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Comparative biodisposition and metabolism of ^{14}C-(±)-fenfluramine in mouse, rat, dog and man

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1. The comparative metabolism of fenfluramine was investigated in mouse, rat, dog and man following a single oral dose of ^{14}C-(±)-fenfluramine hydrochloride (1 mg/kg), and also in rat after eight consecutive 12-h subcutaneous doses (24 mg/kg).

2. Main route of excretion of radioactivity in all species and at all doses was into urine (>80%), with only minor amounts of radioactivity found in faeces.

3. From all species examined a total of 11 metabolites were observed in urine and plasma by t.l.c. and h.p.l.c. analysis and no metabolite was present in the plasma which was not present in urine.

4. All species dealkylate fenfluramine to the active metabolite norfenfluramine, to a relative greater or lesser extent, with plasma metabolic ratios (norfenfluramine/fenfluramine) showing inter-animal variation (rat ≫ dog ≫ mouse = man).

5. These differences are due to the efficient deamination of both compounds to polar inactive metabolites in man, with less dealkylation and lower plasma levels of norfenfluramine compared with the other species studied.

6. In conclusion, major species differences in the metabolism of (±)-fenfluramine, both qualitative and quantitative were observed, and no one species had a similar metabolic profile to that found in man.

Introduction

Although (±)-fenfluramine hydrochloride [1–(*m*-trifluoromethylphenyl)-2-ethylaminopropane hydrochloride) has been used as a safe and effective anti-obesity agent for almost three decades, a detailed knowledge of the metabolism in man and animals has been lacking. This is of particular importance for the interpretation of pharmacological and mechanistic studies when there may be large inter-species differences in metabolism, and active metabolites are formed. Several investigators (Beckett and Brookes 1967, Garattini *et al.* 1979, Caccia *et al.* 1982) have shown that fenfluramine is *N*-de-ethylated rapidly to norfenfluramine with half-lives ranging from 2 to 4 h in rat, mouse and dog, and up to 20 h in man. With the exception of some preliminary studies in man (Richards 1985), few studies have been performed to investigate the subsequent metabolism in different species, and this could be important particularly as fenfluramine and norfenfluramine account for only 10–20% of the administered dose.

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In vitro metabolism studies have been performed in various animal models and some metabolites have been identified, specifically in the guinea-pig, as reviewed by Midha *et al.* (1983), but it is not clear whether they would be found *in vivo*.

In addition, although it has been shown that there are quantitative stereoselective differences in the kinetics of the two enantiomers of fenfluramine in animals (Garattini *et al.* 1979) and man (Caccia *et al.* 1982), there do not appear to be any qualitative differences in the metabolic pathways (Richards 1985). Therefore this comparative metabolic study has been performed using the racemate ^{14}C-(±)-fenfluramine with the purpose of extending the initial *in vivo* kinetic studies of Caccia *et al.* (1982) by examining the biotransformation of (±)-fenfluramine in mouse, rat dog and man. For comparative purposes each of the species was given a low oral dose of 1 mg/kg, approximately equivalent to the dose level used clinically, and in addition a further group of rats were given eight consecutive 12-h subcutaneous doses of 24 mg/kg ^{14}C-(±)-fenfluramine hydrochloride, to examine the metabolism of fenfluramine at toxicological doses.

Materials and methods

Chemicals

Racemic forms of fenfluramine and reference standards, norfenfluramine, *m*-trifluoromethylphenyl propane-1,2-diol, (diol) *m*-trifluoromethylphenyl-1-hydroxy propan-2-one (hydroxyketone), *β*-hydroxynorfenfluramine, *m*-trifluoromethylbenzoic acid (benzoate) and *m*-trifluoromethylhippuric acid (hippurate) were supplied by Technologie Servier (Orleans, France). (±)-Fenfluramine labelled in the *β*-carbon of the molecule (figure 1) was synthesized according to the method of Hawkins and Midgley (1974) and was supplied by Commissariat à l'Energie Atomique, Gif-sur-Yvette, France with a specific activity of 14·0 mCi/mmol and a radiochemical purity >97%, with no single impurity >1%. Purity was assessed using a quantitative radioscan of a thin layer radiochromatogram on silica gel $60F_{254}$ plates which were developed in dichloromethane-methanol-ammonia solution (sp. gr. 0·88) (70:30:1 by vol.).

Scintillation cocktail (OptiPhase Safe) and combuster trapping agent (OptiSorb S+OptiSorb 1) used in liquid scintillation counting were supplied by LKB Scintillation Products (FSA Laboratory Supplies, Loughborough, UK). X-ray film, film developer (G150) and fixer (G334) used in autoradiography were all purchased from Agfa-Gevaert, London, UK.

Silica gel t.l.c. plates, $60F_{254}$, 0·25 mm thickness (Merck Ltd., Darmstadt, Germany), 5 μm C_{18} reverse phase h.p.l.c. column (Thames Chromatography, Berkshire, UK), C_8 Bond Eluts, 6 cm^3 1 g (Jones Chromatography, Mid Glamorgan, UK) were all used as supplied for the chromatographic analysis of samples.

Bacterial *β*-glucuronidase (*Escherichia coli*) Type VII-A, activity 943 000 units/g and sulphatase from *Aerobacter aerogenes*, Type VI were supplied by Sigma Chemical Company Ltd.

H.p.l.c. solvents were h.p.l.c. grade and purchased from BDH Chemicals, (Poole, UK). All other chemicals were of at least standard laboratory reagent grade.

Metabolic studies

Dosing

Human volunteers. Two healthy human, male volunteers (body weights 68 and 80 kg) were entered into the study and refrained from taking any medication, including mild analgesics, for a period of 1

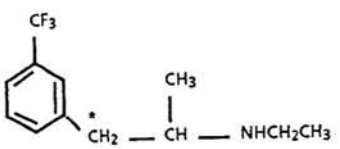

Figure 1. Structure of ^{14}C-(±)-fenfluramine.
*Denotes position of the radiolabel.

week before the start of and during the study. Ethical approval was given for the study and in addition the study was approved by the Administration of Radioactive Substances Advisory Committee (ARSAC). Each volunteer gave their signed, fully informed consent before being admitted into the study. Both volunteers were fasted overnight for a minimum of 12 h before and 4 h after dosing. Neither food nor beverages, apart from water, were allowed. Each volunteeer received an oral dose of 1 mg/kg (±)-fenfluramine hydrochloride, in an aqueous solution, containing an accurately known amount (approximately 35 μCi) of radiolabelled fenfluramine.

