INVESTIGATION OF THE QUANTITATIVE METABOLIC FATE AND URINARY EXCRETION OF 3-METHYL-4-TRIFLUOROMETHYLANILINE AND 3-METHYL-4-TRIFLUOROMETHYLACETANILIDE IN THE RAT

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ABSTRACT:

The urinary metabolites of 3-methyl-4-trifluoromethylaniline in the rat were characterized and quantified using a combination of 19F NMR, HPLC-NMR (1H and 19F), and HPLC-mass spectrometry techniques. The major routes of metabolism were amine N-acetylation and methyl group C-oxidation to the benzyl alcohol (with subsequent glucuronide conjugation) and further to the corresponding benzoic acid derivative. Quantitatively only a small proportion of the urinary metabolites contained the free amino group, and these were products of ortho-hydroxylation (2 and 6 position) with additional conjugation to form the ether sulfates and glucuronides. An N-glucuronide of the parent compound was also identified. 3-Methyl-4-trifluoromethylaniline acetamido (13C-labeled in the acetyl group) gave virtually the same overall metabolite profile as 3-methyl-4-trifluoromethylaniline; however, a significant level of futile N-deacetylation and reacetylation occurred as ca. 50% of the excreted N-acetylated major metabolites contained no 13C-label at the acetyl, having been replaced by an endogenous 12C-acetyl source. This level of futile deacetylation is the highest yet reported for a substituted aniline/acetanilide and indicates a high degree of electronic activation of the amino group toward the acetyltransferase enzymes in vivo.

A comprehensive understanding of the metabolic fate of primary aromatic amines is important because of the insights that such information may provide on the contribution of metabolism to the toxicology of this widely used class of compound. The well known toxicological properties of aromatic amines include hepatotoxicity, nephrotoxicity, carcinogenesis, and methaemoglobinemia (Radomski, 1979). As part of a series of studies aimed at building in vivo metabolic databases of these compounds for the investigation of quantitative structure-metabolism relationships, we have used NMR-based methods to generate data on the metabolic fate of a number of anilines in the rat (Wade et al., 1988; Tugnait, 1994; Scarfe et al., 1998, 1999). In this work we have found 19F NMR spectroscopy provides a useful means of following the metabolic fate of these trifluoromethyl-substituted anilines. The presence of a CF3 group thus enables the simultaneous generation of both excretion balance data and metabolite profiles (Scarfe et al., 1998).

Furthermore, advances in the coupling of NMR to HPLC separations (Lindon et al., 1996) have allowed 1H NMR spectroscopy to become a useful method for the elucidation of the structure of metabolites initially detected using 19F NMR, eliminating the need for prior isolation in many cases. Where 1H NMR spectra alone are insufficient for structure determination, e.g., the production of sulfate conjugates and N-oxides where the new substituents do not contain protons, additional data from HPLC-mass spectrometry (MS) or HPLC with tandem mass spectrometry (Mutlib et al., 1995) generally provides an unequivocal identification. Here we apply this approach to the identification of metabolites of 3-methyl-4-trifluoromethylaniline (MeTFMA) and its acetanilide analog in rat urine after i.p. dose.

Materials and Methods

Test Compounds. MeTFMA hydrochloride (>99% purity) was a gift from Hoechst Marion Roussel, Milton Keynes, UK. 3-Methyl-4-trifluoromethyl-[13C]-acetanilide (MeTFMAC) with the 13C label on the carbonyl, was synthesized by reacting MeTFMA directly with acetyl-1-[13C]-chloride (99 atom % 13C) (Aldrich, Gillingham, UK) in a 1:1.1 M ratio. The resulting solid product was washed with distilled water to remove excess acetyl chloride and then recrystallized from ethanol to give a product that was >95% pure by 1H and 13F NMR. 1H NMR data: δH (CDCl3, Bruker DRX-500 spectrometer operating at 500.13 MHz); 7.52, d, 1H (H5); 7.44, s, 1H (H2); 7.37, d, 1H (H6); 7.19, s, 1H (NH); 2.44, s, 3H (ArCH3); 2.17, d, 3H (13COCH3), where s = singlet and d = doublet.

