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Liquid chromatography—electrospray ionization ion trap mass spectrometry for analysis of mesocarb and its metabolites in human urine

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Abstract

A method is described for the determination of metabolites of mesocarb in human urine by combining gradient liquid chromatography and electrospray ionization (ESI)—ion trap mass spectrometry. Seven metabolites (two isomers of hydroxymesocarb, *p*-hydroxymesocarb, two isomers of dihydroxymesocarb and two isomers of trihydroxymesocarb) and parent drug were detected in human urine after the administration of a single oral dose 10 mg of mesocarb (Sydnocarb®, two tablets of 5 mg). Various extraction techniques (free fraction, enzyme hydrolyses and acid hydrolyses) and their comparison were carried out for investigation of the metabolism of mesocarb. After extraction procedure the residue was dissolved in methanol and injected into the column HPLC (Zorbax® SB-C18 (Narrow-Bore 2.1 × 150 mm i.d., 5 μm particles)) with mobile phase (0.2 ml/min) of methanol/0.2 mM ammonium acetate. Conformation of the results and identification of all metabolites are performed by LC-MS and LC-MS/MS. The major metabolites of mesocarb in urine of the human were *p*-hydroxylated derivative of the phenylcarbamoyl group of the parent drug (*p*-hydrohymesocarb) and dihydroxylated derivative of mesocarb (two isomers of dihydroxymesocarb). This analytical method for dihydrohymesocarb was very sensitive for discriminating the ingestion of mesocarb longer than the parent drug or other metabolites in human urine. The dihydroxymesocarb was detected in urine until 168–192 h after administration of the drug.

Keywords: Mesocarb; Hydroxymesocarb

1. Introduction

Mesocarb (*N*-phenylcarbamoyl-3-(β-phenylisopropyl)sydnoneimine), also known as sydnocarb, was synthesized from amphetamine as a starting material [1–3]. It is a central nervous system stimulant (CNS) [4] and is therefore included in the doping list of forbidden substances indicated by the Medical Commission of the International Olympic Committee [5]. Hence the methods to detect the presence of this compound or its metabolites in human urine are required.

Metabolic studies of mesocarb in rat urine by GC/MS have been first published by Polgar and co-workers [6,7]. These authors have found that the free and conjugated hydroxylated metabolites are the main metabolites. Only traces of unchanged drug were found in rat urine. Three different metabolites such as: *p*-hydroxymesocarb, dihydrohymesocarb and amphetamine were established as metabolites of mesocarb in rat urine.

Many antidoping laboratories performed investigation of the metabolism of mesocarb in human urine. Different methods based on combined chromatographic and mass spectrometric techniques have been applied to the analysis of mesocarb and its metabolites in human urine [8–16]. A two-step analysis, with and without hydrolysis, was carried out to determine indirectly the concentration of conjugated metabolites in the sample.

The method of GC-MS has been widely applied in the doping control. This method is often used for different screening procedure, but for analysis of metabolism of mesocarb is not successful. Mesocarb and its metabolites are highly polar and thermolabile and undergo pyrolysis in the injector block of a gas chromatography and so do its metabolites. The pyrolysis product in both cases was *N*-nitroso-*N*-cyanomethyl-amphetamine. Many technical modifications for the detection of mesocarb have been described recently, such as GC/MS (without derivatization) [8,9], GC/MS (as *N*-fluoroacyl derivative) [10], GC/MS

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(as *N*-trifluoroacetil derivative) [11,12], LC-TS/MS [10], LC-PB/MS [8,14,15] and LC-ESI/MS [16], but only the parent compound and sulfate conjugated *p*-hydroxymesocarb were found. These authors could not to detect other metabolites that were found in rat urine, such as free *p*-hydroxymesocarb, dihydroxymesocarb, unchanged mesocarb and amphetamine [6,7]. All these authors reported that the main metabolite of mesocarb is sulfate conjugated *p*-hydroxymesocarb in human urine, and this compound can be detected in human urine until 48–72 h after intake 10 mg of mesocarb. Therefore information concerning human and animal metabolism of mesocarb and human urinary excretion of mesocarb is limited.

