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An NMR study of the metabolic fate of 2-, 3- and 4-fluorobenzyl alcohols in the rat
Detection of *N*-acetylcysteinyl conjugates as minor metabolites in urine

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Abstract

Following the administration of 2-, 3- and 4-fluorobenzyl alcohols, the major metabolites detected in urine corresponded to the glycine conjugates of the corresponding benzoic acids. Little, or no, unchanged parent compound was detected in the samples. In addition to glycine-conjugated benzoic acids, a small proportion of the urinary metabolites for each of the fluorobenzyl alcohols was found to correspond to *N*-acetylcysteinyl conjugate. These were probably formed as the result of the production of a reactive sulphate ester during metabolism. The overall urinary recoveries of metabolites for the 2- and 3-fluorobenzyl alcohols were lower than that observed for the corresponding benzoic acids whilst that for 4-fluorobenzyl alcohol was similar.
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1. Introduction

^{19}F NMR spectroscopy, in combination with other methods such as ^{1}H NMR spectroscopy and mass spectrometry (MS), provides a practical and readily implemented means to study the metabolic fate of fluorinated xenobiotics without the need for radiolabelled material [1]. We have made extensive use of this methodology in studies of the metabolism of fluorine-substituted aromatic compounds such as substituted anilines, phenols and benzoic acids [2–7]. These investigations have provided a wealth of metabolic data, which have enabled the construction of quantitative structure metabolism relationships (QSMRs) for the prediction of metabolic fate in the rat for a range of substituted aromatic acids [2–5], phenols [6] and

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anilines [7]. As a part of continuing studies aimed at understanding the physicochemical factors that affect the metabolic fate of xenobiotics, we have investigated the metabolic fate of 2-, 3- and 4-fluorobenzyl alcohols (FBALs) for comparison with that of the corresponding 2-, 3- and 4-fluorobenzoic acids (4-FBAs) previously investigated using these NMR spectroscopy-based methods [2–5,8].

2. Experimental

2.1. Chemicals

FBALs and the corresponding substituted benzoic acids were obtained from Fluorochem (Old Glossop, Derbyshire, UK). These compounds were pure by ^{1}H and ^{19}F NMR, and used as received.

2.2. Dosing

Male Sprague–Dawley rats (200–250 g) were divided into groups of three animals each of which was administered one of the substituted benzyl alcohols (100 mg/kg, i.p. in 0.5 ml of 0.9% saline). The animals were housed individually in plastic metabolism cages, designed for the separate collection of urine and faeces, in a well-ventilated room with regular cycles of light and dark (12 h). Food and water were available ad lib.

2.3. Sample collection

Urine was collected over ice for 24 h prior to dosing and for the periods 0–8, 8–24 and 24–48 h post-administration. Urinary volume and pH were recorded on removal of the samples from the collection vessel and the samples were then centrifuged at 3000 rpm for 10 min (4 °C). The samples were then frozen at −20 °C until analysed.

2.4. ^{1}H and ^{19}F NMR spectroscopy

^{1}H NMR spectra of urine and urine extracts were obtained on a Jeol GSX 500 spectrometer operating at a field strength of 11.75 T (500 MHz ^{1}H frequency) at ambient probe temperature. Typically, 128 free induction decays (FIDs) were collected for each sample into 32 768 data points using a spectral width of 6000 Hz and an acquisition time of 2.73 s. A further delay of 2.27 s was used between pulses to ensure that spectra were fully T_1 relaxed. The residual signal for HOD protons was suppressed by applying a gated secondary irradiation field (off during acquisition) at the water resonance frequency. An exponential line-broadening factor of 0.2 Hz was applied prior to Fourier transformation (FT). Chemical shifts were referenced internally to sodium 3-trimethylsilyl-[2,2,3,3,$^{2}H_4$]-1-propionic acid (TSP, 1.0 mg/ml, δ 0.0).

