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Screening and confirmatory analysis of β-agonists, β-antagonists and their metabolites in horse urine by capillary gas chromatography–mass spectrometry

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ABSTRACT

A method for the screening and confirmatory analysis of β-agonists and -antagonists in equine urine is described. Following initial enzymic hydrolysis, the basic drugs and metabolites are extracted using Clean Screen® DAU or Bond Elut Certify™ cartridges, and analysed as their trimethylsilyl ether or 2-(dimethyl) silamorpholine derivatives by capillary gas chromatography–mass spectrometry. The method proved to be very sensitive and selective for basic drugs. After administration of therapeutic doses of propranolol, metoprolol, timolol, isoxsuprine and clenbuterol to thoroughbred horses, the parent compound/metabolites could be detected in urine for upto 14–120 h depending on the drug.

INTRODUCTION

Drugs which act on β-adrenoreceptors fall into two catagories: the sympathomimetic β_2-agonists designed for use as bronchodilators and the β-antagonists (blockers) which are used as antihypertensive drugs. These drugs are chemical modifications of the endogenous catecholamines adrenaline/noradrenaline, which act as neurotransmitters. The chemical structures of the β-agonists are not only related to each other but also to the β-antagonists. The β-agonists are mainly phenethanolamines where substitutions have been made on the aryl moiety and the terminal amino group. The β-antagonists are derivatives of both phenethanolamines and aryloxypropanolamines, the latter being the more cardioselective and potent compounds. In simple chemical terms therefore this diverse group of drugs can be classed as arylhydroxyalkylamines having a common functionality: the presence of a β-hydroxyamino group in the side-chain.

Apart from their clinical use and therapeutic value, these drugs can be misused in human and equestrian sports. The β-antagonists have been listed as prohibited substances by the International Olympic Committee. The β-agonists have recently been classed as "repartitioning agents" and have been used in large doses as growth promoters in animal species reared for human consumption [1]. For reasons of public health and safety government regulations in many countries require that the flesh and organs of treated animals must be free from residues of

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growth promoting agents [2,3]. To monitor this misuse, sensitive and specific methods are therefore required for the detection and confirmation of low or sub-therapeutic levels of these drugs in biological fluids and tissues. In forensic drug detection in equestrian sports in the United Kingdom, it is also essential to screen for and confirm unequivocally the presence of drugs acting on β-adrenoreceptors in post-race urine/blood samples.

The object of the present study was to develop a general method for the screening and confirmatory analysis of drugs which act on the β-adrenergic system. This paper describes the development of a rapid procedure for the detection and identification of these drugs and their major basic metabolites in horse urine and blood plasma based upon solid-phase extraction (SPE), the formation of two types of derivatives [trimethylsilyl (TMS) and cyclic dimethylsilylmethylene or 2-(dimethyl)silamorpholine (DMS)] and analysis by combined capillary column gas chromatography–mass spectrometry (GC–MS). The general applicability of the procedure has been evaluated for the detection of a number of commercially available drugs administered orally or intravenously (i.v.) to thoroughbred horses. The β-agonists clenbuterol and isoxsuprine, commercially available as veterinary preparations, were administered at veterinary therapeutic doses. The β-antagonists were purchased as human formulations and the horses were administered a single dose at human therapeutic levels.

