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Determination of xylazine and its metabolites by GC–MS in equine urine for doping analysis

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Abstract

Xylazine and its main metabolites were detected in equine urine after a single-dose intravenous administration of 0.98 and 1.01 mg/kg body weight xylazine, respectively, in two horses, in order to be used for equine doping control routine analysis. The urine levels of the parent drug and its metabolites were determined using gas chromatography–mass spectrometry (GC–MS). Xylazine is metabolised rapidly, down to a concentration level of about 1.0 μg/ml after 1–3 h administration. Seven metabolites were identified in urine. 4-Hydroxy-xylazine, the major metabolite, could be traced for 25 h and it is regarded as the long-term metabolite of xylazine in horse. 2,6-Dimethylaniline was, for the first time, reported as metabolite in equine.

Keywords: Xylazine; Xylazine metabolites; Equine urine; GC–MS

1. Introduction

Xylazine, $C_{12}H_{16}N_2S$ (Fig. 1), is a powerful tranquilliser, which is commonly used to sedate large animals especially prior to surgical procedures. Xylazine is the first and most widely used α_2-agonist in the horse. It causes sedation primarily by stimulating the central nervous system presynaptic α_2-adrenergic receptors. It is also used or abused in horses because of its calming effect. The drug has also anaesthetic and muscle relaxing properties and the extent of these effects may vary in intensity depending on the administrating dose [1,2]. It produces a profound hypertensive response within 15 s after injection. It also depresses cardiac minute work, mechanical index of myocardial oxygen demand and coronary blood flow in a similar manner [3].

Xylazine is absorbed, metabolised and eliminated extremely rapidly. It is metabolised to many different compounds, the final breakdown products being organic sulphates and carbon dioxide. The main pathway of biotransformation is most likely via 1-amino-2,6-dimethylbenzene, which is formed when the thiazine ring breaks down [7].

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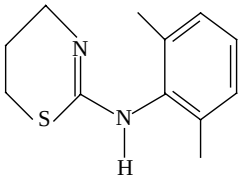

Xylazine, M_r 220

Fig. 1. Structure of xylazine.

The pharmacokinetic disposition of xylazine hydrochloride has been described after both intravenous and intramuscular injection in horse, cattle, sheep and dog. In the horse, following an intravenous administration, the maximum sedative effect occurs within 4–8 min after injection. Following an intramuscular administration, sedation is usually evident within 10 min and reaches its maximum effect by 15 min. Thus, in horses, clinical and pharmacokinetic data suggest that xylazine itself, and not a metabolite, is the active drug [8]. However, xylazine metabolism in racing horses is not fully documented. According to the literature, only Mutlib et al. [9] describe the metabolic fate of xylazine in horses, reporting that xylazine produces a number of metabolites. It is stated therein, that the phenolic metabolites cannot be analysed by gas chromatography–mass spectrometry (GC–MS) even after derivatisation.

Doping control racing laboratories use a variety of screening techniques for drug detection, which typically involve high-performance liquid chromatography (HPLC), gas chromatography, gas chromatography–mass spectrometry and various immunoassay forms. LC–MS/MS is also useful in detecting and confirming xylazine metabolites with the advantage of little sample preparation needed prior to the analysis. However, according to the existing literature, which is quite poor, some of these techniques are used for the determination of xylazine in biological specimens [4–6].

In the framework of the Doping Control Laboratory of Athens preparation project for the doping control analysis in equine, during the 2004 Olympic Games, the present work aimed at the confirmation of xylazine and its metabolites in equine urine using an alternative to LC–MS/MS method, which is a GC–MS method for multi-residue screening procedure for doping control analysis.

2. Experimental

2.1. Materials

Rompun® was a Bayer Leverkusen, Germany, product and xylazine was purchased from Sigma–Aldich, Steinheim, Germany. All reagents and organic solvents were of analytical grade. Methylephedrine (purity 99%) and phenazyne, crystalline were obtained from Sigma–Aldrich (Athens, Greece). β-Glucuronidase/arylsulphatase from *Helix pomatia* (EC 3.2.1.31, type HP-2, crude solution, 127,000 units/ml for β-glucuronidase activity; sulphatase activity is ≥7500 units/ml; Sigma–Aldich, Steinheim, Germany) was used for enzymatic hydrolysis. Derivatisation reagent, *N*-methyl-*N*-trimethylsilyltrifluoro-acetamide (MSTFA), were purchased from Macherey-Nagel, Düren, Germany.

2.2. Instrumentation

A Hewlett-Packard 6890 gas chromatograph coupled with a 5973 quadropole mass spectrometric detector (MSD) with a cross-linked 5% phenylmethylsilicone gum capillary column (HP Ultra2, 12 m × 0.200 mm, 0.33 μm i.d.) or a cross-linked methyl siloxane capillary column (HP Ultra1, 17 m × 0.200 mm, 0.11 μm i.d.) was used. GC was operating at constant flow of helium with a flow rate of 1.0 ml/min. The injector and the transfer line temperatures were set at 250 and 310 °C, respectively. Two microlitres of sample were injected in split mode (1:15).

