Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, Nos 8–12, pp. 939–944, 1990
Printed in Great Britain

0731–7085/90 $3.00 + 0.00
© 1990 Pergamon Press plc

Short Communication

Studies on the metabolism of fluorinated xenobiotics in the rat using ^{19}F-NMR and ^{1}H-NMR spectroscopy*

F.Y.K. GHAURI,‡ C.A. BLACKLEDGE,‡ I.D. WILSON§ and J.K. NICHOLSON†‡

‡ *Department of Chemistry, Birkbeck College, Gordon House, 29 Gordon Square, London WC1H 0PP, UK*
§ *Drug Kinetics, I.C.I. Pharmaceuticals, Mereside, Alderley Park, Macclesfield SK10 4TG, UK*

Keywords: *^{19}F-NMR spectroscopy; trifluoromethyl- and fluoro-benzoic acid; SPEC–NMR; transacylation.*

Introduction

^{19}F-NMR spectroscopy can be used effectively to obtain information on the metabolism of fluorinated xenobiotics without the need for radiolabelling [1–3]. ^{19}F-NMR has several useful features in metabolism studies on fluorodrugs namely, negligible interference in biofluids from endogenous fluorinated compounds (contrasting with ^{1}H-NMR) and a high detection sensitivity (83% of ^{1}H). Furthermore, the large chemical shift range over which the ^{19}F resonances extend, provides high sensitivity to structural and electronic changes in the molecule at sites over eight bonds distant to the ^{19}F atoms. The presence of a ^{19}F atom thus provides a long range probe to monitor structural changes. In the present study, as part of a wide programme investigating structure–metabolism relationships, we have used ^{19}F-NMR to probe the metabolic fate of *o*-, *m*- and *p*-trifluoromethylbenzoic acids (TFMBA) and *p*-fluorobenzoic acid (*p*-FBA) in the rat. A combination of single-pulse ^{19}F and ^{1}H-NMR experiments, chemical modification of the sample and SPEC–NMR [4, 5] methods, were applied for structure elucidation studies on the metabolites present in biofluids.

Materials and Methods

Dosing and sample preparation

Male Sprague–Dawley rats (200–250 g) were dosed with *p*-TFMBA in corn oil; *o*- and *m*-TFMBA in sodium bicarbonate–saline solution (50:50, v/v) and *p*-fluorobenzoic acid in phosphate buffer–saline solution (50:50, v/v), all at 100 mg kg^{-1} i.p. The rats were kept in metabolic cages whilst urine was collected over the following 48 h post-dose. Urinary volumes and pHs were recorded and samples stored at −40°C until analysed.

NMR measurements

Samples of whole urine (0.5 ml) with 0.1 ml of $^{2}H_2O$ added as an internal field frequency lock were made on Varian VXR 400 and Bruker AM 400 spectrometers operating at 9.4 T field strength (376 MHz ^{19}F frequency) and on a JEOL GSX 500 spectrometer operating at 11.75 T field strength (500 MHz ^{1}H frequency).

^{1}H-NMR spectroscopy

^{1}H-NMR spectra (500 MHz) were measured using 45° pulse (3 μs), over a 6000-Hz spectral width. Typically, 128–256 FIDs (free induction decays) were collected into 32,768 computer points; acquisition time of 2.73 s. A further delay of 2.27 s between pulses was used to ensure that the spectra were obtained under fully T_1 relaxed conditions. The FIDs were multiplied by an exponential function corresponding to a 0.2-Hz line broadening prior to Fourier transformation. A gated irradiation field at the water resonance frequency was applied in order to suppress the intense signal due to water protons. Chemical shifts were

* Presented at the "Second International Symposium on Pharmaceutical and Biomedical Analysis", April 1990, York, UK.

† Author to whom correspondence should be addressed.

referenced externally to sodium 3-(trimethylsilyl)-1-propanesulphonate (δ = 0 ppm).