EXPERIMENTAL

Materials

Glass-distilled grade organic solvents were purchased from Rathburn (Walkerburn, U.K.). N-Diethylamine, chloromethyldimethylchlorosilane, N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and propranolol hydrochloride were obtained from Sigma (Poole, U.K.) and *Helix pomatia* digestive juice (mixture of β-glucuronidase and aryl sulphatase enzymes) was purchased from Uniscience (London, U.K.). The Bond-Elut Certify™ LRC (10 ml) solid-phase columns and the vacuum manifold were obtained from Analytichem International (U.S.A.) and supplied by Jones Chromatography (Hengoed, U.K.). The Clean Screen® DAU solid-phase columns (CSDAU506) were obtained from Worldwide Monitoring (Horsham, PA, U.S.A.) and supplied by Technicol (Stockport, Cheshire, U.K.). The β-agonists clenbuterol hydrochloride (Ventipulmin oral granules and aqueous injection ampoules; Boehringer Ingelheim, Bracknell, U.K.) and 3% isoxsuprine hydrochloride in dextrose base (oral powder; Univet, Bicester, U.K.) were obtained from the Department of Clinical Studies, Animal Health Trust (Newmarket, U.K.). The β-antagonists metoprolol tartrate (100-mg Betaloc tablets, Astra Pharmaceuticals, Kings Langley, U.K.) and timolol maleate (10-mg Blocadren tablets, Merck, Sharp & Dohme, Hoddesdon, U.K.) were purchased as human formulations.

Animal administrations

The drug, dose, route(s) and number of administrations are shown in Table I. All urine samples were collected when naturally voided and three urine samples were obtained from each animal before administration of drugs. Following administration urine samples were collected for up to seven days. The voiding time of each sample was recorded and aliquots were stored in polyethylene bottles at −20°C until required for analysis.

TABLE I

DETECTION PERIODS OF β-ADRENERGIC DRUGS AND/OR THEIR METABOLITES IN HORSE URINE

Drug/metabolite	Dose and route	*n*	Excreted as	Derivative	Detection period[c]
Propranolol	0.2 mg kg^{-1} i.v.[a]	3	Conjugated	TMS or	
	1.0 mg kg^{-1} p.o.[a]	2	(95%)	cyclic	
Drug					≈ 12 h
4-Hydroxypropranolol					≈ 60 h
Metoprolol	100 mg with food[a]	2		TMS/cyclic	
Drug			Free		≈ 10 h
			Conjugated		≈ 14 h
Metabolite			Free		≈ 48 h
			Conjugated		≈ 60 h
Timolol	20 mg with food[a]	1	Conjugated	TMS/cyclic	
Drug					ND
Metabolite					≈ 14 h
Clenbuterol	800 μg day^{-1} with food[b]		Free	Cyclic	
	(a) 4 days	2			
	(b) 8 days	1			
Drug					≈ 72–96 h[d]
Isoxsuprine	1.0 mg kg^{-1} with food[a]	1	Free and	TMS	
	0.6 mg kg^{-1} with food[a]	1	conjugated		
Drug					>117 h
Metabolite					>117 h

[a] Single dose.
[b] Divided dose: 400 μg a.m. and 400 μg p.m.
[c] Full-scan EI mass spectra obtained.
[d] After withdrawal of therapy.

Hydrolysis of conjugated metabolites

Blank or post-administration urines (10 ml) were adjusted to pH 4.8 and hydrolysed at 37°C overnight with *Helix pomatia* juices (50 μl).

Sample preparation

SPE cartridges (Certify or Clean Screen DAU) used with a vacuum manifold were preconditioned by washing with methanol (2.0 ml) followed by phosphate buffer (0.1 *M*, pH 6.0, 2.0 ml). Unhydrolysed or hydrolysed urine samples (5.0 ml) were adjusted to pH 6.0 and buffer (2.0 ml) was added. The samples (total volume 7.0 ml) were passed through the activated cartridges at 2 ml min^{-1}. Thereafter the cartridges were rinsed with acetic acid (1.0 *M*, 1.0 ml), dried under full vacuum for at least 5.0 min, washed with methanol (6.0 ml) and re-dried for a further 2.0 min. The basic metabolites were recovered with ethyl acetate (5.0 ml) containing concentrated ammonium hydroxide (2.0%, v/v). The extracts were taken to dryness under nitrogen at 40°C.

Derivatisation

TMS ether derivatives were prepared by addition of MSTFA (50 μl) to the urine extract dissolved in 25 μl of toluene. After vortex-mixing for 5–10 s, the mixture was heated at 60°C for 1 h, solvents were removed under nitrogen at 45°C and the derivatised extract was redissolved in toluene (30–50 μl) for analysis by GC–MS.