Animal Experiments. Three male Sprague-Dawley rats (200–250 g) received a single i.p. injection of MeTFMA hydrochloride in distilled water at a dose of 50 mg/kg. A second group of three rats was dosed using the same dose route and level with MeTFMAC in corn oil. 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 CO2 immediately before dosing and over 48 h postdosing (0- to 8-, 8- to 24-, and 24- to 48-h periods); this was

1 Abbreviations used are: MS, mass spectrometry; MeTFMA, 3-methyl-4-trifluoromethylaniline; MeTFMAC, 3-methyl-4-trifluoromethyl-[13C]-acetanilide.
centrifuged at 2000g for 10 min to remove any food debris. Urinary pH and weights were recorded and samples stored at −20°C until analysis.

19F NMR Spectroscopy of Urine. Samples (500 µl) of whole urine were placed in 5-mm NMR tubes and 100 µl of D2O was added as an internal field frequency lock. A known amount of 4-trifluoroethylmaleimide or 4-trifluoromethylbenzene acid (Fluorochem Ltd, Glossop, UK) was added to each sample as an internal concentration and reference standard before 19F NMR analysis. 19F NMR chemical shifts were referenced to the internal standard at δF = −6.11 or δF = −62.81, respectively, relative to CFCl3 at δF = 0.0. Any 19F–1H couplings were eliminated via the application of composite pulse proton decoupling using the Waltz-16 pulse sequence (Shaka et al., 1983). 19F NMR spectra were measured on a Bruker AM400 spectrometer operating at 376.50 MHz 19F observation frequency, using 90° pulses with a 10,000-Hz spectral width. Typically, 128 scans were collected into 8192 data points with an acquisition time of 0.82 s. A further delay of 6 s between pulses was added to allow T1 relaxation. The free induction decays were multiplied by an exponential apodization function corresponding to a 0.5-Hz line broadening before Fourier transformation.

Quantification of the metabolites in the urine samples was achieved by integration of their 19F 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 19F NMR (assuming the same number of fluorine atoms in the metabolite and standard). 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), the percentage of recovery was determined.

Enzyme Hydrolysis. Aliquots (600 µl) of urine from an appropriate period after dosing were adjusted to pH 7 or pH 5 using 1 M hydrochloric acid or sodium hydroxide solutions and 2500 U of β-gluconuronidase (Escherichia coli type VII-A, sulfatase-free; Sigma Chemical Co., Poole, UK) or 75 to 200 U of aryl sulfatase (Helix pomatia type H-1; Sigma) were added. Fifty microliters of a 0.1 M solution of n-saccharic acid-1,4-lactone (Sigma) in distilled water was added to the sulfatase sample to inhibit β-gluconoridase activity. The samples were incubated at 37°C overnight (~16 h) using a shaking water bath. Control samples of pH-adjusted urine were also incubated. All samples were analyzed by 19F NMR spectroscopy as described above.

Solid-Phase Extraction of Urine. Urine was partially purified and concentrated using solid-phase extraction before analysis by HPLC-NMR and HPLC-MS. Urine samples (2 ml) obtained after dosing with MeTFMA (0–8 and 8–24 h) or 3-methyl-4-trifluoromethyl-[13C]-acetanilide (0–8 h) were adjusted to pH 2 with hydrochloric acid and applied to 3-ml-capacity C18 bonded particles (Hichrom, Reading, UK). This was coupled, via 0.1 M solution of D-saccharic acid-1,4-lactone (Sigma) in distilled water was added to the sulfatase sample to inhibit β-glucuronidase activity. The samples were incubated at 37°C overnight (~16 h) using a shaking water bath. Control samples of pH-adjusted urine were also incubated. All samples were analyzed by 19F NMR spectroscopy as described above.