^{19}F-NMR spectroscopy

Proton-coupled [^{19}F–^{1}H] and decoupled ^{19}F{^{1}H} spectra were measured at 376 MHz using a 45° pulse (15 μs), over a 8000-Hz spectral width. Typically, 128–512 FIDs were collected into 8192 computer points; acquisition time of 0.5 s. A further delay of 4 s (2.5 s for ^{19}F{^{1}H} spectra) between pulses was added to allow T_1 relaxation. The FIDs were multiplied by an exponential function corresponding to 0.5 Hz line broadening prior to Fourier transformation. Chemical shifts were referenced externally to $CFCl_3$ (δ = 0 ppm).

Chemical and enzymatic modification of biofluid samples

(i) In order to identify ester glucuronide conjugates, sodium deuteroxide was added to 200–400 μl of post-dose urine and ^{19}F-NMR spectra recorded.

(ii) In order to identify the presence of ester or ether glucuronide conjugates, 150–350 μl of 0.8 h post-dose urine was incubated with β-D-glucuronidase (*Escherichia coli*) at pH 6.8 and 310 K for 18 h, and analysed by ^{19}F and ^{1}H-NMR.

(iii) In order to characterize further and identify major metabolites, attempts were made to isolate and purify them using solid-phase extraction chromatography followed by NMR detection (SPEC–NMR). Acidified urine (pH 2.2) was loaded onto a preconditioned C_{18} (Bond–Elut^{TM}) column at pH 2 to retain ionizable metabolites. Metabolites were eluted by a step-gradient of methanol (acidified with 2 M HCl to pH 2) from 0 to 100% in 20% subfractions. Solvent was removed using a stream of nitrogen and freeze-drying before being redissolved in $^{2}H_2O$ prior to NMR analysis.

Results and Discussion

^{19}F-NMR spectra of whole biofluids can be obtained rapidly with minimum sample pretreatment [1]. Studies on the metabolism of monofluorinated and trifluoromethylated aromatic compounds using ^{19}F-NMR have shown that it is possible to identify the number and proportion of fluorinated metabolites present in a biofluid [6] from single-pulse ^{19}F-NMR spectra of whole or freeze-dried samples. The ^{19}F atoms present on the trifluoromethyl-group are magnetically equivalent and any couplings between fluorine and protons on the benzyl ring are too small to be measured. Therefore, proton decoupling is not required to obtain singlet resonances for each CF_3 group. In the case of monofluorinated compounds where fluorine is bonded directly to the benzene ring (e.g. *p*-FBA), J_{F-H} couplings are significant and the resulting splitting of the ^{19}F signals is easily detected. Proton decoupling was thus required to obtain single ^{19}F signals. However, these compounds could be used in ^{19}F–^{1}H spin–echo difference and shift correlation multipulse NMR experiments which can provide information on the sites of metabolism.

^{1}H-NMR spectroscopy

Proton NMR spectra of 0–8 h post-dose urine showed resonances due to the metabolite peaks in the aromatic region (δ = 6–8 ppm) but identification of specific signals was difficult due to overlap from endogenous urinary metabolites. However, a resonance at δ = 3.91 ppm was observed due to the glycine conjugates of *m*-TFMBA and *p*-FBA (data not shown).

^{19}F-NMR spectroscopy

^{19}F-NMR spectra of 0–8 h post-dose urine containing metabolites of *o*-, *m*- and *p*-TFMBA are shown in Fig. 1. Chemical shifts due to parent compound were identified by standard addition.

o-Trifluoromethylbenzoic acid

The ^{19}F resonance due to the major metabolite of *o*-TFMBA was observed at δ = −59.67 ppm and accounted for approximately 80% of the total. At least seven minor metabolite signals were also observed and accounted for <5% of the total (Fig. 1A). From the ^{1}H-NMR spectrum of the 0–8 h treated urine, resonances from the α-anomeric proton at δ = 5.2 ppm due to the presence of an ester glucuronide, were identified. Mild alkaline hydrolysis using sodium deuteroxide at 298 K of treated urine, monitored by ^{19}F-NMR, resulted in no change in intensity of the major metabolite signal at δ = −59.67 ppm, but did result in the disappearance of the signal at δ = −59.06 ppm (one of the minor metabolites), suggesting the presence of an ester glucuronide (glucuronic acid conjugate of a carboxylic acid). ^{19}F-NMR