Animals. Two male Beagle dogs, weighing 9·6 and 10·kg were supplied by Interfauna, France and fed on a granular diet (Extra-labo, 270 g daily). Tap water was administered *ad libitum.*

Twelve male Sprague–Dawley rats, weighing between 200 and 250 g were supplied by Harlan Olac Ltd. (Bicester, UK). All animals were fed on R & M Compact 1 laboratory diet as supplied by Lillito and Sons Ltd.

Eight C57BL/6 male mice weighing between 23 and 25 g were supplied by Charles River UK Ltd. (Kent, UK) and were fed on the same diet as rats. All animals were fasted from approximately 12 h beore dosing until 4 h post-dose, and water was available *ad libitum.*

Each mouse, dog and eight rats received, by gavage, a solution of (±)-fenfluramine hydrochloride (dose level 1 mg/kg) containing 34 μCi of radiolabelled fenfluramine in the case of the dog, 1·25 and 12·5 μCi in the case of the mice and rats, respectively.

The remaining four rats each received a subcutaneous injection (24 mg/kg) of (±)-fenfluramine hydrochloride each containing 12·5 μCi of radiolabelled fenfluramine hydrochloride twice a day (every 12 h) for 4 consecutive days. These rats were designated as high dose rats in comparison with those which received the 1 mg/kg oral solution (low dose rats). Amount of radioactivity administered to each animal was determined by the weight difference between the total amount of the dosing syringe and dose solution before, and the dosing syringe following administration of the compound.

Sample collection

Man. Urine samples were collected from human volunteers for a period of 24 h predose and at 3, 6, 24 h post-dose, and then at 24 h intervals until 168 h. Faecal samples were collected as excreted for the same period. Samples were collected separately at room temperature and then frozen immediately and stored at −20°C until use. Blood samples (50 ml) also were taken from the human volunteers by venepuncture at 1, 3, 8 and 24 h post-dose for metabolite profiling. Blood samples were centrifuged immediately to produce plasma which was stored at −20°C.

Animals. Each animal was housed individually in an appropriate metabolism cage, allowing separate collection of the faeces and the urine. Two hours after dosing, four of the low dose rats and four of the mice were killed by cervical dislocation and exsanguinated. Blood samples (10 ml) were collected at 3 h post-dose from each dog for metabolite profiling. Blood was centrifuged to produce plasma which was stored at −20°C until analysis.

Urine, cage wash and faecal samples were collected from the remaining animals as follows: urine samples from mice, dog and low dose rats were collected over solid carbon dioxide for 24 h pre-dose and then at 3,6 and 24 h after dosing and thereafter at 24-h intervals until 168 h. In addition at the end of each urine collection time period, each cage was washed individually with water, the quantity used depending on the size of the cage. At the end of the study each cage was washed individually with water and then methanol to remove any residues of urine. Faecal samples, also collected over solid carbon dioxide, were taken 24 h pre-dose and then at 24-h intervals until 168 h post-dose. All samples were stored at −20°C until analysis. Radioactivity found in cage washings was added to the relevant urine collection to provide a more accurate estimate of the total urinary excretion of radioactivity, although, in general, this did not account for >7% of the dose.

Urine samples also were collected over solid carbon dioxide from high dose rats for a 24-h period pre-dose and then at 12-h intervals until 84 h with a final sample collected from 84 to 86 h. Cages were washed with water at the end of each urine collection and then, at the end of the study, each cage was washed thoroughly with water and then methanol. Total faeces were collected over solid carbon dioxide 24 h pre-dose and then daily until 84 h with a final collection from 84 to 86 h. All samples were stored at −20°C. At the end of the study, 2 h after the final dose, all high dose rats were killed by cervical dislocation and exsanguinated. Blood collected was centrifuged and the plasma stored at −20°C until analysis.

Sample preparation

Since the levels of radioactivity in faeces were low (<5%) indicating good absorption, only the urine and selected plasma samples were used for metabolite profiling.

Urine

Representative urine pools from each species were prepared by combining an equivalent percentage of the total urine weight from each urine sample up to 24 h resulting in a composite 0–24-h urine sample for mouse, dog, man, low and high dose rat. This procedure was repeated for each 12-h urine sample and for the 84–86 h urine sample from the high dose rats. These representative urine pools were used in all subsequent chromatographic analyses unless detailed otherwise.

Before chromatographic analysis the human urine pool was concentrated using solid phase extraction, but this procedure did not in any way alter the metabolic profile. Each sample was diluted with an equivalent volume of phosphate buffer (pH 9, 0·1 M) and passed through a C_8 Bond Elut which had been presolvated with methanol, deionized water and phosphate buffer. The Bond Elut was washed with deionized water and then the metabolites and parent compound eluted with methanol (200 μl per Bond Elut). Methanolic extracts were concentrated by evaporation to dryness under a steady stream of nitrogen and then reconstituted in a small volume of methanol before subsequent chromatographic analysis.

Selected urine samples, low dose rat 6–24 h, high dose rat 72–84 h and a human 0–24-h urine sample, were subjected to enzyme hydrolysis. Samples were hydrolyzed by incubation with both a specific sulphatase and a β-glucuronidase enzyme at 37°C in a shaking water bath for a period of 16 h. An aliquot of each urine sample was incubated with the appropriate enzyme and acetate buffer (pH 5, 0·1 M), together with an additional urine sample which was incubated using the same conditions but excluding the enzyme, to act as a control. Following incubation, the hydrolyzed samples and controls were concentrated using solid phase extraction as detailed above, and after checking that there was no loss or retention of radioactivity, they were analysed by t.l.c. Viability of the β-glucuronidase enzyme was confirmed by incubating phenolphthalein glucuronide and monitoring the production of phenolphthalein. Since specific enzymes had been used no inhibitors of the respective enzymes were incubated as further controls.

Plasma

Plasma samples from mouse, man, dog and rat also required prior concentration. The same solid phase extraction procedure was used as described for the urine, but the plasma buffer mixture was centrifuged and only the supernatant passed through the Bond Elut. Using this concentration procedure, recoveries of radioactivity were between 95 and 100% of the radiolabelled material originally present.