HPLC-NMR Analysis of Urine. The HPLC system consisted of a Bruker (Coventry, UK) LC22 pump with a Bischoff Lambda 1000 variable wavelength UV detector set at 254 nm and a Hypersil BDS C18 (150 × 4.6 mm) column with 5 µm particles (Hichrom, Reading, UK). This was coupled, via the Bruker BPSU-12 collector, to a 1H–19F flow-probe (cell volume 120 µl) of a Bruker DRX-500 spectrometer operating at 500.13 (1H)- and 470.59 (19F)-MHz observation frequencies. For stop-flow HPLC–19F NMR experiments, typically 400 to 1000 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 preirradiation during the relaxation delay of 2.0 s and the mixing time of 0.10 s. For stop-flow HPLC–19F NMR experiments, typically 400 to 5000 free induction decays were collected over a spectral width of 14,120 Hz into 16,384 data points using a relaxation delay of 0.50 s and an acquisition time of 0.58 s. The 19F free induction decays were multiplied by an exponential function corresponding to a 2-Hz line broadening, and the 1H NMR spectra were resolution-enhanced using a Lorentzian-to-Gaussian transformation function before Fourier transformation. 19F NMR chemical shifts were referenced to trifluoroacetic acid at δF = −75.72 relative to CFCl3 at δF = 0.0 and 1H chemical shifts were referenced to the acetonitrile signal at δH = 2.0 relative to trimethylsilanylpromionic acid at δF = 0.0. Two solvent systems were used for HPLC-NMR. Solvent system 1 was formed of D2O (Fluorochem Ltd) containing 0.05 M potassium dihydrogen phosphate (Fisons Ltd, Loughborough, UK) adjusted to pH 2.5 with phosphoric acid and Pestanal grade acetonitrile (Riedel-deHaen, Germany) at a flow rate of 1 ml min⁻¹. Gradient elution was used starting at 5% acetonitrile increasing to 50% after 30 min. Solvent System 2 consisted of D2O containing 0.01 M ammonium formate (Fisons Ltd) adjusted to pH 7.0 with ammonia solution and HPLC grade methanol (Fisons Ltd) at a flow rate of 1 ml/min. Gradient elution was used starting at 5% methanol increasing to 65% after 40 min. Between 10 and 50 µl of the extracted urines were injected onto the column.

HPLC-MS Analysis of Urine. HPLC-MS was performed with a Finnigan LCQ ion-trap mass spectrometer coupled to a Hewlett-Packard 1100 series HPLC system using a Hypersil HSDBS C18 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 acetonitrile (HPLC grade) linear gradient of 5 to 95% over 0 to 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. Ionization was by negative-electrospray and centroid mass spectra were acquired up to m/z = 600 using a cone voltage of 20 to 30 V.

Results

19F NMR Profiling of Urine Samples. Figure 1a and b show the 19F NMR spectra of typical urine samples obtained for the 0- to 8-h period postdosing of MeTFMA (I, δF = −59.98) and 3-methyl-4-trifluoromethyl-[13C]-acetanilide (MeTFMAC, II) (δF = −61.19). Metabolism was extensive with only small amounts of either of the administered compounds detected. In general the 19F NMR metabolite profiles for each time point were very similar irrespective of whether the free amine or the acetalanilide was administered. Up to ten metabolites were detected in the 0- to 8-h urine whereas only two metabolites, at δF = −59.25 and δF = −59.54, were present to any extent in the 8- to 24- and 24- to 48-h samples. It is noteworthy that the major metabolite in the early time point samples, detected at δF = −59.25, declined in importance with time relative to the metabolite at δF = −59.54.