For underivatised sample analysis, the initial oven temperature was 80 °C for 1.0 min, then ramped at 20 °C/min to 290 °C held at this level for 4.0 min. The MSD was acquiring data in the full scan mode (mass range 40–300) at 2.9 scans/s, with a multiplier voltage of 2000 V and ionisation energy of 70 eV. MS source and MS quad temperatures were 230 and 150 °C, respectively.

For the derivatised sample analysis, initial oven temperature was 100 °C, then ramped at 20 °C/min to 290 °C and held at this temperature for 5.0 min. The MSD was acquiring data in the full scan mode (mass range 69–400).

2.3. Drug administration

For experimental purposes, two mares from the Greek island of Skyros aged 2.5 years (horse A) and

3.5 years (horse B) were used, respectively. The mares had both good performance, excellent nourishing conditions and were not pregnant. Prior to receiving medication, urine was collected with an elastic catheter within glass tubes. The two mares, weighting 158 and 215 kg body weight, were each injected with 160 and 210 mg xylazine intravenously. Following administration, urine samples were collected using the same procedure and in a regular time span, specifically after: 1, 3, 5, 9, 13, 25, 37, 49, 61, 73 and 85 h. The pH of urine varied from 4.5 to 5.5, while no preservatives were added. After each collection, the urine samples were placed in a freezer (−20 °C) and kept until the end of the experiment. After the completion of the excretion study, the samples were transferred to the analysis laboratory within an isothermal vessel for temperature preservation.

2.4. Sample preparation

Seven millilitres of horse urine were hydrolysed with 100 μl of β-glucuronidase/aryl sulphatase from *H. pomatia*, overnight at 37 °C, after addition of 1.0 ml of 1.0 M acetate buffer, pH 5.2. Internal standard of methylephedrine or phenazyne was also added. The hydrolysed urine was extracted twice with a mixture of diethyl ether/ethyl acetate/dichloromethane (4:3:3.5) at pH 9.5–10, using a saturated solution of NH_4Cl/concentrated NH_3 at pH 10, in the presence of 2 g anhydrous sodium sulphate, after shaking for 20 min. The samples were centrifuged at 1900 × *g* for 10 min, the organic phase was evaporated to dryness under nitrogen stream and re-dissolved in 100 μl ethyl acetate. Two microlitres were subjected to GC–MS analysis.

