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Detection of fenspiride and identification of in vivo metabolites in horse body fluids by capillary gas chromatography–mass spectrometry: administration, biotransformation and urinary excretion after a single oral dose

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Abstract

Studies related to the in vivo biotransformation and urinary excretion of fenspiride hydrochloride in the horse are described. After oral administration, the drug is metabolised by both phase I functionalisation and phase II conjugation pathways. Following enzymatic deconjugation, fenspiride and its phase I metabolites were isolated from post-administration biofluids using bonded co-polymeric mixed mode solid-phase extraction cartridges to isolate the basic compounds. Following trimethylsilylation (TMS), the parent drug and metabolites were identified by capillary gas chromatography–mass spectrometry (GC–MS). Fenspiride (**A**) and seven metabolites (**B→G**) arising from oxidation on both the aromatic and heterocyclic substructures were detected in urine. The positive ion electron ionisation mass spectra of the TMS derivatives of fenspiride and its metabolites provided useful information on its metabolism. Positive ion methane chemical ionisation–GC–MS of the derivatives provided both derivatised molecular mass and structural information. Unchanged fenspiride can be detected in post-administration plasma and urine samples for up to 24 h. Maximum urinary levels of 100–200 ng ml^{-1} were observed between 3 and 5 h after administration. After enzymatic deconjugation, the major phenolic metabolite (**G**) can be detected in urine for up to 72 h. This metabolite is the analyte of choice in the GC–MS screening of post-race equine urine samples for detection of fenspiride use. However, a distinct difference was observed in the urinary excretion of this metabolite between the thoroughbred horses used in UK study and the quarterbred and standardbred horses used for the USA administrations.

Keywords: Fenspiride

1. Introduction

Fenspiride hydrochloride {8-(2-phenylethyl)-1-oxa-3,8-diazaspiro[4,5]decan-2-one.HCl} is a whitish water-soluble microcrystalline solid. It is marketed in European countries in tablet, syrup or injectable solution forms in proprietary formulations (Decaspir, Espiran, Fenspir, Pneumorel, Respiride, Tegencia and Viarespan) for human use. Fenspiride is reported

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to have bronchodilator and anti-inflammatory properties [1–3]. It is not licensed for veterinary use in the UK.

The pharmacokinetics, absorption, metabolism and elimination of orally administered [^{14}C]fenspiride in the rat have been reported [4]. The drug is rapidly absorbed and between 74 and 89% of the dose is excreted in urine in the first 24 h. Three metabolites were detected in urine; the major metabolite was tentatively identified as phenylacetic acid. In a separate study performed in both rat and man following oral administration of ^{14}C-labelled fenspiride, approximately 75–80% of the dose was excreted in rat and approximately 66% in human urine in the first 24 h [5]. The parent drug was the main component present in the plasma and urine of both species. In rat urine, three unidentified ^{14}C-labelled metabolites and in human urine, two hydroxylated metabolites were detected. The major metabolite was tentatively identified as the glucuronic acid conjugate of fenspiride hydroxylated at the *para*-position on the phenyl ring and the minor metabolite as an isomer, hydroxylated at either the *ortho*- or *meta*- positions. A study on the absolute bioavailability of fenspiride after a single oral or intravenous administration of 80.0 mg of fenspiride HCl to 12 healthy human volunteers has also been reported [6].

A method for the determination and quantification of fenspiride in human plasma and urine using high-performance liquid chromatography (HPLC) with electrochemical and ultraviolet detection has been reported [7]. Fenspiride is a basic drug and the reported pre-treatment of biological samples involved liquid–liquid extraction of the drug from the basified biological matrices with a mixture of organic solvents, washing with aqueous acid and back-extraction of the drug at alkaline pH with an organic solvent. The organic layer was reduced to dryness and reconstituted in the HPLC mobile phase for analysis.

The current study was carried out in order to develop a confirmatory method for fenspiride based upon gas chromatography–mass spectrometry (GC–MS) and to establish the metabolic profile of the parent drug and its phase I metabolites in equine plasma and urine. Following the administration of a single oral dose of fenspiride hydrochloride (50.0 mg) to thoroughbred horses, the drug and its metabolites were extracted from post-administration (PA) equine plasma and urine by a targeted solid phase extraction (SPE) method for basic drugs using co-polymeric mixed mode sorbents [8]. Developed in 1986, mixed mode Bond-Elut Certify® cartridges contain both non-polar C_8 and strong cation exchanger SCX [propylbenzenesulphonyl (H^+ form)] bonded sorbents. Following solvation of the sorbent bed, the prepared sample (e.g. plasma or hydrolysed urine) is applied at pH 6. Organic compounds present in the aqueous biological matrix are retained on the sorbent by a combination of non-polar and ionic interactions. Following the addition of acetic acid (1 *M*) the basic (cationic) compounds are strongly bound to the sorbent by the cation-exchange interactions. The non-polar, polar and anionic organics (neutrals and weak and strong acids) retained by polar hydrophobic interactions are selectively removed by washing the sorbent bed with methanol. The retained basic organic compounds are then eluted from the sorbent by suppression of ionisation with a strong base in ethyl acetate.

The basic isolates obtained from post-fenspiride plasma and urine samples were derivatised (TMS) and analysed by capillary column GC–MS in the EI+ and methane CI+ modes. Metabolite profiles (urinary) of the two thoroughbred horses were compared with that from a set of pooled equine urine samples obtained after administration of the drug to two standardbred and three quarterbred female horses (mares). A preliminary report on this work has been presented previously [9]. This paper reports on the isolation of the in vivo phase I metabolites of fenspiride detected in equine plasma and urine, the tentative elucidation of their structures by GC–MS and semi-quantification of the parent drug and two of the metabolites in post-administration urine.

2. Experimental

2.1. Solvents and chemicals

Glass distilled grade organic solvents were purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland, UK). Fenspiride hydrochloride (F6145, pure drug), β-glucuronidase from *Helix pomatia* [G

7017, type HP2; a mixture of β-glucuronidase (EC 3.2.1.31); 100 000 U/ml and aryl sulfatase enzymes, ≥7500 U] and *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) were obtained from Sigma (Poole, Dorset, UK). Bond-Elut Certify® mixed-mode solid-phase extraction cartridges were obtained from Anachem (Luton, Bedfordshire, UK). A 20-port SPE vacuum manifold for sample extraction was obtained from Whatman International Ltd (Kent, UK). Pentazocine ($C_{19}H_{27}NO$; pure drug) used as the internal standard was a gift from Sterling Winthrop Group Ltd (Guildford, UK).

2.2. Drug administration to animals, collection and storage of biofluids

2.2.1. HFL (UK) study

Two thoroughbred racehorses, an entire male (bodyweight, 518 kg) and a male castrate (482 kg) were used. Naturally voided blank urine samples ($n=2$) were collected from each horse prior to administration. Pre-administration blood samples (25 ml) were collected directly in Monovette tubes containing lithium heparin (Sarstedt, Numbrecht, FRG) via indwelling cannulae inserted into the vena jugularis of each horse. Following overnight fasting, the horses were fed fenspiride hydrochloride (pure drug, 50.0 mg=43.92 mg free base) sprinkled on a damp mixture (250 g) of oats, bran mash and barley. Following ingestion of the drug, timed blood samples (25 ml) were collected at hourly intervals for up to 7 h and then at 24 h PA. The tubes were centrifuged (1250 *g*, 15 min), the plasma aspirated into polypropylene vials, labelled and stored at −20 °C until analysed. The animals were kept in metabolism stalls for the first 24 h after administration with a harness attached for the collection of urine. During the day, naturally voided urine samples were collected in individual bottles and overnight on a modified fraction collector. The horses were then placed in looseboxes and three samples were collected daily between 08:00 h and 17:00 h from each animal for the next 2 days making a total collection period of 72 h PA. The void times and volumes were recorded, and aliquots of each urine were stored in labelled polypropylene bottles at −20 °C until required for analysis.

