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SHORT COMMUNICATIONS

Species differences in the metabolism of ^{14}C-*p*-trifluoromethylaniline: Production of an oxanilic acid as the major metabolite by the rat

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The metabolic fate of primary aromatic amines is of considerable toxicological interest because of the adverse effects which may result from exposure to such compounds. Examples of the toxicity associated with this class of compound include methaemoglobinaemia following exposure to aniline, and the development of tumours resulting from the effects of substances such as benzidine or 2-naphthylamine [1].

Studies on the metabolism of ^{14}C-*p*-trifluoromethylaniline (^{14}C-TFMA) in rat and dog revealed that each converted the compound to quite different products. In the rat the majority of an oral dose of ^{14}C-TFMA was excreted in the form of an unusual oxanilic acid (Fig. 1). No trace of this compound could be detected following metabolism by dog. As this finding may be of toxicological significance, the metabolism of ^{14}C-TFMA and related compounds was investigated further in order to explain this species difference.

Results and discussion

Following oral administration of ^{14}C-TFMA (50 μCi, 4.5 mg/kg as a solution in ethanol–propylene glycol 1:1, 20 mg/ml) to a male beagle dog the majority of the radiolabel was excreted in the urine, with nearly 50% of the dose recovered in the first 24 hr. A total of 75% of the administered radioactivity was excreted in the urine over the whole of the 8 day collection period (Table 1). Faeces formed a very minor route for the excretion of radioactivity, accounting for only 3% of that dosed.

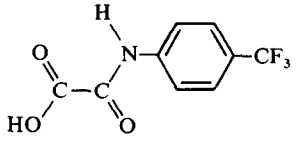

Fig. 1. The structure of *p*-trifluoromethylaninline. The TFMA used in this study was non-specifically labelled with ^{14}C in the ring.

The determination of radioactivity in plasma and whole blood samples revealed peak concentrations of 4.16 and 14.4 μg equiv./ml at 20 min and 4 hr post dose respectively. A specific analytical method for TFMA [2] was employed to measure concentrations in plasma and red blood cells (RBCs). This showed that the plasma profile of TFMA was similar to that of total radioactivity but with concentrations some 5–10 times lower. The maximum plasma concentration of TFMA was 1.14 μg/ml and coincided with the peak of total plasma radioactivity. Thereafter plasma levels declined in a biexponential manner with initial and terminal half lives of 19 and 475 min respectively. In RBCs, TFMA was still easily detectable long after its disappearance from plasma. The plasma and whole blood radioactivity profiles, together with those for TFMA in plasma, are shown in Fig. 2.

On TLC (silica gel) the bulk of the urinary radioactivity was found to be associated with a single component. This metabolite had R_f values of 0.91 and 0.15 using solvent systems consisting of acetonitrile–ethanol–ammonia (s.g. 0.88) 3:1:1 (solvent system A) and chloroform–ethanol–ethyl acetate–water–ammonia (s.g. 0.88) 65:30:5:1:1 (solvent system B) respectively. The major ^{14}C-metabolite was isolated and partially purified by preparative scale TLC using solvent system A. This ^{14}C-metabolite was then characterized by combined gas chromatography–mass spectrometry (GC–MS) as a silyl derivative, (prepared by reaction of the metabolite with *N*,*O*-bis-(trimethylsilyl)acetamide in pyridine (1:1 v/v) at room temperature for 90 min). This suggested that the metabolite was a monohydroxylated form of TFMA, probably 2-amino-5-trifluoromethylphenol. The structure was confirmed by the synthesis of an authentic standard and comparison of the GC properties and GC–MS of standard and ^{14}C-metabolite. The standard was prepared by nitration of 3-hydroxy

Table 1. Excretion of radioactivity following an oral dose of ^{14}C-TFMA to the dog (4.5 mg/kg)

