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Liquid chromatography electrospray ionization tandem mass spectrometry for the detection of mesocarb abuse in horse doping

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A method is described for the determination of mesocarb abuse in equestrian sport by combining gradient liquid chromatography and electrospray ionization tandem mass spectrometry. Mesocarb was administrated orally to two horses at a dose of 50 μg/kg. Urine samples were collected up to 120 h post administration. Hydrolyzed and conjugated urine fractions were handled using liquid-liquid extraction (LLE). The identity of the parent drug and metabolites was confirmed using liquid chromatography combined with tandem mass spectrometry (MS/MS). Mesocarb and seven metabolites were detected in horse urine. Mono- and two di-hydroxylated metabolites were the main metabolites observed in horse urine samples. Based on the differences in MS/MS spectra it was supposed that these metabolites were been formed by the hydroxylation of the phenylisopropyl moiety of mesocarb whilst the main process of hydroxylation of mesocarb in human occurred in the phenylcarbamoyl moiety. The main metabolites were almost completely glucuroconjugated. Minor metabolites such as p-hydroxymesocarb and three di-hydroxylated metabolites together with parent mesocarb were also presented in the free urine fraction. This study has shown that two mono- and two di-hydroxylated metabolites are useful for controlling the abuse of mesocarb in horses. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: mesocarb; metabolism; horse; doping control; liquid chromatography-mass spectrometry

Introduction

Mesocarb (Sydnocarb) is the central nervous system (CNS) stimulant that was developed in 1970s.[1–3] As the psychostimulant with antidepressant and anticonvulsant properties, mesocarb was added to the list of banned compounds in sports in 1991.[4] It is prohibited by the World Anti-Doping Agency (WADA)[5] and by the rules of Federation Equestre International (FEI).[6]

Early metabolic studies were performed by Polgar *et al*. in rat urine revealed that free mesocarb, free and conjugated hydroxylated metabolites (*p*-hydroxymesocarb and dihydroxymesocarb) were the main products of biotransformation.[7] Besides, amphetamine was also detected as the product of mesocarb metabolic pathway in rat urine[7] and human plasma.[8] Later some anti-doping laboratories performed investigations on mesocarb metabolism in human urine. The implementation of high pressure liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) was the method of choice for metabolic screening activities.[9–12] The use of gas chromatography-mass spectrometry (GC-MS) that has been widely applied in doping control purposes was limited since mesocarb and its metabolites are highly polar and thermolabile.

Phase I metabolism of mesocarb in humans produces one or more hydroxy groups in the phenylisopropyl, phenylcarbamoyl moiety, or both. The sulfate conjugate of p-hydroxymesocarb seems to be the main phase II metabolite, and a small amount of β-D-glucuronide conjugate has also been detected.[10] Early studies of our group described the presence of several mono-, di- and tri-hydroxylated metabolites in human urine.[13] However, the precise regiochemistry of these metabolites has remained unknown for a long period of time. The use of chemical synthesis for the precise characterization of mesocarb metabolites by Vahermo *et al*.[14] and following HPLC-MS/MS analysis investigations by Gómez *et al*.[15] based on reference standards data, molecular mass and product ion mass spectra identified the exact structures of 19 metabolites of mesocarb in human urine, including mono-, di- and tri-hydroxylated metabolites excreted free as well as conjugates with sulfate and glucuronic acid. All metabolites were detected up to 48 h after a single oral dose of mesocarb administration to healthy volunteers. However dihydroxymesocarb metabolites in work *Appolonova et al*.[13] were detected in human urine up to 168–192 h.

Although metabolic pathways for mesocarb have been reported previously in humans, little is known about its biotransformation in equine. This paper describes the investigation of *in vivo* mesocarb metabolism based on urine excretion analysis in horses' urine with an objective to detect the most appropriate target for detecting mesocarb administration in horse doping.

