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Identification and quantification of metabolites of 2,3,5,6-tetrafluoro-4-trifluoromethylaniline in rat urine using ^{19}F nuclear magnetic resonance spectroscopy, high-performance liquid chromatography–nuclear magnetic resonance spectroscopy and high-performance liquid chromatography–mass spectrometry

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Abstract

The urinary excretion profile and identity of the metabolites of 2,3,5,6-tetrafluoro-4-triflouromethylaniline, following i.p. administration to the rat at 50 mg kg^{-1}, were determined using a combination of ^{19}F-NMR, HPLC–NMR and HPLC–MS. A total of 38% of the dose was eliminated in the urine as five metabolites. The major routes of metabolism were *N*-glucuronidation, sulfation and oxidation with a significant amount of metabolic defluorination to give a number of *ortho*-ring hydroxylated metabolites. The oxidised metabolites were excreted as glucuronide and/or sulfate conjugates.

Keywords: 2,3,5,6-Tetrafluoro-4-trifluoromethylaniline

1. Introduction

Understanding the metabolic fate of primary aromatic amines is important for understanding of the toxicology of a group of compounds to which there is widespread human exposure resulting from their use as intermediates in the production of drugs, pesticides and bulk chemicals. Some of the toxicity of aromatic amines is thought to be mediated by the production of metabolites. These effects include hepatotoxicity, nephrotoxicity, carcinogenesis and methaemoglobinaemia [1]. As part of a series of studies aimed at building metabolic databases, from which predictive in vivo models of drug metabolism will be constructed [2–6], we have used nuclear magnetic resonance (NMR) spectroscopy-based methods to generate data on the metabolic fate of a number of fluorinated anilines [7–10]. The presence of fluorine in a xenobiotics enables metabolites to be rapidly detected and quantified, with high specificity using ^{19}F-NMR providing a useful and convenient alternative to the use of radiolabelled compounds in metabolism studies. Recent advances in the hyphenation of chromatographic and spectroscopic tech-

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niques [e.g., high-performance liquid chromatography–mass spectrometry (HPLC–MS), HPLC–MS–MS, HPLC–NMR, HPLC–NMR–MS] have enabled the rapid and efficient identification of metabolites detected in such studies [7,8,11–16]. Here the use of ^{19}F-NMR, HPLC–NMR and HPLC–MS is described for the identification and the quantitative determination of the metabolites of 2,3,5,6-tetrafluoro-4-trifluoromethylaniline (TFTFMA) in rat urine following intraperitoneal (i.p.) administration.

2. Experimental

2.1. Animal experiments

Three male Sprague–Dawley rats (200–250 g) received a single i.p. injection of TFTFMA (98% chemical purity, Aldrich, Gillingham, UK) in ethanol–water (50:50, v/v) at a dose of 50 mg kg^{-1}. The rats were housed individually in metabolism cages and permitted free access to food (rat maintenance diet TRM 9607, Harlan Teklab, Bicester, UK) and water throughout the study. Urine was collected over solid CO_2 immediately prior to dosing and over 48 h post-dosing (0–8, 8–24 and 24–48 h periods) and samples were centrifuged at 2000 *g* for 10 min to remove any food debris. Urinary weights were recorded and samples stored at −20°C until analysis.

2.2. ^{19}F-NMR spectroscopy of urine

Samples (500 μl) of whole urine were placed in 5-mm NMR tubes and 100 μl of D_2O was added as an internal field frequency lock. A known amount of 2-trifluoromethylbenzoic acid (Fluorochem, Glossop, UK) was added to each sample as an internal concentration and reference standard prior to ^{19}F-NMR analysis. ^{19}F-NMR chemical shifts were referenced to the internal standard at δ_F −59.9 relative to $CFCl_3$ at δ_F 0.0. ^{19}F-NMR spectra were measured on a Bruker AMX600 spectrometer operating at 564.62 MHz ^{19}F observation frequency using 90° pulses with a 76 453 Hz spectral width. Typically 512 scans were collected into 131 072 data points with an acquisition time of 0.85 s. A further delay of 9 s between pulses was added to allow T_1 relaxation. The free induction decays were multiplied by an exponential apodization function corresponding to a 5 Hz line broadening prior to Fourier transformation (FT).

Quantification of the metabolites in the urine samples was achieved by integration of their ^{19}F-NMR signals relative to those of the internal standard. Multiplication of this integral value by the number of moles of internal standard added gave the number of moles of metabolite in the urine aliquot taken for ^{19}F-NMR. The number of moles of metabolite in the whole urine sample could then be determined and dividing this by the number of moles of the substrate dosed to the rat (multiplying by 100), a percentage recovery could be deduced.