2.2.2. USA (Iowa) study

Five further oral drug administrations were performed at the Iowa State University (USA) at a dose level of 0.1 mg kg^{-1} per horse. Two standardbred and three quarterbred horses (all mares; body weights, 394, 395, 472, 509 and 541 kg) were used. Naturally voided pre-administration urine samples were obtained from each animal. Following oral administration of fenspiride, timed urine samples were collected by use of Foley end-dwelling catheters at 2, 4, 8, 24, 48 and 72 h. Urine collections from each animal were pooled by time intervals (i.e. 5×2 h, etc.). The total pooled urine at each time point was thoroughly homogenised, dispensed into aliquots (100 ml) and stored in labelled bottles at −20 °C until required for analysis.

2.3. Solid phase extraction of pre- and post-administration biofluids

2.3.1. Preparation of plasma and urine samples for the extraction of unconjugated metabolites

Blank and sequential PA samples (5.0 ml) were adjusted to pH 5.8–6.0 with HCl (2.0 *M*) and any solid precipitation was removed by centrifugation (1250 *g*, 10.0 min) prior to SPE of the analytes using the method described below.

2.3.2. Preparation of urine samples for the extraction of total (free and conjugated) metabolites

Blank and sequential PA urine samples (5.0 ml) were adjusted to pH 5.0 and incubated at 50 °C for 2 h or overnight at 37 °C with *Helix pomatia* enzymes (50 μl) to hydrolyse the glucuronide and/or sulphate conjugates. After hydrolysis the samples were readjusted to pH 6.0 and centrifuged as above prior to SPE of the analytes.

2.3.3. Determination of recovery of fenspiride from fortified urine

Blank urine samples (5.0 ml) were adjusted to pH 5.0 with HCl (2.0 *M*), fortified with authentic fenspiride at a levels of 75 and 130 ng ml^{-1} ($n=5$ each). After hydrolysis the samples were readjusted to pH 6.0 and centrifuged as above prior to SPE of the analytes.

2.3.4. SPE of basic compounds from plasma and urine

Bond-Elut Certify® columns, used with a vacuum manifold, were preconditioned for use by washing successively with methanol (4.0 ml) and phosphate buffer (0.1 *M*; pH 6.0; 4.0 ml). The clear supernatant biofluids (5.0 ml) obtained after centrifugation were passed through the columns at about 2 ml min^{-1}. The columns were rinsed with acetic acid (1.0 *M*; 2.0 ml), dried under full vacuum suction for 5.0 min, washed with methanol (5.0 ml) and re-dried for a further 2.0 min. The retained basic metabolites were recovered by elution with ethyl acetate/concentrated ammonia mixture [98:2 by vol; 5.0 ml]. The isolates were taken to dryness under N_2 at 40 °C and derivatised.

2.4. Formation of trimethylsilyl (TMS) derivatives

TMS derivatives were prepared by the addition of MSTFA in toluene (1:1 by vol, 50 μl). After vortexing, the mixture was heated at 80 °C for 0.5 h, transferred directly to conical auto-sampler vials and analysed by GC–MS in the EI+ and methane CI+ modes. A 1.0 μl aliquot of the reaction mixture was injected directly in the heated injector unit for pyrochemical derivatisation of the 1-oxa-3,8-diaza-spiro[4,5]decan-2-one heterocycle.

2.5. Semi-quantification of urinary fenspiride and two metabolites

Selected sequential urine samples from the two administrations (HFL) and the pooled urine samples ($n=7$) collected at Iowa State University were analysed by single ion monitoring (SIM)-GC–MS on the Fisons MD800 GC–MS instrument using a semi-quantitative method. Since no stable isotope-labelled drug or any authentic metabolites of fenspiride are available, pentazocine was added to the urine as the internal standard to establish a calibration line and quantify the selected analytes.

2.5.1. Preparation of calibration line

The calibration line (in duplicate) was constructed in normal (drug-free) hydrolysed equine urine (5.0 ml) adjusted to pH 6.0, fortified with fenspiride (as free base) at 100, 200 300, 400 and 500 ng ml^{-1} and analysed alongside the blank (0 ng ml^{-1}) samples. The internal standard pentazocine was added to all samples at a constant level (200 ng ml^{-1}).

2.5.2. Preparation of pre- and post-administration urine samples

Aliquots (5.0 ml) of the pre-administration blanks and selected PA urine samples were analysed. All samples were adjusted to pH 5.0, the internal standard pentazocine added, thoroughly mixed and hydrolysed (*Helix pomatia* 50 μl; 50 °C; 2 h).

2.5.3. Semi-quantification of fenspiride and selected metabolites in urine

The prepared calibrators, quality controls, recovery study samples and the unknowns were extracted by the Certify® SPE method as described. The basic isolates were derivatised and analysed by SIM-GC–MS in the EI+ mode (Fisons MD800 instrument) monitoring the ions m/z 169, m/z 241, m/z 329 (analytes) and m/z 289 (internal standard).

Peak area integration, ion ratios, the calibration line and the concentration of analytes in pre- and PA urine samples were obtained using the Mass Lynx™ automated quantification software program. The calibration line was plotted for the peak area ratio (m/z 241/289; fenspiride/pentazocine) against the amount (ng ml^{-1}) of fortified fenspiride. The non-weighted linear regression line of the peak area ratios was fitted over the entire concentration range to include the origin (0 ng point). From the ratio of the peak areas of ions m/z 241 [Fenspiride (**A**) and metabolite **G**; Table 2] and m/z 329 (metabolite **C**) to m/z 289 (internal standard), the urinary levels (ng ml^{-1}) of fenspiride and the two metabolites (expressed as fenspiride equivalents) were determined from the regression line. The urinary excretion profile (apparent concentration against time after administration) was plotted for each administration using the Fig-PWIN scientific processor (Biosoft Cambridge, UK).

2.6. Capillary column GC–MS analysis

Structure elucidation of metabolites was carried out by combined GC–MS using either a Finnigan MAT TSQ-700 triple quadrupole mass spectrometer (methane CI+-MS) or a Fisons MD800 bench top

GC–MS system (EI+-MS). The Finnigan MAT TSQ-700 mass spectrometer was interfaced to a Varian 3400 gas chromatograph and operated in both EI+ and CI+ modes. A fused capillary column (BPX5; 25 m×0.32 mm I.D.; 0.25 μm film thickness; SGE (UK) Ltd., Milton Keynes, UK) was used with helium as carrier gas (linear gas velocity 40 cm s^{-1}). The mass spectrometer was operated in the repetitive scan mode with a scan time of 1.5 s for the mass range between 60 and 550 amu. The GC was temperature programmed as follows: initial temp. 70 °C, initial hold 1.0 min, then at 15 °C min^{-1} to the final temp. 320 °C, final hold 3.0 min. The injector and transfer line temperatures were 240 and 280 °C, respectively. Aliquots (1.0 μl) of the urine isolates were injected in the splitless mode using an autosampler (CTC model A200S, Zwingen, CH). Full scan EI+ and methane CI+ mass spectra were recorded at 70 eV. The ion source temperature was 150 °C.