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Materials and methods

Chemical and reagents

Mesocarb (Sydnocarb), 3-(1-methyl-2-phenylethyl-5-[[9phenylaminocarbonyl]-amino]-1,2,3-oxadiazolium was received from Pharmacological Committee (Moscow, Russian Federation) as pure substance. Sydnocarb® was obtained from a Russian pharmacy (Pharmacon, St Petersburg, Russian Federation). Reference standard of p-Hydroxymesocarb was supplied by United Medix laboratories Ltd (Helsinki, Finland). Diphenylamine (internal standard – ISTD), ammonium acetate, formic acid, hydrochloric acid, acetic acid, cysteine and isopropanol were purchased from Sigma (St Louis, MO, USA). HPLC grade methanol, potassium hydroxide (p.a.), sodium hydroxide (p.a.), sodium dihydrogen phosphate monohydrate (p.a.), and β-Glucuronidase for enzymatic hydrolysis from *Escherichia Coli K12* were acquired from Merck (Darmstadt, Germany). Distilled water for HPLC-MS/MS analysis was purified using the Milli-Q Elix System (Millipore, Milford, USA). Hydroxyl ammonium chloride and ammonium sulfate were purchased from Panreac Quimica SA (Barcelona, Spain). Potassium carbonate, sodium bicarbonate and diethyl ether were purchased from Khimmed (Moscow, Russian Federation).

Instrumentation

Thermo Finnigan TSQ Quantum AM triple stage quadrupole mass spectrometer (Thermo Electron Corp., San José, CA, USA) equipped with an HESI (heated electrospray ionization) source and Surveyor autosampler plus and MS-pump plus was used in all experiments.

All separations were performed at 30 °C on Phenomenex Luna C18 column (150 × 2 mm i.d., 5 μm; Phenomenex, Torrance, CA, USA) connected to the HPLC guard cartridge system (12.5 × 2.1 mm i.d., 5 μm; Phenomenex, Torrance, CA, USA). The mobile phase was composed of 0.05 % formic acid with 20 mM ammonium acetate (pH 3.0) as solvent A and Methanol as solvent B. A liner gradient was run at 0.2 ml/min, with 80% solvent A and 20% solvent B at the start ($t = 0$ min), decreasing to 40% solvent A at $t = 20$ min, and held for 5 min (until $t = 25$ min). The gradient then returned to the initial mobile phase composition (80% solvent A and 20% solvent B) at $t = 30$ min, and stabilized until $t = 35$ min before starting the next injection.

The MS interface was operated in positive ion electrospray ionization mode with a capillary voltage of +4.5 kV, nebulizer pressure of 30 psi, lens voltage of 0.2 V, source temperature of 200 °C. The collision gas was argon with the collision gas pressure of 1.5 mTorr. To find metabolites of mesocarb, parent ion scan MS/MS mode over the range *m/z* 50–380 were performed to detect any ions fragmenting to *m/z* 177 and 135, as well as to *m/z* 193 and 151 that should reflect hydroxylation at the phenylisopropyl, phenylcarbamoyl moiety or at the both. The MS/MS product ion scans were performed for each metabolite at CE = −10 eV. Excretion study of post administration urinary samples was quantified in the SRM mode and two transitions were monitored per compound (Table 1). Data were collected and processed with the Xcalibur 2.0 SR2 software package (Thermo Electron Corp., San José, CA, USA).

Sydnocarb® administration

Sydnocarb ® (4 x 5 mg each) was administrated to two healthy thoroughbred geldings by stomach tubing. Urine samples were collected before administration and then at least twice a day for up to six days post administration. All collected samples were kept in frozen state till the analysis.

Standard solutions

Stock solutions were prepared by dissolving mesocarb in methanol (1 mg/ml). Working solutions were obtained by diluting adequate amounts of stock solutions in water. All stock and working solutions were stored at −20 °C.

Sample preparation

Free fraction

5.0 ml of horse urine was fortified with 10 μl of the internal standard solution (diphenylamine – ISTD, 1000 μg/ml). Solid buffer containing potassium carbonate and sodium bicarbonate (1:2 *w/w*) was added to adjust the urine pH to 9.5. Liquid-liquid extraction was performed twice by adding 5 ml of diethyl ether and shaking for 2 min after addition of 500 mg of ammonium sulfate. After centrifugation at 1200 *g* for 5 min, the organic layer was transferred into a clean glass tube and evaporated to dryness under nitrogen flow at room temperature. Free fraction was analyzed by dissolving the solid residue in 50 μl of methanol.