2.3. Solid-phase extraction of urine

Urine was partially purified and concentrated using solid-phase extraction prior to analysis by HPLC–NMR and HPLC–MS. Urine samples (2 ml) were adjusted to pH 2 with hydrochloric acid and applied to 3 ml capacity C_{18} bonded cartridges (500 mg of sorbent) (BondElut, Varian, Jones Chromatography, Hengoed, UK). The cartridges had been conditioned with methanol (2 ml) and water (2 ml, at pH 2 using 0.1 *M* HCl). Following extraction, the cartridges were washed with acidified water (2 ml), and then eluted with methanol (2 ml). The methanol then removed by evaporation under a stream of nitrogen at room temperature following which the extracted sample was reconstituted in 0.5 ml distilled water.

2.4. HPLC–NMR analysis of urine extracts

The HPLC system consisted of a Bruker (Coventry, UK) LC22 pump with a Bischoff Lambda 1100 variable-wavelength UV detector set at 254 nm and a Hypersil BDS C_{18} (250×4.6 mm) column with 5 μm particles (Hichrom, Reading, UK). Separation was effected at 25°C using D_2O (Fluorochem) containing 0.01 *M* ammonium formate (Fisons, Loughborough, UK) adjusted to pH 7.0 with ammonia solution and ''Pestanal'' grade acetonitrile (Riedel-de Haen, Germany) at a flow-rate of 1 ml min^{-1}. Gradient elution was used starting at 5% acetonitrile (ACN) and increasing linearly to 65% ACN after 40 min, the chromatographic sequence being controlled by Bruker Chromstar software. Typically 100 μl of the

extracted urine samples was injected onto the column.

The HPLC system was coupled, via the Bruker BPSU-12 collector, to a ^{1}H-NMR flow-probe (cell volume 120 μl) of a Bruker DRX-500 spectrometer operating at a 500.13 MHz observation frequency. This probe was manually tuned to 470.59 MHz in order to observe ^{19}F signals. For stop-flow HPLC–^{19}F-NMR experiments, typically 1024 free induction decays were collected over a spectral width of 66 007 Hz into 65 536 data points using a relaxation delay of 0.50 s and an acquisition time of 0.50 s. For stop-flow HPLC–^{1}H-NMR experiments, typically 1024 free induction decays were collected over a spectral width of 8278 Hz into 16 384 data points using an acquisition time of 0.99 s. The residual solvent resonances were suppressed using the standard one-dimensional solvent suppression NOESYPRESAT pulse sequence (Bruker) with pre-irradiation during the relaxation delay of 2.0 s and the mixing time of 0.10 s. The ^{19}F-free induction decays were multiplied by an exponential function corresponding to a 5 Hz line-broadening and the ^{1}H-NMR spectra were resolution enhanced using a Lorentzian-to-Gaussian transformation function prior to FT. ^{19}F-NMR chemical shifts were referenced to trifluoroacetic acid at δ_F −75.7 relative to $CFCl_3$ at δ_F 0.0 and ^{1}H chemical shifts were referenced to the acetonitrile signal at δ_H 2.0 relative to trimethylsilylpropionic acid at δ_H 0.0.

2.5. HPLC–MS analysis of urine extracts

HPLC–MS was performed with a Finnigan LCQ ion-trap mass spectrometer coupled to a Hewlett-Packard 1100 series HPLC system using a Hypersil H5BDS C_{18} column (150×2.1 mm) with 5 μm particles. Separation was effected at ambient temperature using 0.01 *M* ammonium formate at pH 2.4 (adjusted with formic acid) with an ACN (HPLC grade) linear gradient of 5–95% over 0–40 min. The flow-rate used with this narrow bore column was 200 μl min^{-1} and this was reduced by a factor of 10 using a stream splitter before entering the mass spectrometer. Ionisation was by negative-ion electrospray and centroid mass spectra were acquired up to m/z=600 using a cone voltage of 20–30 V. Typically 10 μl of the extracted urine samples was injected onto the column.