The Fisons MD800 was fitted with a BPX5 column (25 m×0.2 mm I.D.; 0.25 μm film thickness; SGE (UK) Ltd.) with helium as carrier gas. The GC oven was programmed as follows: initial temp. 90 °C, initial hold 1.0 min, then at 15 °C min^{-1} to final temp 320 °C, final hold 2.0 min. Full scan EI+ mass spectral data were acquired in the repetitive scan mode (scan interval 0.8 min) for the mass range 60–550 amu. The injector and transfer line temperatures were maintained at 250 °C. Aliquots (1.0 μl) of the derivatised isolates were injected in the splitless mode using the autosampler as described above.

3. Results

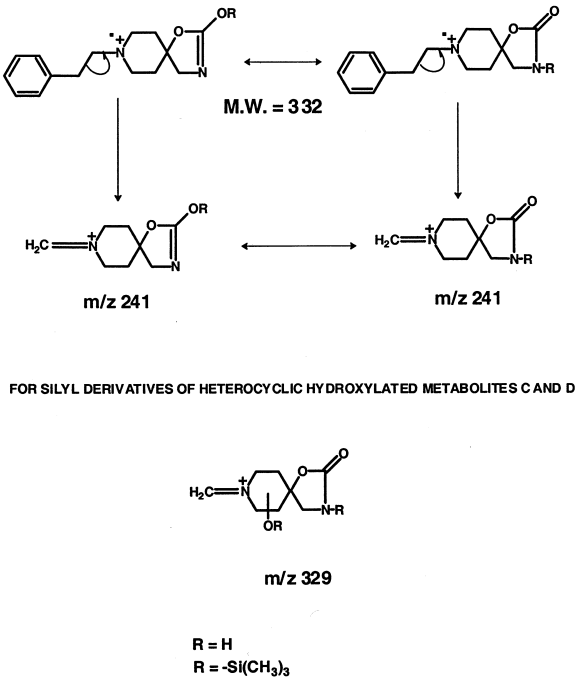
The basic isolates obtained from plasma and unhydrolysed/hydrolysed urine samples were analysed as TMS derivatives by GC–MS in both EI+ and methane CI+ modes to detect, identify and quantify the in vivo phase I metabolites. The EI+ mass spectrum of fenspiride is dominated by the α-cleavage ions at *m/z* 169 (underivatised) or *m/z* 241 (as either 2-enol-*O*-TMS or 3-*N*-TMS derivative). In both cases this ion arises from the loss of the $C_6H_5CH_2$ group as a radical (Scheme 1). Where oxidation has occurred on the carbocyclic (aromatic) substructure, the ion remains at *m/z* 241. However, oxidation on the 1-oxa-3,8-diazaspiro[4,5]decan-2-one heterocycle results in an increase in mass of 88 amu for this ion to *m/z* 329, corresponding to the additional *O*-TMS group. The EI+ and CI+ mass spectral data for fenspiride and its metabolites are summarised in Table 1.

3.1. GC–MS detection and identification of phase I metabolites in urine

The total ion chromatogram (TIC) and mass chromatograms for the ions *m/z* 169, *m/z* 241, *m/z* 329 and *m/z* 405 obtained from the GC–MS analysis of the TMS-derivatised isolates of the same unhydrolysed and enzyme-hydrolysed post-administration urine sample are illustrated in Fig. 1a and 1b, respectively. In the unhydrolysed urine isolate only two substrate-related compounds (peaks **A** and **B**) were detected. In the enzyme hydrolysed isolate, eight compounds (peaks **A**→**H**) were detected and tentatively identified (Table 2), compound **G** being the major urinary component.

The EI+ mass spectrum (Fig. 2a) of the component **A** (RT 14.83 min; Fig. 1b) as the TMS derivative shows a base peak at *m/z* 241 (α-cleavage ion) and fragment ions at *m/z* 317 $[M^{+\cdot}-15]$, *m/z* 154, *m/z* 91 (tropylium species) and *m/z* 73 (TMS reagent). The methane CI+ spectrum of this compound (Fig. 2b) shows a $[M+H]^+$ ion at *m/z* 333 (base peak), fragment ions at *m/z* 317, *m/z* 261 [loss of 72 amu≡$(CH_3)_2SiCH_2$], *m/z* 241 and the adduct ions at *m/z* 361 $[M+29]^+$ and *m/z* 373 $[M+41]^+$. From a comparison of the RT and mass spectral data of the urinary component with those of authentic fenspiride-TMS derivative (data not shown), this compound was identified as the unconjugated parent drug (**A**; Table 2) excreted in equine urine.

The EI+ mass spectrum (Fig. 3a) of the component **B** (RT 15.16 min; Fig. 1b) as the bis-TMS derivative shows a base peak at *m/z* 241, with fragment ions at *m/z* 405 $[M^{+\cdot}-15]$ and *m/z* 179 (hydroxylated tropylium species). The methane CI+ mass spectrum (Fig. 3b) of this compound shows a $[M+H]^+$ ion at *m/z* 421 (base peak), with fragment ions at *m/z* 405 $[M^{+\cdot}-15]$, *m/z* 349 (loss of 72 amu), *m/z* 331 $[(M+H)^+-90]$, *m/z* 241 and the adduct ions



Scheme 1. EI+ fragmentation pathways of the tautomeric putative keto/enol trimethylsilyl derivatives of fenspiride and its metabolites detected in equine urine.

Table 1
EI+ and methane CI+ mass spectral data for the TMS derivatives of fenspiride and its metabolites

Compound (Fig. 7)	EI+ mass spectral data		CI+ mass spectral data	
	Base peak (*m*/*z*)	Fragment ions and intensity (%)	Base peak (*m*/*z*)	Fragment ions and intensity (%)
Fenspiride mono-TMS	241	317 (3), 154 (4), 91 (7)	333 $[M+H]^+$	317 (21), 241 (56) [methane adduct ions 361 (20), 373 (7)]
Metabolite B bis-TMS	241	405 (6), 179 (2)	421 $[M+H]^+$	405 (38), 349 (4), 331 (4), 241 (23) [methane adduct ions 449 (17), 461 (5)]
Metabolite C bis-TMS	329	405 (5), 330 (26), 214 (17), 184 (18)	421 $[M+H]^+$	405 (41), 349 (15), 329 (45), 306 (14) [methane adduct ions 449 (19), 461 (5)]
Metabolite D tris-TMS	329	493 (6), 179 (7)	393	509 $[(M+H)^+$; 41], 493 (22), 329 (24) [methane adduct ion 537 (7)]
Metabolite E, F and G bis-TMS	241	405	421 $[M+H]^+$	405, 349, 241 [methane adduct ions 449, 461]
Metabolite H bis-TMS	241	435 (5), 209 (2)	451 $[M+H]^+$	435 (40), 241 (45) [methane adduct ions 479 (16), 491 (4)]