^{19}F NMR spectra were measured on a Bruker AM 400 NMR spectrometer operating at a field strength of 9.4 T (376 MHz ^{19}F frequency) using 45° pulses with a 10 000 Hz spectral width. Typically, 128 scans were collected into 8192 data points with an acquisition time of 0.819 s. A further delay of 2.5 s was used between pulses to allow T_1 relaxation. An exponential line-broadening factor of 0.5 Hz was applied prior to FT. Chemical shifts were referenced externally to $CFCl_3$ (δ 0.0) and to the secondary reference compound trifluoroethanol (δ 77.0). ^{19}F–^{1}H couplings were eliminated using broadband ^{1}H decoupling. For quantification, a known amount of 4-fluorophenol was added to each sample to act as an internal standard and a delay of $5T_1$ between pulses was used to ensure complete T_1 relaxation.

2.5. Metabolite identification

2.5.1. NMR-monitored solid-phase extraction/chromatography (SPEC-NMR)

In order to concentrate and (partially) purify the metabolites, selected urine samples were subjected to solid-phase extraction followed by stepwise gradient elution with NMR detection [9]. Samples (pH 2.2 with 0.1 M HCl) were applied to C18 Bond Elut® columns (Jones Chromatography, Hengoed, UK) containing 500 mg of sorbent. The cartridges had been conditioned with methanol (5 ml) and 0.1 M HCl (1 ml). Following the sample application, the cartridges were washed

with 0.1 M HCl (1 ml), and then sequentially with 1 ml aliquots of methanol–water mixtures of increasing eluotropic strength (20:80, 40:60, 60:40, 80:20 and 0:100). The column eluates were collected and methanol was removed using a stream of nitrogen (room temperature). Any remaining water was removed by freeze-drying with samples reconstituted in 600 μl of D_2O prior to spectroscopy.

2.5.2. Enzymic hydrolysis

Urine samples (0.5 ml) were adjusted to pH 5.0 using 0.2 M sodium acetate buffer (0.55 ml). Samples were then incubated with either type B10 β-glucuronidase (bovine liver, Sigma, Poole, UK) or type-V sulphatase activity (ex. *Helix pomatia*, Sigma). A concentration of 1 mg/ml of the enzyme was used. Incubations, including a control urine containing only acetate buffer, a sample containing saccharolactone (0.05 ml of a 100 mM solution) to inhibit glucuronidase, and samples containing either phenolphthalein glucuronide or 4-nitrocatechol sulphate as positive controls to monitor enzyme activity, were carried out overnight (16 h) at 37 °C in a thermostated waterbath. The hydrolysis of conjugated metabolites was monitored by NMR.

3. Results and discussion

3.1. 2-Fluorobenzyl alcohol

Examination of the 500 MHz ^{1}H NMR spectra obtained for urine for the periods 0–8, 8–24 and 24–48 h post-administration of 2-fluorobenzyl alcohol (2-FBAL) clearly revealed the presence of compound-related material in the samples obtained for the period to 0–8 h post-dose (pd). These compound-related signals were not visible in samples from the later time points. In addition, changes were observed in the endogenous metabolite profile, most notably the presence of large amounts of acetate, benzoic acid, and lactate in the 24–48 hpd sample. ^{19}F NMR confirmed that the 0–8 hpd sample contained the majority of the compound-related material, with small amounts also present in the 8–24 hpd sample. The 24–48 hpd sample was free of detectable quantities of 2-FBAL or its metabolites. As shown in Fig. 1a, the 0–8 hpd sample contained a series of singlets at δ −118.8, −118.4, −116.2 and a split resonance (see below) at δ −114.6 and −114.65. 2-FBAL itself (δ −120.2) was not detectable in any of the samples. Identification of the peaks was accomplished by a combination of standard addition, enzymic hydrolysis and isolation using solid-phase extraction chromatography (SPEC) followed by NMR. Standard addition indicated that the peak at δ −116.2 was probably due to the presence of a 2-fluorobenzoic acid (2-FBA). Incubation of urine samples with β-glucuronidase revealed that the minor singlet at δ −118.8 was a glucuronide. There was no evidence for sulphate conjugates following incubation with sulphatase. SPEC followed by ^{19}F and ^{1}H NMR spectroscopy revealed the presence of compound-related material in the 20:80, 60:40, 40:60 and 80:20 methanol–water eluates. On the basis of the ^{1}H NMR data, the major component of the 20:80 eluate corresponded

