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Disposition of human drug preparations in the horse. II. Orally administered fencamfamine

F.T. DELBEKE* and M. DEBACKERE

Labo voor Farmacologie en Toxicologie, Faculteit Diergeneeskunde, Universiteit Gent, Casinoplein, 24, B-9000 Gent, Belgium

Abstract: A gas chromatographic method to measure urinary levels of the central nervous system stimulant fencamfamine and some of its metabolites is described. When 100 mg fencamfamine was given orally to four horses the parent drug could not be detected in the urine. After enzymatic hydrolysis of the urine the major human metabolite, N-desethylated fencamfamine, only accounted for 1% of the dose in 12 h. The major equine metabolites were conjugated parahydroxylated compounds representing 18% of the dose. With regard to horse doping control and analysis, the injudicious use of human doping routine methods for the detection of fencamfamine in equine urine could lead to false negative results.

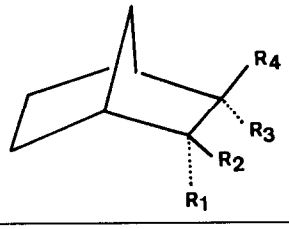
Keywords: *Doping; fencamfamine; horse; metabolism; urinary excretion; CNS stimulant.*

Introduction

Due to the continuing improvement in analytical methods and equipment, most racing laboratories are able to detect typical veterinary drugs in horse urine and plasma. The continuing challenge for the analyst has shifted towards the detection of the misuse of endogenous substances, very small amounts of tranquillizers or narcotic analgesics and to the detection in equine body fluids of drugs developed for use in humans. Recently the disposition of the anti-inflammatory drug indomethacin was studied in the horse after the administration of a human suppository preparation [1]. In this work the excretion and metabolism of fencamfamine in the horse is reported.

Fencamfamine, *N*-ethyl-3-phenyl-8,9,10-trinorbornan-2-ylamine, (Fig. 1) is a sympathomimetic central stimulant widely abused in sports. This drug is considerably less basic than amphetamine but at the same time more lipophilic [2]. Consequently, as horse urine is generally slightly alkaline, the suppression of the renal excretion of fencamfamine might lead

Figure 1
Structure of fencamfamine and some of its principal metabolites.



	R_1	R_2	R_3	R_4
Fencamfamine	H	C_6H_5	NHC_2H_5	H
N-desethylfencamfamine	H	C_6H_5	NH_2	H
M1	H	*p*-OH-C_6H_4	NH_2	H
M2	H	*p*-OH-C_6H_4	HNC_2H_5	H
M3	*p*-OH-C_6H_4	H	H	NHC_2H_5
I.S.*	H	*p*-OH-C_6H_4	$NHCH_3$	H

*Internal standard for the determination of the parahydroxylated metabolites.

*Author to whom correspondence should be addressed.

to urinary concentrations below levels detectable by routine doping analysis methods.

Materials and Methods

Experimental animals

Four standard bred mares were fasted from the afternoon preceding the day of the experiment. Tablets corresponding to a total dose of 100 mg were mixed with oats and given to the horses.

A balloon-tipped catheter was placed in the bladder of each mare. The total volume in the bladder was collected during a 12 h period after drug administration with sampling times at 0, 1, 2, 3, 4, 6, 9 and 12 h. Urinary pH and volume were measured and aliquots analysed in duplicate.

Reagents

The reference substances fencamfamine.HCl and N-desethylated fencamfamine.HCl were obtained through Merck (Brussels, Belgium). The parahydroxylated metabolites were synthesized by Janssen Pharmaceutica (Beerse, Belgium), mepivacaine hydrochloride was obtained from Astra (Södertälje, Sweden).

Dichloromethane, methanol, diethylether and ethyl acetate, analytical grade was obtained from Merck (Darmstadt, Germany). Trifluoroacetic anhydride was from Macherey–Nagel (Düren, Germany).

The enzyme preparation Suc *Helix pomatia* (SHP) containing 100,000 Fishman units per ml of β-glucuronidase and 1,000,000 Roy units per ml of arylsulphatase was obtained from IBF (Villeneuve, France). The ammonia buffer was prepared by adjusting a saturated ammonium chloride solution to pH 9.5 with ammonia.