Sample	Hours post dose	% Dose excreted	Cumulative excretion (%)
Urine	0–6	30.4	30.4
	6–24	19.0	49.4
	24–48	14.6	64.0
	48–120	6.8	70.8
	120–144	2.8	73.6
	144–168	1.1	74.7
	169–192	0.5	75.2
Total		75.2	
Faeces	0–192	3.00	3.00
Total urine and faeces		78.2	
Cage wash		0.4	
Total		78.6	

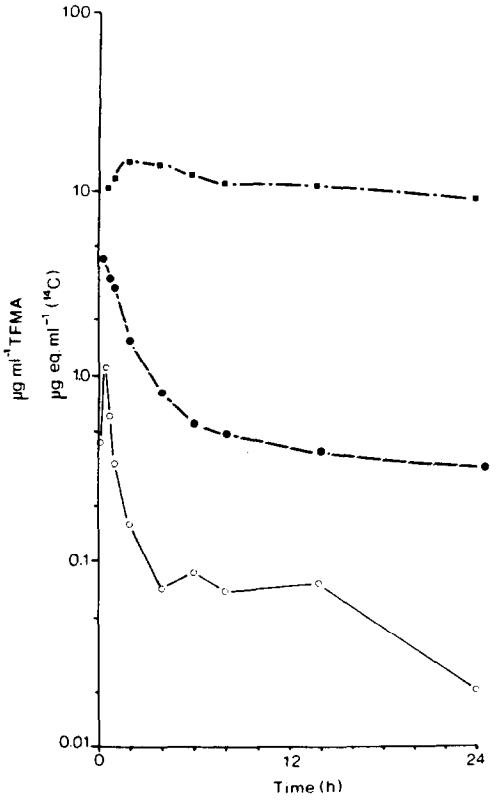

Fig. 2. Concentrations of radioactivity in whole blood (– – –) and plasma (——), and TFMA in plasma (———), of a male beagle dog following an oral dose of 4.5 mg/kg of ^{14}C-TFMA.

benzotrifluoride followed by catalytic reduction of the products, and was then isolated from the mixture of isomers by column chromatography (silica gel). The mass spectrum of the bis-trimethylsilyl derivative of ^{14}C-2-amino-5-trifluoromethylphenol isolated from dog urine is shown in Fig. 3.

On the basis of enzymic hydrolysis with β-glucuronidase (*Helix pomatia*, pH 5.0, 37°), and specific saccharolactone inhibition of that hydrolysis, it was concluded that the metabolite was probably present in the urine in the form of a β-D-glucuronide. In similar experiments involving oral dosing of male Sprague–Dawley rats with ^{14}C-TFMA (5 µCi per animal; 16 mg/kg) plasma concentrations of 11.45 µg equiv./ml were achieved 0.5 hr post dose. The amount of radioactivity in the plasma then fell rapidly until by 24 hr only 0.1 µg equiv./ml remained. Whole blood concentrations fell more slowly following a peak of 17.4 µg equiv./ml attained 1 hr after dosing, and at 24 hr were still at 6.4 µg equiv./ml, resulting in a blood to plasma ratio of 64.9. These results are shown in Fig. 4. Specific analysis for TFMA was not performed.

Like dog, the rat excreted the major part of the radiolabel in the urine, with nearly 90% of the dose excreted within the first 24 hr. Faecal excretion accounted for less than 5% of the dose. By 48 hr post-dose an essentially complete recovery (> 95%) of the administered radioactivity had been achieved (Table 2).

The urine was then analysed by TLC in solvent system A. This revealed that unlike dog only a small amount of radioactivity (< 10%) was present with an R_f of 0.91 (subsequently identified as 2-amino-5-trifluoromethylphenol by GC–MS). The majority of the urinary radioactivity was located in a single band with an R_f of 0.51. Treatment of urine samples with β-glucuronidase-aryl sulphatase was without effect on this ^{14}C-metabolite implying that it was not a glucuronide or sulphate conjugate.