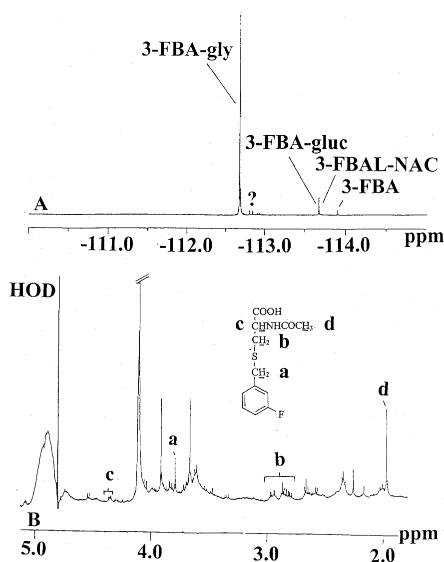

Fig. 1. (A) A typical ^{19}F NMR spectrum for a 0–8 hpd rat urine sample obtained following i.p. administration of 2-FBAL at 100 mg/kg. Key: 2-FBA-gly = glycine conjugate of 2-FBA, 2-FBAL-NAC = *N*-acetylcysteinyl conjugate of 2-FBAL, 2-FBAL-gluc = glucuronide conjugate of 2-FBAL. (B) The ^{1}H NMR spectrum of the 80:20 methanol–water SPEC eluate with the signals corresponding to the *N*-acetylcysteinyl moiety (a–d) of 2-FBAL-NAC highlighted. HOD = residual water.

to the glycine conjugate of 2-FBA. In urine samples, but not extracts, this material gave a split resonance in the ^{19}F NMR spectrum as noted above. This was probably dependent upon the degree of protonation of the nitrogen in the glycine moiety as the signal collapsed to a singlet when the pH was increased [8]. The 40:60 eluate also contained some of this material, but in addition also contained the 2-FBAL ether glucuronide which was also present in the subsequent 60:40 eluate. Both the 60:40 and 80:20 eluates contained a metabolite with resonances indicative of the presence of *N*-acetylcysteinyl conjugate (Fig. 1b). Given that the resonances for the aromatic ring and the benzylic CH_2 were readily identifiable, the only remaining site for this conjugation was the position occupied originally by the benzyl alcohol. The structure of this putative metabolite is given as an inset to Fig. 1b. Quantification of these metabolites (Table 1) highlighted the fact that excretion in the urine was a relatively minor route for the elimination of 2-FBAL (ca. 9.0%). This contrasts with the metabolic fate of 2-FBA in the rat, where 76.6 $\pm$ 7.8% of the dose was eliminated via this route [3,8]. An *N*-acetylcysteinyl metabolite accounted for some 10% of the urinary metabolites.

3.2. 3-Fluorobenzyl alcohol

Following administration of 3-fluorobenzyl alcohol (3-FBAL), ^{1}H NMR spectroscopy of the resulting urine samples revealed the presence of compound-related signals in the 0–8 hpd-dose urine. Samples for later time points, however, did not appear to contain metabolites of 3-FBAL. As seen with 2-FBAL, changes were noted in the excretion of endogenous metabolites. Thus, all the post-administration samples contained large amounts of lactate and acetate. The presence of 3-hydroxybutyrate, alanine, glutamine, glutamate and benzoic acid was also noted together with increases in formate, succinate and glycine. ^{19}F NMR spectroscopy also showed the presence of 3-FBAL-related material in the 0–8 hpd urine sample and confirmed the absence of large quantities of metabolites in subsequent samples. The ^{19}F NMR spectrum obtained for a 0–8 hpd urine sample is shown in Fig. 2a. This spectrum is dominated by a signal at δ −112.68, with a range of minor signals at δ −112.83, −112.84, −113.78 and −113.90. The latter signal was identified as 3-fluorobenzoic acid (3-FBA) on the basis of standard addition. Incubation of samples with β-glucuronidase resulted in the loss of the signal at δ −113.78 with a concomitant increase in the signal at δ −113.90 (3-FBA by standard addition), indicating the presence of an ester glucuronide. No changes were observed on incubation with sulphatase. Using SPEC the urine was fractionated as described above with the 40:60 methanol–water eluate containing the majority of the 3-FBAL metabolites. On the basis of the ^{1}H NMR spectrum of this fraction, the major urinary-excreted metabolite of 3-FBAL was the glycine conjugate of 3-FBA. The subsequent 60:40 methanol–water fraction also contained small amounts of this metabolite. The 40:60 and 60:40 SPEC fractions contained a metabolite giving a ^{1}H NMR spectrum consistent with that of the 3-FBA ester glucuronide that had been indicated by enzymic hydrolysis on the neat urine sample. Also present in the ^{1}H NMR spectrum of the 60:40 SPEC eluate were resonances characteristic