This work presents LC-MS(-MS) method for qualitative identification of metabolites of mesocarb in human urine. Structural assignments of metabolites were based on changes in molecular masses and spectral patterns of product ions. The sensitive and specific method for the confirmation of mesocarb and its metabolites in human urine was developed. Quantitative analyses of mesocarb and its metabolites are not part of the routine doping control testes.

2. Experimental

The laboratory is accredited by ISO 17025.

2.1. Reagents and chemicals

Mesocarb (*N*-phenylcarbamoyl-3-(β-phenylisopropyl)sydnoneimine) was received from Pharmacological Committee (Moscow, Russia) as pure substance. Sydnocarb® was obtained from a Russian pharmacy (Manufacturer: Pharmacon®, St. Petersburg, Russia). Diphenylamine was used as internal standard (ISTD), purchased from Sigma (St. Louis, MO, USA). HPLC-grade methanol was acquired from Merck (Darmstadt, Germany). Ammonium acetate was purchased from Sigma (St. Louise, MO, USA). β-Glucuronidase (from *Helix Pomatia*) was used for enzymatic hydrolysis, purchased from Boeringer Mannheim (Mannheim, Germany). Distilled water for LC-MS ion trap was purified using a Milli-Q Elix System (Millipore, Milford, MA, USA).

2.2. LC-ESI/MSD ion trap-analysis

The 1100 Series LC/MSD Trap "SL" system was an Agilent Technologies (Palo Alto, CA, USA) equipped with a diode array detector, autosampler and autoinjector. The analytical column was Zorbax® SB-C18 (2.1 × 150 mm i.d., 5 μm) from Agilent Technologies (Palo Alto, CA, USA) connect with a guard column (cartridge 2.1 mm × 12.5 mm) filled with the same packing material. The temperature of the column was 30 °C. The gradient program is shown in Table 1.

Table 1
The gradient programme

Time (min)	Ammonium acetate: 0.2 mm (mobil phase A, %)	Methanol (mobil phase B, %)	Flow (ml/min)
0.0	80	20	0.2
20	40	60	0.2
35	40	60	0.3

The Agilent Technologies "SL" ion trap mass spectrometer (LC/MSD ion trap "SL") with atmospheric pressure electrospray ionization (API-ES) source was used for quantification in a positive ionization mode. The operating conditions were: dry temperature (350°C), capillary voltage (−4000 V), nebulizer (40 psi), dry gas (helium, 9 l/min). Ion trap full scan analyses were conducted from *m/z* 85 to 450 with an upper fill time of 200 ms. Multiple reaction monitoring (MRM) experiment used helium in the ion trap. MS–MS experiments utilized a retention time (RT)-dependent LC-MS/MS program where the retention time and parent ion mass was programmed following review of the full scan LC-MS chromatogram.

A 1-μl sample volume was injected. Complete system control and data evaluations were done on the HP ChemStation for LC/MS.

2.3. Sample preparation

2.3.1. Administration

Two healthy volunteers gave their informed consent to participate in the study. Just before drug administration the urine morning voids (blank) were collected to analyzed for absence of mesocarb. Volunteers without any medication and poppy seeds in diet 3 days before, fasting since evening, were administrated the peroral therapeutic dose of 10 mg mesocarb (Sydnocarb®, two tablets of 5 mg). Excreted urine fractions were collected for a period of 240 h. Urine samples were stored at −20 °C until analyses.

2.3.2. Standard solutions

Stock solutions were prepared by dissolving the mesocarb in methanol (1 mg/ml).

2.3.3. Urine extraction

2.3.3.1. Free fraction. To 5 ml of urine samples were added 10 μl diphenylamine (ISTD, 1000 μg/ml) and 0.1 g amount of solid buffer ($NaHCO_3/K_2CO_3$, 2:1 mixture) to adjust the pH to 9.5. Then were added 100 mg of anhydrous sodium sulfate and the mixture were extracted twice with 5 ml of diethyl ether. After shaken (2 min) and centrifugation (5 min, 1000 g), the organic layer was separated and taken to dryness at 60 °C.