Acidic hydrolysis

10 μl of the internal standard solution (diphenylamine – ISTD, 1000 μg/ml), 1 ml of 6 M HCl and 100 mg of cysteine were added

Table 1. Retention time (RT), precursor ion (PI), product ion (DI) and collision offset voltage (CE) for mesocarb and its metabolites by HPLC-MS/MS

Code	Compounds	RT (min)	MW	PI (*m/z*)	DI (*m/z*)	CE (eV)
	Mesocarb	21.8	322	323	**177***	5
					119	5
					91	40
M1	p-Hydroxymesocarb	15.0	338	339	**193**	5
					135	10
					91	15
M2	Hydroxymesocarb	18.6	338	339	**205**	5
					177	5
					135	15
					119	15
					107	20
M3	Dihydroxymesocarb	9.2	354	355	221	5
					193	5
					135	10
					107	25
M4, M5	Dihydroxymesocarb (*2 isomers*)	11.0/11.7	354	355	221	5
					193	5
					135	15
M6, M7	Dihydroxymesocarb (*hydroxylation only in phenylisopropyl moiety*)	13.5/16.2	354	355	205	5
					177	5
					151	5
					123	25
					119	25

* Product ions for SRM mode

to 5.0 ml of horse urine. The mixture was heated at 100 °C for 60 min. After cooling, it was neutralized with 2 ml of 5 M NaOH and liquid-liquid extraction without addition of solid buffer and the following procedure was performed according to previously described free fraction analysis sample preparation.

Enzyme hydrolysis

A few drops of glacial acetic acid were added to 5.0 ml of horse urine to adjust pH to 5.5–6.0. Then 1 ml of phosphate buffer solution and 30 μl of β-Glucuronidase from *E. Coli K12* were added. The mixture was incubated for 60 min at 57 °C. After incubation, the mixture was cooled at room temperature and the following procedure was performed as it was described in free fraction sample preparation.

The solid residue was dissolved in 50 μl of methanol in all cases of sample preparation procedures. 5 μl of final solution was injected into the HPLC-MS/MS system.

Results and discussion

Previous studies on the mesocarb and its metabolites identification reported the presence of parent mesocarb, two mono-, three di- and two tri-hydroxylated metabolites of mesocarb in human plasma and urine.[8,13] A proposed scheme for the metabolic pathway of mesocarb in human and horse urine is illustrated in Figure 1. After analysis of samples obtained in an excretion study of mesocarb in horses, detection of mesocarb and several metabolites was performed. The protonated molecular ions $[M+H]^+$, retention times and product ions of mesocarb and its metabolites are presented in Table 1. As it is seen from Figure 2, mesocarb produces the informative mass spectrum. The ions at *m/z* 119 and 177 are structurally important as they correspond to the fragments with phenylisopropyl and phenylcarbamoyl moieties.

Using liquid chromatography combined with a tandem mass spectrometry eight peaks with $[M+H]^+$ corresponding to the molecular weight of the mesocarb (*m/z* 323), monohydroxylated metabolites (*m/z* 339) and dihydroxylated metabolites (*m/z* 355) were observed on the chromatograms (Figures 3–5). The MS/MS product ion scans were performed for each metabolite at CE = 10 eV (Figure 2). It was found that ion transitions $[M+H]^+ \rightarrow$ *m/z* 119, 135, 177 and $[M+H]^+ \rightarrow$ *m/z* 123, 193, 151 provide a high selectivity of analysis.

The exact structure identity of only one metabolite **M1** (p-hydroxymesocarb) was confirmed with reference standard. The structures of other metabolites (**M2 - M5**) were identified by comparing their mass spectra with literature data reported in humans after an oral administration.[13–15] **M6** and **M7** metabolites were tentatively identified as dihydroxylated metabolites of mesocarb, where both of the hydroxy groups are located in phenylisopropyl part of molecule (ring A). Both **M6** and **M7** have not been reported previously as metabolites of mesocarb in any species.

The presence of mesocarb in urine samples was confirmed by HPLC-MS/MS in comparison to the reference standard. Mesocarb has the nominal mass of 322. The characteristic ions at *m/z* 177, 119 and 91 were detected both for drug standard and post-administration samples. The *m/z* 177 ion is characteristic fragment due to fragmentation of the sydnone ring.[8,13] Extracted ion chromatograms for mesocarb of pre- and post-administration samples are shown in Figure 3.