3. Results and discussion

3.1. Profiling of metabolites by ^{19}F-NMR spectroscopy

The ^{19}F-NMR spectrum of TFTFMA (I) shows two distinct regions with the signal for the aliphatic CF_3 group can be observed at δ_F −54.6 as a triplet (J_{F-F}=21 Hz) due to spin–spin coupling to the aromatic fluorines at positions 3 and 5. The CF_3 was used to determine the number and quantity of metabolites present in the urine after dosing TFTFMA to rat. Further resonances at δ_F −146.1 and δ_F −162.1 were due to the chemically equivalent fluorines at the 3 and 5, and the 2 and 6 positions, respectively. These signals appeared as multiplets due to spin–spin coupling to each other and to the CF_3 group forming a complex $AA'MM'X_3$ spin system. The pattern that this provides, typical of *para* substitution, is useful in that it enables structural information on the metabolites of TFTFMA to be deduced from their ^{19}F-NMR spectra.

After dosing rats with TFTFMA, the collected urine samples were profiled by ^{19}F-NMR spectroscopy. A typical set of ^{19}F-NMR spectra of urine samples collected up to 48 h after dosing TFTFMA are shown in Fig. 1a–c for the aliphatic CF_3 region of the spectrum. This clearly shows resonances for five metabolites, but no unchanged TFTFMA (δ_F −54.6) was detected in the urine. In the aromatic region of the spectrum at least eleven separate ^{19}F resonances were observed (not shown) and, in order to deconvolute the structural information contained in this region, HPLC–NMR was performed on the urine sample. In addition to these fluorine resonances, a broad signal centered at δ_F −119 was observed in the post-dose urine samples due to the presence of the fluoride ion (F^-), probably present as a result of metabolic defluorination.

3.2. Identification of metabolites by HPLC–NMR and HPLC–MS

In order to identify these metabolites solid-phase extracts of urine were initially investigated by HPLC–NMR with postulated structures then confirmed by HPLC–MS. The UV–HPLC profile for a 24–28 h post-dose sample is shown in Fig. 2. The stop-flow HPLC–^{19}F-NMR spectrum for the aliphat-

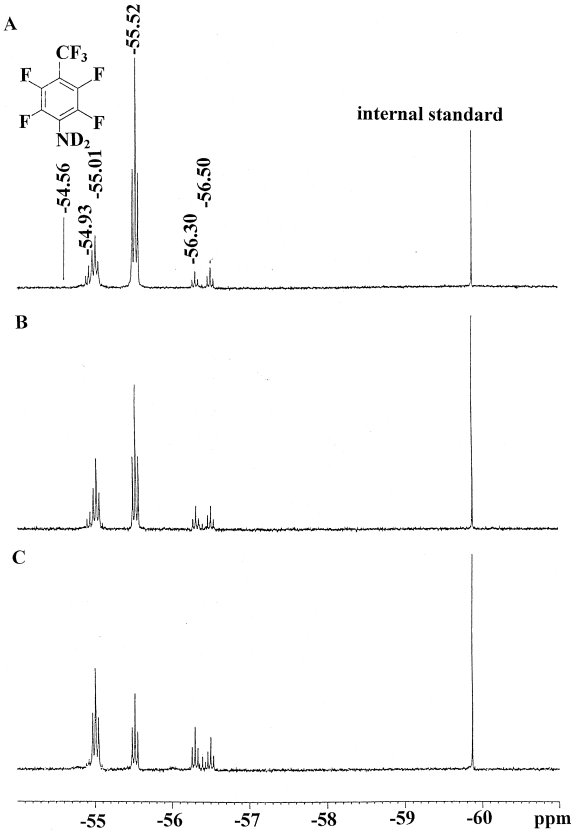

Fig. 1. 565 MHz ^{19}F-NMR spectra (aliphatic trifluromethyl region) of urine samples obtained for the periods (a) 0–8 h, (b) 8–24 h and (c) 24–48 h after dosing to the rat.

ic and aromatic regions of the ^{19}F spectrum of a peak with the retention time of 14.4 min is shown in Fig. 3a and b. The characteristic triplet resonance for the CF_3 group gave an immediate indication that this UV absorbing peak contained a metabolite of TFTFMA and its chemical shift (δ_F −56.5) showed it to be metabolite II in the ^{19}F-NMR spectrum of the whole urine sample (Fig. 1b). In the aromatic region of the ^{19}F-NMR spectrum of this minor metabolite there were two resonances at δ_F −143.3 and δ_F −146.1 corresponding to the aromatic fluorines at positions 3 and 5, and 2 and 6, respectively. The ^{19}F-NMR spectrum of this metabolite showed that all the aromatic fluorines and the CF_3 group were intact and therefore metabolism must have occurred at the amino group. A stop-flow HPLC–^{1}H-NMR spectrum of this peak did not contain any observable proton signals that could be attributed to the metabolite. This metabolite was therefore postulated to be an *N*-sulfate conjugate and this was confirmed by HPLC–MS which gave a molecular ion $[M-H]^-$ at $m/z=312$ (Fig. 3c). Metabolite II was thus identified as 2,3,5,6-tetrafluoro-4-trifluoromethylaniline-*N*-sulfate ($M=313$).