Table 1
The urinary recovery (% of the dose) of the metabolites of 2-FBAL following i.p. administration to the rat at 100 mg/kg

Time point (h)	2-FBA-glycine	2-FBA	2-FBAL-*N*-acetylcysteinyl	2-FBAL-glucuronide	Total (%)
0–8	5.1 ± 0.4	0.5 ± 0.2	0.7 ± 0.1	1.3 ± 0.1	7.5 ± 0.3
8–24	0.5 ± 0.0	1.1 ± 0.7	0.2 ± 0.1	0.2 ± 0.0	1.8 ± 0.3
24–48	ND	ND	ND	ND	–
Total (%)	5.5 ± 0.4	0.9 ± 0.2	0.9 ± 0.2	1.5 ± 0.1	9.3 ± 1.0

ND: not detected.

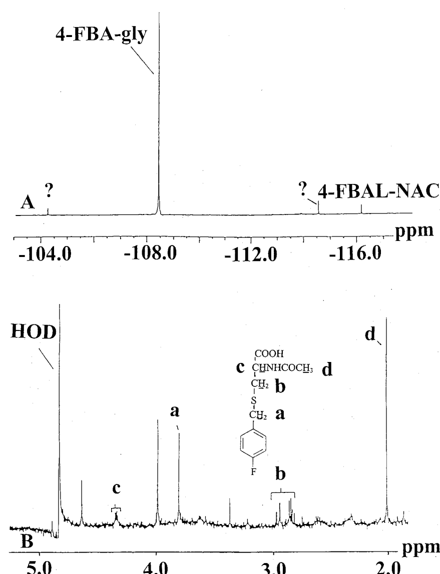

Fig. 2. (A) A typical ^{19}F NMR spectrum for a 0–8 hpd rat urine sample obtained following i.p. administration of 3-FBAL at 100 mg/kg. Key: 3-FBA-gly = glycine conjugate of 3-FBA, 3-FBA-gluc = ester glucuronide conjugate of 3-FBA, 3-FBAL-NAC = *N*-acetylcysteinyl conjugate of 3-FBAL, ? = unknown. (B) The ^{1}H NMR spectrum of the 60:40 methanol–water SPEC eluate with the signals corresponding to the *N*-acetylcysteinyl moiety (a–d) of 3-FBAL-NAC highlighted. HOD = residual water.

of *N*-acetylcysteinyl conjugate as well as a further glucuronide, possibly of 3-FBAL itself (Fig. 2b).

A total of 20.3 ± 2.2 of the administered dose was eliminated via the urine over the collection period with the bulk of this excreted in the first 0–8 hpd (Table 2). This compares with 39.07 ± 1.7% of an equivalent dose of 3-FBA [3,8].

3.3. 4-Fluorobenzyl alcohol

The administration of 4-fluorobenzyl alcohol (4-FBAL) was accompanied by the clear presence of compound-related peaks in ^{1}H NMR spectrum of the 0–8 hpd urine sample. It was not possible to observe the presence of 4-FBAL metabolites in subsequent spectra. However, unlike the situation observed with 2- and 3-FBAL, the administration of 4-FBAL was not accompanied by major disturbances in the endogenous metabolite profile, with the only noteworthy change being a transient increase in taurine concentration in the 0–8 hpd samples. ^{19}F NMR spectroscopy of these urine samples showed that fluorinated metabolites were present in both the 0–8 and 8–24 hpd urine samples with the bulk of the urinary metabolites excreted in the first 0–8 hpd sample. A total of four resonances were observed in the 0–8 hpd sample (Fig. 3a) with the major signal at δ −108.4, and three smaller peaks detected at δ −104.2, −114.5 (present in a single sample) and −116.11. Standard addition of 4-FBAL and 4-FBA to the samples revealed that none of the signals present in the urine corresponded to either of these compounds. Enzymic hydrolysis was without effect indicating an absence of glucuronide or sulphate metabolites. The SPEC experiments resulted in the elution of the 4-FBAL metabolites in the 40:60 and 60:40 methanol–water fractions. The main peak was identified by its ^{1}H NMR spectrum as the glycine conjugate of 4-FBA. The 60:40 methanol–water fraction contained a 4-FBAL metabolite that was identified as *N*-acetylcysteinyl conjugate (Fig. 3b). The remaining two minor peaks seen in the 0–8 hpd sample were not