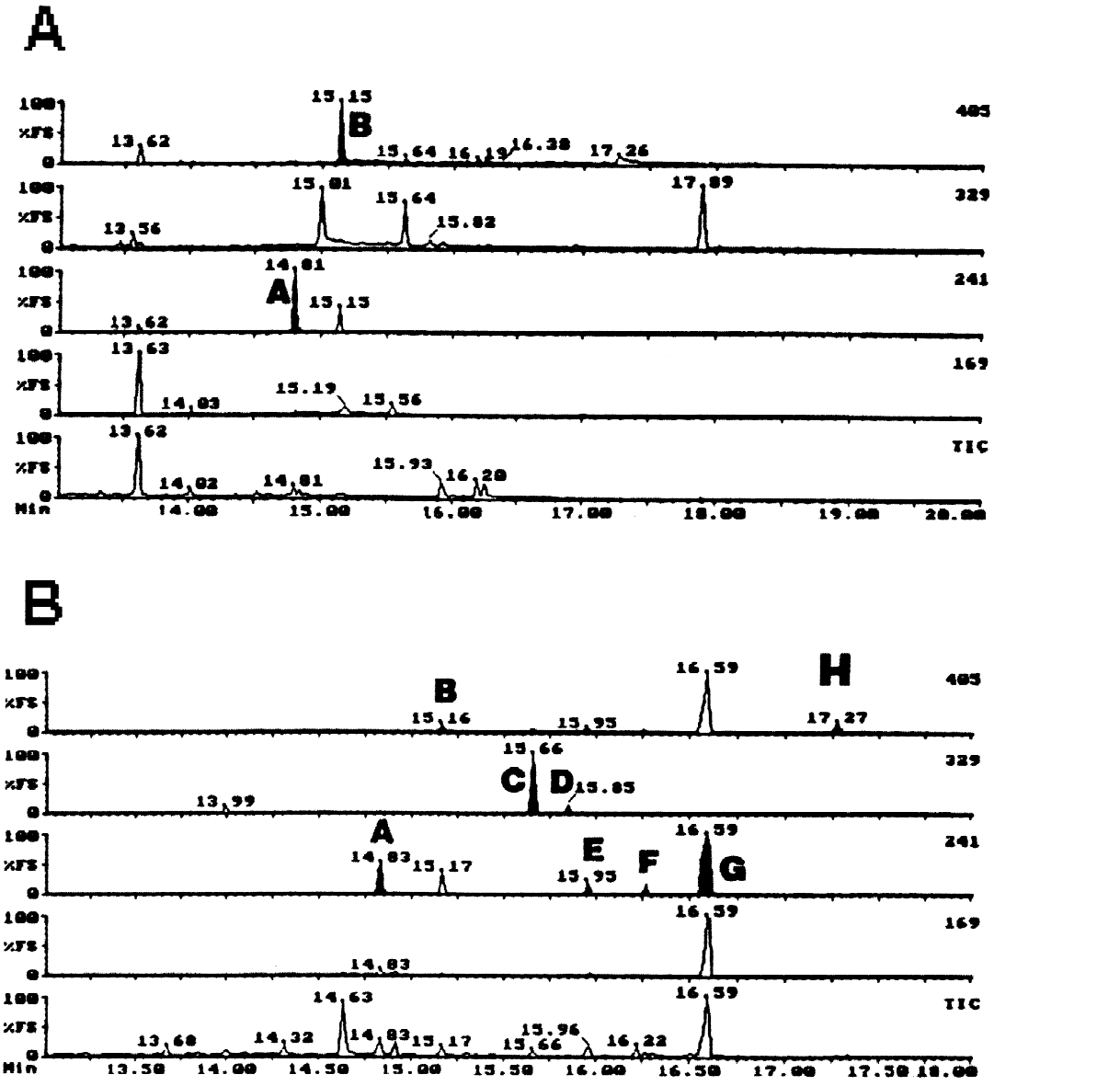


Fig. 1. The total ion chromatogram (TIC) and mass chromatograms for the ions m/z 169, m/z 241, m/z 329 and m/z 405 obtained from the TMS-derivatised isolates of (A) unhydrolysed and (B) enzyme hydrolysed post-fenspiride administration urine sample.

m/z 449 $[M+29]^+$ and m/z 461 $[M+41]^+$. Based upon the above data this compound was tentatively identified as the bis-TMS derivative of a metabolite of fenspiride, hydroxylated on the α-carbon of the phenethyl substructure resulting in the formation of chiral (*R*)-(+)- or (*S*)-(−)-*sec*-phenethyl carbinol(s). The formation of either a single enantioselective isomer or both enantiomers cannot be confirmed. No attempt was made to resolve the possible enantiomers by HPLC due to the non-availability of authentic reference compounds. This compound, classified as 8-[2-(phenyl-2-hydroxyethyl)]-1-oxa-3,8-diaza-spiro[4,5]decan-2-one (**B**; Table 2) is excreted in the unconjugated form in equine urine.

Table 2
Chemical abstracts nomenclature of fenspiride and its metabolites (**A**→**H**) tentatively identified by positive ion EI and methane CI mass spectrometry in post-administration equine urine

Metabolite no.	Chemical nomenclature	Excreted in urine as
A	8-(2-Phenylethyl)-1-oxa-3,8-diazaspiro[4,5]decan-2-one	F
B	8-[2-(Phenyl-2-hydroxyethyl)]-1-oxa-3,8-diazaspiro[4,5]decan-2-one	F
C	8-(2-Phenylethyl)-1-oxa-3,8-diazaspiro[4,5]hydroxydecan-2-one	C
D	8-[2-(Hydroxyphenyl)ethyl]-1-oxa-3,8-diazaspiro[4,5]hydroxydecan-2-one	C
E[a]	8-[2-(Hydroxyphenyl)ethyl]-1-oxa-3,8-diazaspiro[4,5]decan-2-one	C
F[a]	8-[2-(Hydroxyphenyl)ethyl]-1-oxa-3,8-diazaspiro[4,5]decan-2-one	C
G[b]	8-[2-(Hydroxyphenyl)ethyl]-1-oxa-3,8-diazaspiro[4,5]decan-2-one	C
H	8-[2-(Hydroxymethoxyphenyl)ethyl]-1-oxa-3,8-diazaspiro[4,5]decan-2-one	C

F, unconjugated; C, conjugated.

[a] For these two minor isomeric aromatic hydroxylated metabolites, the sites of oxidation at the 2′ (*ortho*) or the 3′ (*meta*) could not be assigned without the availability of authentic reference compounds.

[b] Most likely to be the 4′ (*para*)-hydroxy metabolite previously reported in human urine.

The EI+ mass spectrum of the component **C** (RT 15.66 min; Fig. 1b) as the bis-TMS derivative shows a base peak at m/z 329, with fragment ions at m/z 405 [$M^{+\cdot}$-15], m/z 330 [$M^{+\cdot}$-90], m/z 214 [loss of O=C=N–Si$(CH_3)_3$, (115 amu), from the base peak] and m/z 184. The methane CI+ mass spectrum of this compound shows a $[M+H]^+$ ion at m/z 421 (base peak), with fragment ions at m/z 405, m/z 329 (α-cleavage ion) and m/z 306 [$(M+H)^{+\cdot}$-115], and the adduct ions m/z 449 and m/z 461. Based upon these data this compound was tentatively identified as the bis-TMS derivative of a metabolite of fenspiride, hydroxylated on the piperidine moiety of the diazaspirodecan heterocycle. This compound, characterised as 8-(2-phenylethyl)-1-oxa-3,8-diazaspiro[4,5]hydroxydecan-2-one (**C**; Table 2) is excreted as a phase II conjugate in equine urine.