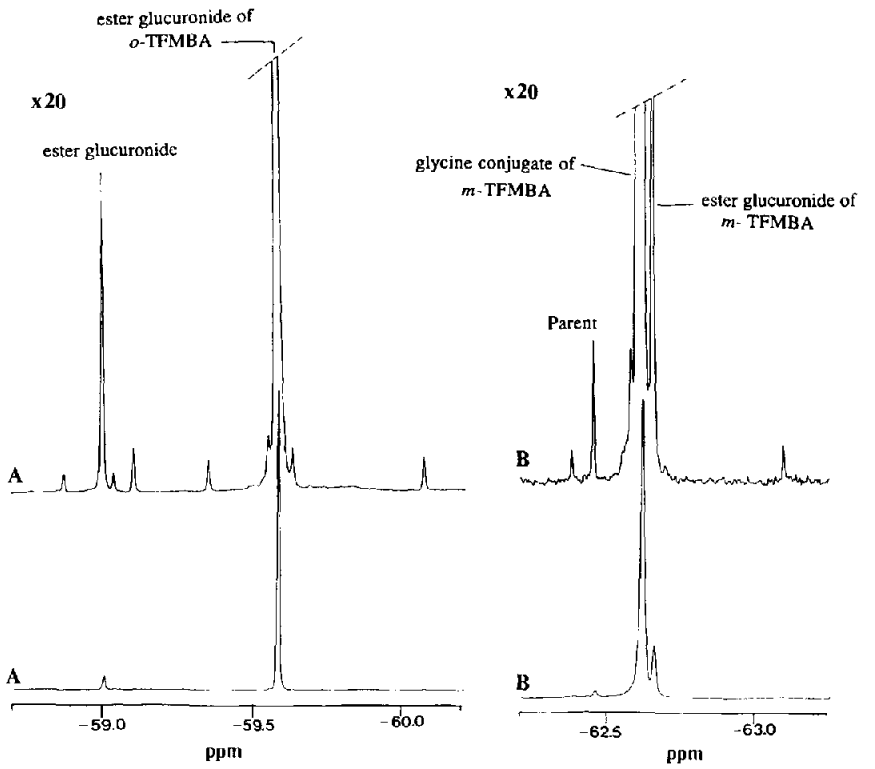

Figure 1
^{19}F–^{1}H-NMR spectra (376 MHZ) of 0–8 h post-dose (100 mg kg^{-1}) rat urine: A, *o*-TFMBA; and B, *m*-TFMBA.

spectra obtained from treated urine incubated with β-D-glucuronidase enzyme at 310 K showed the complete disappearance of the signal at δ = −59.06 ppm, providing further evidence to suggest the presence of a β-D-ester glucuronide, but was seen to have no effect on the major metabolite at δ = −59.67 ppm. The failure of mild alkaline or enzymic hydrolysis to effect cleavage of the conjugated metabolite at δ = −59.67 ppm, suggests that this was probably due to steric hindrance effects between the —CF_3 group and the carboxylic group. Solid-phase extraction chromatography provided relatively pure (by ^{1}H-NMR) extracts of the metabolites. From the ^{1}H-NMR spectra of the 60 and 80% acidified methanol extracts, resonances, in particular that of the α-anomeric proton at δ = 5.2 ppm due to the presence of an ester glucuronide, were identified. Fast atom bombardment (FAB) mass spectrometry of these extracts confirmed the identification of the ester glucuronide (unpublished data).

m-*Trifluoromethylbenzoic acid*

The ^{19}F resonance due to the major metabolite was observed at δ = −62.59 ppm and accounted for 70% of the total. The signal for unmetabolized parent compound appeared at δ = −62.4 ppm (Fig. 1B) with an additional signal at δ = −62.64 ppm which accounted for <10% of the total. Urine incubated at 310 K with β-D-glucuronidase at pH 6.8 resulted in the complete disappearance of the signal at δ = −62.64 ppm and a corresponding increase in that of the parent compound at δ = −62.4 ppm. This suggested that the signal at δ = −62.64 ppm was an ester β-D-glucuronide, of the parent acid. Solid-phase extracts of treated urine showed the major metabolite at δ = −62.59 ppm, to be spectroscopically pure by ^{1}H-NMR. From the ^{1}H-NMR spectra, resonances at δ = 3.91 ppm due to glycine conjugation of *m*-TFMBA were identified.