Two monohydroxylated metabolites **M1** and **M2** were detected during the excretion study of samples. The ESI(+) MS/MS spectra for the metabolites **M1** and **M2** are given in Figure 2. Chromatograms of pre- and post-administration samples are shown in Figure 4. p-Hydroxymesocarb (**M1**) was detected in free fraction analysis as well as after acidic and enzymatic hydrolysis. p-Hydroxymesocarb has the nominal mass of 338. The characteristic ions at *m/z*

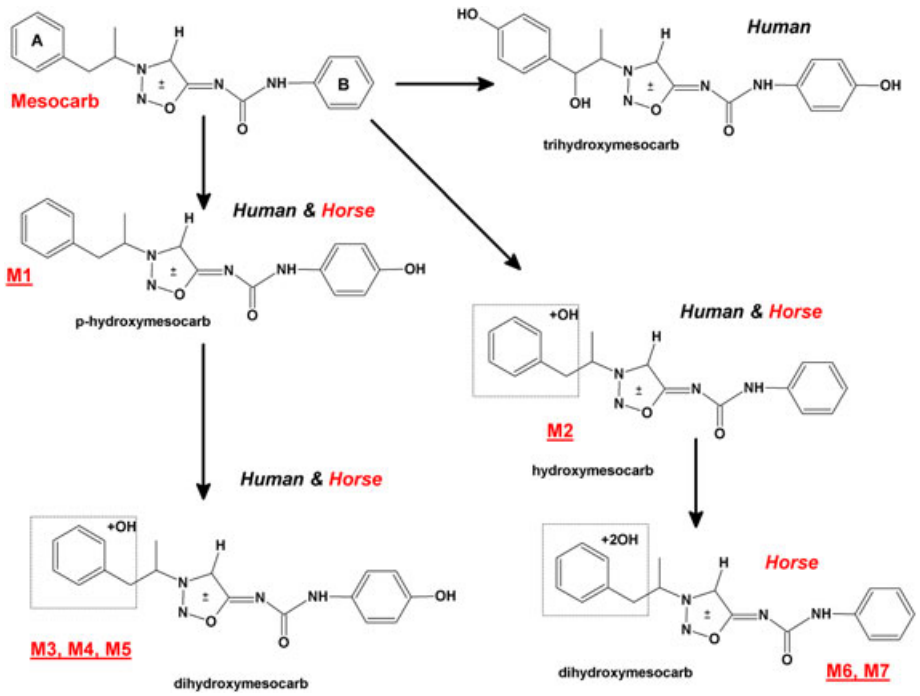

Figure 1. A proposal mesocarb metabolic pathway in equine and human.