Radiochemical analysis

Amount of radioactivity in all individual urine and cage wash samples was determined directly by liquid scintillation counting using a Packard Tri-Carb 1500 or 2000CA (Canberra Packard, Berks, UK). All faecal samples were homogenized after the addition of a known amount of water and weighed aliquots were combusted using a Packard Tri-Carb Sample Oxidiser (Canberra Packard, Berks, UK). Radiolabelled carbon dioxide liberated from the oxidation of the faecal sample was trapped and quantified by liquid scintillation counting.

Total radioactivity in each sample type for each species was compared with the dose administered to provide total recovery of administered radioactivity.

Thin layer chromatographic (t.l.c.) analysis

Composite 0–24-h urine samples from each individual animal and a pool from each species were analysed by t.l.c. Subsequent 12 and 84–86-h composite urine pools from the high dose rats also were analysed using this t.l.c. technique, to provide radioactive metabolite profiles.

An aliquot (approximately 20 000 d.p.m. of each sample) was either applied directly to a t.l.c. plate or, in the case of human samples, applied as a methanolic solution following solid phase sample concentration. Samples were applied as a 1·5-cm band using a Linomat (Camag, Muttenz, Switzerland) automatic t.l.c. spotter. Reference standards of possible metabolites were also applied by direct spotting of methanolic solutions alongside each urine aliquot for comparison. T.l.c. plates were developed in a solvent system of dichloromethane-methanol-ammonia soln (sp. gr. 0·88) (70:30:1 by vol.), since previous work using reference standards had shown that this gave optimum separation. Separated radioactive metabolites were located and quantified using a Berthold LB2842 Automatic t.l.c. Linear Analyser (Berthold Ltd., Herts, UK) with Berthold Chroma Software, but a metabolite was not quantified if it accounted for $<1\%$ total of the radioactivity. Non-labelled reference compounds were located using quenching of background fluorescence at a wavelength of 254 nm. Following quantification each t.l.c. plate also was subjected to autoradiography for a period of at least 1 month before development of the film in order to confirm the metabolic profile. Concentrated plasma samples and hydrolysed urine samples also were analysed by t.l.c. in the same manner.

High performance liquid chromatographic (h.p.l.c.) analysis

Pooled urine samples (0–24 h) for each species were analysed by h.p.l.c. Rodent urine was analysed directly but human and dog samples required prior concentration using solid phase extraction. A

Table 1. H.p.l.c. solvent gradient programme

Time (min)	%A	%B	%C	%D
Initial	30	70	0	0
20	0	0	0	100
25	70	30	0	0
60	70	30	0	0
61	30	70	0	0

Solvent A = methanol.
Solvent B = methanol 5%; phosphoric acid (0·05 M) 95% v/v.
Solvent C = methanol 5%; water 95% v/v.
Solvent D = methanol 70%; water 30% v/v.
Total flow rate remained constant at 1 ml/min.

quaternary liquid chromatographic pump (Waters 600, Waters Associate Ltd., Watford, UK) and an autosampler (Waters 710B) were linked to a Ramona 5 Radiochemical detector (Raytest Ltd., South Yorkshire, UK) to monitor radiolabelled metabolic products in the h.p.l.c. eluate. Non-labelled reference compounds were monitored using an LC90 UV spectrophotometric detector (Perkin Elmer Ltd., Bucks. UK). The mobile phase consisted of four solvents: 30% methanol in deionized water; *o*-phosphoric acid (0·05 M); deionized water and methanol, the composition of which was varied with time according to a predefined gradient (table 1). Flow rate remained constant at 1·0 ml/min.

Results

Excretion balance

Averaged cumulative radioactivity and total radiolabelled material excreted for each species is shown in figure 2. Total recovery of radioactivity in the urine and faeces of all animal species and human dosed orally with radiolabelled (±)-fenfluramine hydrochloride (1 mg/kg) was within the accepted range of 100 ± 10% with the majority being recovered in the first 24 h in all species except man, where a slightly slower rate of elimination of total radiolabelled material was observed, but the majority of excretion still occurred within the first 48 h. However in all species radioactivity was excreted predominantly into urine (>80%) and metabolic profiling was performed therefore only on urine samples.

T.l.c. metabolite profiles

Urine samples. Between the species, 11 metabolites in total were resolved using the t.l.c. method detailed. Comparing the relative Rf values of the available reference compounds with the radioactive metabolites (figure 3), metabolites 1–3 were probably conjugates, 4–7 acidic moieties, components 8 and 9 norfenfluramine and fenfluramine respectively, and metabolites 10 and 11 were probably deaminated neutral products. However, it was noteworthy that analysis of the 0–24-h urine pools from each species and the 84–86-h urine pool from the high dose rat showed that there were important species differences in the metabolism of fenfluramine as detailed below and summarized in table 2.

Man. The metabolic profile of man indicated that one major metabolic route of fenfluramine existed producing the conjugate U2 accounting for 54% of the radioactivity in the 0–24-h composite urine sample. Other metabolites which also appeared in the profile were U1 (2%), U3 (15%), which were possibly additional