Enzyme Hydrolysis Studies. To gain additional information on the presence of sulfate and glucuronide conjugates, enzyme hydrolysis experiments analyzed by 19F NMR were performed on urine samples collected 0- to 8-h after dosing with MeTFMA; the urine samples were deemed to be representative of both MeTFMA- and MeTFMAC-dosed animals. After β-glucuronidase treatment, the major peak at δF = −59.25 disappeared, indicating it to be a glucuronide conjugate, with concomitant production of the aglycone giving a signal at δF = −59.45. The minor peak at δF = −59.93 was lost in both control and enzyme incubations, showing that this metabolite was labile at pH 7. The two minor, unresolved, peaks at approximately δF = −60.6 also disappeared, suggesting that they also were glucuronides. However, there were no new or enhanced peaks that could be attributed to their liberated aglycones. Arysulfatase treatment resulted in the loss of the two small peaks at δF = −59.67 and δF = −60.21, suggesting the presence of sulfate conjugates although no peaks corresponding to the aglycones could be detected. This type of behavior is often noted for both sulfates and glucuronides when the aglycones are volatile. Once again, the minor peak at δF = −59.93 was seen to be labile at pH 5. The result of these incubations suggested that the major metabolite present in the 0- to 8-h urine (δF = −59.25) was a glucuronide conjugate and two other minor glucuronides were detected along with two minor sulfate conjugates. An N-glucuronide of MeTFMA was implied by the lability of the peak at δF = −59.93 at pH 7 and 5. The major metabolite in the later time point samples (δF = −59.54) did not change after incubation with

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either β-glucuronidase or arylsulfatase, indicating that it was not a glucuronide or sulfate conjugate.

Metabolite Identification Using HPLC-NMR and HPLC-MS. To determine the identities of these metabolites, HPLC-NMR studies were performed on a 0- to 8-h urine sample obtained after dosing MeTFMA using solvent system 1. The UV-detected chromatogram of this sample is shown in Fig. 2. The major peak with a retention time of 15.7 min was analyzed under stop-flow conditions, by $^{19}$F/$^1$H NMR. This peak was a single metabolite with a $^{19}$F NMR chemical shift matching that of the major metabolite in the 0- to 8-h urine ($\delta_F = 59.25$). The stop-flow $^1$H NMR spectrum of this peak is shown in Fig. 3a. Signals at $\delta_H 4.6$ and $\delta_H 2.0$ were due to the residual water and acetonitrile from the HPLC mobile phase and the signals at $\delta_H 3.3$ and $\delta_H 2.4$ were from methanol and propionitrile contamination of the acetonitrile. The remaining signals in this spectrum were due to the metabolite, and it could clearly be seen that it was a glucuronide conjugate with doublets at $\delta_H 4.51$ and $\delta_H 3.88$ from the H1′ and H5′ sugar protons. The coupling constant ($^3J_{H-H}$) of the anomeric proton (H1′) was 7.7 Hz, indicating that it was a β-D-glucuronide. This result was consistent with the enzyme hydrolysis studies using β-glucuronidase, which also showed that this metabolite was a β-D-glucuronide conjugate. The other sugar ring protons could also be seen as triplets at $\delta_H 3.53$ (H4′), $\delta_H 3.44$ (H3′), and $\delta_H 3.34$ (H2′). The aromatic methyl, if present, would have been expected to give a resonance at approximately $\delta_H 2.2$ to 2.4 but this was absent. Instead, there was a resonance centered at $\delta_H 4.92$ comprising an AB-quartet from two nonequivalent protons. The chemical shift and splitting are consistent with a benzylic CH$_2$ attached to a chiral moiety. The aromatic region of the spectrum of this metabolite showed that all the aromatic protons of the parent compound were present. The chemical shifts of these protons (H2 at $\delta_H 7.78$, H5 at $\delta_H 7.68$, and H6 at $\delta_H 7.62$) were similar to those of MeTFMAc rather than the free aniline (MeTFMA), suggesting that this metabolite might also have been N-acetylated. The acetyl methyl signal at approximately $\delta_H 2.1$ (if present) could not be observed due to the acetonitrile resonance from the HPLC mobile phase. In this solvent system this metabolite had a retention time of 23.9 min. The stop-flow $^1$H NMR spectrum, although still dominated by an acetonitrile resonance due to residual solvent in the system, did, however, show a resonance from the putative N-acetyl methyl protons as a singlet at $\delta_H 2.12$ (between the acetonitrile signal and its $^{13}$C-satellite). In all, the evidence for the structure of this metabolite leads to 2-trifluoromethyl-5-acetamidobenzylglucuronide (III). This was further supported by the analysis of samples after the dosing of $^{13}$C-labeled MeTFMAc. When the methanol-based solvent system was used, the $^1$H NMR
The spectrum of the acetyl methyl signal showed a doublet (due to the presence of the $^{13}$C-label), with a coupling constant ($\Delta_j^\text{C-H}$) of 6.2 Hz, confirming the presence of the N-acetyl group. Within this doublet the singlet of the acetyl methyl of the $^{12}$C-labeled metabolite, i.e., containing the naturally occurring endogenous acetyl moiety, was also observed. For this metabolite to be produced after dosing only with $^{13}$C-MeTFMAc, the compound must have undergone $N$-deacetylation then reacetylation, i.e., a futile deacetylation cycle. The proportion of $^{13}$C- and $^{12}$C-labeled metabolite was determined by estimation of the corresponding peak areas and it was found that the ratio was approximately 1:1. HPLC-MS of this metabolite, after dosing MeTFMA and MeTFMAc, gave the expected $[M - \text{H}]^-$ ion for this metabolite at either $m/z = 408$ (MeTFMA) or $m/z = 408/409$ (50:50) (MeTFMAc). The observed ratio of the relative proportions of $^{12}$C and $^{13}$C labeled in the metabolite was in agreement with those estimated by NMR.