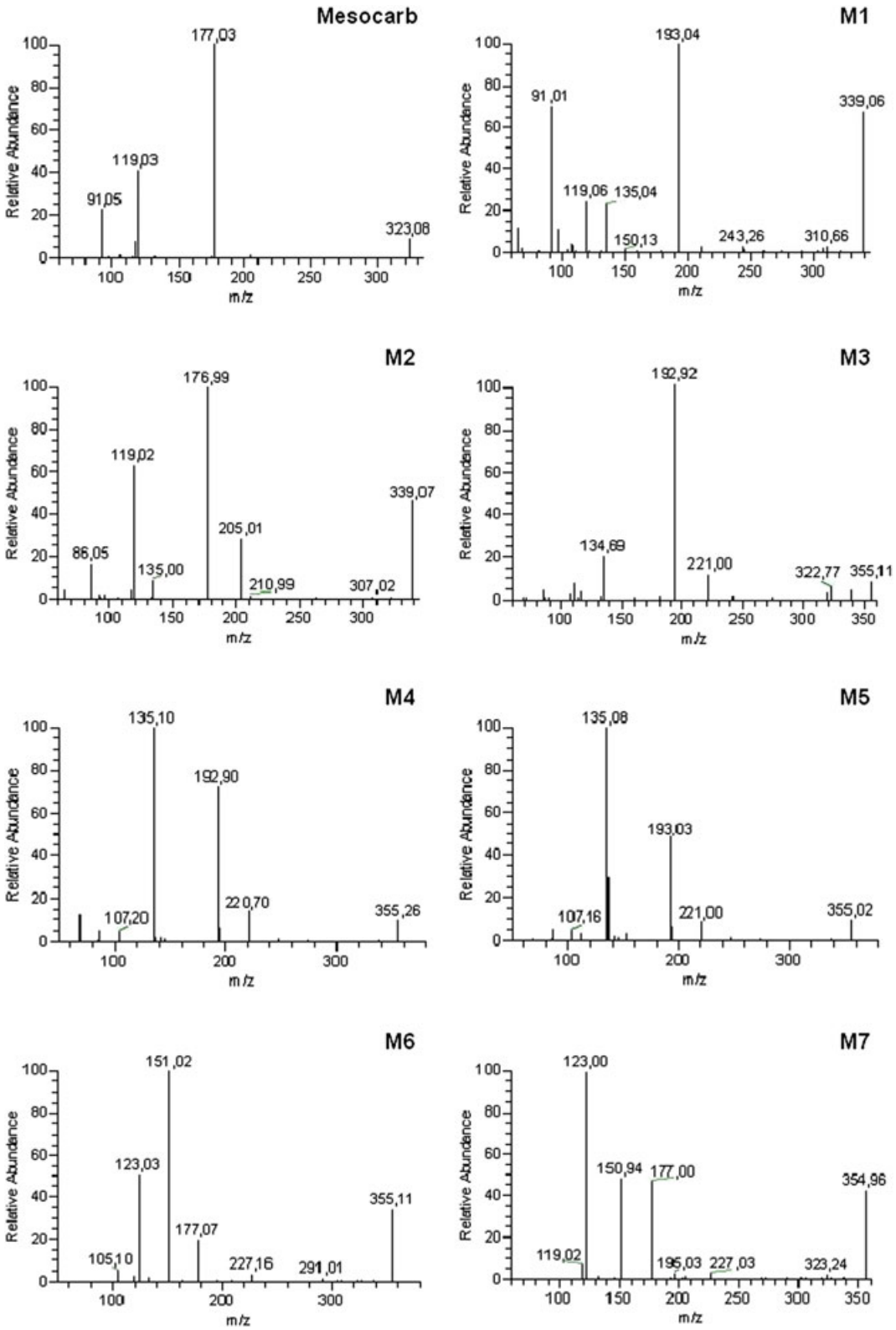

Figure 2. ESI(+) MS/MS spectra of the mesocarb and its metabolites (M1-M7), CE=-10 eV.

193, 135, 119, and 91 were detected both in reference standard and post-administration samples. The ions at *m/z* 135 and *m/z* 193 resulted from fragmentation of the phenylcarbamoyl moiety similar to the ions *m/z* 119 and *m/z* 177 for mesocarb, with addition of hydroxyl group.[15] Monohydroxylated metabolite of mesocarb (**M2**) was evaluated only after performing the hydrolysis procedures and was not observed during free urine fraction analysis. The exact position of hydroxy group (ortho-, para- or α-position) in **M2** was not precisely evaluated. The presence of ion at *m/z* 177 indicates that the hydroxy group is located in the phenylisopropyl moiety.