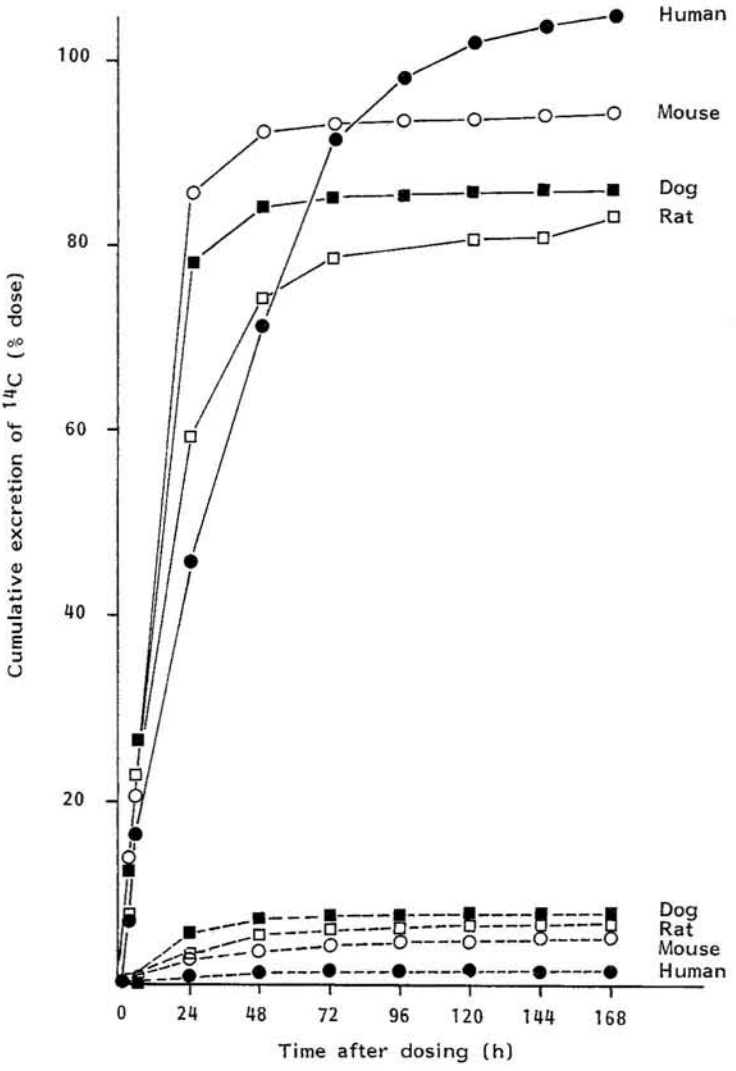

Figure 2. Cumulative excretion of radioactivity into urine and faeces following oral administration of ^{14}C-(±)-fenfluramine to mouse, rat, dog and man.
For mouse, rat and dog the urine profiles includes the cage washings. The dose was 1 mg/kg.
Data are shown for urines (—) of human (●), mouse (○), dog (■) and rat (□); and faeces (- - -) of human (●), mouse (○), dog (■), and rat (□)

conjugates; U5 (8%) which had the same Rf as the hippurate and U8 (4%) and U9 (9%) which corresponded to norfenfluramine and fenfluramine respectively.

Hydrolysis of the urine indicated that U2, the major metabolite, was the glucuronide conjugate of the diol metabolite. The metabolic profile thus indicated that man is highly efficient at the deamination of fenfluramine to then one major component, the diol, which is excreted as its glururonide in urine.

Mouse. The major urinary components in the mouse were U8 and U9 representing norfenfluramine and fenfluramine, and accounted for 31 and 29% of the

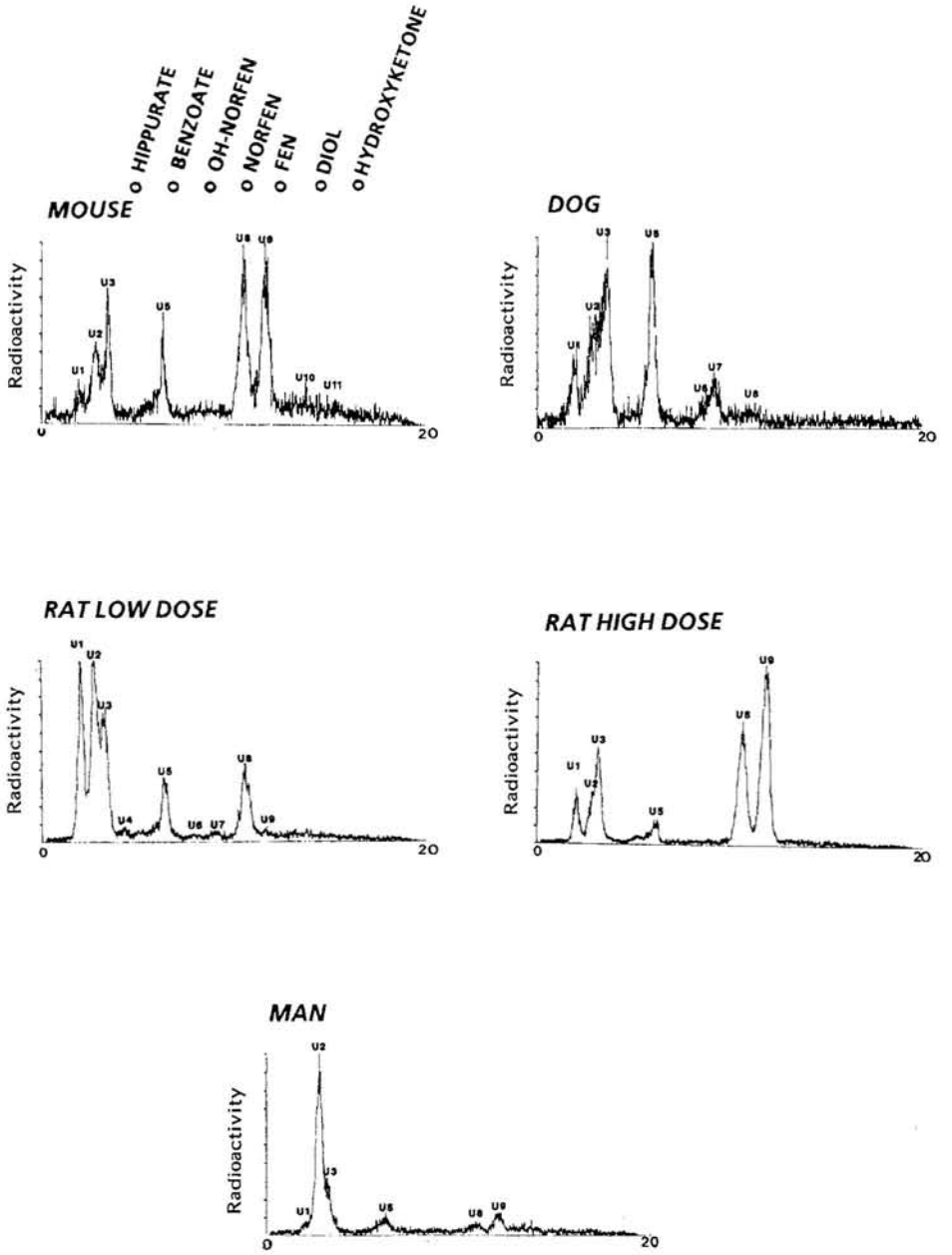

Figure 3. T.l.c. radiochromatograms of urines of man and animals dosed with ^{14}C-fenfluramine. Reference compounds are: benzoate = *m*-trifluoromethylbenzoic acid; hippurate = *m*-trifluoromethylhippuric acid; OH-NORFEN = β-hydroxynorfenfluramine; norfen = norfenfluramine; fen = fenfluramine; diol = *m*-trifluoromethylphenylpropane-1,2-diol; hydroxyketone = *m*-trifluoromethylphenylpropan-1-hydroxy-2-one.