The cyclic DMS derivatives were prepared as follows: to *n*-hexane (2.0 ml) in a screw-capped vial were added N-diethylamine (150 μl) and chloromethyldimethylchlorosilane (150 μl). The mixture was vortex-mixed and centrifuged at 1250 *g* for 5 min. The supernatant (100–150 μl) was added to the urine extract dissolved in toluene (25 μl). After 30 min at 60°C, the reagents were removed under nitrogen and the derivatised extract was redissolved in toluene (30–50 μl) for analysis by GC–MS.

Capillary column GC–MS

Combined GC–MS was carried out on a Finnigan MAT TSQ-70 mass spectrometer in the electron-impact (EI) mode. A fused-silica capillary column (OV-1; 18 m × 0.3 mm I.D.; 0.25 μm film thickness; Thames Chromatography, Maidenhead, U.K.) 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 650 u. The oven temperature was programmed as follows: initial temperature, 70°C; initial hold, 1.0 min; temperature programming rate, 15°C min^{-1}; final temperature, 320°C; final hold, 3.0 min. Injector and transfer line temperatures were 250 and 300°C respectively. Injections (1–2 μl) were made in the splitless mode. EI mass spectra were recorded at 70 eV.

RESULTS AND DISCUSSION

In drug detection in sport, it is often essential to develop rapid screening methods for large sample throughput and confirmatory methods based upon

GC–MS for the unequivocal identification of the drug(s) and its major metabolite (s). These requirements have not yet been satisfactorily met for the analysis of β-agonists and antagonists, which form a large diverse group of basic compounds containing highly polar functional groups. High-performance liquid chromatography is frequently used for the determination of β-antagonists in biofluids [4], but the technique lacks the sensitivity and specificity required in forensic detection. More promising is the use of GC–MS which has already been used in a number of methods for screening for β-antagonists, each one utilizing different extraction, derivatisation and ionisation techniques [5–8].

SPE is fast gaining acceptance in doping analysis as a rapid technique for pretreatment of biological samples to give optimal trace enrichment, the non-polar phases having the advantage of high capacity with low affinity and selectivity. The recent introduction of copolymeric bonded-phase silica columns such as Bond-Elut Certify or Clean Screen DAU cartridges which have both lipophilic and ion-exchange properties have provided a simple, rapid, efficient and selective method for the extraction of a broad range of basic drugs and their basic metabolites from horse urine. In preliminary studies, this method has been used to elucidate the *in vivo* biotransformation pathways and urinary excretion of propranolol after oral or i.v. administration to horses [9,10]. The procedure has now been shown to be equally applicable to the detection and identification of a number of other drugs and their metabolites in horse urine (Table I).

The derivatisation of functional groups for GC and GC–MS analysis, although not always essential, is mainly used to overcome problems associated with low volatility, high polarity and thermal instability. Some of the derivatives have the additional advantage in that they direct fragmentation pathways under EI-MS and thus aid structure elucidation. The EI mass spectra of the TMS ether derivatives of β-hydroxyamines containing a *tert.*-butylamino or isopropylamino substituent show a base peak formed by α-cleavage ions at *m/z* 86 or 72, respectively, other common fragment ions being $[M-15]^+$ and the rearrangement ion $[M-116]^+$ [5].

The formation of cyclic DMS derivatives of the bifunctional β-hydroxyamino group was first reported by Hammar [11], using a slightly different procedure to that described in this paper. The mechanism is an aliphatic nucleophilic substitution reaction resulting in intramolecular alkylation and the formation of a heterocycle (silamorpholine) with concomittant loss of a molecule of HCl. The derivatives are stable for over a week in solution at 4°C, and show a good molecular ion with fragment ions at $[M-15]^+$ and $[M-43 \text{ or } 57]^+$ for the loss of isopropyl or *tert.*-butyl group, respectively. For the β-antagonists containing the 3-aryloxypropan-2-ol element, the cleavage of the side-chain containing the heterocycle provides the base peak at *m/z* 186 or 200 depending upon the alkyl substituent on the amine. The cleavages from the heterocyclic ring giving rise to the other diagnostically important fragment ions are shown in Fig. 1.