p-*Trifluoromethylbenzoic acid*

For ^{19}F-NMR spectra of 0–8 h post-dose urine, four metabolite signals (Fig. 2A) and a signal for the parent compound at δ = −64.2 ppm, were observed. Alkaline hydrolysis of treated urine (Figs 2D and 2E) using sodium deuteroxide at 298 K monitored by ^{19}F-NMR, resulted in the total disappearance of signals at δ = −64.72, δ = −64.76 and δ = −64.8 ppm,

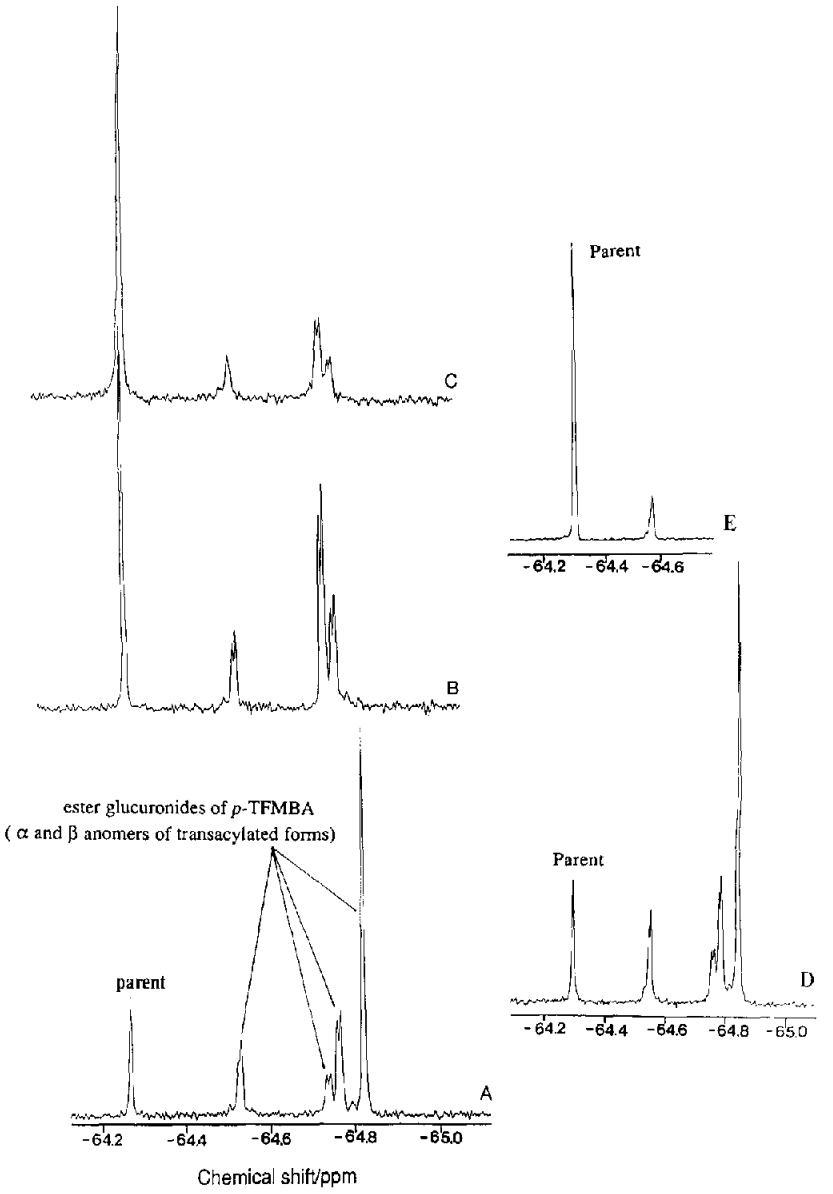

Figure 2
^{19}F–^{1}H-NMR spectra (376 MHZ) of urine from a rat dosed with *p*-TFMBA: A, 0–8 h post-dose urine; B, urine (pH 6.8) incubated at 310 K; C, urine incubated with β-D-glucuronidase (pH 6.8) at 310 K; D, 0–8 h post-dose urine; and E, urine after alkaline hydrolysis (pH 10) at 293 K.