radioactivity respectively in the 0–24-h urine sample (table 2). Other metabolites present in the mouse urine sample were U1 (2%), U2 (9%) and U3 (11%) all believed to be conjugates. Other minor components also present were U5 (9%) which had the same Rf as the hippurate and U10 (3%) and U11 (2%) which were possibly by comparison with the Rf values of the known standards, the diol and

Table 2. Comparison of metabolite profiles observed in urine samples of animals and man dosed with ^{14}C-(±)-fenfluramine

Metabolite	Mouse (0–24 h)	Rat high dose (84–86 h)	Rat high dose (0–24 h)	Rat low dose (0–24 h)	Dog (0–24 h)	Man (0–24 h)
U1	2	3	6	22	7	2
U2	9	17	4	24	18	54
Diol glucuronide						
U3	11	17	12	16	24	15
U4	ND	ND	ND	1	ND	ND
U5	9	3	3	10	21	8
Hippurate						
U6	ND	7	ND	ND	3	ND
U7	ND	ND	ND	1	8	ND
U8	31	27	30	18	4	4
Norfenfluramine						
U9	29	18	42	1	ND	9
Fenfluramine						
U10	3	2	ND	ND	ND	ND
diol						
U11	2	2	ND	ND	ND	ND
Hydroxyketone						

Data are expressed as percentage of total ^{14}C in urine samples.

ND denotes not detected in <1% of total radioactivity.

All animals were given 1 mg/kg ^{14}C-(±)-fenfluramine orally except high dose rats which were given 24 mg/kg b.i.d. for 4 days subcutaneously.

Table 3. Comparison of metabolite profiles observed in plasma samples of animals and man dosed with ^{14}C-(±)-fenfluramine

Metabolite	Mouse (2 h)	Rat low dose (2 h)	Rat high dose (2 h)	Dog (3 h)	Man (3 h)	Man (8 h)
P1	11	10	3	ND	6	ND
P2	10	15	1	14	34	16
P3	9	5	9	32	ND	ND
P4	7	ND	ND	ND	ND	ND
P5	1	ND	ND	15	ND	ND
P6	4	ND	ND	27	ND	ND
P7	3	ND	ND	ND	ND	ND
P8	21	57	33	4	22	12
Norfenfluramine						
P9	27	5	38	1	24	38
Fenfluramine						
P10	2	ND	5	ND	ND	17
P11	6	ND	13	ND	ND	ND

ND denotes not detected in <1% of total radioactivity.

All animals were given 1 mg/kg ^{14}C-(±)-fenfluramine orally, except high dose rats which were given 24 mg/kg b.i.d. for 4 days subcutaneously.

Sampling time are given in parenthesis under each species.

hydroxyketone metabolite respectively. This metabolic profile suggests that the mouse is capable of *N*-dealkylation as indicated by the formation of norfenfluramine, but less efficient in the deamination of fenfluramine than man.

Rat. The major urinary metabolites in the rat which had been given an oral dose of 1 mg/kg were conjugates (U1 (22%), U2 (24%) and U3 (16%)). Other significant metabolites were the possible hippurate U5 (10%) and U8 (18%) which was presumed to be norfenfluramine by comparison of Rf with reference compounds. Minor metabolic components also observed in the profile were U4, U7 and U9 (fenfluramine) representing individually $\leqslant 1\%$ of the radioactivity. This profile indicated that the rat efficiently and rapidly biotransforms fenfluramine producing a number of metabolites, of which norfenfluramine predominates.

In contrast when rats were given eight consecutive 12-h doses (24 mg/kg) of fenfluramine subcutaneously, a very different 0–24-h urine metabolic profile was observed. The most apparent difference in the profile was the predominance of fenfluramine in the sample, accounting for 42% of the radioactivity in the sample and the reduction in polar compounds U1–U3 indicating that the metabolism of fenfluramine and to a lesser extent norfenfluramine was saturated at high doses. Qualitatively the metabolic profiles of the two dose levels were similar in that both indicated the presence of the conjugates U1, U2 and U3 as well as U5 and norfenfluramine (U8). Chromatographic analysis of the 84–86-h urine composite pool from high dose rats, (that is 0–2 h after the last subcutaneous dose of fenfluramine) again showed a qualitatively similar metabolic profile however, three additional minor metabolites appeared with time: U6 (7%), U10 (2%) and U11 (2%).

In addition, the rat high dose 72–84-h composite urine sample was subjected to enzyme hydrolysis. This urine sample was chosen since it was quantitatively similar to the 84–86-h sample, but there was a larger volume. Results indicated that U2 and U3 were indeed conjugates and by comparison with reference compounds were possibly the glucuronides of the diol and hydroxyketone. From these data alone it was not possible to assign either of these aglycones to the respective conjugates.

Dog. The dog demonstrated a very different urinary metabolic profile with very little norfenfluramine (4%) being produced as a metabolite and no detectable fenfluramine. This would indicate that the dog has both the capability to *N*-deethylate fenfluramine and deaminate norfenfluramine. The major components in this profile were U1 (7%), U2 (18%), U3 (24%) which by comparison with the rat profiles were conjugates and U5 (21%) which corresponded to the reference hippurate standard. Minor metabolites, U6 (3%) and U7 (8%) were also apparent. This metabolic profile demonstrated that the dog had a variety of routes for metabolizing fenfluramine with no specific biotransformation pathway predominating.

Plasma samples

Man. Unfortunately, only in the 3 and 8-h plasma was there sufficient radiolabelled material to produce metabolic profiles and as these samples also had relatively low levels of radioactivity, it was not possible to quantify separately P2 and P3 on the chromatograms as it had been on urine chromatograms (table 3).

However, the plasma profile indicated that the conjugated metabolites P1, P2 and P3 were present as were P8 and P9 representing norfenfluramine and fenfluramine. Interestingly, the 8-h plasma sample also contained P10 (unconjugated diol) (17%) which was observed when a human urine 0–24-h sample was incubated with a specific β-glucuronidase. Plasma profiles therefore supported the urine profiles indicating that there was no metabolite in the plasma which could not be accounted for in the urine.