The major metabolite in the later time point samples ($\delta_p - 59.54$), retention time 18.4 min in HPLC solvent system 1, gave the stop-flow $^1H$ NMR spectrum shown in Fig. 3b, which shows only two metabolite-related signals for aromatic protons with no evidence for an aromatic methyl signal. As signals attributable to a -CH$_2$OR moiety were also absent, it seemed most likely that the methyl group had been completely oxidized to a carboxylic acid. From the lack of sugar protons an ester glucuronide could be discounted and there was no evidence for amino acid conjugation in the NMR spectrum either. Integration of the two peaks in the aromatic region showed that they were in the ratio of 2:1, therefore, the signal at $\delta_H 7.76$ contained two unresolved chemical shifts corresponding to protons H5 and H6. The singlet at $\delta_H 7.96$ corresponded to proton H2. The chemical shifts of these aromatic protons were downfield from the parent compound, due in part to the electron-withdrawing carboxyl group but may be also indicating that the amino group was probably N-acetylated. This was confirmed by HPLC with the methanol-based solvent system (both on this and the corresponding MeTFMAc-derived metabolite). Separate HPLC-MS studies were performed to provide confirmation of this result. The negative-ion electrospray mass spectra of this metabolite, produced after dosing either MeTFMA or MeTFMAc were essentially the same except that doublet isotope patterns were observed after dosing MeTFMAc due to the presence of both $^{13}$C- and $^{12}$C-labeled material. The peak, corresponding to 2-trifluoromethyl-5-acetamidobenzoic acid [MW = 247 ($^{12}$C) or 248 ($^{13}$C)] showed a molecular ion $[M - \text{H}]^-$ at $m/z = 246$ or 247. Thus, using a combination of NMR and MS this metabolite was identified as 2-trifluoromethyl-5-acetamidobenzoic acid (IV). HPLC-NMR of a urine sample obtained 0 to 8 h after administration of MeTFMAc with stop-flow $^1H$ NMR confirmed that a futile deacetylation/reacetylation reaction had occurred. As with HPLC-MS it was estimated by NMR that approximately 50% of the material had undergone this metabolic process, as the ratio of $^{12}$C-$^{13}$C-labeled isotopomers was 1:1.