The EI+ mass spectrum of a minor component **D** (RT 15.85 min; Fig. 1b) as the tris-TMS derivative shows a base peak at m/z 329, with fragment ions at m/z 493 [$M^{+\cdot}$-15] and m/z 179. The methane CI+ mass spectrum of this metabolite shows a $[M+H]^+$ ion at m/z 509, a base peak at m/z 393 [$M^{+\cdot}$-115], fragment ions at m/z 493 and m/z 329 [$M^{+\cdot}$-179] and an adduct ion at m/z 537 $[M+29]^+$. Based upon these data, this compound was tentatively identified as the tris-TMS derivative of a metabolite of fenspiride, hydroxylated at both the carbocyclic (phenyl) and heterocyclic substructures. This compound, characterised as 8-[2-(hydroxyphenyl)ethyl]-1-oxa-3,8-diazaspiro[4,5]hydroxydecan-2-one (**D**; Table 2) is excreted as a phase II conjugate in equine urine.

The EI+ mass spectra of the three components **E**, **F** and **G** [RTs 15.95 min (minor), 16.27 min (minor) and 16.6 min (major), respectively; Fig. 1b] all show a base peak at m/z 241 and fragment ions at m/z 405 [$M^{+\cdot}$-15] and m/z 73. The methane CI+ mass spectra of these three regioisomers show a $[M+H]^+$ ion at m/z 421, with fragment ions at m/z 405, m/z 349 (loss of 72 amu) and m/z 241 and the adduct ions m/z 449 and m/z 461. The EI+ and methane CI+ mass spectra of the bis-TMS derivative of metabolite **G** are illustrated in Fig. 4a and 4b, respectively as being representative for the compounds. Based upon these data, the compounds are tentatively identified as three regioisomeric monohydroxylated metabolites of fenspiride, the sites of oxidation being the *ortho*-, *meta*- and *para*- positions on the phenyl ring. These compounds, characterised as isomeric 8-[2-(hydroxyphenyl)ethyl]-1-oxa-3,8-diazaspiro[4,5]decan-2-one (Table 2) are excreted as phase II conjugates. Metabolite **G** (probably the *para*-hydroxylated isomer [5]) was the major compound detected in post-administration urine of the thoroughbred horse following oral administration of the pure drug at the stated dose.

The EI+ mass spectrum of the component **H** (RT 17.3 min; Fig. 1b) as the bis-TMS derivative shows a base peak at m/z 241 and fragment ions at m/z 435

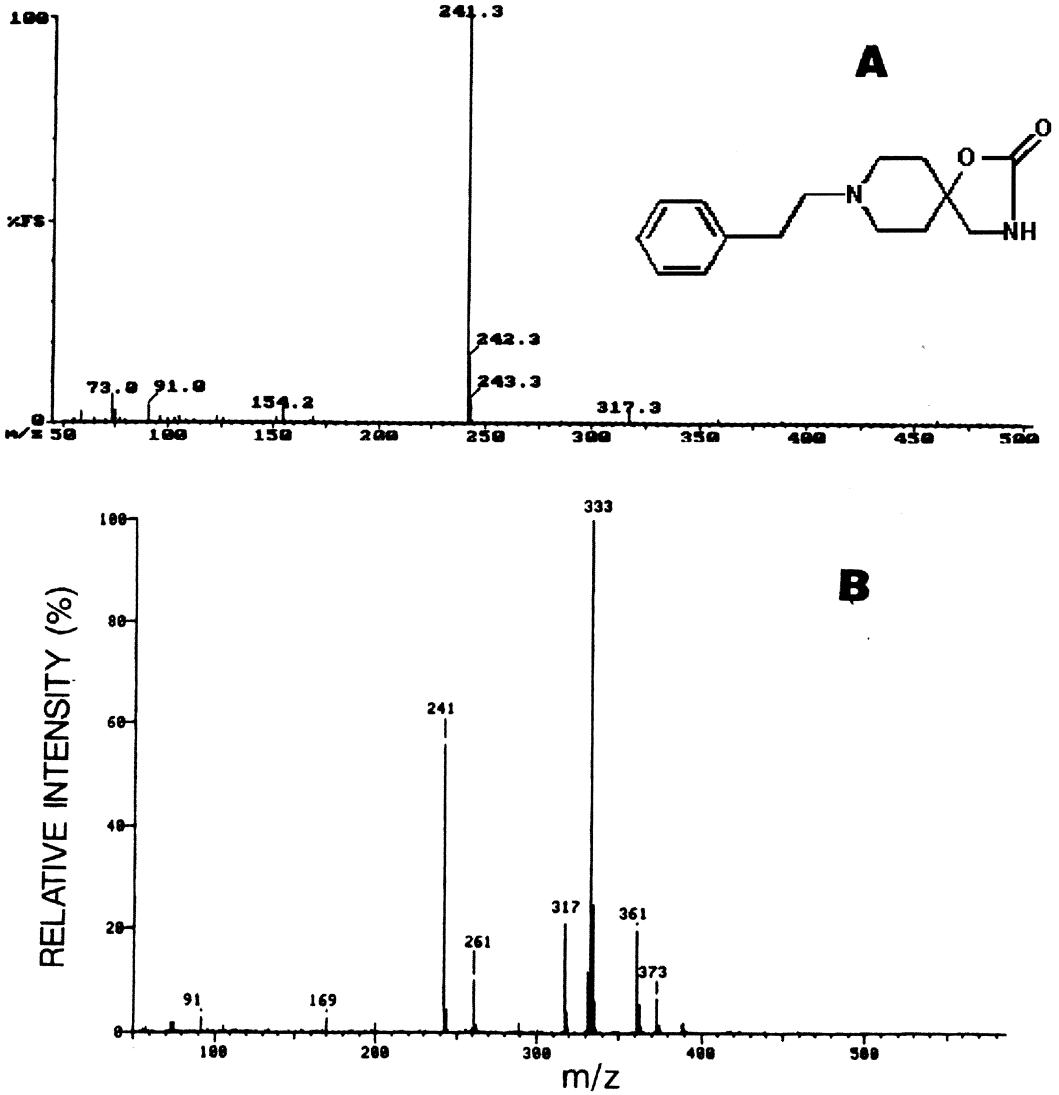


Fig. 2. The positive ion (A) EI and (B) methane CI mass spectra of the TMS ether derivative of unconjugated fenspiride (metabolite **A**) isolated from post-administration horse urine.

($M^{+\cdot}$-15) and *m*/*z* 209. The methane CI+ mass spectrum of this compound shows a $[M+H]^+$ ion at *m*/*z* 451, fragment ions at *m*/*z* 435 and *m*/*z* 241 and adduct ions at *m*/*z* 479 $[M+29]^+$ and *m*/*z* 491 $[M+41]^+$. Based upon this data this compound was tentatively identified as the bis-TMS derivative of a secondary (phase II) metabolite of fenspiride having a guaiacolic functionality (vicinal hydroxy and methoxy groups on the phenyl moiety). This compound was characterised as 8-[2-(hydroxymethoxyphenyl)ethyl]-1-oxa-3, 8-diazaspiro[4, 5]decan-2-one [**H**; Table 2]. It is further conjugated on the phenolic OH group with either glucuronic or sulphuric acid and then excreted in equine urine. In the present study, only one isomer of this metabolite was detected in the PA urine isolates.