The selected ion profiles for the TMS ethers and the cyclic DMS derivatives of

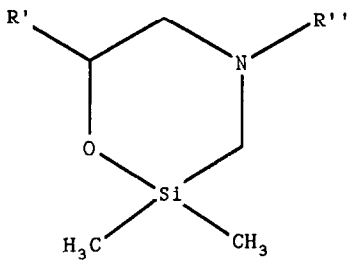

Fig. 1. Structure and electron-impact fragmentations of 2-(dimethyl)silamorpholine derivatives of β-adrenergic drugs containing the β-hydroxyamino group in the side-chain. R′ = Aryl (benzylic) or aryloxy (oxymethylene linked) group. R″ = Terminal substituent on the secondary amine: alkyl groups: isopropyl, *tert.*-butyl etc. Electron-impact fragmentation ions: *m/z* 186, 200 = loss of the 2-(dimethyl)silamorpholine heterocycle from drugs containing the aryloxy group; *m/z* 128 = loss of the terminal alkyl group + a methyl group from the heterocycle; *m/z* 100 = $(CH_3)_2$–Si=CH–$\overset{+}{N}$H=CH_2.

seven drugs spiked at the 0.5–1 ng ml^{-1} level in blank horse urine, extracted, derivatised and analysed as described, are shown in Fig. 2. For the TMS ethers, drugs such as propranolol and betaxolol having the isopropylamino group are distinguished by the ion *m/z* 72 whereas those with the *tert.*-butylamino group have the ion at *m/z* 86. The ions at *m/z* 128 and the heterocycle cleavage ion *m/z* 100 present in the EI mass spectra of the cyclic DMS derivatives are common to all β-adrenergic drugs containing the β-hydroxyamino group, the intensities varying with different compounds, being particularly low for the β-antagonists having the 3-aryloxypropan-2-ol structure (Fig. 3a and b). However, these ions can also be used to screen horse urine samples for the presence of β-adrenergic drugs.

The use of the method is demonstrated in Fig. 4 which shows the total ion chromatograms and selected ion profiles of the α-cleavage ions for the TMS ether derivatives of isoxsuprine (*m/z* 178) and its side-chain hydroxylated metabolites (*m/z* 266) isolated by SPE from hydrolysed post-administration horse urine samples obtained 4.5 h (Fig. 4a) and 117 h (Fig. 4b) after a single oral dose (0.6 mg kg^{-1}). The mass spectra of the TMS ethers of isoxsuprine and the major isomer of the side-chain hydroxylated metabolite isolated from horse urine are shown in Fig. 5a and b.

Using the method, the partial *in vivo* biotransformation and the urinary excretion times (detection periods) of a number of drugs were studied after administration of therapeutic or low doses to thoroughbred horses. The drugs, doses, routes and the detection periods of their major analytes (parent drug and/or metabolite) are shown in Table I.

In conclusion, for the analysis of β-hydroxyamines (β-adrenergic drugs) in horse urine, a rapid, simple and sensitive method based upon SPE and GC–MS has been developed. This method can be used for both screening and confirmatory analysis using a combination of derivatisation techniques and provides unequivocal identification of the parent drugs and/or their metabolites excreted in