HPLC–NMR of the major peak with the retention time of 16.0 min gave the spectrum shown in Fig. 4a. The CF_3 region of the ^{19}F-NMR spectrum revealed that this peak contained two unresolved metabolites as there were two separate triplets. These corresponded to the metabolites III (δ_F −55.5) and VI (δ_F −54.9) in the whole urine ^{19}F-NMR spectrum (Fig. 1). The aromatic fluorine region of the stop-flow HPLC–^{19}F-NMR spectrum of this peak also showed signals from two metabolites. Major signals at δ_F −143.9 and δ_F −157.5 (Fig. 4b) corresponded to the metabolite III, with the intact *para*-disubstituted pattern of these aromatic fluorine signals clearly indicating that metabolism must have occurred at the amino group of this metabolite. The remaining aromatic ^{19}F-NMR signals were due to metabolite VI and this substitution pattern showed, in contrast, that metabolism had occurred on the aromatic ring resulting in the loss of a fluorine atom as there were now signals from three non-equivalent fluorines. The fact that the CF_3 resonance for this metabolite (δ_F −54.9) remained a triplet would suggest that the fluorine atoms at positions 3 and 5 were still present and therefore a fluorine *ortho* to the amino group had been lost. A ^{1}H-NMR spectrum was also acquired on this chromatographic peak (Fig. 4c) and this revealed that both metabolites were glucuronide conjugates showing characteristic β-anomeric proton signals (one-proton doublets, $^3J_{H-H}=7.7$ Hz) at δ_H 4.89 and 4.73. Other signals from the sugar protons of the glucuronic acid moieties of these metabolites could also be observed in the ^{1}H-NMR spectrum between δ_H 3.4 and 3.7 thus confirming them as glucuronide conjugates. It was postulated from these data that one of the metabolites was conjugated directly with glucuronic acid at the amino position and the other was a product of *ortho*-hydroxylation (resulting in the loss of fluorine) followed by glucuronic acid conjugation. This was confirmed by HPLC–MS analysis where molecular ions at $m/z=408$ and 406 were observed

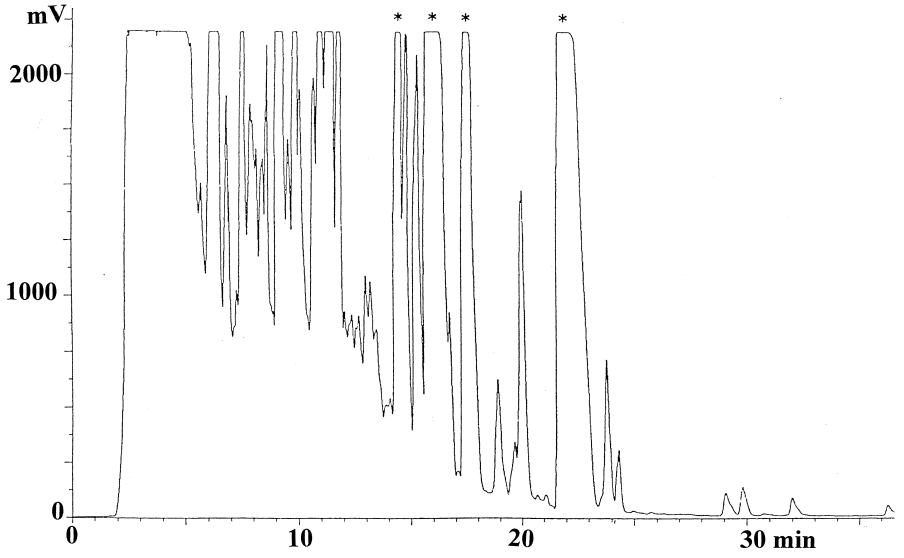

Fig. 2. HPLC–UV (254 nm) chromatogram for a solid-phase extract of a 24–48 h urine sample obtained from a rat after administration of TFTFMA. Peaks indicated with * were those on which stopped-flow NMR analysis was performed.