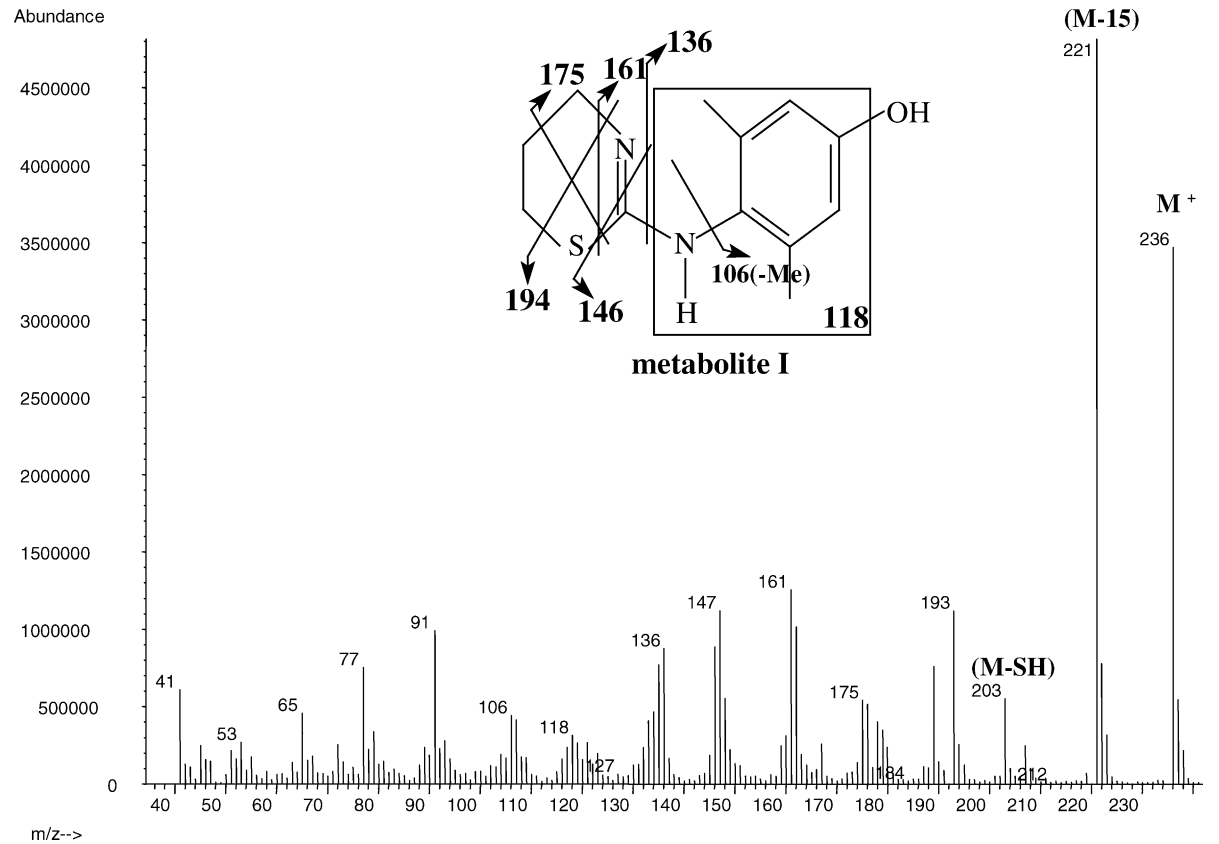

Fig. 2. Mass spectrum and fragmentation pattern of underivatised metabolite I identified in horse urine, after xylazine administration, prepared according to Section 2.4 (basic fraction).

For derivatised samples, a 100 μl volume of MSTFA was added to the dried residue, vortexed and kept at 80 °C for 30 min. Two microlitres of the mixture were subjected to GC–MS analysis.

After the basic extraction, acidic extraction was performed to the remaining urine with ethyl acetate at pH 4.0 using orthophosphoric buffer, in order to detect possible acidic metabolites.

3. Results and discussion

Experiments show the presence of a quantifiable level of the parent drug in urine. In horse A, xylazine was fully excreted at 3 h giving a concentration of 1.5 μg/ml, while in horse B at the first hour post-administration giving a concentration of 0.5 μg/ml. This fact suggests an extensive metabolism of xylazine and therefore a high probability for urinary metabolites. The quantification of xylazine was based on a standard sample of xylazine 109 ng/ml prepared by spiking blank horse urine with xylazine standard solution. Relative areas (area for ion 220 (underivatised xylazine)/area for ion 72 (internal standard, methylephedrine)) in horse urine were compared to those of the standard sample of xylazine.

In addition, calibration curve of xylazine, using spiked urine samples, proved to be linear for the range of 50–1500 ng/ml, with a correlation coefficient of 0.9993. The equation for calibration curve is $y = (-0.0247 \pm 0.0106) + (0.00140 \pm 0.0000206)x$, $N = 5 \times 2$ (5 scalar points × 2 injections). Taking into consideration the percentage difference between the theoretical concentration of xylazine and the experimental concentration of the standards, percentage mean analytical error was found to be −4.4%.

Statistical method was used to estimate limit of detection and limit of quantification for xylazine. Limit of detection was defined as the xylazine concentration giving a signal equal to the blank signal plus three standard deviations of the blank and was found to be 35 ng/ml. A value of three times the limit of detection

