conjugates, accounted for approximately 53.0 and 31.5% of the dose, respectively. The total urinary recovery of 2-TFMA and metabolites of approximately 96% indicate urinary excretion as the major elimination pathway. The 48 h collection period appeared to be adequate for the 2-TFMA and 2-TFMAc metabolites.

This study demonstrates that (with minimal sample preparation) on-flow ^{19}F detection of the 2-TFMA metabolites could be achieved and the majority of the metabolites could be identified by subsequent sequential stop-flow ^{1}H NMR experiments. Additional identification was provided by the results from the enzyme hydrolysis experiments. Even without optimisation of the HPLC

Table 1
Percentage of the dose of 2-TFMA (50 mg kg^{-1}) recovered in the urine of male rats ($n = 3$, ±S.D.)

Metabolite (δ 19_F)	%0–8 h	%8–24 h	%24–48 h	Total 0–48 h
2-TFMA (−62.75)	1.1±0.2	0.1±0.1	0.0±0.0	1.2±0.2
2-TFMA-*N*-glucuronide (−62.62/5)	43.4±5.6	8.0±1.3	1.6±1.0	53.0±5.8
TFMA-phenyl-glucuronide (−62.96)	2.7±0.2	0.6±0.4	0.0±0.0	3.3±0.5
TFMA-phenyl-sulphate (−63.23)	26.9±1.8	2.6±1.3	2.0±1.5	31.5±2.7
Minor unknowns	5.6±0.2	1.8±1.4	0.0±0.0	7.4±1.4
Total	79.7±5.9	13.1±2.3	3.6±1.8	96.4±6.6

separation, none of the HPLC fractions were found to be contaminated with endogenous urinary material, hence, enabling their structural identification. The detection of a di-hydroxylated-metabolite (with glucuronidation of one of the hydroxy-groups) was an unexpected finding and emphasises the strength of the technique.

HPLC-NMR spectroscopy requires minimal sample preparation, hence, it is generally recommended as a suitable method for the analysis of volatile, light and air-sensitive analytes. However, this study has highlighted some of the problems, which can be encountered with (acid) labile metabolites. In previous related work, SPEC (at pH 2) has been routinely utilised as a rapid sample purification and concentration method prior to analysis by HPLC-NMR/–MS [7–9]. Despite the technical advances, it has to be accepted that this approach would not have been suitable for the analysis of the compounds in this present study. The attention in future and repeat studies will have to be focussed on ensuring that the sample preparation and analyses are carried out using pH conditions which ensure the stability of the parent compound and any metabolites.

4. Conclusions

The overall urinary metabolic profiles of both the parent aniline and its acetanilide were the same with the major metabolites for both 2-TFMA and 2-TFMAc identified as *N*-glucuronide and hydroxysulphate conjugates. *N*-acetylation did not seem to be a major pathway for the metabolism of 2-TFMA as indicated by the rapid deacetylation of the acetanilide. 2-TFMA, as a representative of both compounds dosed, appeared to be rapidly and extensively metabolised, and almost entirely cleared by urinary excretion.

HPLC-NMR provided a rapid and suitable means for the direct identification of the majority of the 2-TFMA metabolites utilising the ^{19}F handle during HPLC-separation and subsequent structural characterisation by stop-flow ^{1}H NMR.

NMR silent conjugates, such as sulphate, could be additionally positively identified by ^{19}F NMR-monitored enzyme hydrolysis. Glucuronide conjugates were easily identified in the ^{1}H NMR spectra by the presence of the anomeric proton signals and could be additionally confirmed by enzyme hydrolysis with -glucuronidase. Neither of the HPLC fractions suffered from coelution with endogenous urinary metabolites, despite the lack of optimisation of both HPLC conditions and sample purification prior HPLC-NMR analysis.

Detection of a di-hydroxylated metabolite by HPLC-NMR was unexpected, as it could not be not characterised by prior enzyme hydrolysis. Equally surprising was the long retention of the mono-hydroxy-metabolite, presumably due to inter- and/or intramolecular hydrogen bonding.

This analysis has also highlighted the lability of *N*-conjugated glucuronide signals. The acid- catalysed decomposition could be clearly observed in the pH titration experiment.

Great care will have to be taken in choosing the optimal pH and sample preparation conditions in future studies with compounds of this nature.

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