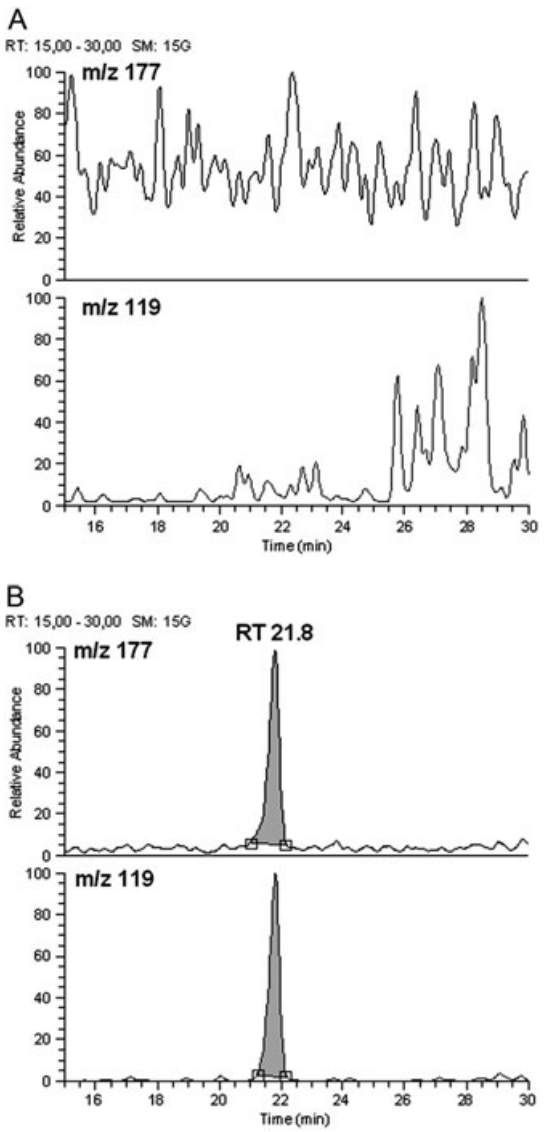

Figure 3. Extracted ion chromatograms for mesocarb of (A) blank urine and (B) obtained from a urine sample collected 3.5 h after an administration of 20 mg of mesocarb to a thoroughbred gelding (323 → m/z 119, 177).

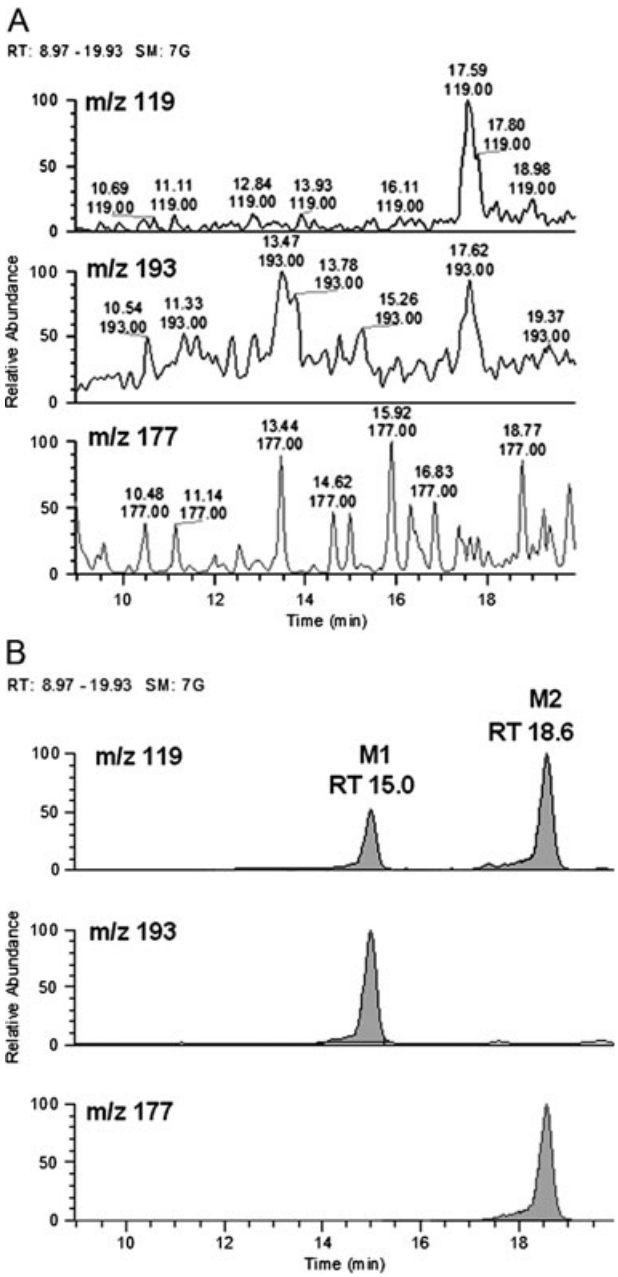

Figure 4. Extracted ion chromatograms for hydroxylated metabolites of mesocarb of (A) blank urine and (B) obtained from a urine sample collected 3.5 h after an administration of 20 mg of mesocarb to a thoroughbred gelding (339 → m/z 119, 177, 193).