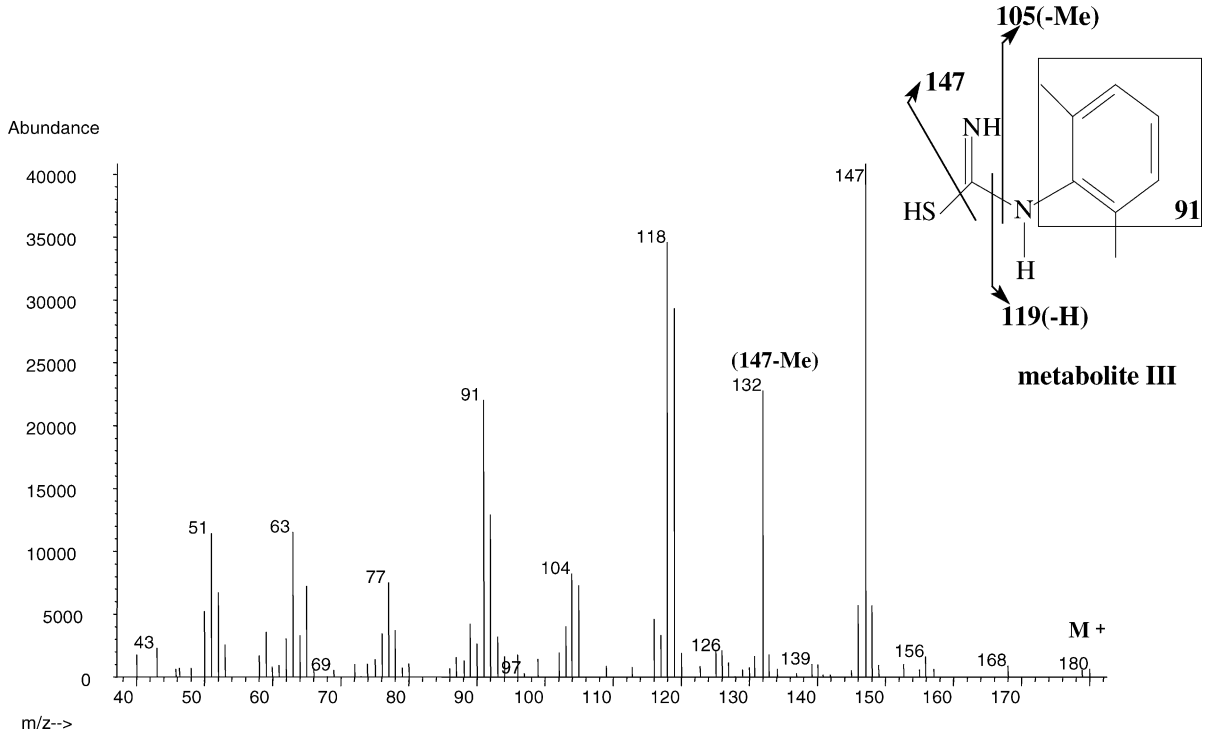

Fig. 3. Mass spectrum and fragmentation pattern of underivatised metabolite III identified in horse urine, after xylazine administration, prepared according to Section 2.4 (basic fraction).

is suggested for the limit of quantification, meaning 105 ng/ml. In fact, although the first standard of the calibration curve (51 ng/ml < 105 ng/ml) showed a value of −9.4% analytical error, the signal to noise (S/N) ratio was >10. Recovery was satisfactory for xylazine and was estimated at 94% ($N = 2$).

Ion chromatography of full scan (*m/z* 40–300) acquisitions of extracted hydrolysed and underivatised urine showed seven metabolites. Two metabolites that have been assigned the structures 4′-hydroxy-xylazine (metabolite I) and 3′-hydroxy-xylazine (metabolite II) based primarily on the presence of the molecular ion *m/z* 236 and ion *m/z* 221 ($M-CH_3$). These metabolites are formed by oxidation on the aromatic ring. As it is expected—the two metabolites possess the hydroxyl group in adjacent positions—the corresponding peaks

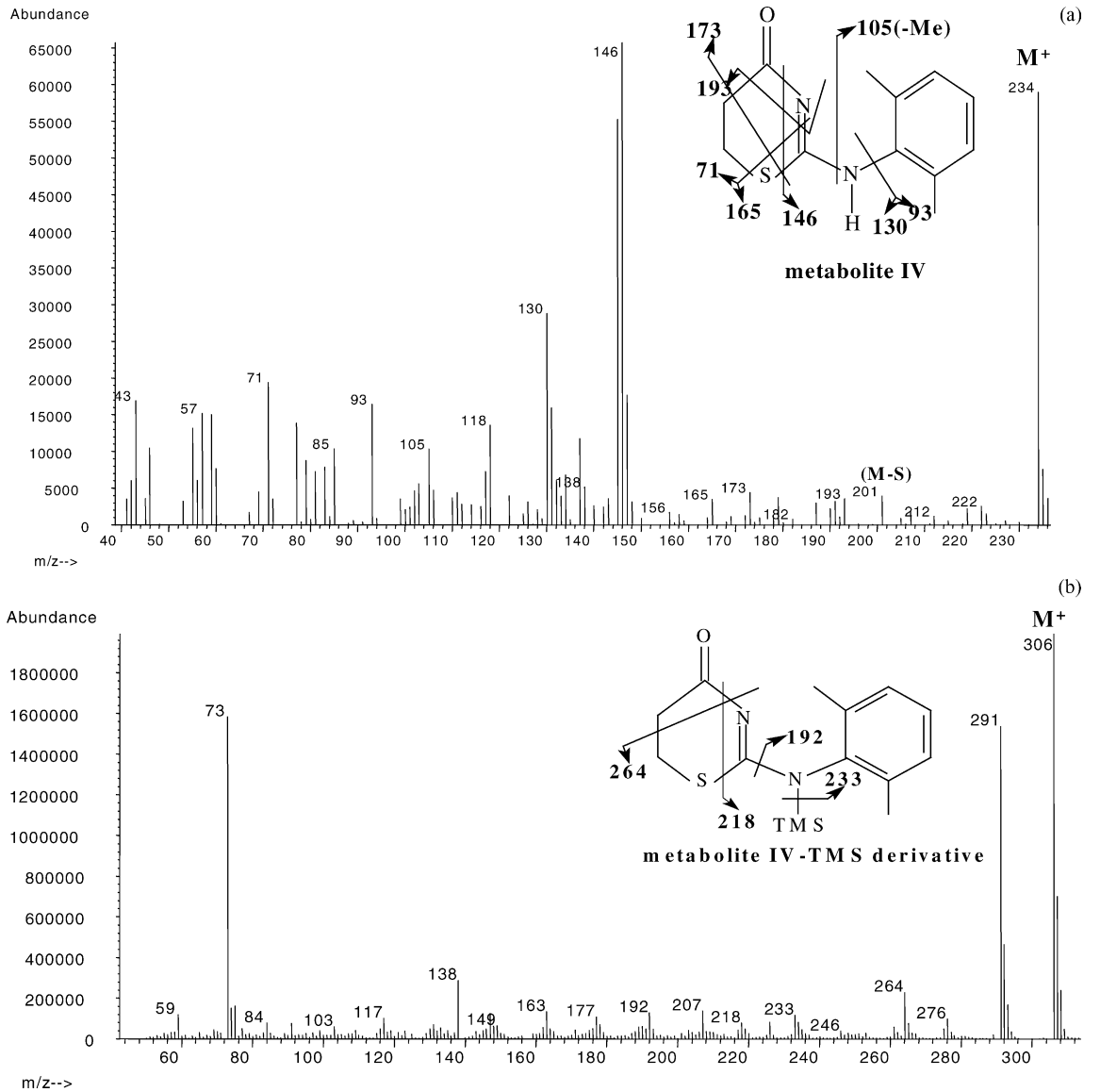

Fig. 4. Mass spectra and fragmentation patterns of underivatised metabolite IV (a) and metabolite IV–TMS derivative (b) identified in horse urine, after xylazine administration, prepared according to Section 2.4.