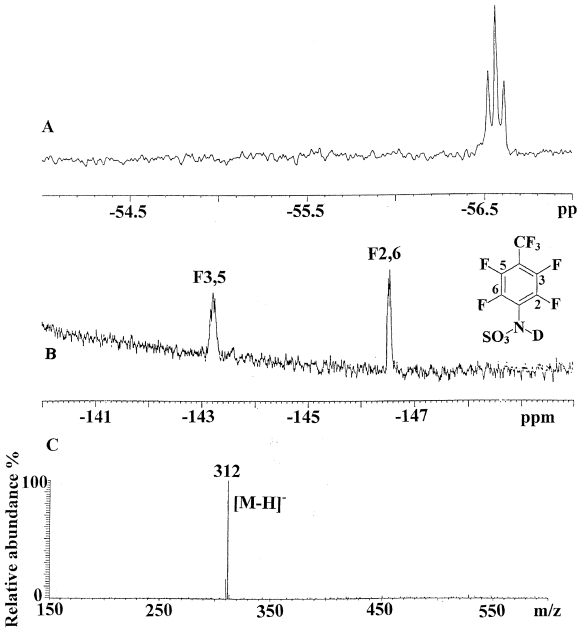

Fig. 3. The stop-flow ^{19}F-NMR spectrum of the peak with a retention time of 14.4 min (see Fig. 2) showing (a) the aliphatic and (b) the aromatic portions of the spectrum and (c) the corresponding negative ion electrospray mass spectrum for this peak.

corresponding to 2,3,5,6-tetrafluoro-4-trifluoromethylaniline-*N*-glucuronide (III, M_r=409) and 2-amino - 3,4,6 - trifluoro - 5 - trifluoromethylphenyl-glucuronide (VI, M_r=407), respectively.

In Fig. 5 the stop-flow HPLC–^{19}F-NMR spectrum of a UV absorbing chromatographic peak with a retention time of 17.5 min is shown. The CF_3 resonance of this metabolite (δ_F −56.3, Fig. 5a) showed it to be the minor metabolite IV in the whole urine sample (Fig. 1). The aromatic region of the stop-flow HPLC–^{19}F-NMR spectrum revealed that all the aromatic fluorines were intact as a *para*-disubsituted pattern was observed. The ^{1}H-NMR spectrum of this metabolite (not shown) indicated it to be a glucuronide conjugate with doublets at δ_H 4.83 and 3.67 from H1′ and H5′ and triplets at δ_H 3.47, 3.42 and 3.28 from H4′, H3′ and H2′ of the glucuronide moiety, which must be attached at the amino group of TFTFMA. The molecular ion of this metabolite, as determined from HPLC–MS experiments, was at m/z=424 and was 16 u greater than the previously identified *N*-glucuronide of TFTFMA (metabolite III) indicating oxidation as well as glucuronidation had occurred in this metabolite to give 2,3,5,6-tetrafluoro-4-trifluoromethylphenyl-hydroxylamine glucuronide.

The stop-flow HPLC–^{19}F-NMR spectrum of a

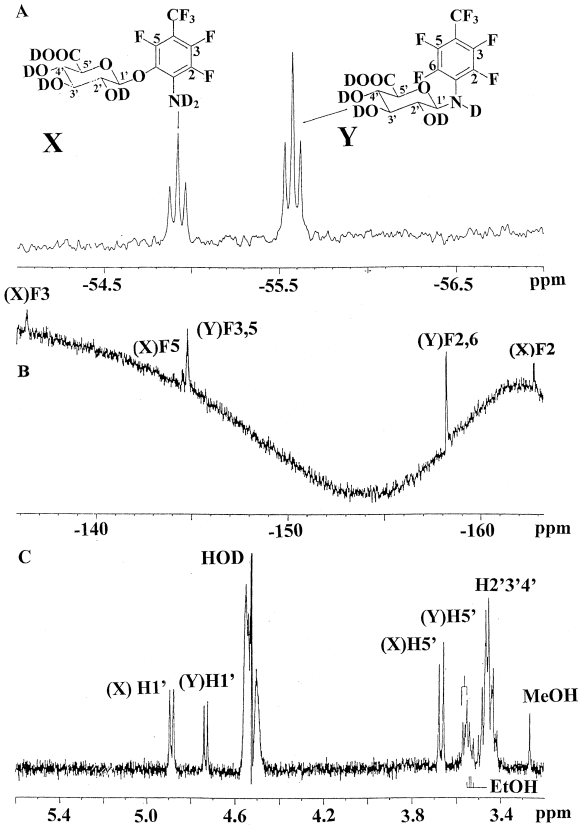

Fig. 4. The stop-flow ^{19}F- (a, b) and (c) ^{1}H-NMR spectra of the UV absorbing peak eluting at 16.0 min (see Fig. 2).