Mouse. The plasma metabolite profile of the mouse showed that all eleven metabolites were present. However, like the urine profile, the major metabolites were P8 (21%) and P9 (27%) representing norfenfluramine and fenfluramine respectively. Again the postulated conjugates, P1 (11%), P2 (10%) and P3 (9%) were present in the sample. Overall the plasma metabolite profile was a very good reflection of the 0–24-h composite urine profile.

Rat. The low dose rat plasma metabolite profile had one major metabolite P8 (57%) which had the same Rf value as norfenfluramine. A small amount of fenfluramine (P9, 5%) was also present in the plasma. The only other metabolites present in the plasma were the conjugates P1, P2 and P3. Qualitatively the plasma and urine profiles of the low dose were similar, although quantitatively there were less conjugates in the plasma, as would be expected from these more polar compounds.

In contrast the plasma profile from the high dose rats contained a large amount of P9 (38%) representing fenfluramine as well as P8 (33%), norfenfluramine. Other metabolites present were P1 (3%), P2 (1%), P3 (9%), P10 (5%) and P11 (13%). Again the high dose rat plasma profile was a good representation of the urine metabolite profile, indicating the saturation of fenfluramine biotransformation when compared to the low dose rat plasma profile.

Dog. The dog plasma metabolite profile was very similar to the urine profile, both matrices containing very little norfenfluramine and fenfluramine. In addition, like the urine, there would appear to be no single major metabolite, but four metabolites, P2 (14%), P3 (32%), P5 (15%) and P6 (27%) of approximately equivalent importance. It was interesting to note that as with the other species studied the possible conjugates P2 and P3 were present in the plasma. The only differences between the dog urine and plasma profiles were the absence of P1 in the plasma and the higher levels of P6 in the plasma when compared with urine. This could indicate that P6 is a metabolic precursor of P1. However, overall, the metabolite profiles from the two biological media are very similar.

H.p.l.c. metabolite profiles of urine samples

H.p.l.c. analysis of 0–24-h urine samples was performed to support species differences in the metabolism of ^{14}C-(±)-fenfluramine which had been observed using t.l.c. In addition the h.p.l.c. system was developed to aid isolation and identification of fenfluramine and its urinary metabolites in man, which are reported elsewhere (Brownsill *et al.* 1991).

Qualitatively, in all species, the h.p.l.c. profiles were representative of the t.l.c. chromatograms with respect to the quantities of norfenfluramine and fenfluramine. The other metabolites were also present in each of the samples although it was not possible to assign possible metabolite structures to these peaks (figure 4).

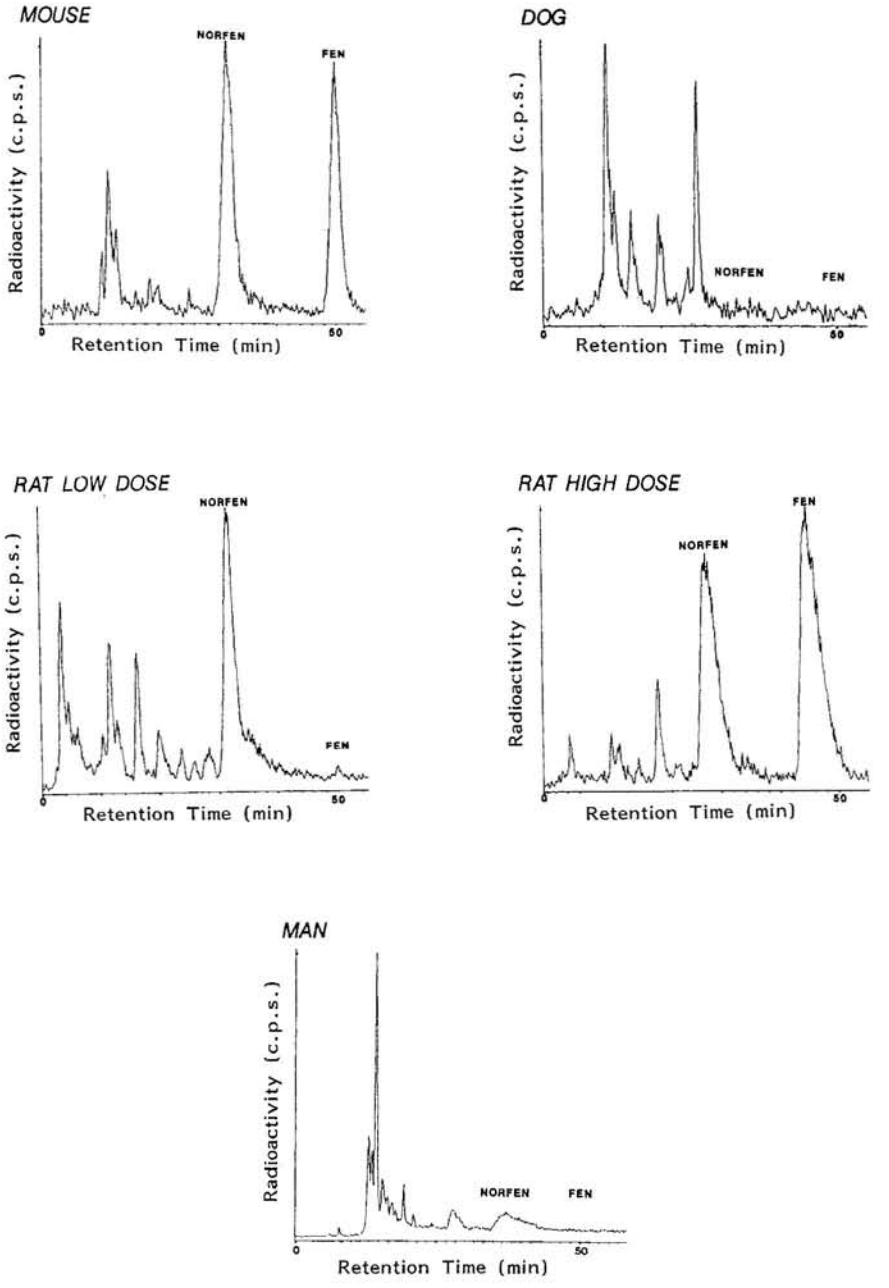

Figure 4. H.p.l.c. radiochromatograms of urines of man and animals dosed with ^{14}C-fenfluramine.