are characterised by poor resolution (retention times 8.89 and 8.96 min), especially in high concentration. In addition, their mass spectra are very similar, this making difficult to distinguish these two metabolites. Fig. 2 shows the mass spectrum and fragmentation pattern corresponding to these metabolites.

Fig. 3 presents the mass spectrum and the diagnostic ions of the Mr 180 metabolite III, *N*-(2,6-dimethylphenyl)thiourea. Metabolites IV and VI, which have been tentatively assigned the structures 4-oxo-xylazine and 2-(2′,6′-dimethylphenylamino)-4-oxo-5,6-dehydro-1,3-thiazine, revealed by chromato-

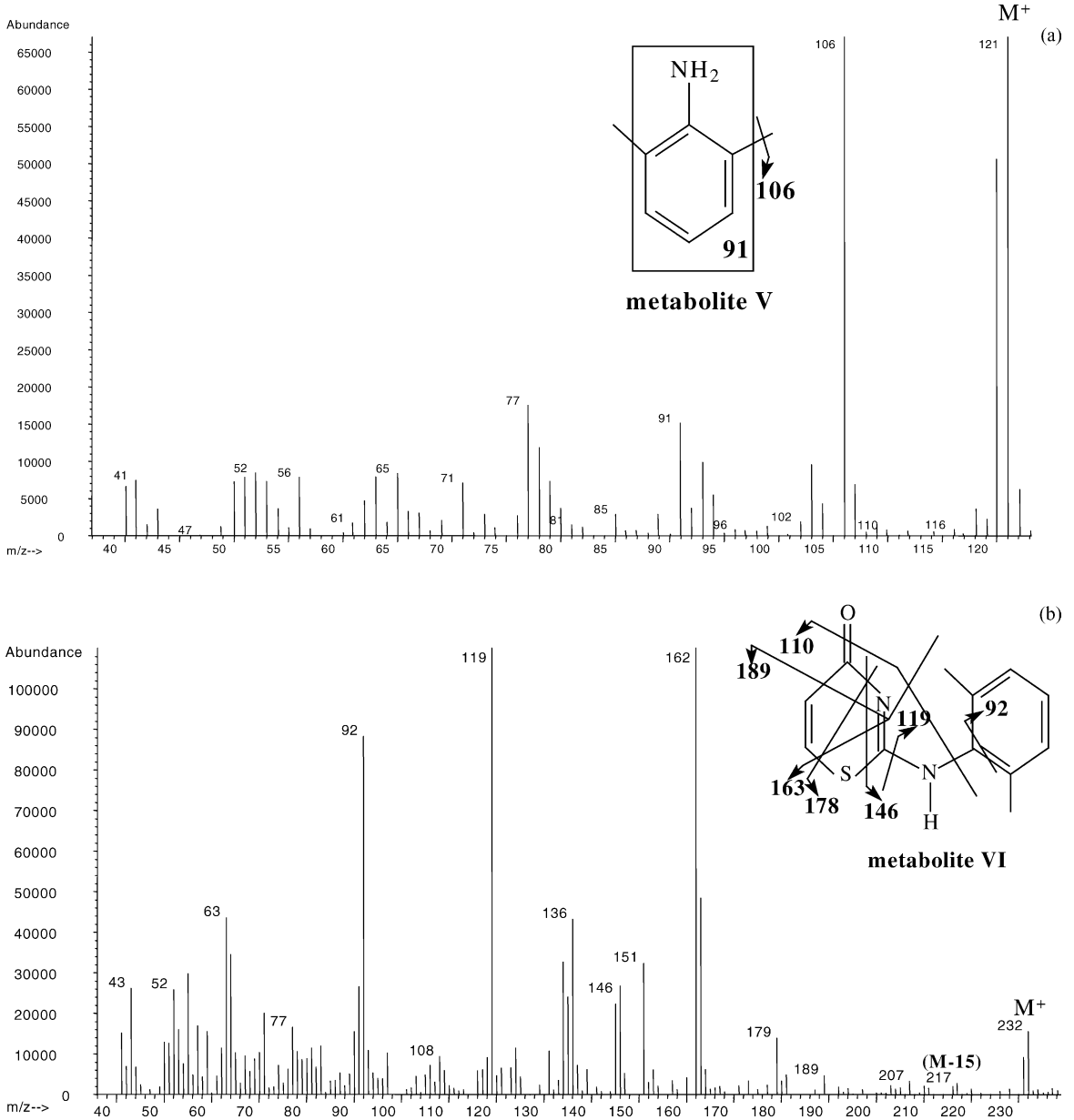

Fig. 5. Mass spectra and fragmentation patterns of underivatised metabolite V (a) and metabolite VI (b) identified in horse urine, after xylazine administration, prepared according to Section 2.4 (basic fraction).

graphy for the diagnostic ion *m/z* 146 and for ions *m/z* 234 and 232, the molecular masses, respectively (Figs. 4a and 5b). Metabolite VI was a minor metabolite. The mass spectrum of the TMS derivative metabolite IV is shown in Fig. 4b.