Equipment

All chromatograms were generated in the split mode (split ratio 1:10) on a Varian 3400 gas chromatograph (Walnut Creek, CA, USA) fitted with a 25 m × 0.25 mm i.d. fused silica Permabond OV-1 column (Macherey–Nagel) with a film thickness of 0.25 μm. The GC was equipped with an autosampler, a nitrogen-selective detector and interfaced with an IBDH data processor. Injector and detector temperatures were maintained at 280°C. The oven temperature was programmed as follows. Initial temperature: 160°C; initial hold, 1 min; temperature program rate, 10°C min^{-1}; final temperature, 280°C.

Helium was used as the carrier gas at an inlet pressure of 1.1 bar. Detector make-up flow rate was 25 ml min^{-1}.

GC–MS was carried out on a Hewlett–Packard (Palo Alto, CA, USA) 5993 instrument equipped with a HP Ultra-performance fused silica column (25 m × 0.20 mm i.d., methylsilicone cross-linked). Injections were made with an all-glass moving needle; the column was directly coupled to the MS source. GC conditions were: injector temperature, 270°C; oven temperature program from 140 to 280°C at a rate of 10°C min^{-1}; transfer line temperature, 250°C. Helium was used as carrier gas (0.8 ml min^{-1}). Electron impact (E.I.) spectra were recorded at 70 eV.

Analytical procedure

Fencamfamine and N-*desethylfencamfamine. Without hydrolysis.* Urine (5 ml) in a screw-capped tube was made alkaline with 0.5 ml of ammonia buffer. After adding 50 μl of the internal standard solution (mepivacaine 50 μg ml^{-1}) the urine was extracted by rolling with 5 ml of dichloromethane–methanol (9:1, v/v) for 15 min. Back-extraction was performed by vortexing the organic layer with 1 ml 0.1 mol l^{-1} HCl for 0.5 min. Afterwards the aqueous layer was made alkaline and extracted with 5 ml CH_2Cl_2–MeOH (9:1, v/v).

The organic layer was dried with Na_2SO_4 and one drop of diethylether saturated with HCl was added. After evaporation under nitrogen the residue was dissolved in 200 μl ethylacetate–methanol (9:1, v/v) and 1 μl injected.

Enzymatic hydrolysis. Urine (5 ml) was buffered with 1 ml 1 mol l^{-1} sodium acetate buffer (pH 5.2) and 50 μl of the enzyme preparation SHP added. Hydrolysis was performed for 2 h at 56°C. After cooling, the hydrolysate was made alkaline with 0.5 ml ammonia buffer and subsequently analysed as described above.

Parahydroxylated metabolites. A 5 ml volume of urine was hydrolysed as above. After cooling, 50 μl of the internal standard [2-methylamino-3-(parahydroxyphenyl)norbornane hydrochloride, 50 μg ml^{-1} in water] was added. The hydrolysate was buffered with ammonia buffer and extracted as described above. After evaporation, the residue was

redissolved in 150 µl ethylacetate and 50 µl TFAA added. Derivatization was performed at 80°C for 30 min. Afterwards, the solvent and excess TFAA were evaporated under nitrogen at 40°C, the residue dissolved in 200 µl ethylacetate; 1 µl was injected for GC or GC–MS analysis.

Quantitative determination. Standard curves were obtained by subjecting spiked urine samples (0, 0.1, 0.25, 0.5, 1 and 2 µg ml^{-1}) to the appropriate extraction method in quadruplicate at each concentration. The accuracy of the assay was measured for two different (0.25 and 1 µg ml^{-1}) concentrations of fencamfamine and its N-desethylated metabolite.

Results and Discussion

Under the chromatographic conditions described, fencamfamine, *N*-desethylfencamfamine and the internal standard mepivacaine gave symmetrical peaks with retention times of, respectively, 4.80, 4.07 and 9.82 min. The standard curves were linear over the range 0.1–2 µg ml^{-1} (σ_R = 0.999 and 0.997 for, respectively, fencamfamine and *N*-desethylfencamfamine). Starting with 5 ml urine, a concentration of 0.1 µg ml^{-1} could easily be measured. However, in order to obtain reproducible results, fencamfamine and the desethylated metabolite should be converted into the less volatile salts by the addition of one drop of diethylether saturated with HCl before evaporation. In this way, good accuracy was obtained as illustrated in Table 1. The structure of each of the synthesized parahydroxylated metabolites M1, M2 and M3 (Fig. 1) was elucidated by NMR analysis [J. Bracke, personal communication]. These metabolites required derivatization before GC analysis. Trifluoroacetylation at room temperature resulted in the formation of monoderivatives. The characteristic ions were: m/z = 186; m/z = 158 (base peak); m/z = 107; and m/z = 91. The mass spectrum of the mono-derivatized metabolite M1 is illustrated in Fig. 2.