Five dihydroxylated metabolites of mesocarb excreted in free and conjugated forms were identified in horse urine (**M3-M7**). The ESI(+) MS/MS spectra for this metabolites are given in Figure 2. Chromatograms of pre- and post-administration samples are shown in Figure 5. While **M3** metabolite was observed after free and hydrolyzed fractions analysis, **M4-M7** metabolites were detected only after performing the hydrolysis procedures and were not detected as unconjugated forms.

Metabolite **M3** is dihydroxylated form of mesocarb with the position of hydroxy group in ring A and *para*-hydroxy group in ring B. The exact position of ring A hydroxy group in **M3** was not evaluated. However, dihydroxylated metabolites described by Gómez *et al.*[15] in human urine showed the presence of hydroxy groups in *para* position of both phenylisopropyl moiety (*m/z* 135) and phenylcarbamoyl moiety (*m/z* 193).

Metabolites **M4** and **M5** showed very close retention times and similar product ions that may correspond to the diastereomeric metabolites. The presence of ion at *m/z* 193 indicates that one hydroxy group is located in the phenylcarbamoyl moiety (ring B), and the second is located in the phenylisopropyl moiety (*m/z* 135, ring A). We suggest that second hydroxy group may be located in the α position of the phenylisopropyl moiety. This data is in concordance with work of Gómez *et al.*[15] that the introduction of a hydroxyl group in the α position of the phenylisopropyl moiety creates a second chiral center in the molecule.

According to the product ion spectra of **M6** and **M7** metabolites of mesocarb, both of the hydroxy groups are located in phenylisopropyl part of molecule (Figure 2). The presence of *m/z* 177 in the mass spectra of **M6** and **M7** provided a useful clue to the position of hydroxyl groups. **M6** and **M7** had mass spectra almost identical but with different retention times. The protonated molecular ion $[M+H]^+$ (m/z 355) of these metabolites was increased