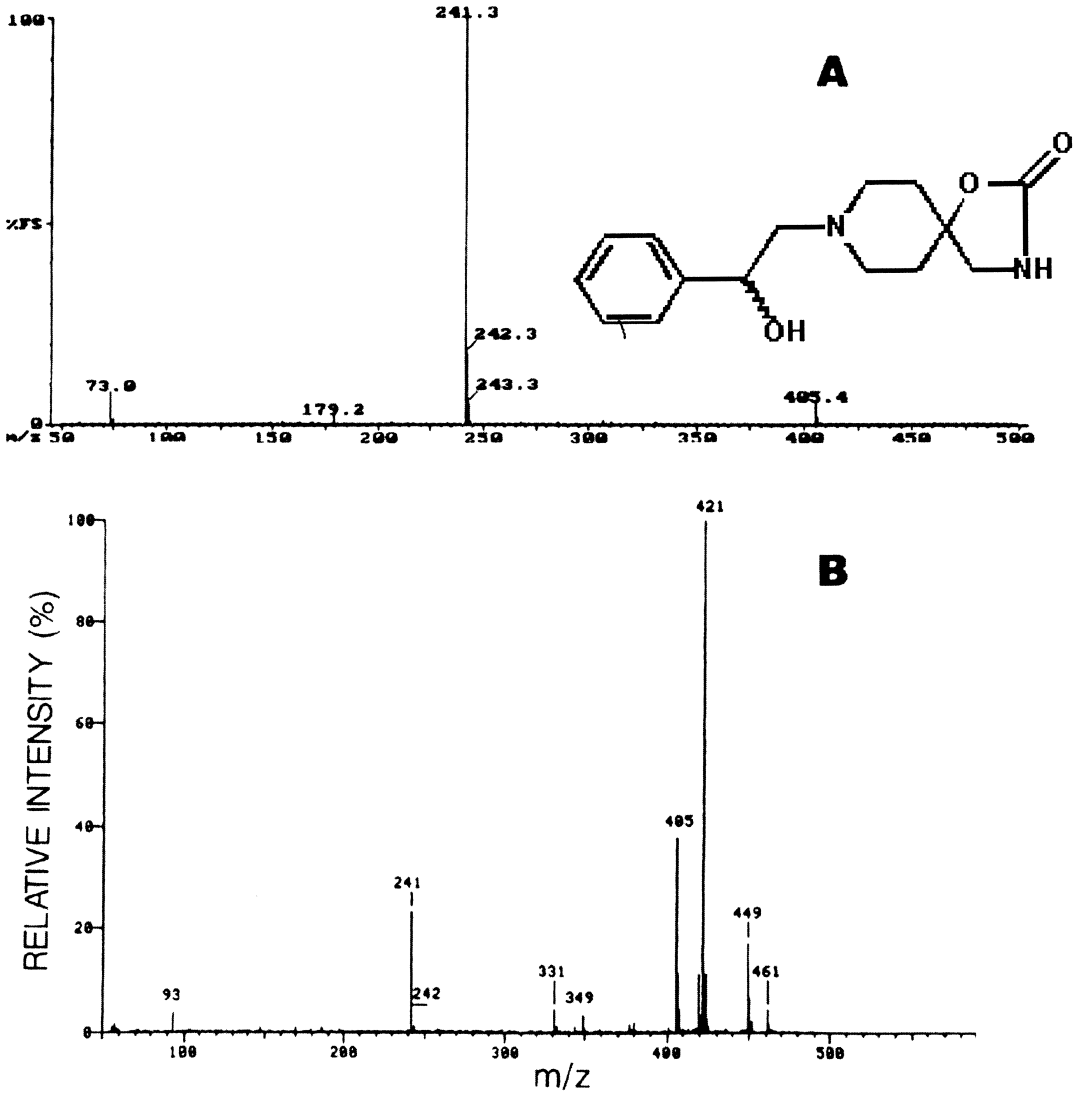


Fig. 3. The positive ion (A) EI and (B) methane CI mass spectra of the bis-TMS ether derivative of an unconjugated metabolite identified as 8-[2-(phenyl-2-hydroxyethyl)]-1-oxa-3,8-diazaspiro[4,5]decan-2-one (metabolite **B**) isolated from post-administration horse urine.

3.2. GC–MS detection and identification of phase I metabolites in post-administration plasma samples

Following extraction of unhydrolysed plasma (5.0 ml) samples, the basic isolates were derivatised and analysed by GC–MS in the full scan positive ion EI and methane CI modes. Only the parent drug (**A**, Table 1) was detected in plasma for up to 24 h. The RIC and mass chromatograms generated from the blank and the 24-h plasma isolates are shown in Fig. 5a and 5b, respectively.

3.3. Semi-quantification of fenspiride and two metabolites by SIM-GC–MS

Following preparation of the calibration, recovery study and pre- and post-administration samples, the