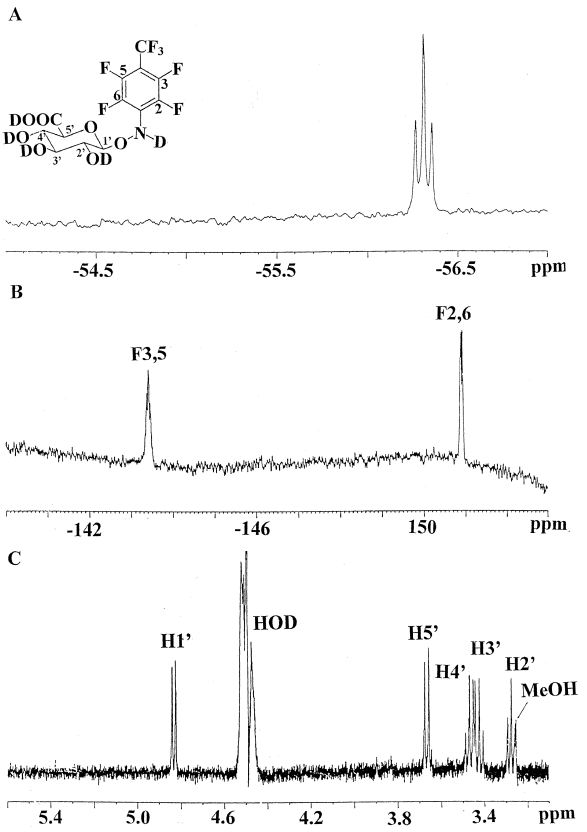

Fig. 5. The stop-flow ^{19}F- (a, b) and (c) ^{1}H-NMR spectra of the UV absorbing peak eluted at 17.5 min (see Fig. 2).

chromatographic peak with a retention time of 22.0 min is shown in Fig. 6a and b. The CF_3 region of the spectrum showed that this peak contained one metabolite with a ^{19}F-NMR chemical shift of δ_F −55.0 corresponding to the major urinary metabolite V shown in Fig. 1. The *para*-disubstituted aromatic fluorine pattern of the parent compound has been lost, indicating metabolic defluorination giving three separate signals for the non-equivalent fluorine atoms at δ_F −133.6 (F5), −143.9 (F3) and −161.2 (F2). The triplet resonance of the CF_3 group again showed that substitution had occurred *ortho* to the amino group. The ^{1}H-NMR spectrum of this metabolite contained no signals that could be attributed to the metabolite implying that it was either simply ring hydroxylated or a ring hydroxylated sulfate conjugate. The identity of this metabolite was confirmed as the latter by HPLC–MS which gave a molecular ion at m/z=310 corresponding to 2-amino-3,4,6-trifluoro-5-trifluoromethylphenylsulfate (V, M_r=311) (Fig. 6c).

3.3. Quantification of metabolites

Table 1 shows the mean percentage of the dose of TFTFMA recovered in urine up to 48 h post-dose. These data were derived from the CF_3 region of the ^{19}F-NMR spectra of neat urine samples (Fig. 1b). Comparison of the ^{19}F-NMR chemical shifts with those of the identified metabolites by HPLC–NMR (Fig. 2a–d) enabled the two sets of data to be compared. A mean total recovery of 38.4±5.9% of the dose was achieved in urine up to 48 h after dosing TFMTFA to rat. Excretion of metabolites into urine was slow with only 7.8±0.9% recovered between 0 and 8 h and then a further 15% of the

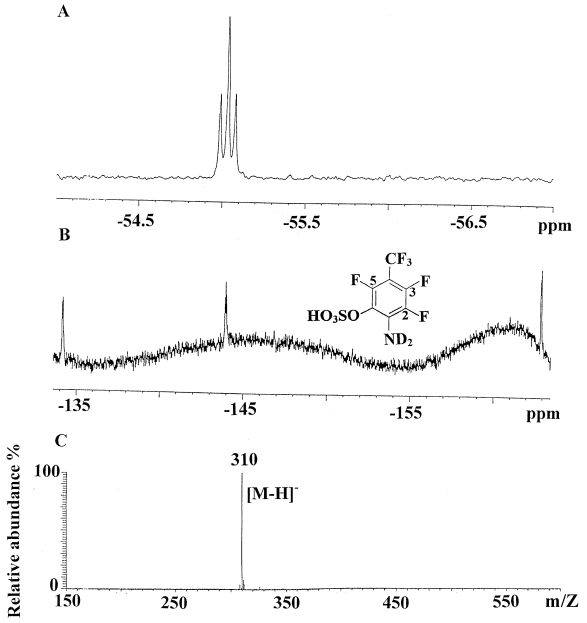

Fig. 6. The stop-flow (a) ^{19}F- and (b) ^{1}H-NMR spectra of the UV absorbing peak eluted at 22.0 min (see Fig. 2) and (c) the corresponding negative ion electrospray mass spectrum for this peak.