and a corresponding increase in the intensity of the parent compound with only the signal at $\delta = -64.52$ ppm remaining, suggesting the presence of ester glucuronides. Incubation of treated urine at 310 K resulted in the complete disappearance of the signal at $\delta = -64.8$ ppm which accounted for 10% of the total dose, suggesting the presence of an unstable ester glucuronide (Fig. 2B). Hydrolysis with β-D-glucuronidase at 310 K (Fig. 2C) also caused the disappearance of the peak at $\delta = -64.8$ ppm, with a corresponding increase in parent signal intensity at $\delta = -64.2$ ppm. A decrease in the ^{19}F signals at $\delta = -64.52$ and $\delta = -64.76$ ppm, and an increase in the ^{19}F signals at $\delta = -64.72$ ppm were also observed, suggesting that these metabolites are probably rearranged conjugates of β/D-glucuronic acid. Resistance of ester glucuronides to enzymic hydrolysis but not to alkaline hydrolysis has previously been reported [8, 9] and was found to occur with ester glucuronides which have undergone transacylation from the 1-*O*-acyl to the 2-*O*-acyl, 3-*O*-acyl and the 4-*O*-acyl

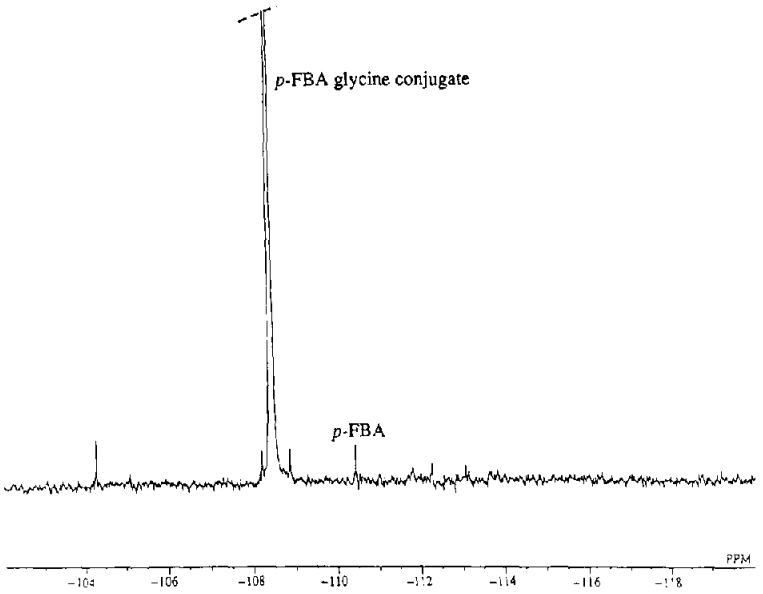

Figure 3
^{19}F{^{1}H}-NMR spectrum (376 MHZ) of 0–8 h urine from a rat dosed with *p*-FBA (100 mg kg^{-1}).