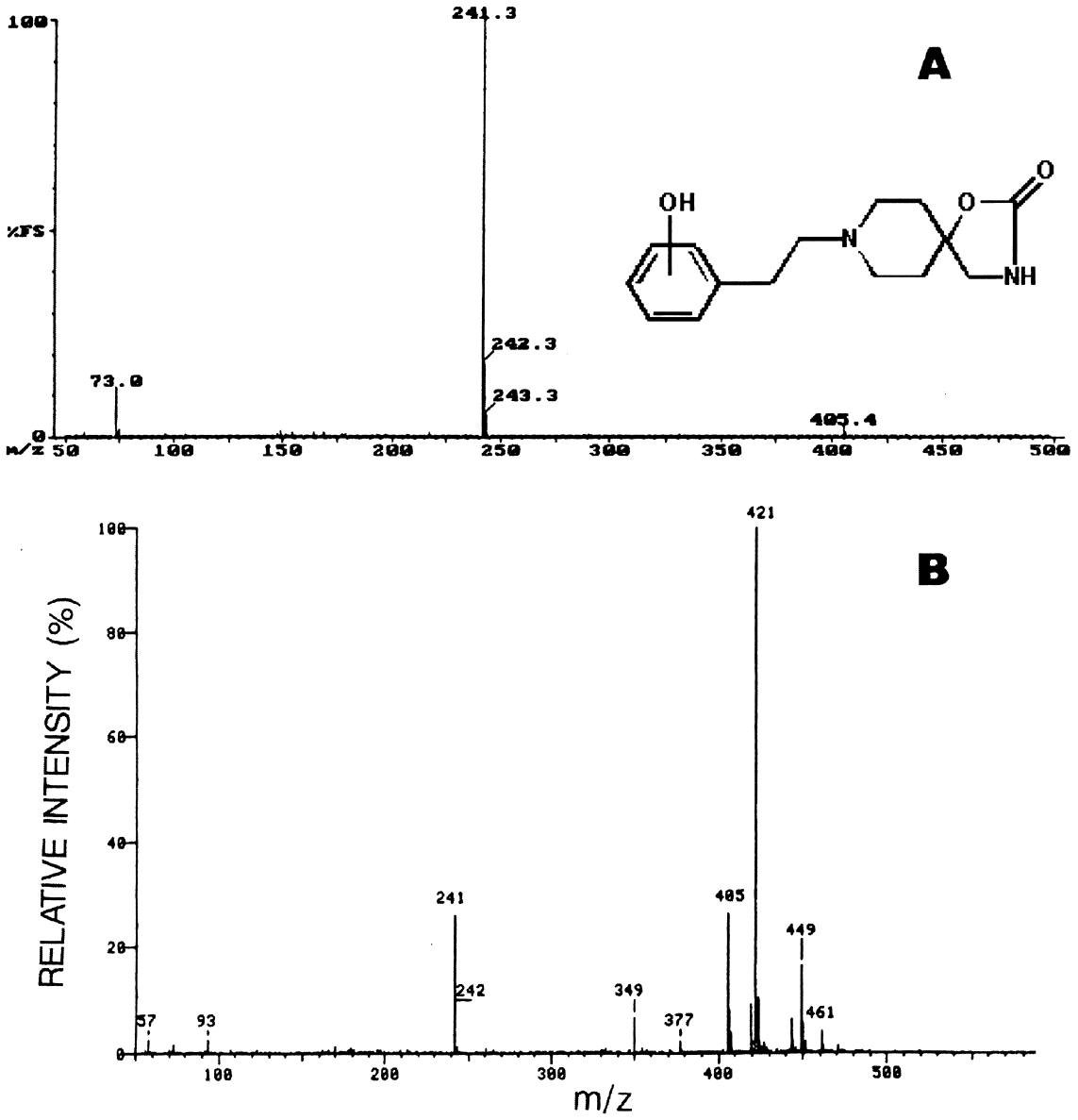


Fig. 4. The positive ion (A) EI and (B) methane CI mass spectra of the bis-TMS ether derivative of an isomer (metabolite **G**) of three conjugated metabolites identified as regioisomeric 8-[2-(hydroxyphenyl)ethyl]-1-oxa-diazaspiro[4,5]decan-2-one isolated from hydrolysed post-administration horse urine.

isolates were derivatised (TMS) and analysed by GC–MS in the SIM mode as described. With respect to the internal standard, the calibration plot showed linearity in the range between 0 and 500 ng ml^{-1} (correlation coefficient 0.9985). From the data, the extraction efficiency of fenspiride extracted from fortified urine was determined to be 80%. The levels (ng ml^{-1}) of the three analytes in sequential urine samples was also determined and the urinary excretion profiles of apparent concentration against time after drug administration were plotted for all experiments (Fig. 6a–c).

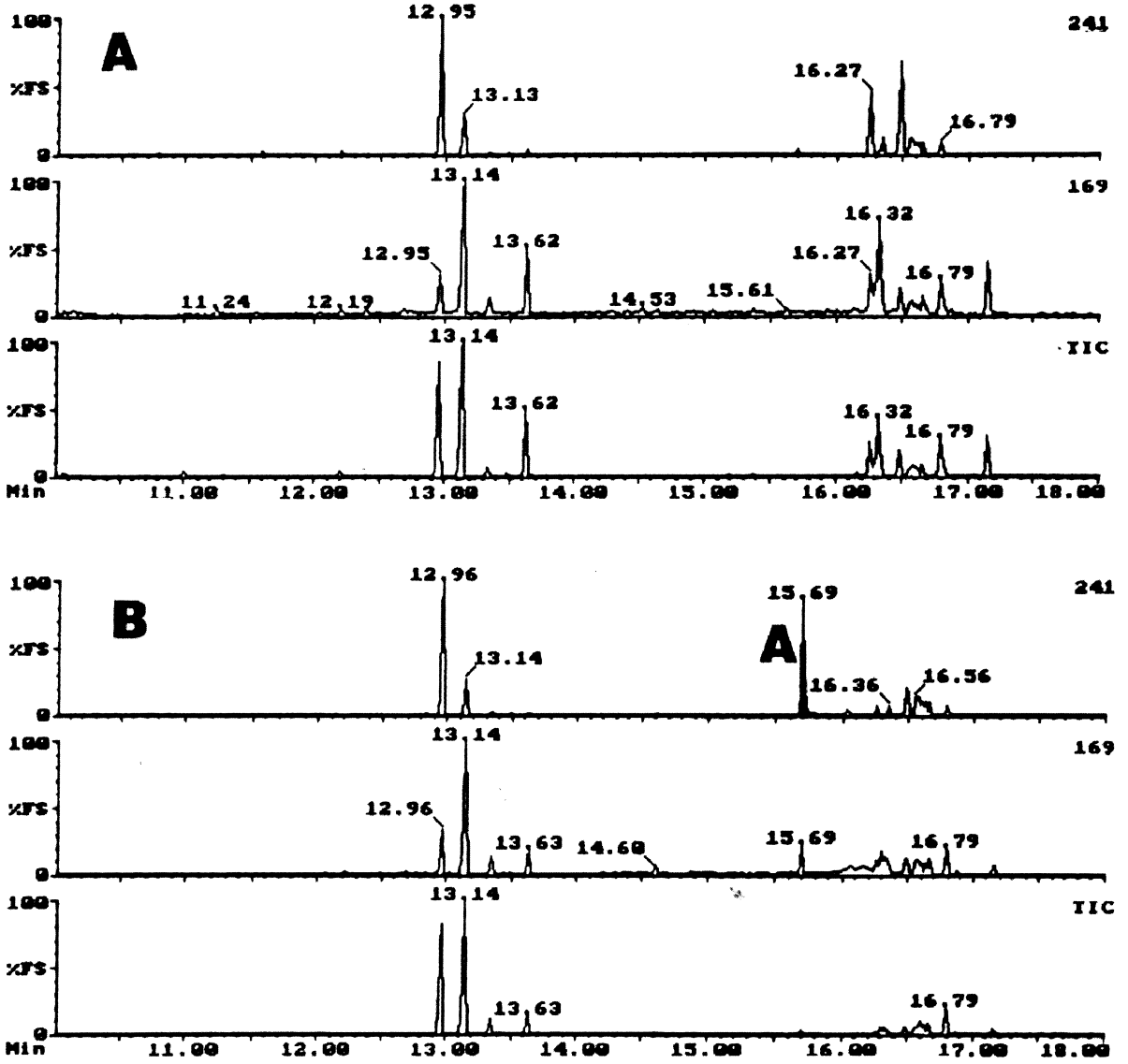


Fig. 5. The total ion current (TIC) and the mass chromatograms for the ions m/z 169 and m/z 241 obtained from the TMS-derivatised isolates of (A) a pre-administration blank plasma and (B) a post-administration plasma sample showing the detection of unchanged fenspiride (**A**) for 24 h.

4. Discussion

The partial in vivo biotransformation of fenspiride.HCl was studied for the first time in two thoroughbred, two standardbred and three quarterbred horses after administration of a single oral dose (0.1 mg kg^{-1}). The basic metabolites were isolated from PA urine before and after enzymatic deconjugation using mixed mode SPE columns. Any neutral or acidic metabolites (viz, phenylacetic acid formed by *N*-dealkylation) would be removed in the methanol wash and therefore not detected by the present method.