The major urinary ^{14}C-metabolite of TFMA produced by the rat was therefore isolated using repeated preparative TLC in solvent system A. further purification was then effected by reversed phase HPLC using ODS Spherisorb (5 µm, 15 cm by 4 mm i.d. stainless steel column) with a mobile phase of distilled water at pH 5 (1 ml/min). The metabolite was most strongly retained under acidic conditions suggesting that it was acidic in nature. Analysis of the purified metabolite by GC–MS as the methyl and ethyl

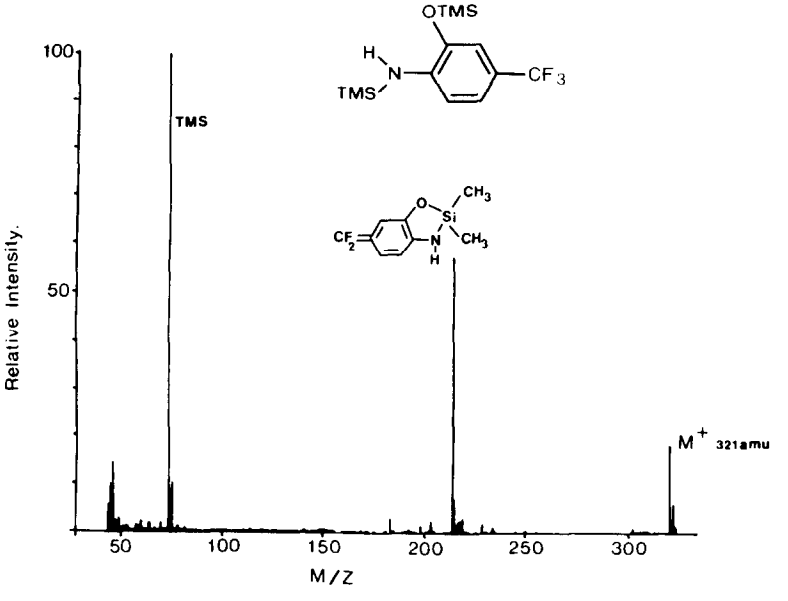

Fig. 3. A mass spectrum (electron impact, 70 eV) obtained for the bistrimethylsilyl derivative of ^{14}C-2-amino-5-trifluoromethylphenol, the major urinary metabolite of ^{14}C-TFMA of the dog.

Table 2. Excretion of radioactivity following an oral dose of ^{14}C-TFMA (16 mg/kg) to 3 male rats

Sample	Hours post dose	% Dose ± S.D. excreted	Cumulative excretion (%)
Urine	0–6	49.6 ± 7.6	49.6
	6–24	38.3 ± 12.7	87.9
	24–48	3.27 ± 0.6	91.2
Faeces	0–24	1.4 ± 0.1	1.4
	24–48	2.9 ± 0.5	4.28
Cage Wash		1.8 ± 1.2	
Total		97.3 ± 17.3	

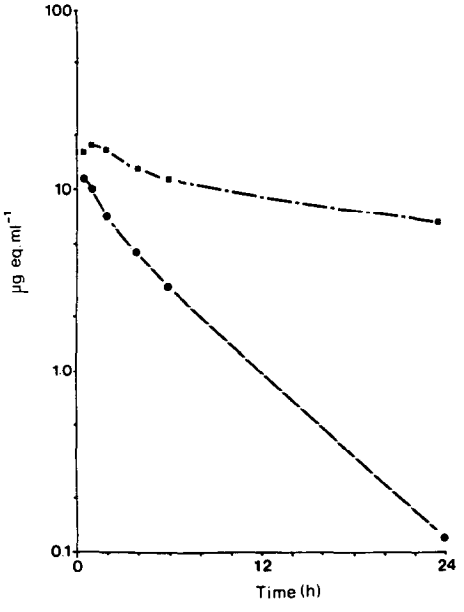

Fig. 4. Concentrations of radioactivity in whole blood (———), and plasma (——), following an oral dose of ^{14}C-TFMA to the rat.

derivatives was then performed. Esterification was accomplished by incubation of the metabolite in methanolic or ethanolic HCl solution (1 M) at 60° for 15 min. A mass spectrum of the methyl derivative obtained on the GC–MS of the metabolite is shown in Fig. 5. On the basis of the spectroscopic data the metabolite was identified as the oxanilic acid of TFMA. This structure was confirmed by comparison of the mass spectra obtained following GC–MS of its ester derivatives with that of an authentic standard. The latter was obtained as a gift from ICI Plant Protection PLC (Jealotts Hill, U.K.).