The human h.p.l.c. metabolite profile was particularly interesting with one major radiolabelled metabolite being present and only small amounts of norfenfluramine and fenfluramine, as observed in the t.l.c. profile. Analysis of the mouse urine indicated that, as with t.l.c., the major peaks corresponded to norfenfluramine and fenfluramine with some other minor metabolites. The low dose rat

metabolite profile, however, confirmed that a large percentage of the sample was norfenfluramine as indicated in the respective t.l.c. profile, with no detectable peak for fenfluramine. Again, other radiolabelled peaks were apparent in the chromatogram as indicated in the t.l.c. profile. In comparison the high dose rat 0–24-h h.p.l.c. metabolite showed that there were high levels of both norfenfluramine and fenfluramine as well as other radiolabelled metabolites. This again supported t.l.c. analysis of the 0–24-h urine sample indicating that at these dose levels, 24 mg/kg b.i.d. subcutaneously, the metabolism of fenfluramine became saturated.

In contrast, the h.p.l.c. metabolite profile of the dog 0–24-h urine sample was interesting by the virtual absence of both norfenfluramine and fenfluramine as was indicated in t.l.c. analysis of the same sample. There were, however, at least five other metabolites present in the profile.

Overall, the h.p.l.c. results confirmed the results found by t.l.c., that the metabolic profiles in all the species studied were essentially different to that shown in man.

Discussion

The metabolism of *N*-substituted *β*-phenylethylamines, which are structurally similar to fenfluramine, has been extensively reviewed by Caldwell (1976) who reported that this group of compounds can undergo aromatic or aliphatic hydroxylation, *N*-dealkylation, oxidative deamination, *N*-oxidation and conjugation. However, marked species differences occur in the biotransformation of these *N*-substituted *β*-phenylethylamines.

In the rat deamination is not a major metabolic route for this group of compounds. In fact no intermediary metabolites of this biotransformation have been observed in the urine of this species and the major route of metabolism reported is hydroxylation and de-alkylation. Metabolic studies with the dog have shown *N*-dealkylation and deamination are both important in the biotransformation of this class of compounds. However, it was observed that the dog excretes a significant amount of these chemical moieties in the unchanged form but the extent depends on the hydrophilicity of the drug. The mouse is also capable of deamination, excreting both benzoic acid and its conjugates (Dring *et al.* 1970); although like the dog a significant amount is excreted as the unchanged *β*-phenylethylamine.

Deamination of *N*-substituted *β*-phenylethylamines is also observed in man with the excretion of benzoic acid as its glycine conjugate. Indirect evidence (Parli *et al.* 1971) indicates that oxidative deamination occurs via hydroxylation of the carbon atom bearing the amino group, resulting in an unstable carbinolamine. The chemical instability of this metabolic intermediate leads to its breakdown to the corresponding ketone and amine. The resulting ketone can be further metabolized in a variety of ways; it can be reduced to the corresponding alcohol; oxidized to produce the benzoic acid derivative; or less commonly undergo sulphate conjugation of the enol. In addition it is also believed that *N*-dealkylation also occurs via this mechanism (Beckett 1972), but that in this instance the carbinolamine is formed on the other carbon atom adjacent to the amino group. Again, an unstable carbinolamine is formed which breaks down to produce the *N*-dealkylated product and an aldehyde. However, fenfluramine is substituted with a CF_3 group and therefore the metabolism will be different, particularly in those animals which hydroxylate the phenyl ring.

Our studies with fenfluramine generally substantiate the summary of Caldwell with one or two minor divergencies, in particular, the CF_3 substitution produces a higher lipid solubility, allowing less to be excreted unchanged and steric hindrance of ring hydroxylation. Following oral administration of fenfluramine (1 mg/kg) all species extensively metabolize the drug (although less so in the mouse). Man appears to metabolize efficiently fenfluramine principally by de-amination, producing one major metabolite, U2, which recently has been identified using mass spectrometry as the glucuronide conjugate of the diol (Brownsill *et al.* 1991). Earlier workers (Bruce and Maynard 1968) identified the major urinary metabolite in man as the hippurate accounting for 66–93% of the dose, but used only g.l.c. retention parameters to quantify and characterize the metabolite. Brownsill *et al.* (1991) did observe the hippurate as a urinary metabolite but it accounted for only 8% of the radioactivity in the 24-h urine.

However, the rat, in contrast to man, that would normally hydroxylate a phenylethylamine is unable to do so, due to the CF_3 substitution causing steric hindrance, and dealkylates fenfluramine producing norfenfluramine. This biotransformation is relatively extensive in the rat, unlike *N*-deamination, as indicated by the relatively high levels of norfenfluramine in the urine. Enzyme hydrolysis studies further highlighted the differences between rat and man. Following incubation with a specific β-glucuronidase two conjugated metabolites were cleaved, whereas in man only one product of hydrolysis was observed.

Following administration of repeat high doses of fenfluramine to rats the difference between the metabolic capability of man and this animal species is especially apparent. A predominance of fenfluramine was present in urine indicating that at this high dose level the metabolism of fenfluramine had become saturated, confirming the work of Caccia *et al.* (1981) who found that at doses above 40 mg/kg the rat had a limited capacity for metabolism of fenfluramine.

In contrast, the dog extensively metabolizes fenfluramine by a variety of routes producing a number of metabolites of similar importance. Although little unchanged drug or norfenfluramine is found in urine or plasma, the relative amount of norfenfluramine indicates a comparatively greater process of dealkylation than deamination, as shown for other β-phenylethylamines (Axelrod 1954, Walkenstein *et al.* 1955). By comparison in the mouse deamination is less efficient leading to higher levels of the parent compound and norfenfluramine in urine. Caccia *et al.* (1982) found similar results when studying the metabolism of β-phenylethylamines in the mouse.

A metabolic pathway indicating all the biotransformations proposed in this study is given in figure 5.