Following derivatisation (TMS), the compounds were analysed by GC–MS. The structure of the silylated derivative could be rationalised as either

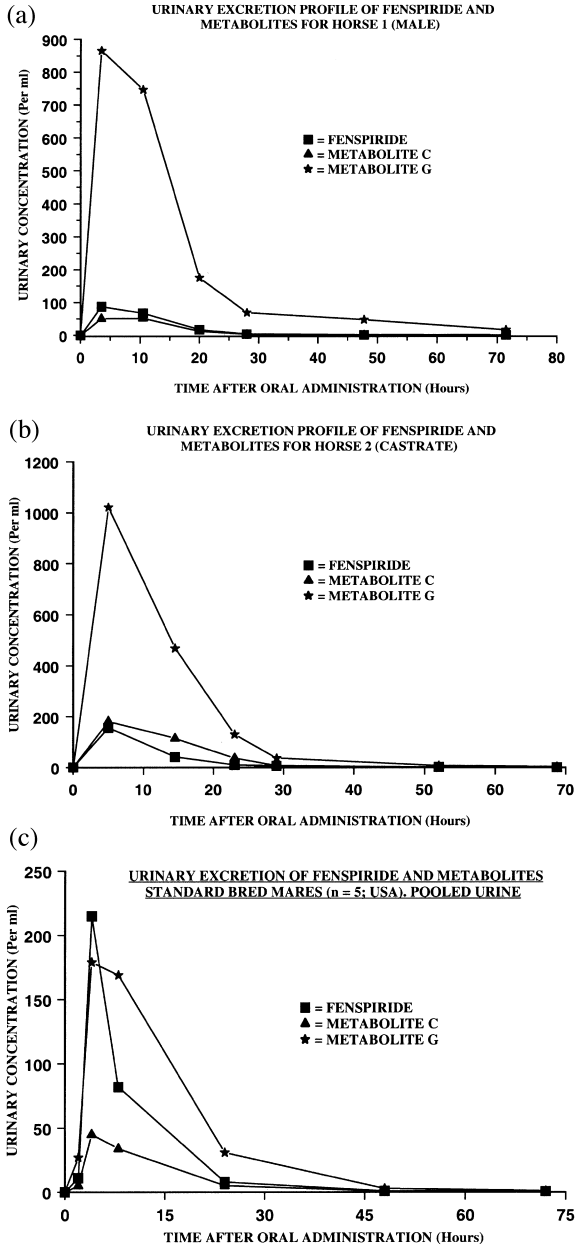


Fig. 6. The urinary excretion profiles of fenspiride (**A**) and of metabolites **C** and **G** following oral administration of fenspiride to two horses [(a) and (b); HFL (UK) study] and of the pooled samples from USA (Iowa) study (c).

N-TMS or as enol-*O*-TMS ether of the tetrahydro oxazole-2-one functionality (keto-enol tautomerism; Scheme 1). Due to the unavailability of authentic reference compounds or any previously published data on the metabolites of fenspiride in other species, tentative structure elucidation of the metabolites is based primarily on the interpretation of their EI+ and CI+ mass spectral data which is summarised in Table 1.

Following administration, the parent drug and seven basic substrate-derived compounds (**B→H**, Fig. 7 and Table 2) were detected and identified by GC–MS in PA urine. Fenspiride is extensively metabolised by both the phase I and phase II pathways in the horse. Biotransformation is mainly confined to the cytochrome P-450 mediated oxidations on both the phenylethyl and the 1-oxa-3,8-diazaspiro[4,5]decan-2-one sub-structures resulting in the formation of regioisomeric phenolic metabolites, catechol, guaiacol (methoxyphenol), benzylic alcohol (α-carbinol), heterocylic monohydroxy and mixed carbocyclic plus heterocyclic dihydroxy me-

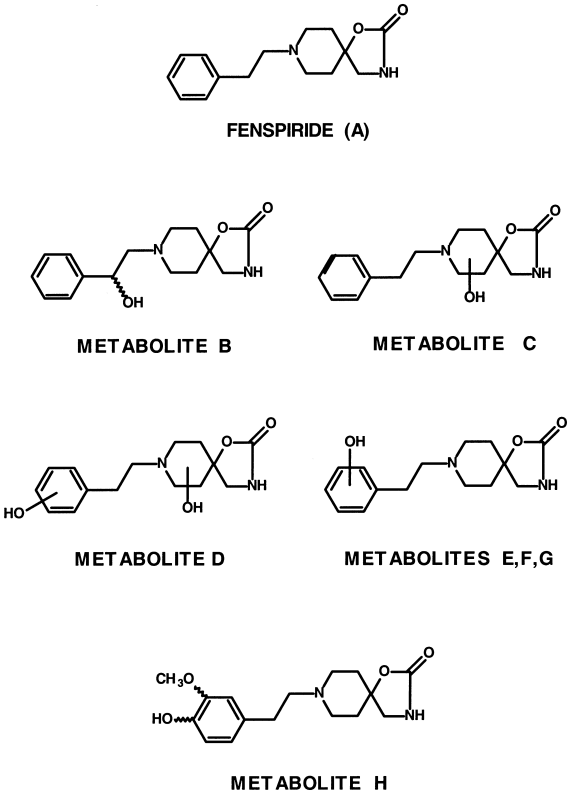


Fig. 7. Chemical structures of fenspiride and its putative in vivo metabolites identified in equine urine.

tabolites. The phase II guaiacholic metabolite **H** is formed by the action of the enzyme catechol *O*-methyl transferase on the cytochrome P-450 mediated primary catecholic metabolite, dihydroxy fenspiride, hydroxylated on the adjacent carbon atoms (1,2-diol) of the phenyl moiety. The catechol *O*-methyltransferase conjugation is a phase II reaction involving a direct transfer of the methyl group from the sulfur of *S*-adenosylmethionine to the oxygen of a catechol in an SN2 process, without formation of a methylated enzyme intermediate. Although normally two isomers are formed, only one isomer of this metabolite was detected in the PA urine isolates in the present study. Although the catechol metabolite was not detected in the present study, this does not preclude its excretion in the post-administration urine samples, as catecholamines are difficult to isolate by the described method.

The parent drug (Fig. 6) was detected in unhydrolysed post-administration equine plasma and urine samples for up to 24 h. The major aromatic (phenolic) monohydroxy metabolite (**G**, Fig. 6) could be detected in urine for up to 72 h, only after enzymatic deconjugation. The other metabolites can be detected at low levels for about 18–24 h in post-administration urine samples. Hydroxylation at the α-carbon of the phenylethyl group (metabolite **B**; Fig. 7) introduces a chiral centre with the formation of enantiomers containing the benzenecarbinol functionality similar to the sympathomimetic amines. This is the only phase I metabolite of fenspiride to be excreted in the unconjugated form in equine urine.

The availability of authentic analytes and suitable internal standards is essential for the development of quantification methods. Since authentic reference compounds were unavailable, a simple, rapid method for the simultaneous SIM semi-quantification of fenspiride and its two metabolites in equine urine was developed using pentazocine as the internal standard. The detector response was linear over the range of 0–500 ng ml^{-1}. The apparent concentrations of the analytes determined empirically were useful for graphical representation of the urinary excretion profiles. The urinary excretion of fenspiride and the two metabolites increased rapidly following drug administration, reached its peak at about 3–4 h and declined to very low levels by 30 h. The major analyte (**G**) could still be detected for up to 72 h when collection was discontinued. The concentration–time profiles (Fig. 6a,b) for the two single experiments were very similar. However, differences were quite clear in the biotransformation and urinary excretion of fenspiride and its major metabolite (**G**) in the samples obtained from Iowa State University (Fig. 6c). Whether this difference is due to the breed of the horses (quarterbred and standardbred) or the gender of the animals (mares) is not known.

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