Table 1
Accuracy of the fencamfamine and *N*-desethylfencamfamine assay

Sample conc. (µg ml^{-1}) (n = 4)	Concentrations found (RSD, %)	
	Fencamfamine	N-desethylated metabolite
0.25	0.240 (2.9)	0.258 (5.8)
1	0.995 (2.3)	1.076 (2.0)

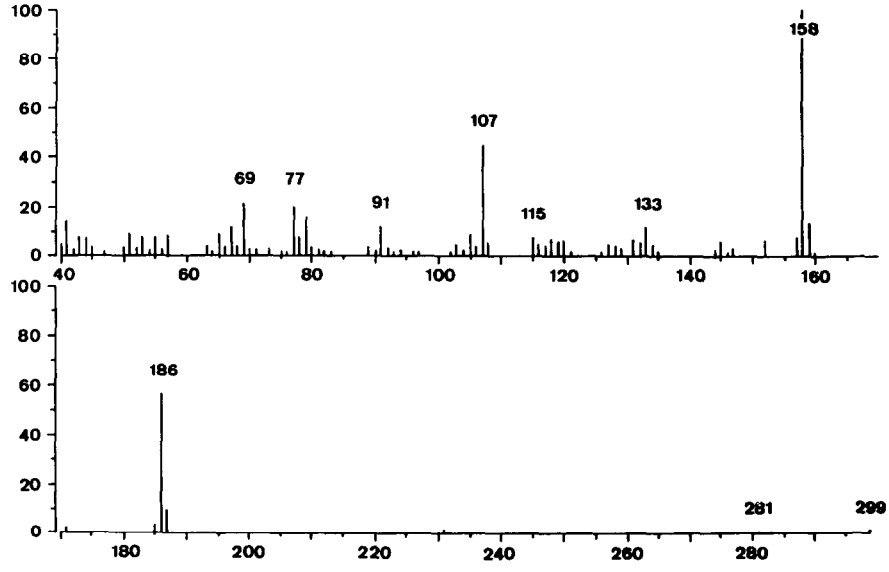


Figure 2
Mass spectrum of mono-TFA-derivatized M1 metabolite of fencamfamine.

However, derivatization at room temperature was not complete, giving rise to irreproducible quantitation. Therefore, the extraction residue was derivatized at 80°C. Using different reaction times (0.5, 1 and 2 h), the reaction appeared to be complete after 30 min. Under these conditions the N,O derivatized parahydroxylated metabolites gave symmetrical peaks, while the standard curves were linear over the range 0.1–2 μg ml^{-1} (σ_R = 0.998 for M1, M2 and M3).

Major ions in the mass spectrum of the parahydroxylated metabolites were: m/z = 254 (base peak); m/z = 282; m/z = 169; and m/z = 203. The mass spectrum of the bis-derivatized metabolite M3 is illustrated in Fig. 3.

The therapeutic dose of fencamfamine in humans is 10–20 mg. Taking into account the respective body weights, 100 mg fencamfamine was given to the horses. When the routine human doping analysis method (fencamfamine and N-desethylated metabolite) was used to detect the misuse of fencamfamine in horses, no parent drug could be detected. This is probably due to the alkaline character of equine urine. Indeed, the excretion of weak basic drugs and especially fencamfamine is strongly dependent on the urinary pH [3]. As can be seen from Table 2, the same is true for the N-desethylated metabolite which was not detected during the first 4 h in horse 4, for which the urinary pH range was 7.8–8.7 during this period.

The urinary concentrations of fencamfamine and the major human metabolite in equine urine were far below the values found in humans [3]. Hence the injudicious use of analytical methods developed for doping in man, for the detection of fencamfamine in horse urine, could lead to false negative results. The maximum detection period (i.e.