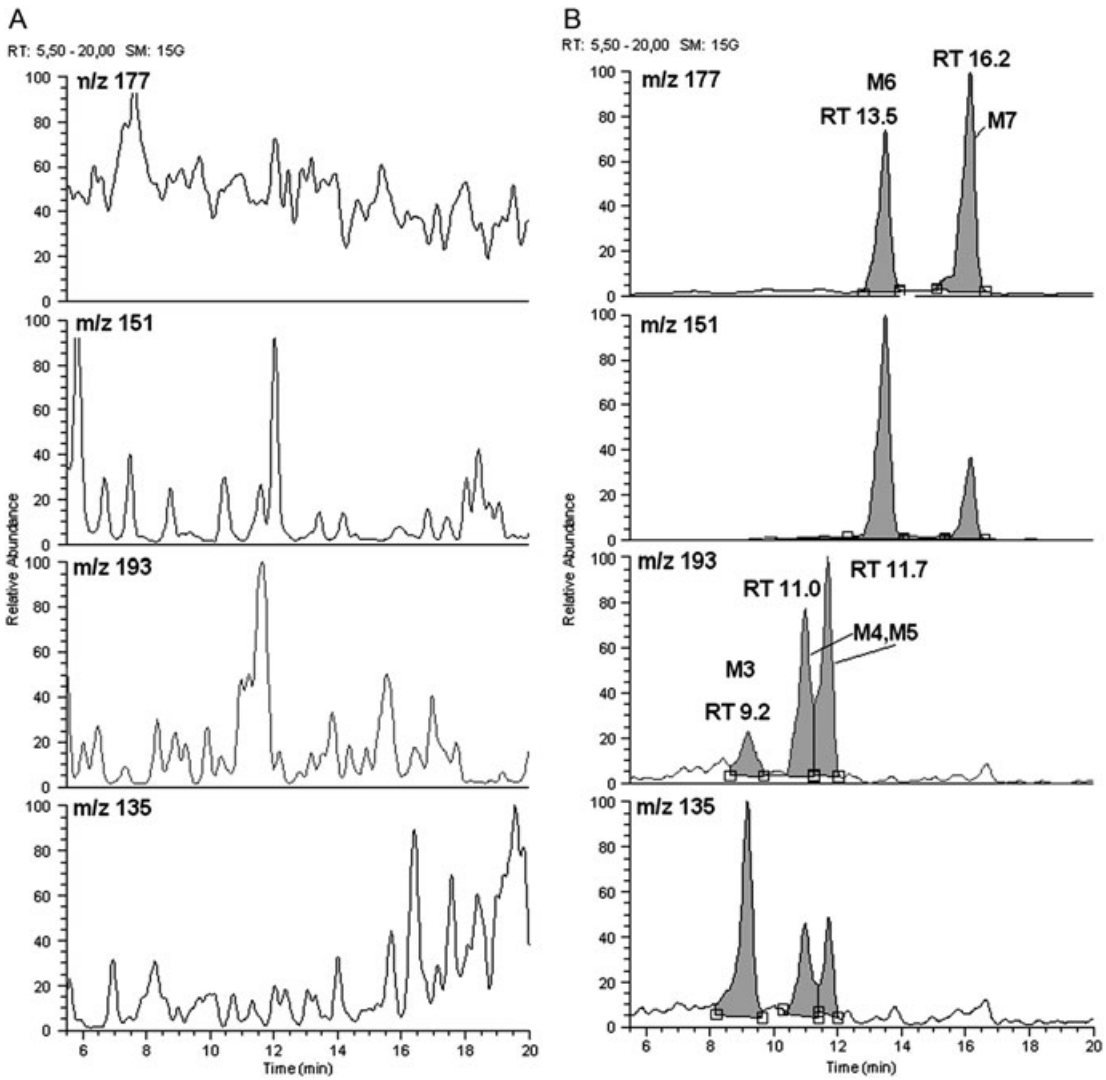

Figure 5. Extracted ion chromatograms for dihydroxylated metabolites of mesocarb of (A) blank urine and (B) obtained from a urine sample collected 3.5 h after an administration of 20 mg of mesocarb to a thoroughbred gelding (355 → m/z 135, 151, 177, 193)

by a mass 32 as compared to that of mesocarb. This assumes that **M6** and **M7** are the dihydroxylated metabolites of mesocarb. As the chromatographic peaks of **M6** and **M7** are long-way from each other, we can suppose that they are not diastereomeric metabolites and possibly both hydroxy groups are located in ring A. Moreover, previous mesocarb metabolic pathway investigations did not showed the presence of **M6** and **M7** in human and animals, so these metabolites are specific and unique to horse biotransformation.

Due to our experiment we could not detect trihydroxylated metabolites of mesocarb in horse urine that were detected in post-administration human urine by Appolonova *et al.*[13] and Gómez *et al.*[15]

One of the objectives of the present study was the identification of potential screening targets for controlling misuse of mesocarb in racehorses. It is therefore of great interest to determine the detection window of these metabolites in urine. For how long these metabolites can be detected in the post-administration urine by LC-MS/MS is summarized in Table 2. All identified metabolites were detected up to 12–120 h after administration of a single dose of 20 mg mesocarb. **M1** metabolite was detected up to 90 h after administration in both horses. Among these metabolites, **M2** was detected for the longest (up to 120 h). **M6** and **M7** metabolites were the substances of interest not only owning the specificity but also due to the excretion time up to 96 h. Metabolites **M1**, **M2**, **M6** and **M7** are therefore the best screening targets for detecting mesocarb administration.

Table 2. Detection window of the urinary mesocarb and its metabolites in horses

Compounds	Horse1 (h)	Horse2 (h)
Mesocarb	6	12
M1 (p-Hydroxymesocarb)	90	90
M2 Hydroxymesocarb	96	120
M3 Dihydroxymesocarb	7	12
M4, M5 Dihydroxymesocarb (*2 isomers*)	3	3
M6, M7 Dihydroxymesocarb (*hydroxylation only in phenylisopropyl moiety*)	96	96

Conclusion

The *in vivo* metabolism of mesocarb was studied. Mesocarb and seven (**M1 – M7**) metabolites were detected in horse urine. A metabolic pathway for mesocarb in equine was postulated. The main metabolites (**M2**, **M6**, and **M7**) in equine were formed by the hydroxylation of the phenylisopropyl moiety of mesocarb whilst the main process of hydroxylation of mesocarb in human was carried out in the phenylcarbamoyl moiety. Two mono- (**M1**, **M2**) and two di-hydroxylated (**M6**, **M7**) metabolites were detected for 90–120 h and are useful for the abuse of mesocarb administration in equine.

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