2.3.3.2. Acidic hydrolysis. Diphenylamine (10 μl) (ISTD, 1000 μg/ml), 1 ml of 6 M HCl and 100 mg of cysteine were

added to 5 ml of urine samples. Mixture was heated at 100 °C (60 min). After cooling, it was neutralized 5 M NaOH and extracted twice with 5 ml of diethyl ether. After shaken mechanically (2 min) and centrifugation (5 min, 1000 g), the organic layer was separated and taken to dryness at 60 °C.

2.3.3.3. Enzyme hydrolyses. To 5 ml of urine samples a few drops of glacial acetic acid was added to adjust pH to 5.0–5.5. Then 1 ml of acetate buffer and 30 μl of β-glucuronidase from *Helix Pomatia* are added prior to enzymatic hydrolyses. It takes 3 h at 57 °C. After cooling pH is adjusted to 9.5 of solid buffer (Na_2CO_3/$NaHCO_3$, 1:1 mixture). Vortex extraction is performed with 5 ml diethyl ether/isopropanol mixture (9:1). 500 mg of Na_2SO_4 is added during vortex mixing. After centrifugation organic layer is separated and overprized to dryness. Isopraponol is removed in rotary evaporator.

In the all these cases the residue was dissolved with 50 μl of methanol and 1 μl of this solution was injected into the LC-MS ion trap.

3. Results and discussion

3.1. MS–MS analysis of standard

The fragmentation of mesocarb and its metabolites was investigated using a quadrupole ion trap mass spectrometer with ammonium acetate as the buffer for positive ion mode operation. This study employed multiple MS which involved repeated trapping and fragmentation of ions.

The first step of this work involved the characterization of the mass spectral properties of the parent drug. Mesocarb was determined to be >98% pure by LC-UV-ESI/MS; no impurities or degradation products were detected. Full scan mass spectral analyses of mesocarb showed protonated molecular ion of *m/z* 323 (Fig. 1A). The MS–MS spectrum of the protonated molecular ion (*m/z* 323) and suggested fragmentation pattern are presented in Fig. 1B. Fragmentaion of protonated molecular ion of mesocarb in the ion trap leads to three product ions *m/z*: 91, 119 and 177. The ion at *m/z* 91 is resulted in a cleavage of by methylbenzene moiety $[C_6H_5CH_2]^+$, *m/z* 119 is formed by cleavage of both the isopropyl benzene moiety $[C_6H_5(CH_2)_2CH_2]^+$ and phenylcarbamoyl moiety $[CONHC_6H_5]^+$. The base ion at *m/z* 177 is a result of ring fragmentation. The mass spectrum pattern (Fig. 1) served as templates in the elucidation of the structures of the proposed metabolites.

Visual examination and comparison of the LC-MS/MS ion chromatograms of blank urine, mesocarb standard and sample urine showed the fact that the method has good specificity for mesocarb. The analyte has good chromatographic peak shape and no significant interferences from endogenous material at the retention time of mesocarb was observed. Fig. 2 shows the representative ion chromatogram of *m/z*: 193, 177 and 170 of mesocarb standard, blank urine in comparison with sample urine.

3.2. MS–MS analysis of metabolites

The LC-ESI/MS method was applied to the analysis and confirmation testing of mesocarb and its metabolites in human urine. Various extraction techniques (free fraction, enzyme hydrolyses and acid hydrolyses) and their comparison were carried out for investigation of the metabolism