Stop-flow $^1H/^{19}$F NMR spectra of the minor metabolites of MeTFMA and MeTFMAc were also obtained to determine their structures. A peak eluting at 29.5 min in solvent system 2 (not shown) contained two metabolites with $^{19}$F NMR chemical shifts of $\delta_F - 59.7$ and $\delta_F - 60.2$. The stop-flow $^1H$ NMR spectrum of this peak showed two aromatic methyl signals, at $\delta_H 2.27$ and $\delta_H 2.30$, respectively. The largest component gave rise to two signals in the aromatic region at $\delta_H 7.38$ and $\delta_H 6.73$, which were both singlets, indicating that oxidation had occurred at the 6-position. The minor metabolite also had two aromatic signals, at $\delta_H 7.24$ and $\delta_H 6.70$, present as doublets, indicating that oxidation had occurred at the 2-position. The chemical shifts of the ortho protons H2 and H6 were similar to those of MeTFMA, indicating that both of these metabolites still contained a free amino group and there was no evidence of acetyl methyl protons in the spectra. These metabolites were shown to be sulfate conjugates by enzyme hydrolysis studies and correspond to 2-amino-4-methyl-5-trifluoromethylphenylsulphate (V) and 2-methyl-3-trifluoromethyl-6-aminophenylsulphate (VI). HPLC-MS gave the mass spectra with...
an [M–H]⁻ at m/z = 270, corresponding to a molecular weight of 271 consistent with the sulfate structure proposed by the NMR and enzyme hydrolysis studies.

Figure 4a shows the ¹H NMR spectrum of a MeTFMA metabolite peak that contained a ¹⁹F NMR signal at δF = –60.6. This was obtained from stop-flow analysis in HPLC solvent system 1 of the peak with a retention time of 17.5 min (Fig. 1). In the aromatic region, signals from two metabolites could be seen with a pattern similar to those observed for the previously described sulfate conjugates. A pair of singlets, corresponding to one metabolite with substitution at the 6-position and a pair of doublets, were also observed, indicating that the other metabolite was substituted at the 2-position. Two separate aromatic methyl signals could be seen at approximately δH 2.4, showing this moiety was intact in both metabolites. The results of the enzyme hydrolysis studies described above implied that these compounds were both glucuronide conjugates [2-amino-4-methyl-5-trifluoromethylphenylglucuronide (VII) and 2-methyl-3-trifluoromethyl-6-aminophenylglucuronide (VIII) respectively]. However, only one set of signals from the sugar protons of the glucuronic acid moiety were observed using HPLC-NMR (Fig. 4a) which, based on relative peak areas, were probably due to 2-substituted metabolite. The metabolite substituted at the 6-position may have undergone hydrolysis before acquisition of the ¹H NMR spectrum. HPLC-MS of this peak gave an [M–H]⁻ ion at m/z = 366 consistent with these metabolites being the proposed glucuronides (MW = 367).