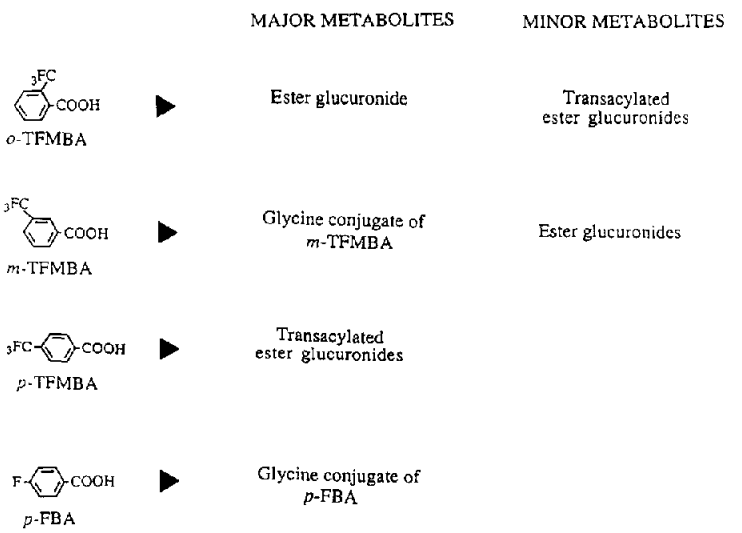

Figure 4
Metabolic routes of *o*-, *m*- and *p*-TFMBA and *p*-FBA in the rat.

glucuronide; the most resistant ester glucuronide being the 4-*O*-acyl glucuronide, in this case occurring at $\delta = -64.52$ ppm. The splitting of the two resonances at $\delta = -64.72$ and $\delta = -64.76$ ppm also observed by ^{19}F-NMR was probably due to the presence of two stereochemical forms of the α- and β-anomers of the 2-*O*-acyl and 3-*O*-acyl glucuronides caused by mutarotation of these ester glucuronic acid conjugates.

p-Fluorobenzoic acid. The ^{19}F{^{1}H}-NMR spectra of urine containing *p*-FBA is shown in Fig. 3. Three resonances from metabolites were observed, the major resonance occurring at $\delta = -108.4$ ppm (accounting for 70% of the total) was due to glycine conjugation of *p*-FBA. This was confirmed by ^{1}H-NMR coupled with SPEC–NMR and FAB mass spectrometry on extracts of these samples. A summary of the major metabolic pathways in the rat of the above compounds is shown in Fig. 4.

Conclusions

These studies show that ^{19}F-NMR provides a

powerful tool for the investigation of the fate and disposition of fluorinated xenobiotics. Chemical modification and/or enzymatic hydrolysis of the fluoroxenobiotic metabolites *in situ* coupled with SPEC using ^{19}F and ^{1}H-NMR detection can be used for identification and structure elucidation of fluorinated metabolites in biofluids. Thus ester glucuronides of *p*-TFMBA and *o*-TFMBA were identified as major metabolites whereas the major metabolite of *m*-TFMBA was identified as the glycine conjugate of the parent acid. ^{19}F was able to distinguish the α- and β-anomers of the transacylated forms of the ester glucuronides. Clearly, the electronic effect of the fluorine atom(s) at various positions on the molecule strongly influences the metabolic fate of these compounds. The NMR approach allows rapid identification of metabolites in biomaterials, and as such will be useful in the generation of metabolic data bases for families of fluorinated compounds where structure–metabolism relationships are being studied.

References

[1] J.R. Everett, K. Jennings and G. Woodnutt, *J. Pharm. Pharmacol.* **37**, 869–873 (1985).
[2] J.P. Beteille, A. Lopez, M. Bon, M.C. Malet-Martino and R. Martino, *Anal. Chim. Acta* **171**, 225–231 (1985).
[3] M.C. Malet-Martino and R. Martino, *Xenobiotica* **19**, 583–607 (1989).
[4] I.D. Wilson and J.K. Nicholson, *Anal. Chem.* **59**, 2830–2832 (1987).
[5] I.D. Wilson and J.K. Nicholson, *J. Pharm. Biomed. Anal.* **6**, 151–165 (1987).
[6] K.E. Wade, J.A. Troke, C.M. Macdonald, I.D. Wilson and J.K. Nicholson, *Bioanalysis of Drug and Metabolites*, Vol. 18, pp. 383–388. Plenum Press, New York (1988).
[7] H.P.A. Illing and I.D. Wilson, *Biochem. Pharmacol.* **30**, 3381–3384 (1981).
[8] I.D. Wilson, A. Bhatti, H.P.A. Illing, T.A. Bryce and J. Chamberlain, from *Drug Metabolite Isolation and Detection* (E. Reid and J.P. Leppard, Eds). Plenum Press (1983).

[*Received for review 30 April 1990*]