dose obtained in both the 8–24 and 24–48 h samples. There were two major metabolites; 2-amino-3,4,6-trifluoro-5-trifluoromethylphenylsulfate (V) and 2,3,5,6-tetrafluoro-4-trifluoromethylaniline-*N*-glucuronide (III) accounting for 15.8±1.7 and 13.0±3.9% of the dose, respectively. The other three identified metabolites; 2,3,5,6-tetrafluoro-4-trifluoromethylphenylhydroxylamine glucuronide (IV), 2,3,5,6-tetrafluoro-4-trifluoromethylaniline-*N*-sulfate (II) and 2-amino-3,4,6-trifluoro-5-trifluoromethylphenylglucuronide (VI) were minor accounting for less than 5% of the dose each.

The metabolic fate of TFTFMA, based on that proportion of the dose excreted via the urine, in rat is summarised in Fig. 7. Clearly a major route of metabolism was direct phase II conjugation at the amino group to give glucuronide (III, 13% of the dose) and sulfate (II, 3% of the dose) conjugates. This level of direct phase II conjugation was higher than observed for structurally-related trifluoromethylanilines [7,8,10] and maybe a result of the usual major route of metabolism of this class of compound (namely phase I aromatic ring hydroxylation) being blocked by fluorine substituents. However, phase I aromatic ring hydroxylation of TFTFMA did still occur to some extent resulting in metabolic defluorination. Thus, the sulfate (V, 16% of the dose) and glucuronide (VI, 1% of the dose) conjugates of 2-amino-3,4,6-trifluoro-5-trifluoromethylphenol were detected in post-dose urine together with the oxidatively removed fluoride ion. This type of metabolic process has previously been observed in that the fluorine atom *para* to the amino group in 4-fluoroaniline was oxidatively removed [9]. A minor metabolite identified (4% of the dose) was 2,3,5,6-tetrafluoro-4-trifluoromethylphenylhydroxylamine glucuronide (IV). As discussed previously [7,8], this type of metabolite may exist either conjugated with glucuronic acid directly on to the *N*-

Table 1
Percentage of dose recovered as the metabolites identified in rat urine up to 48 h after dosing with 2,3,5,6-tetrafluoro-4-trifluormethylaniline (derived from the CF_3 resonances in the ^{19}F-NMR spectra, Fig. 1)[a]

Metabolite identified	CF_3 δ_F/ppm (Fig. 1b)	% Dose in 0–8 h urine	% Dose in 8–24 h urine	% Dose in 24–48 h urine	Total % dose (0–48 h)
2-Amino-3,4,6-trifluoro-5-trifluoromethylphenyl sulfate	−55.0	2.2±0.3	5.7±0.9	7.9±1.2	15.8±1.7
2,3,5,6-Tetrafluoro-4-trifluoromethylaniline-*N*-glucuronide	−55.5	4.1±1.3	5.8±1.7	3.1±1.3	13.0±3.9
2,3,5,6-Tetrafluoro-4-trifluoromethylphenylhydro-xylamine glucuronide.	−56.3	0.6±0.3	2.0±1.0	1.8±0.2	4.4±1.1
2,3,5,6-Tetrafluoro-4-trifluoromethylaniline-*N*-sulfate	−56.5	0.4±0.2	1.1±0.2	1.5±0.4	3.0±0.6
2-Amino-3,4,6-trifluoro-5-trifluoromethylphenyl glucuronide	−54.9	0.5±0.0	0.6±0.1	ND	1.3±0.4
Minor metabolites[b]		ND	0.3±0.1	0.7±0.1	1.0±0.2
Cumulative totals		7.8±0.9	15.4±3.0	15.2±2.9	38.4±5.9

[a] Values are mean data (n=3)±SD.
[b] *Comprised of two minor unidentified metabolites.
ND=Not detected.