Figure 4b shows the ¹H NMR spectrum of another minor metabolite with a ¹⁹F NMR chemical shift of δF = –59.93. This was acquired using solvent system 1 by stop-flow HPLC-NMR on the peak (retention time 21.2 min, Fig. 2) detected in a 0- to 8-h MeTFMA sample. The spectrum clearly shows the metabolite to be a glucuronide conjugate with doublets at δH 4.74 (partially obscured by contamination) and δH 3.91 attributable to the H1⁹ and H5⁹ sugar protons, respectively. The triplets corresponding to the other glucuronide protons were also observed at δH 3.44 to 3.55. A signal due to the aromatic methyl group was present at δH 2.39 (on top of the quartet from the propionitrile contamination), showing that this group was intact in this metabolite. In the aromatic region of the spectrum, all three aromatic protons of the parent compound were present, showing that this metabolite must result from N-glucuronidation of the amino group. The lability of this metabolite in the enzyme hydrolysis studies also implies a primary N-glucuronide of the parent compound, identifying it as 3-methyl-4-trifluoromethylaniline-N-glucuronide (IX). HPLC-MS studies failed to detect an ion corresponding to this metabolite identified; however, given the lability of this type of metabolite, this is perhaps not surprising. Based on these studies, the metabolic fate and urinary excretion of MeTFMA and MeTFMAc is summarized in Fig. 5.
Quantification of the Metabolites of MeTFMA and MeTFMAc.
The mean percentages of the dose recovered as the metabolites identified in urine up to 48 h after dosing MeTFMA and MeTFMAc are shown in Table 1 as determined by $^{19}$F NMR spectroscopy. Total recoveries of 51.1 ± 3.2 and 68.3 ± 0.2% of dose were achieved in urine up to 48 h after dosing MeTFMA and MeTFMAc, respectively. The higher value associated with MeTFMAc was due a greater recovery of 2-trifluoromethyl-5-acetamidobenzylglucuronide (III) (34.0 ± 0.5% of the dose, compared with only 18.6 ± 3.5% after dosing MeTFMA). The other major metabolite, 2-trifluoromethyl-5-acetamidobenzoic acid (IV), represented approximately 20% of the dose for each test compound. The $\text{ortho}$-hydroxylated sulfates [2-amino-4-methyl-5-trifluoromethylphenylsulphate (V) and 2-methyl-3-trifluoromethyl-6-aminophenylsulphate (VI)] accounted for approximately 5% of dose for each compound whereas the corresponding glucuronide conjugates [2-amino-4-methyl-5-trifluoromethylphenylglucuronide (VII) and 2-methyl-3-trifluoromethyl-6-aminophenyl glucuronide (VIII)] only accounted for approximately 2%. Recovery of the $N$-glucuronide of MeTFMA (IX) was quite variable but was less than 1% of the dose in each case. Overall urinary excretion was more rapid after administration of MeTFMAc, with 38.9 ± 7.6% recovered in the 0- to 8-h urine, compared with 15.3 ± 12.0% after dosing MeTFMA. Approximately 30% was recovered 8 to 24 h after dosing each compound with an additional 3.1 ± 1.0 and 1.5 ± 0.7% found in the 24- to 48-h samples after dosing MeTFMA and MeTFMAc, respectively.

Discussion

The metabolites identified in urine after dosing MeTFMA and MeTFMAc were identical. $N$-acetylation/deacetylation/reacetylation (futile acetylation) and oxidation of the methyl group were the major routes of metabolism for both compounds, followed by further metabolism to form glucuronide and sulfate conjugates. This study once again confirms the value of stable isotope labeling at the $N$-acetyl group to determine the extent of futile acetylation or deacetylation reactions in this class of compounds. Approximately 50% of the $N$-acetylated metabolites recovered after dosing MeTFMAc were shown to have undergone futile deacetylation. After dosing the structurally related 3- and 4-trifluoromethylacetanilide to rat, 40 and 23% of the respective metabolites with the $N$-acetyl residue intact were shown to have undergone futile deacetylation (Tugnait, 1994; Wilson et al., 1985). The proportion of futile deacetylation after dosing MeTFMAc was also very high in comparison with results with paracetamol (acetaminophen), where approximately 10% of the paracetamol sulfate and glucuronide metabolites had undergone futile deacetylation (Nicholls et al., 1997a). This futile deacetylation and subsequent transient flux through the metabolic intermediate and potent nephrotoxin (Green et al., 1969), 4-aminophenol, is believed to
FIG. 5. Summary of the urinary metabolic fate of MeTFMA and MeTFMAc in the rat.

Values in brackets refer to the mean percentage of the dose metabolized by that route.