Table 2
The urinary recovery (% of the dose) of the metabolites of 3-FBAL following i.p. administration to the rat at 100 mg/kg

Time point (h)	3-FBA-glycine	3-FBAL	3-FBA	Unk. 1	Unk. 2	3-FBAL–glucuronide	Total (%)
0–8	10.3 ± 4.1	0.3 ± 0.1	6.7 ± 5.1	0.3 ± 0.1	0.4 ± 0.2	0.5 ± 0.0	18.4 ± 2.2
8–24	0.7 ± 0.6	ND	1.2 ± 0.4	ND	ND	ND	1.9 ± 0.2
24–48	ND	ND	ND	ND	ND	ND	–
Total (%)	11.0 ± 3.4	0.3 ± 0.1	7.9 ± 5.4	0.3 ± 0.1	0.4 ± 0.2	0.5 ± 0.0	20.3 ± 2.0

ND: not detected, parent compound was not detected in any of these samples. Unk. 1 = unknown metabolite at δ*F* −112.83. Unk. 2 = unknown metabolite at d*F* −112.84.

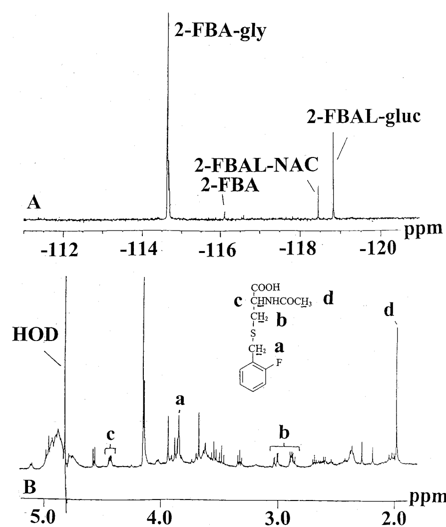

Fig. 3. (A) A typical ^{19}F NMR spectrum for a 0–8 hpd rat urine sample obtained following i.p. administration of 4-FBAL at 100 mg/kg. Key: 4-FBA-gly = glycine conjugate of 4-FBA, 4-FBAL-NAC = *N*-acetylcysteinyl conjugate of 4-FBAL, ? = unknown. (B) The ^{1}H NMR spectrum of the 60:40 methanol–water SPEC eluate with the signals corresponding to the *N*-acetylcysteinyl moiety (a–d) of 4-FBAL-NAC highlighted. HOD = residual water.

identified. Quantification of these metabolites (Table 3) accounted for a total of 31.0 ± 2.7% of the administered dose in the urine compared with 31.9 ± 5.7% of the dose of 4-FBA [3,8].

4. Discussion

For all the three benzyl alcohols, the predominant metabolic fate, revealed by the urinary metabolites, was oxidation to the benzoic acid followed by glycine conjugation. No unchanged parent compound was detected in these samples. However, because only 10–40% of the dose was excreted by this route, depending upon the compound, it was not possible to fully define the metabolic fate of these compounds in the rat. However, it may be possible to make an estimate of the extent of the oxidation of the alcohol moiety to the corresponding acid by comparison with the results seen with the corresponding benzoic acids dosed under the same conditions [3,8]. This comparison reveals some interesting similarities and differences. Thus in the case of 4-FBAL, the quantity of material excreted in the urine (ca. 31% of the dose) was essentially the same as that seen for 4-FBA (ca. 32% of the dose). One interpretation of this result is that there was essentially complete conversion of the benzyl alcohol via oxidative metabolism to the benzoic acid (and thence to the glycine conjugate). For 3-FBAL, ca. 20% of the dose was eliminated via the urine compared with ca. 40% for 3-FBA, implying a somewhat lower degree of conversion of the alcohol to the glycine conjugate of the acid. The largest discrepancy between the excretion of the metabolites of the benzyl alcohol and the benzoic acid was seen with 2-FBAL. Thus for this compound only ca. 10% of the dose was seen in the urine whereas for 2-FBA nearly 80% was eliminated by this route. This implies, all other things being equal, that the metabolic fate of 2-FBAL was not dominated by oxidation to the benzoic acid.