Metabolite V, 2,6-dimethylaniline, previously mentioned as xylazine metabolite in bovine and swine kidney [6], shows a peak with retention time of 2.59 min. This is presented in Fig. 5a with the relative mass spectrum. Metabolite V was detected in both the basic and the acidic fraction of samples, despite the difficulty in the determination of the compound, due to its great volatility. According to the literature, this metabolite is reported for the first time in horse urine.

Oxidation on the heterocyclic ring leads to the structure assigned as metabolite VII, 4-hydroxy-xylazine, which is proposed by Mutlib et al. [9] and proved to be the major and the long-term metabolite, lasting up

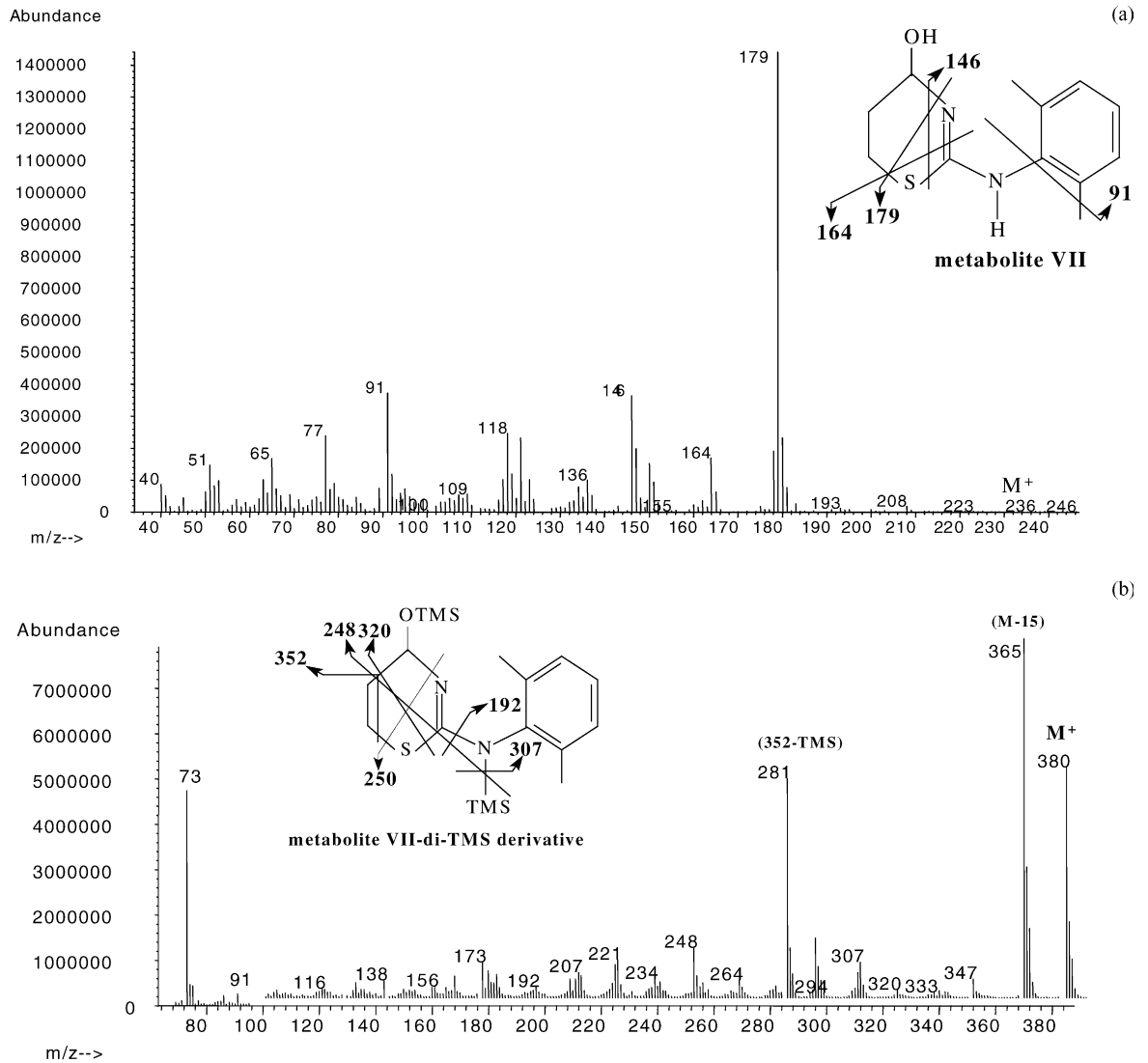

Fig. 6. Mass spectra and fragmentation patterns of underivatised metabolite VII (a) and metabolite VII–di-TMS derivative (b) identified in horse urine, after xylazine administration, prepared according to Section 2.4.