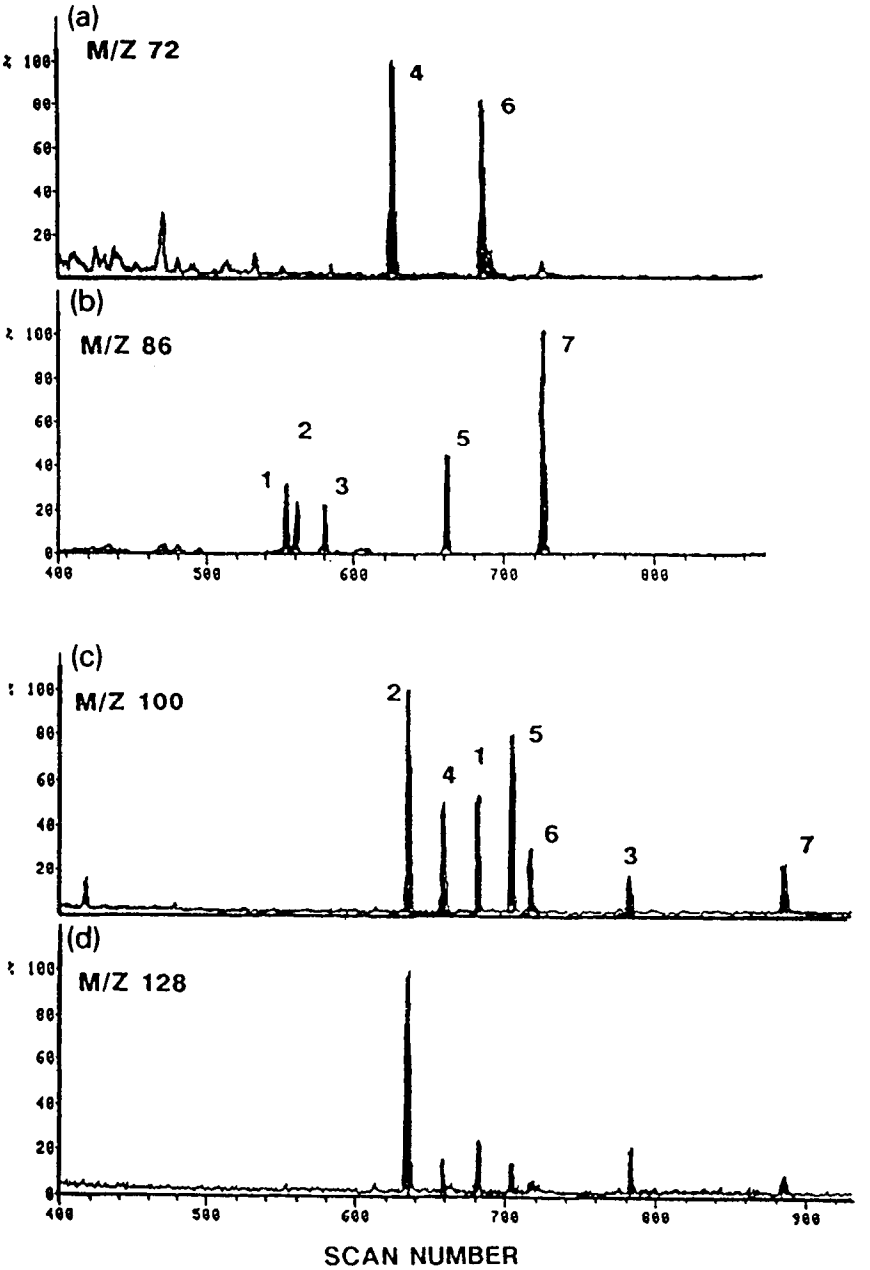

Fig. 2. Ion chromatograms of some β-adrenergic drugs isolated from spiked (0.5–1.0 ng ml^{-1}) horse urine using Certify cartridges. (a) and (b) TMS ether derivatives; (c) and (d) 2-(dimethyl)silamorpholine derivatives. Drugs: 1 = terbutaline; 2 = clenbuterol; 3 = salbutamol; 4 = propranolol; 5 = timolol; 6 = betaxolol; 7 = nadolol.