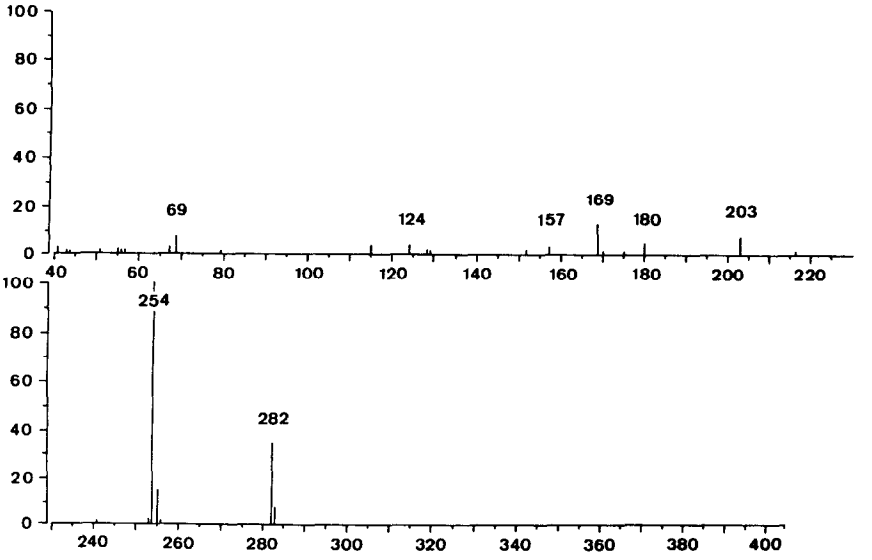


Figure 3
Mass spectrum of bis-TFA-derivatized M3 metabolite.

Table 2
Urinary concentration* of N-desethylated fencamfamine in four horses

t (h)	Horse 1	Horse 2	Horse 3	Horse 4
1	0.28 (0.95)	—	—	—
2	0.43 (1.32)	—	—	—
3	0.31 (0.76)	0.10 (0.16)	—	—
4	0.17 (0.26)	0.17 (0.41)	0.10 (0.13)	—
6	—	0.12 (0.25)	0.30 (0.88)	0.11 (0.67)
9	—	0.11 (0.24)	0.22 (0.40)	0.11 (0.83)
12	—	—	0.09 (0.12)	— (0.17)

*Concentrations in μg ml^{-1}; the figures in brackets represent values after enzymatic hydrolysis with SHP.

the time during which the drug could be detected) for the N-desethylated metabolite varied from 4 to 12 h and was strongly dependent on the urinary pH. The mean percentage of the dose excreted as N-desethylated fencamfamine in 12 h was 0.28 ± 0.10% when the urine was not hydrolysed. Enzymatic hydrolysis resulted in slightly higher values (0.98 ± 0.42%), ranging from 0.7 to 1.6%.

Hydroxylation of the phenyl group followed by conjugation is a frequent metabolic pathway in the horse. When equine urine was enzymatically hydrolysed and subsequently derivatized with TFAA, three additional GC peaks were noticed (Fig. 4). GC–MS analysis revealed the presence of the metabolites M2 and M3 resulting from parahydroxylation of fencamfamine, and of M1 arising from parahydroxylation of the desethylated metabolite. The resolving power of capillary GC enabled the detection of both the *trans*-iosmers, M2 and M3. The product M3 most probably occurred by hydroxylation of the enantiomer present in small amounts in the fencamfamine preparation. Retention time data and mass spectral data obtained for the urinary metabolites were identical to those for the synthetic material.

The concentrations of the parahydroxylated metabolites are summarized in Table 3. Generally, when the urine was not hydrolysed, only small amounts of the hydroxylated metabolites were detected. After hydrolysis most of these metabolites could be detected from 1 to 12 h after dosing. Inter-subject variations re-

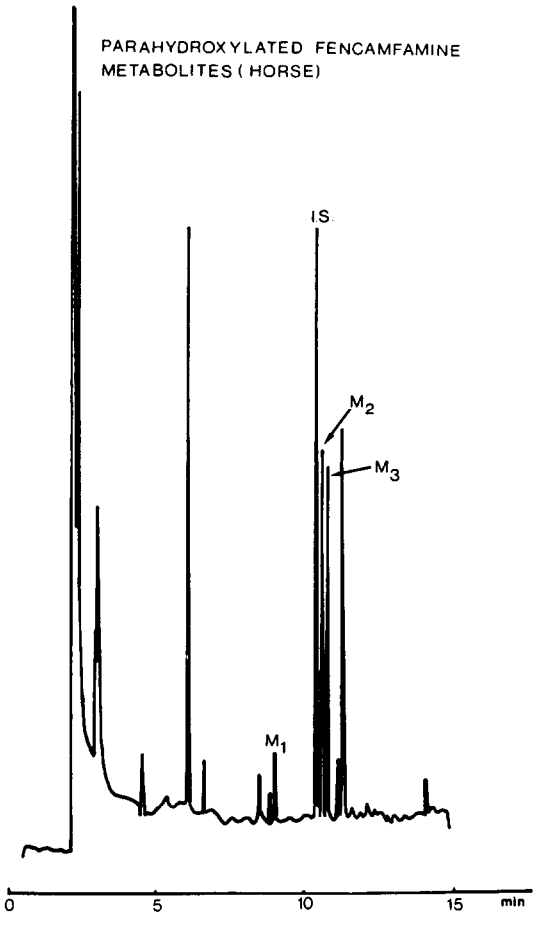


Figure 4
GC detection of parahydroxylated fencamfamine metabolites (bis-TFA derivatives) in a spiked horse urine extract.