TABLE 1

Percentage of dose recovered as the metabolites identified in urine up to 48 h after dosing MeTFMA and MeTFMAc (derived from $^{19}$F NMR spectroscopic data, Fig. 1)

<table>
<thead>
<tr>
<th>Metabolite Identified</th>
<th>$\delta$/ppm</th>
<th>% Dose in 0- to 8-h Urine</th>
<th>% Dose in 8- to 24-h Urine</th>
<th>% Dose in 24- to 48-h Urine</th>
<th>Total % Dose (0- to 48-h Urine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Trifluoromethyl-5-acetamidobenzoic (IV)</td>
<td>−59.54</td>
<td>1.7 ± 1.2</td>
<td>5.6 ± 2.2</td>
<td>21.4 ± 3.3</td>
<td>28.7 ± 4.5</td>
</tr>
<tr>
<td>2-Trifluoromethyl-5-acetamidobenzylglucuronide (III)</td>
<td>−59.25</td>
<td>6.9 ± 5.2</td>
<td>22.7 ± 3.8</td>
<td>10.7 ± 3.3</td>
<td>39.4 ± 5.5</td>
</tr>
<tr>
<td>2-Amino-4-methyl-5-trifluoromethylphenylsulphate (V)</td>
<td>−60.21</td>
<td>2.5 ± 2.5</td>
<td>3.7 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>2-Amino-4-methyl-5-trifluoromethylphenylglucuronide (VII)</td>
<td>−61.60</td>
<td>1.7 ± 1.7</td>
<td>2.0 ± 1.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2-Methyl-3-trifluoromethyl-6-aminophenylglucuronide (VIII)</td>
<td>−59.67</td>
<td>0.7 ± 0.7</td>
<td>0.9 ± 0.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3-Methyl-4-trifluoromethyl aniline-N-glucuronide (IX)</td>
<td>−59.93</td>
<td>0.6 ± 0.9</td>
<td>1.2 ± 1.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MeTFMA (I)</td>
<td>−59.98</td>
<td>0.2 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>ND</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>MeTFMAc (II)</td>
<td>−61.19</td>
<td>ND</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.6</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Minor metabolites*</td>
<td>0.9 ± 0.7</td>
<td>2.1 ± 0.8</td>
<td>2.7 ± 0.9</td>
<td>3.3 ± 0.5</td>
<td>3.6 ± 0.5</td>
</tr>
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</table>

Total 15.3 ± 12.0  38.9 ± 7.6  32.7 ± 12.8  27.9 ± 6.8  3.1 ± 1.0  1.5 ± 0.7  51.1 ± 3.2  68.3 ± 0.2

Values are mean data (n = 3) ± S.D.

* Comprise five minor unidentified metabolites. ND = not detected.
play a role in the toxicity of paracetamol. The futile deacetylation after dosing phenacetin (a more potent nephrotoxin) was found to be approximately 30% (Nicholls et al., 1997b), also lower than MeTFMAc. The major metabolites thus identified were 2-trifluoromethyl-5-acetamidobenzylglucuronide (III) and 2-trifluoromethyl-5-acetamidobenzoic acid (IV), each accounting for approximately 20% of the dose. The importance of acetylation in the case of MeTFMA contrasts strongly with the metabolic fates of trifluoromethylanilines substituted with a halogen (chloro or bromo), rather than a methyl group, at the 2-position where acetylation did not occur to any significant extent (Scarfe et al., 1998, 1999). The presence of the methyl also seems to have prevented the formation of measurable quantities of N-oxanilic acids of the type produced in the case of simple para-halogen substituted (chloro, trifluoromethyl, and bromo) anilines (Wilson et al., 1985; Ehlhardt and Howbert, 1991; G.B.S., I.D.W. and J.K.N., manuscript in preparation). We are currently using computational chemistry and pattern recognition techniques to investigate the structure-metabolism relationships of substituted anilines with respect to N-acetylation and subsequent o-hydroxylation to produce N-oxanilic acid metabolites (G.B.S., I.D.W. and J.K.N., manuscript in preparation). The in vivo metabolic data obtained from the studies on MeTFMA and MeTFMAc were an important addition to the database used for these investigations.

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References