Table 1
Parent drug and basic metabolites identified in hydrolysed horse urine after xylazine administration using GC–MS.

Chemical nomenclature	RT (min)	MW	Base peak	Mass fragments (*m*/*z*)
2-(2′,6′-Dimethylphenylamino)-5,6-dihydro-4*H*-1,3-thiazine (xylazine)	7.45	220	205	118, 120, 145, 177
2-(4′-Hydroxy-2′,6′-dimethylphenylamino)-5,6-dihydro-4*H*-1,3-thiazine (metabolite I)	8.89	236	221	118, 147, 161, 193
2-(3′-Hydroxy-2′,6′-dimethylphenylamino)-5,6-dihydro-4*H*-1,3-thiazine (metabolite II)	8.96	236	221	118, 147, 161, 193
N-(2,6-Dimethylphenyl)thiourea (metabolite III)	2.64	180	147	91, 118, 132
2-(2′,6′-Dimethylphenylamino)-4-oxo-5,6-dihydro-1,3-thiazine (metabolite IV)	7.75	234	146	118, 130, 193
2,6-Dimethylaniline (metabolite V)[a]	2.59	121	106	65, 77, 91
2-(2′,6′-Dimethylphenylamino)-4-oxo-5,6-dehydro-1,3-thiazine (metabolite VI)	8.40	232	162	119, 136, 146, 179
2-(2′,6′-Dimethylphenylamino)-4-hydroxy-5,6-dihydro-1,3-thiazine (metabolite VII)[a]	6.26	236	179	118, 146, 164

[a] Metabolites not documented before in equine.

to 25 h. As far to our knowledge, no analytical data for this metabolite have been published. Fig. 6a presents the mass spectrum of the proposed metabolite based on the presence of *m*/*z* 146 and 179 diagnostic ions, and Fig. 6b illustrates the mass spectrum of the TMS derivative with the presence of molecular ion *m*/*z* 380 and ion *m*/*z* 365 ($M - CH_3$).

It is noteworthy that all metabolites, except 2,6-dimethylaniline, showed the characteristic diagnostic ion *m*/*z* 146, which is related to the same common

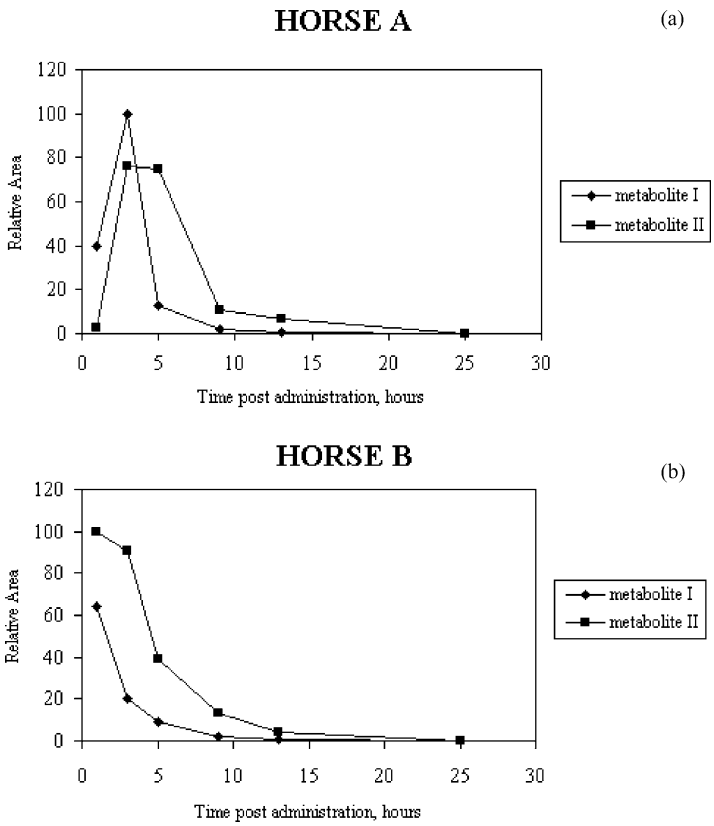

Fig. 7. The excretion profile of metabolites I and II following intravenous single-dose administration of xylazine: (a) 2.5-year-old horse; (b) 3.5-year-old horse.