The formation of oxanilic acids has been described as a minor pathway for the metabolism of *p*-chloroaniline in several species [3]. This conversion probably proceeds via an initial *N*-acetylation, followed by ω-hydroxylation and oxidation to the carboxylic acid [4]. The metabolism of doubly labelled ^{13}C,^{14}C-*N*-acetyl TFMA (^{13}acetyl, ^{14}C-TFMA) was therefore investigated, the double labelling allowing unequivocal evidence for the retention of the *N*-acetyl group during subsequent metabolism to be obtained. The ^{13}C,^{14}C-*N*-acetyl TFMA was synthesised from the reaction of ^{14}C-TFMA and 2-^{13}C-acetyl chloride (91 atom%, Prochem, BOC, U.K.) in pyridine. Purification was effected by column chromatography (silica gel) and recrystallization to give ^{13}C,^{14}C-*N*-acetyl TFMA with a radiochemical purity of greater than 99%.

The ^{14}C-TFMA-oxanilic acid, isolated from rat urine following an oral dose of ^{13}C, ^{14}C-acetyl-TFMA, was found on analysis to be ^{13}C-enriched, thus confirming that the

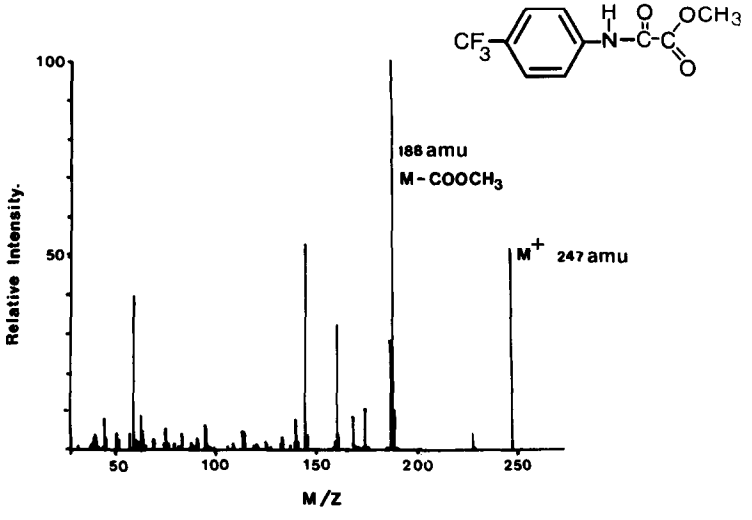

Fig. 5. A mass spectrum (electron impact, 70 eV) of the methyl derivative of the *N*-oxanilic acid of TFMA, the major urinary metabolite of ^{14}C-TFMA of the rat.

formation of the TFMA-oxanilic acid via *N*-acetyl-TFMA can occur. However, it was observed that whilst the *N*-acetyl-TFMA dosed to the animals was over 90% ^{13}C-enriched, the *N*-oxanilic acid isolated was only 67% enriched (as determined by isotope ratios on GC–MS of the esterified metabolite). This indicates that deacetylation of a portion of the *N*-acetyl-TFMA occurred before metabolism to the oxanilic acid (presumably via re-acetylation).

The requirement for *N*-acetylation prior to formation of the oxanilic acid of TFMA probably explains why this metabolite is not formed by the dog, given the well known metabolic deficiency of *N*-acetylation in this species [5]. When dosed with ^{13}C, ^{14}C-N-acetyl-TFMA (16 mg/kg) the dog also produced the expected TFMA oxanilic acid. In this case the stable isotope ratio of the metabolite was the same as that of the *N*-acetyl TFMA dosed to the animal.