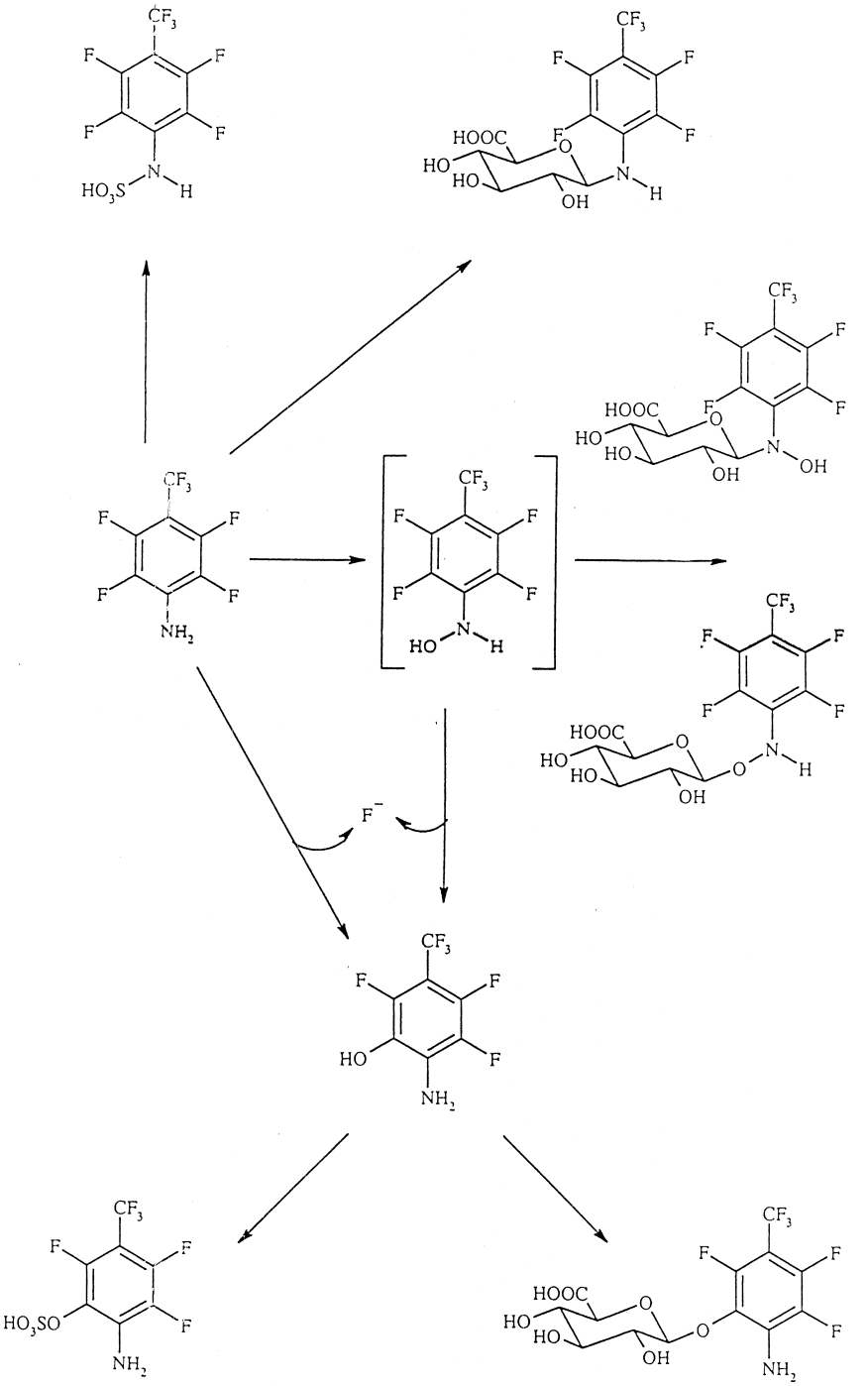

Fig. 7. The postulated metabolic pathway for 2,3,5,6-tetrafluoro-4-trifluormethylaniline based on the metabolites excreted in the urine.

or via the *O*-group (see Fig. 3) and the structural techniques used cannot distinguish between these. The metabolic route to the generation of this metabolite, phase I *N*-oxidation, is generally thought to be responsible for the toxic effects of these compounds [1,17]. As with other structurally related compounds [7,8], it is again postulated here that the unstable hydroxylamine intermediate may rearrange to the more stable *ortho*-aminophenol, the logical precursor for metabolites V and VI (see Fig. 7). Therefore, the amount of the dose metabolised via *N*-oxidation maybe higher than implied by the detection of the hydroxylamine glucuronide (IV).

4. Conclusions

The use of HPLC–NMR and HPLC–MS enabled the rapid and efficient elucidation of the metabolic fate of TFTFMA with a minimum of sample preparation. This allowed the resonances present in the ^{19}F-NMR spectra of the whole urine samples to be assigned and the metabolites quantified. This study has also further highlighted the metabolic lability of aromatic fluorines.

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