Table 3
The urinary recovery (% of the dose) of the metabolites of 4-FBAL following i.p. administration to the rat at 100 mg/kg

Time point (h)	4-FBA glycine	4-FBAL-*N*-acetylcysteinyl	Unk. 1	Unk. 2	Total (%)
0–8	28.5 ± 3.6	0.6 ± 0.1	0.3 ± 0.3	0.2 ± 0.1	29.6 ± 3.1
8–24	1.4 ± 0.9	ND	ND	ND	1.4 ± 0.9
24–48	ND	ND	ND	ND	–
Total (%)	29.9 ± 3.1	0.6 ± 0.1	0.03	0.2 ± 0.1	31.0 ± 2.7

ND: not detected, parent compound was not detected in any of these samples. Unk. 1 = unknown metabolite at δ*F* −114.5. Unk. 2 = unknown metabolite at δ*F* −104.2.

In this context, the excretion via the urine of quantities of the corresponding *N*-acetylcysteinyl conjugates of these compounds may have some relevance. The formation of such conjugates represents a common "detoxification" mechanism following the administration of substituted benzaldehydes and alcohols to the rat, rabbit and dog [10–14]. Metabolism via this route probably proceeds via the formation of a sulphate ester conjugate as an intermediate followed by nucleophilic attack by glutathione. Chidgey et al. [15] have shown that pre-treatment of rats with pentachlorophenol (a sulphotransferase inhibitor) abolished the excretion of the *N*-acetylcysteinyl conjugate that was otherwise formed from benzyl acetate. Rietveld et al. [14] demonstrated, albeit with a limited data set, that the substitution pattern on the aromatic ring and the size of the substituent greatly influenced the extent of urinary excretion of the *N*-acetylcysteinyl conjugates following the administration of range of benzaldehydes. These authors concluded that *ortho* substitution had the greatest effect in increasing *N*-acetylcysteinyl conjugate formation, whilst *para* substitution had the least.

It is tempting to conclude that the pattern of excretion of metabolites following the administration of these FBALs reflects the propensity of the compounds to form sulphate esters (and thence glutathione adducts) as an alternative to oxidation to the corresponding FBAs. Such a metabolic fate could result in more extensive elimination via the bile as a result of an increased molecular mass of the metabolites taking them into the range where such a route of elimination is favoured (ca. 300–350 Da in the rat). This could explain the low-urinary recovery of 2-FBAL-related material. Alternatively, it is also possible that the production of reactive sulphate metabolites could result in high levels of covalent tissue binding (with all the potential toxicological sequelae that this implies), and hence low recoveries. In this context, it is perhaps noteworthy that the largest disturbances in the pattern of endogenous metabolites present in urine were seen for the 2- and 3-substituted compounds, whilst only minor changes were observed with 4-FBAL.

5. Conclusions

Excretion of FBAL-related material in the urine was rapid but accounted for less than 50% of the dose in all cases. In the case of 4-FBAL, the extent of urinary excretion was comparable with that of the corresponding benzoic acids. However, for 3-FBAL urinary excretion was reduced by ca. 50% compared with 3-FBA and for 2-FBAL urinary excretion of metabolites was much lower than that observed for 2-FBA. The major metabolites of the three FBALs detected in urine were the glycine conjugates of the corresponding benzoic acids. Small quantities of other metabolites including the unconjugated FBAs, glucuronide conjugates of either the FBAs or the FBALs and the benzylic *N*-acetylcysteinyl conjugates were also detected. The presence of the latter indicates the formation of reactive metabolites.

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