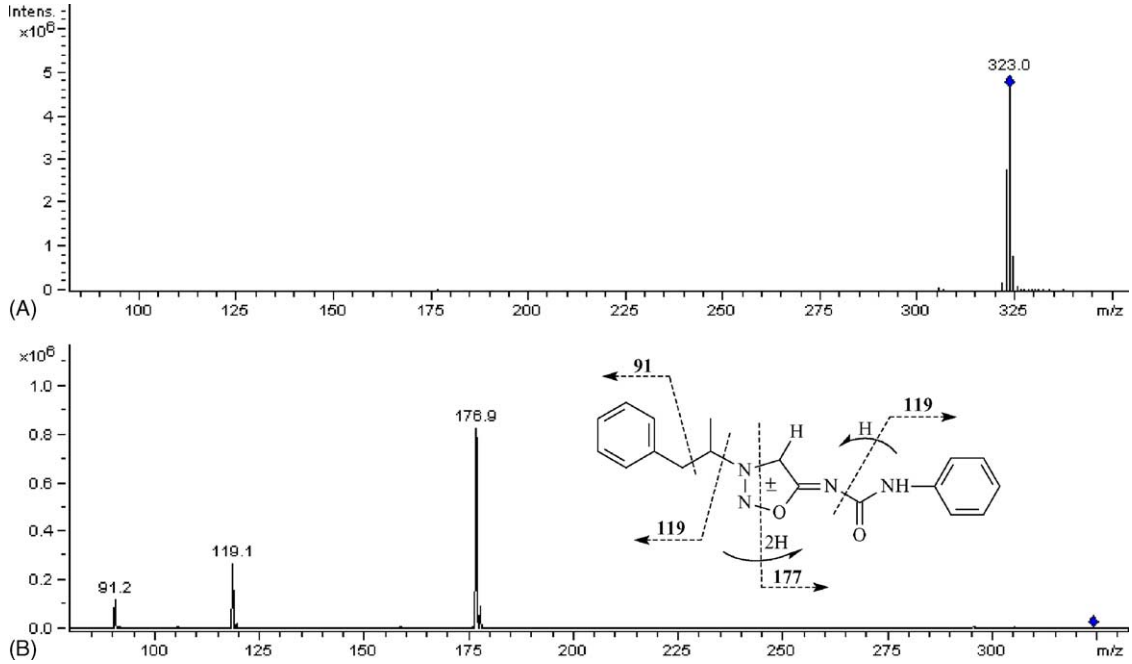

Fig. 1. (A) ESI mass spectrum of mesocarb. (B) MS–MS spectrum of the mesocarb molecular ion (*m/z* 323).