By comparing the plasma ratio of norfenfluramine to fenfluramine in the different species studied (figure 6), the difference in deaminating capability between the rat and man is highlighted, whereas the mouse produces a similar plasma ratio to man, confirming the work of Garattini *et al.* (1979) who found that following administration of anorectic doses of *d*-fenfluramine to rat, dog, mouse and man, plasma AUC ratios (norfenfluramine/fenfluramine) of 2·8, 5·4, 0·3 and 0·7, respectively, were observed. Interestingly, Garattini (1986) also observed that the mouse had a lower norfenfluramine/fenfluramine brain ratio than the rat (0·15 compared with 1·75), which would indicate that the human brain ratio would also be very low, and indeed this has been confirmed in a case of fenfluramine overdosage (Fleisher and Campbell 1969) where a ratio of 0·13 was measured.

There is no doubt that significant species differences in the metabolism of fenfluramine do occur, as demonstrated in this study, particularly in the extent of deamination and dealkylation, and this could be very important in view of the different pharmacological effects of norfenfluramine and the parent compound. Both compounds have been shown to have a similar activity (Hirsh and Wurtman 1981), but Garattini *et al.* (1989) have shown that fenfluramine produces its anorectic effect by inhibiting the re-uptake of 5-HT into serotonergic neurones, whilst norfenfluramine predominantly increases the release of 5-HT.

The conclusion reached by Caldwell (1976) in his review was that no species metabolizes the *N*-substituted β-phenylethylamines in the same way as man and that the rat is probably the poorest indicator of the metabolism of this class of compounds. Indeed, he recommends that the use of the rat should be contraindicated in any studies which purport to have relevance to the human situation. Our investigations of the metabolism of fenfluramine certainly support these findings, as although mouse, dog, the low and high dosed rat and, from recent unpublished results in our laboratories, the cynomolgus and squirrel monkey, all have common

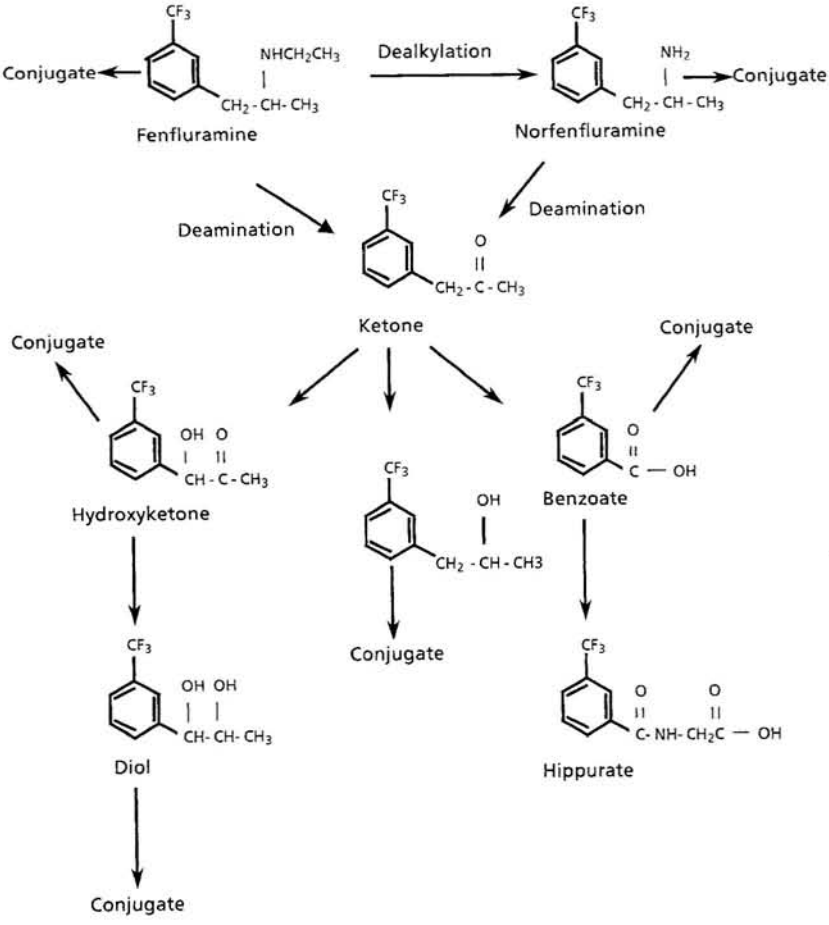

Figure 5. Postulated metabolic pathway of ^{14}C-(±)-fenfluramine.

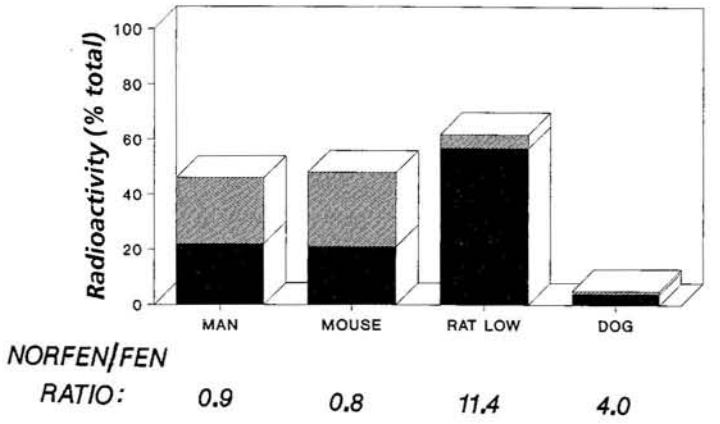

Figure 6. Ratios of norfenfluramine to fenfluramine in plasma of mouse, rat, dog and man.

metabolites, no one species is truly representative, either qualitatively or quantitatively, of the human metabolic profile.

In man, there is efficient deamination of fenfluramine and the dealkylated active metabolite, norfenfluramine, to inactive polar metabolites which are excreted into the urine. In rat, there is poor deamination, whilst in dog, there is relatively greater dealkylation, both leading to an excess of norfenfluramine and a high plasma norfenfluramine/fenfluramine metabolic ratio. In contrast, for mouse, there is poor dealkylation and less deamination, producing relatively high combined levels of both fenfluramine and norfenfluramine, but with a plasma metabolic ratio similar to that seen in man.

These species differences in the metabolism of fenfluramine and norfenfluramine clearly make it difficult to interpret pharmacological studies, particularly when the mechanism of activities of these two active compounds are not the same, and this should be borne in mind when using animal models to predict the effect of fenfluramine in man.

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