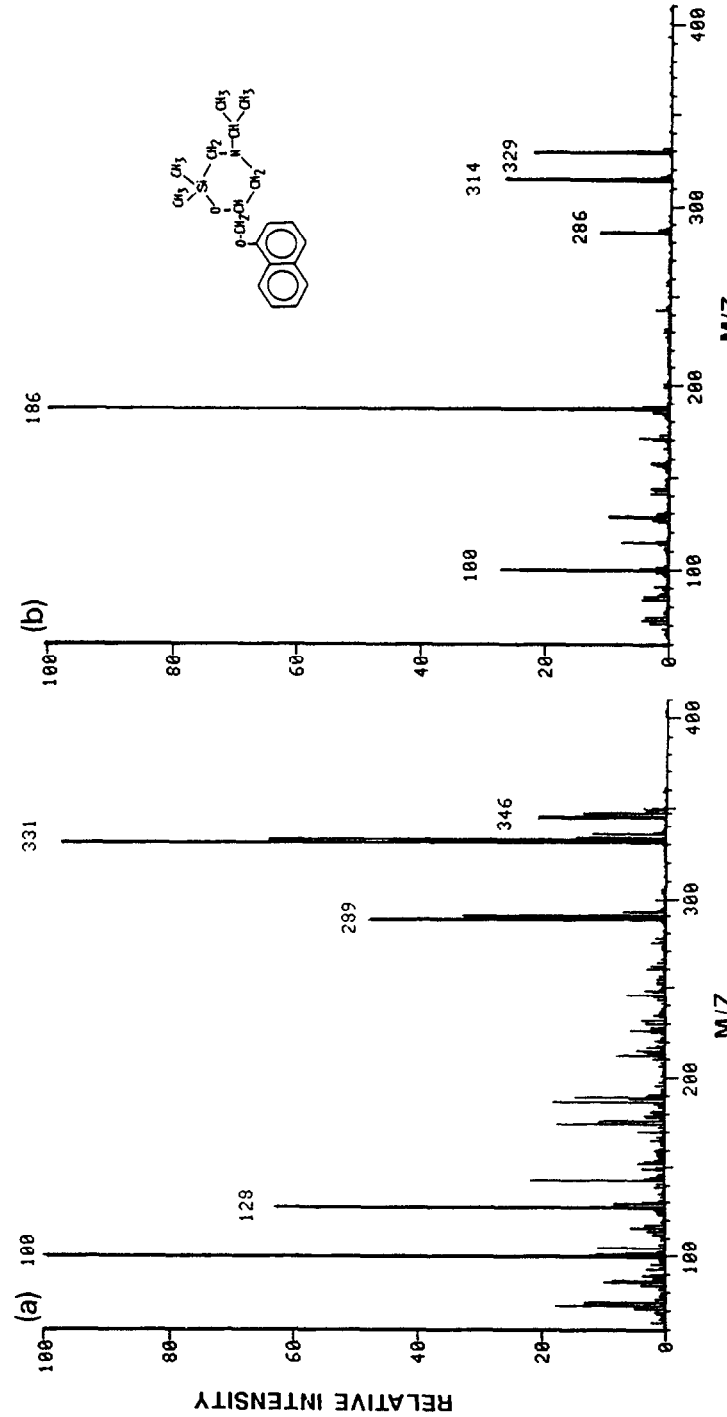

Fig. 3. EI mass spectra of the 2-(dimethyl)silamorpholine derivatives of (a) clenbuterol and (b) propranolol.