Table 3
Urinary concentration* of parahydroxylated metabolites after the oral administration of 100 mg fencamfamine to four horses

		Urine collection period (h)						
		0–1	1–2	2–3	3–4	4–6	6–9	9–12
Horse 1	M1	4.69 (—)	18.00 (0.30)	4.37 (0.24)	3.48 (0.20)	0.40 (—)	0.30 (—)	0.27 (—)
	M2	4.59 (—)	16.03 (0.10)	8.69 (0.10)	6.38 (0.06)	0.82 (0.07)	0.49 (—)	0.31 (—)
	M3	0.22 (—)	1.05 (—)	1.16 (—)	0.80 (—)	0.12 (—)	0.10 (—)	0.09 (—)
Horse 2	M1	— (—)	0.54 (—)	2.50 (—)	4.43 (—)	—	2.32 (—)	0.97 (—)
	M2	— (—)	0.45 (—)	2.11 (—)	3.53 (—)	0.11 (—)	5.00 (—)	2.56 (—)
	M3	0.44 (0.13)	0.30 (0.10)	0.44 (0.11)	0.72 (0.12)	0.44 (—)	1.41 (0.23)	0.88 (0.17)
Horse 3	M1	—	—	0.55 (—)	1.20 (0.28)	3.33 (0.25)	3.66 (0.28)	0.48 (—)
	M2	—	0.15 (—)	0.62 (—)	1.73 (0.45)	3.59 (—)	4.90 (—)	3.57 (—)
	M3	—	—	—	0.14 (—)	0.65 (—)	0.47 (—)	0.36 (—)
Horse 4	M1	—	—	0.20 (—)	0.23 (—)	2.50 (—)	2.41 (—)	0.78 (—)
	M2	—	—	0.25 (—)	0.28 (—)	9.42 (—)	6.62 (—)	3.75 (—)
	M3	—	—	—	—	0.32 (—)	0.45 (—)	0.29 (—)

*Concentration in μg ml^{-1}; the figures in brackets refer to the values obtained without enzymatic hydrolysis.

sulted in different maximum concentration times and peak excretion rates for metabolite M1, varying from 2 to 9 h. The mean percentage of the dose excreted as M1 in the four horses after 12 h was 6.6 ± 2.6 (range 3.6–9.6%). Generally, higher amounts of the parahydroxylated metabolite M2 were found in equine urine. The percentage of the dose excreted in 12 h varied from 5.4 to 15.5 (mean: 10.3 ± 4.2%). Maximum concentrations and peak excretion rate were both obtained after completely different time periods in the four horses, ranging from 2 to 9 h after dosing. The excretion of metabolite M3 in a 12 h period accounted for only 1.5 ± 1.1% of the dose (range 0.6–3.2%). In summary, the metabolism and disposition of the central nervous system stimulant fencamfamine in the horse differ from the data found in man [3], where the cumulative excretion of fencamfamine and its N-desethylated metabolite was found to be nearly equal after 80 h, and altogether represented 20% of the administered dose. In the horse, however, no parent drug was detected and N-desethylated fencamfamine only accounted for 1% of the dose after 12 h, this metabolite was not detectable after this period. Using analysis methods developed for doping in man as methodology in horse doping could therefore lead to false negative results.

It was found that three parahydroxylated fencamfamine metabolites were excreted as conjugates in the horse, representing nearly 20% of the dose after 12 h. Further studies in man are needed in order to establish whether or not these compounds are typical equine metabolites. Moreover, nearly 80% of the orally administered dose was not renally excreted in the horse. Multiple effects such as unknown and/or more polar metabolites, biliary excretion, drug metabolism by the gastro-intestinal flora and accumulation of the lipophilic drug in adipose tissue, could account for the fact that only a small percentage of the administered dose was recovered in horse urine.

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