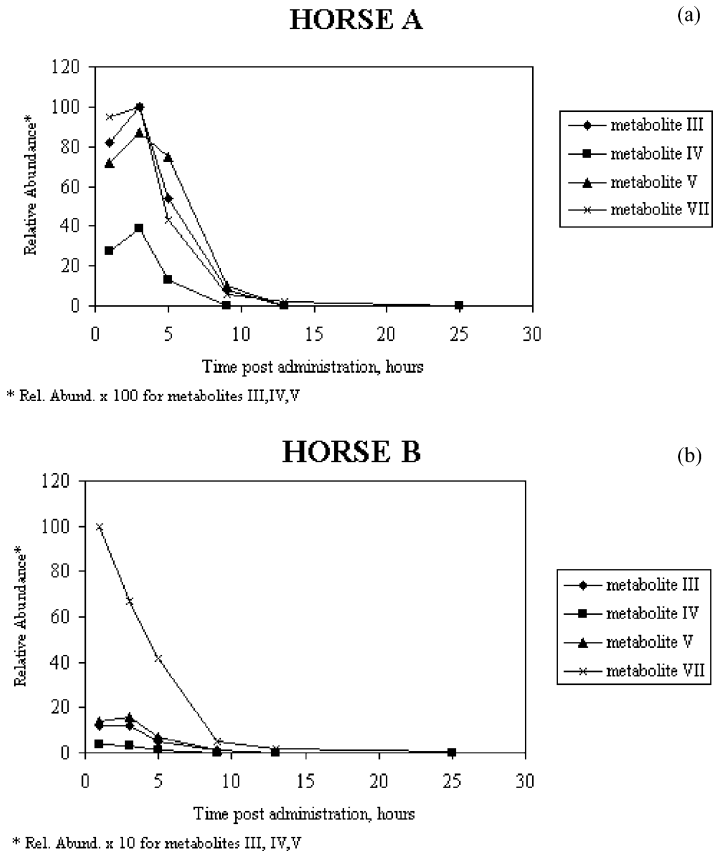

Fig. 8. The excretion profile of metabolites III–V and VII following intravenous single-dose administration of xylazine: (a) 2.5-year-old horse; (b) 3.5-year-old horse.

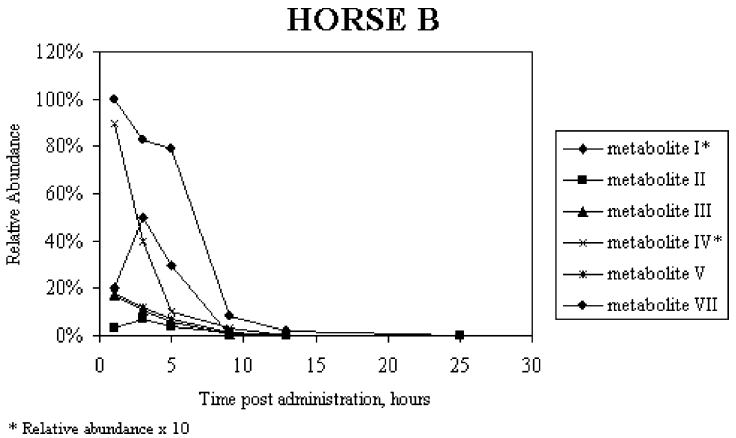

Fig. 9. The excretion profile of the acidic fraction of hydrolysed urine following intravenous single-dose administration of xylazine for metabolites I–V and VII (3.5-year-old horse).

fragment presented in metabolites structure (see fragmentation patterns in Figs. 2, 3, 4a, 5b and 6a).

Peak assignment with the aid of Mass Spec Calculator Pro version software (Quadtech Associates, 1998) enabled interpretation of all spectra. The chemical nomenclature, the molecular weight, the base peak and other diagnostic ions in the mass spectra of xylazine and metabolites are summarised in Table 1.

Excretion curves for basic metabolites I and II are presented in Fig. 7, for both horses. In horse A, metabolites I and II followed the excretion profile of metabolites III–V and VII (see further) and they were detectable up to 13 h. In horse B, metabolite II was more abundant than metabolite I and both diminished rapidly after the first hour of administration.

Fig. 8 illustrates the profile for xylazine basic metabolites III–V and VII excreted in urine following intravenous administration for horses A and B, by plotting the relative areas derived from ion chromatograms for each of their mass spectral base peaks against the post-administration time. The peak level for metabolites III–V and VII was observed in the second void obtained at 3 h for horse A. Levels then declined rapidly to no detectable level at 13 h and to trace level at 25 h for metabolite VII.

In the acidic fraction of the hydrolysed urine, metabolites I–V and VII were present. Excretion curve is shown in Fig. 9. The excretion profiles showed no significant inter-animal variation in the urinary excretion of the metabolites.

4. Conclusions

In this paper, the detection of xylazine and its metabolites in equine for doping control analysis is discussed. Excretion profiles after intravenous administration of 160 and 210 mg xylazine in horses A and B are also presented. It is apparent that, in the horse, drug screening should be directed toward detection of the phenolic metabolites of xylazine rather than the unchanged drug, which can be detected for 3 h in maximum. Seven metabolites were detected in the hydrolysed urine. Major metabolite VII, 4-hydroxy-xylazine, was detected in both the basic and the acidic fraction up to 25 h post-administration and could be used as a long-term metabolite for screening of the drug abuse in horses. Metabolite V, 2,6-dimethylaniline, was first time detected as metabolite in equine.

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