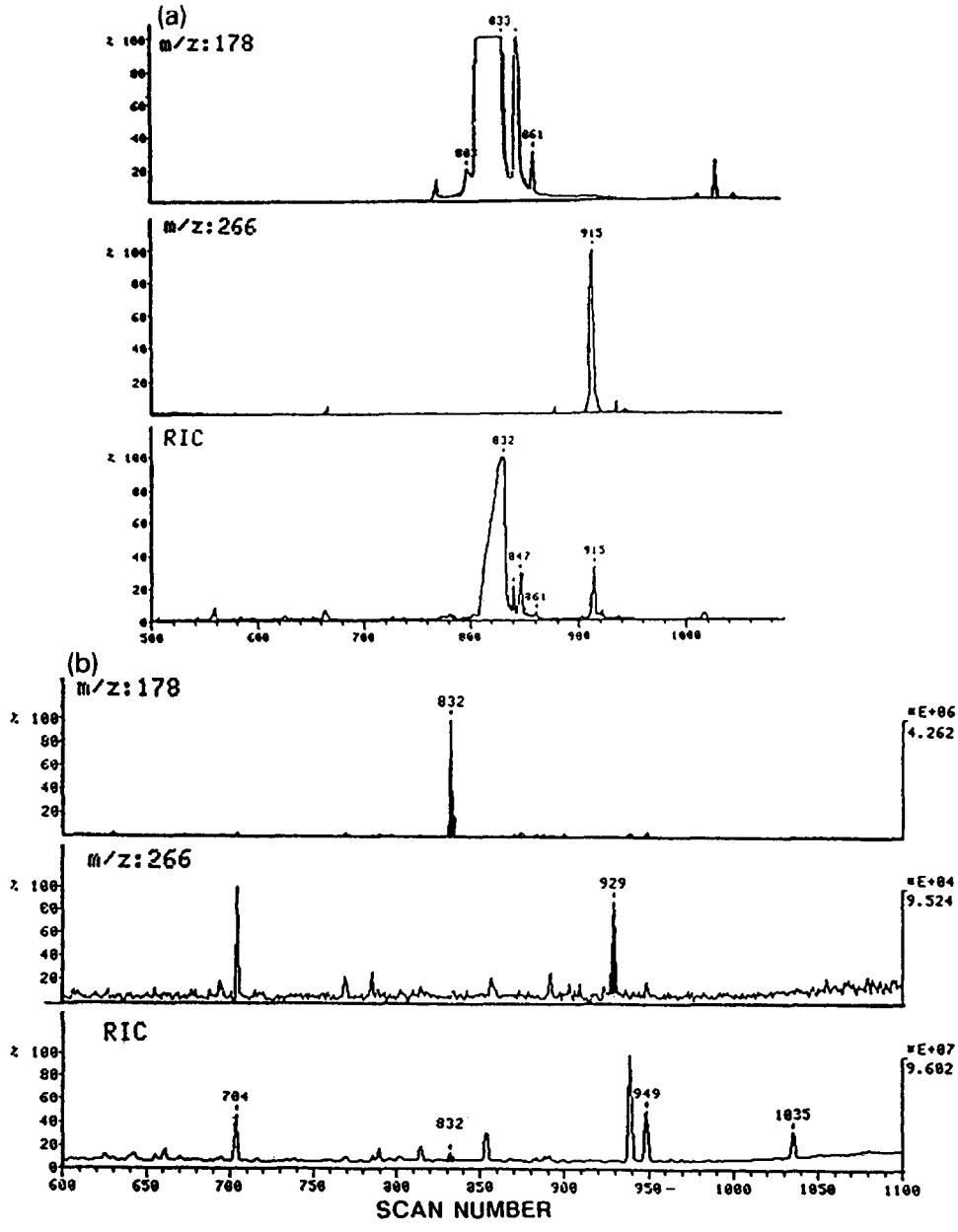

Fig. 4. Reconstructed ion chromatograms and mass fragmentograms for isoxsuprine (*m/z* 178) and its side-chain hydroxylated metabolites (*m/z* 266) as TMS ethers. Basic extracts (using Clean Screen DAU cartridges) of post-administration urines obtained (a) 4.5 h and (b) 117 h after administration (0.6 mg kg^{-1}).