As referred to in the text, the formation of oxanilic acids as minor metabolites of certain aromatic amines and their derivatives has been reported [3, 4]. Thus up to 21% of the administered 4-chloroacetanilide was recovered from the urine of rabbits as the 4-chloro-oxanilic acid metabolite within 24 hr after dosing [3]. Rabbits have also been shown to convert up to 14% of a dose of [^{35}S] sulphanilamide to *N*-(4-sulphamoylphenyl)-oxanilic acid [6]. However, the metabolism by rat of virtually the whole of an oral dose of TFMA to an oxanilic acid is unprecedented.

The species difference observed between rat and dog in the metabolism of ^{14}C-TFMA is most easily explained if formation of the oxanilic acid metabolite requires *N*-acetylation, a process at which the dog is deficient. Although we have not demonstrated that *N*-acetylation of TFMA occurs *in vivo*, the retention of the stable isotope label in the TFMA-*N*-oxanilic acid formed from [^{13}C, ^{14}C]-*N*-acetyl-TFMA provides additional support for the involvement of acetylation in this transformation.

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Influence of species and drug pretreatment on the metabolic oxidation of cimetidine and metiamide

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Cimetidine and its forerunners, buramamide and metiamide, are specific H_2-receptor antagonists but only cimetidine is currently used in the treatment and prophylaxis of gastro-intestinal ulcer disease and reduction of gastric hyperacidity states in man [1, 2]. Cimetidine and metiamide are mainly metabolized by oxidation to the sulfoxide and 5-hydroxymethyl derivatives [3, 4], but recently the *N*-glucuronide conjugate has been reported as the major metabolite of cimetidine [5]. Drug interactions with cimetidine have been well documented [6], and this paper reports the influence of species and drug pretreatment on the metabolic oxidation of cimetidine and metiamide.

Materials and methods

Cimetidine, metiamide and their derivatives were supplied by Smith Kline & French Research Limited, U.K. Phenobarbitone, anthracene and ethanol were obtained from the British Drug House, while -pregnen-3-OL-20-one and methimazole were from Sigma. Metyrapone was a gift from Ciba-Geigy, Lagos, Nigeria.

Four non-mammalian and three mammalian species (male) were dosed with 50 mg/kg of cimetidine or metiamide, and housed in appropriate metabolic cages. The excreta and urine were collected daily. Sets of rats were also pretreated with chemicals before the administration of cimetidine or metiamide.

Assay procedure. Urine samples and the excreta were made alkaline with 1.25 N NaOH and then extracted with 2 × 5 ml isopropanol–dichloromethane (1:1, v/v). The pooled extracts were evaporated to dryness on a water bath at 45° and reconstituted in ethanol (40 μl). Portions (20 μl) were examined by TLC (silica gel $60F_{254}$ 0.25 mm thick 5 × 10 cm, E. Merck, Darmstadt, West Germany) and developed in the appropriate solvents (Table 1). The compounds and their metabolites were located under u.v. light and by exposure for 15 sec in iodine vapor. The spots were scraped off and eluted into 1 N HCl (6 ml) and then quantified using a Varian 634 u.v.-visible scanning spectrophotometer.

Results and discussion

Sulfoxidation of xenobiotics has been shown to be both species and substrate dependent [7, 8]. The results (Table 2) clearly show that the rates of appearance of the sulfoxide and 5-hydroxymethyl metabolites and unchanged drug were species dependent for both cimetidine and metiamide, although by 72 hr the total amounts of dose excreted were similar except for the rat.

The induction inhibition experiment in the rat (Table 3) also demonstrated selective effects with regard to compound and specific metabolites. The induction of cimetidine sulfoxidation by alcohol, phenobarbitone, and pregnenolone, and its inhibition by methimazole rather than metyrapone tend to suggest the involvement of a flavoprotein-mediated monooxygenase in this oxidation reaction.* Metiamide sulfoxidation appears different since it was only

* R. A. Prough and R. N. Hines, "Eighth European Workshop on Drug Metabolism", Abstr. No. 20, p. 64 (1982).