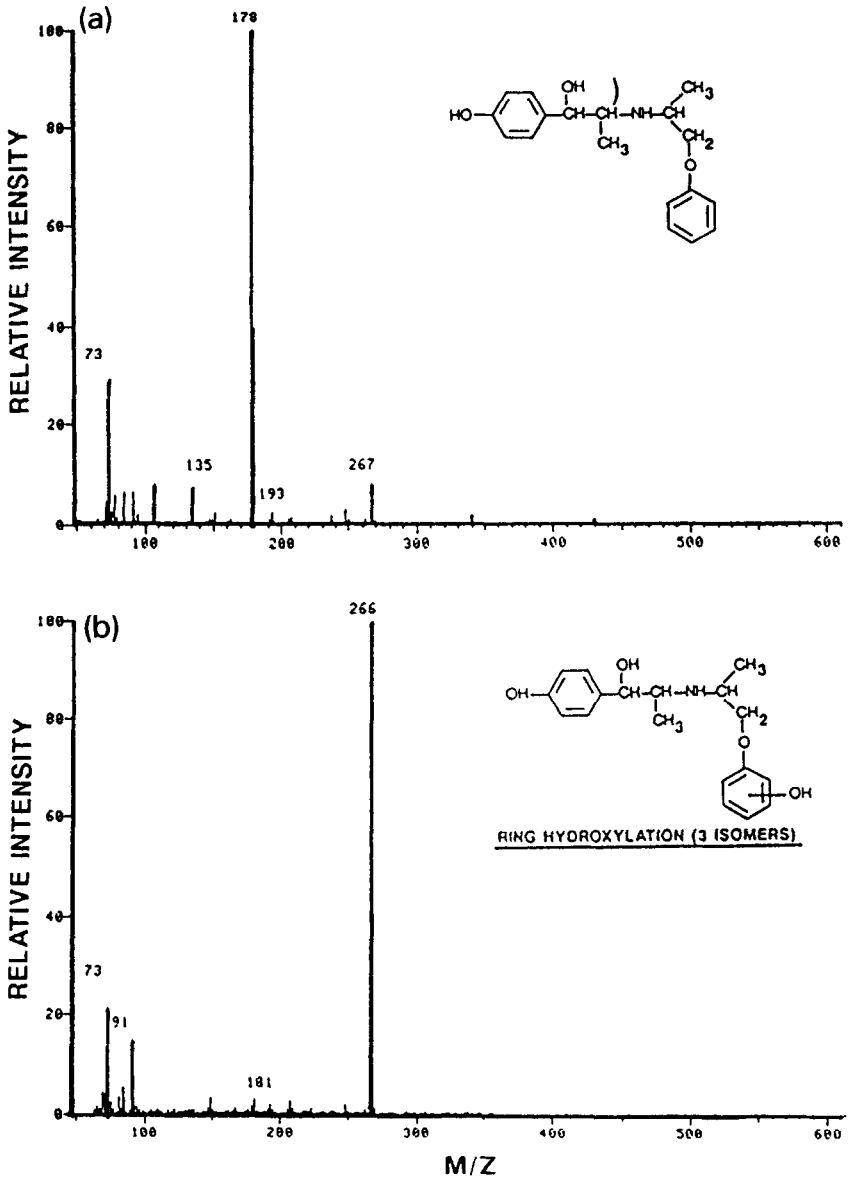

Fig. 5. EI mass spectra of TMS ether derivative of (a) isoxsuprine and (b) major isomer of side-chain monohydroxy metabolite isolated from horse urine 117 h after administration.

horse urine. The studies relating to the *in vivo* biotransformation and the identification of metabolites of these drugs in horse urine will be reported elsewhere.

APPENDIX

Generic and chemical abstracts names of drugs used in this paper: propranolol, (±)-1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol; betaxolol, (±)-1-(isopropylamino)-3-[*p*-(cyclopropylmethoxyethyl)phenoxy]-2-propanol; metoprolol, (±)-1-(isopropylamino)-3-[*p*-(β-methoxyethyl)phenoxy]-2-propanol; timolol, (*S*)-1-[(1,1-dimethylethyl)amino]-3-[[4-(4-morpholinyl)-1,2,5-thiadiazol-3-yl]oxy]-2-propanol; clenbuterol, (±)-4-amino-3,5-dichloro-α-[[(1,1-dimethylethyl)amino]methyl]benzenemethanol; isoxsuprine, (±)-4-hydroxy-α-[1[(1-methyl-2-phenoxyethyl)amino]ethyl]benzenemethanol.

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