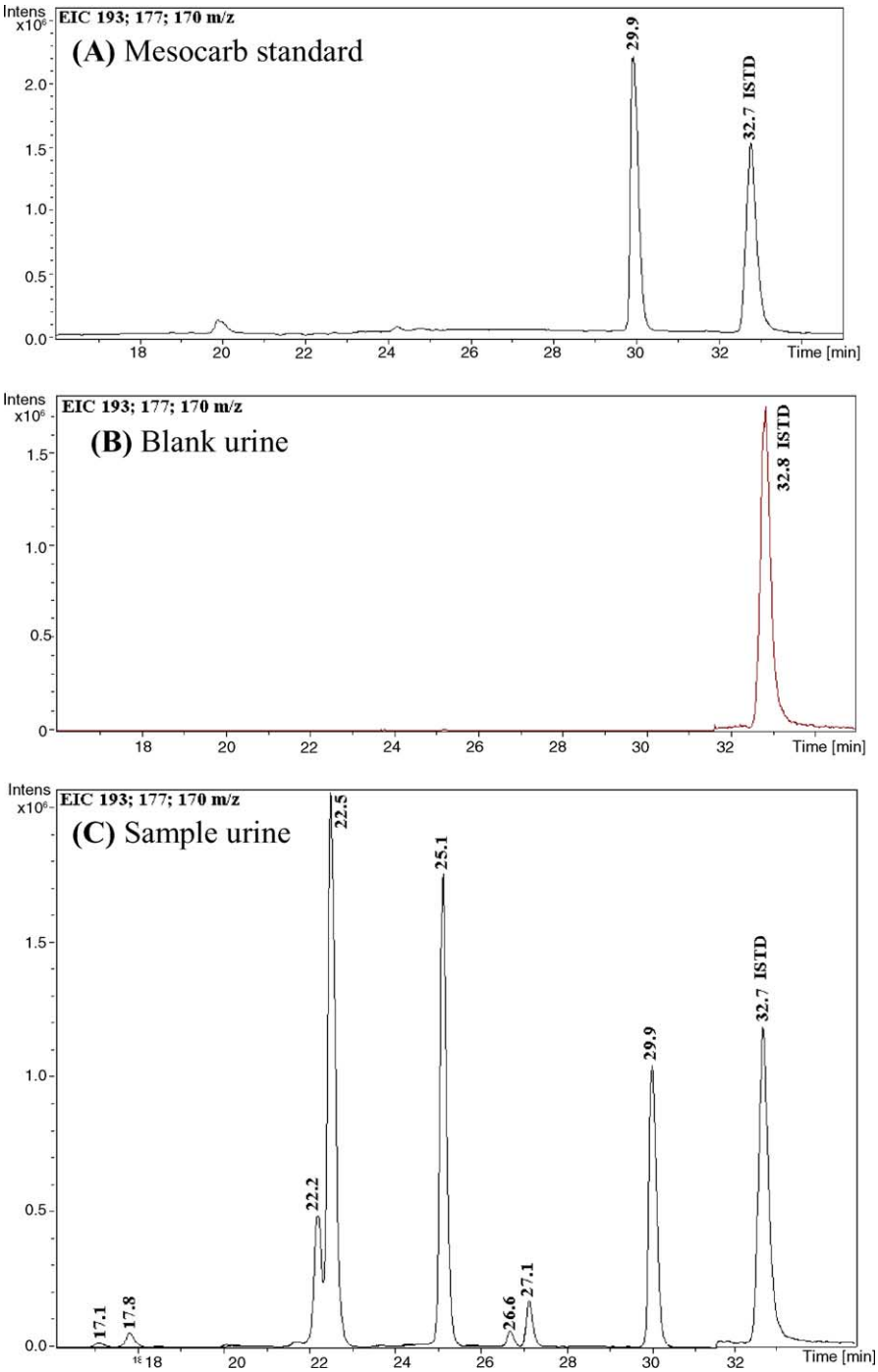

Fig. 2. Ion chromatograms of *m*/*z* 193, 177, 170 in (A) mesocarb standard (50 ng/ml); (B) blank urine; (C) after administration of a single oral dose (10 mg), after 8 h.

of mesocarb. For the free fraction of urine the mean recoveries ($n = 4$) were 76.9 and 81.4% at concentrations of 1 and 2 ng/ml, respectively.

Analysis by LC-ESI/MS of extracts from free fraction urine (Fig. 2C) gave the chromatogram of urine sample that show a number of products appearing at retention times shorter than those of the parent drug (i.e. most polar compounds). These results have shown that besides the unchanged parent drug (**I**), the following seven metabolites were detected: two isomers of hydroxymesocarb (**II**, **III**), *p*-hydroxymesocarb (**IV**), two isomers of dihydroxymesocarb (**V**, **VI**) and two isomers of trihydroxymesocarb (**VII**, **IIX**). Most of the metabolites were detected from human urine for the first time.

The protonated molecular ions ($[M + H]^+$) and changes in observed mass (ΔM) for these proposed metabolites are

Table 2
Product ions for mesocarb and its metabolites, protonated molecular ions $[M+H]^+$, changes in observed mass for the metabolites (ΔM) and fragmentation results from MS–MS spectrum were obtained via fragmentation of molecular ions

		M_W	$[M+H]^+$	ΔM	MS–MS
(I)	Mesocarb	322	323		**323** → **177**, 119, 91
(II)	Mesocarb-M (OH–), isomer-1	338	339	+16	**339** → 205, **177**, 135, 119, 108
(III)	Mesocarb-M (OH–), isomer-2	338	339	+16	**339** → 205, **177**, 135, 119, 108
(IV)	Mesocarb-M (*p*-OH–), isomer-3	338	339	+16	**339** → **193**, 135, 119, 91
(V)	Mesocarb-M (di-OH–), isomer-1	354	355	+32	**355** → 221, **193**, 135, 108
(VI)	Mesocarb-M (di-OH–), isomer-2	354	355	+32	**355** → 221, **193**, 135, 108
(VII)	Mesocarb-M (tri-OH–), isomer-1	370	371	+48	**371** → 221, **193**, 151, 135, 123
(IIX)	Mesocarb-M (tri-OH–), isomer-2	370	371	+48	**371** → 221, **193**, 151, 135, 123

presented in Table 2. The postulated structural assignments for metabolites of mesocarb are presented in Fig. 3. The structures of metabolites could not be determined conclusively by mass spectrometry alone, but partial identification was made.

The identification of the metabolites of mesocarb is based on the LC-MS, and LC-MS/MS spectra (Fig. 4). LC-MS/MS mass spectra were obtained via fragmentation of protonated molecular ions that used for more precise structural identification of metabolites. The molecular ions of the metabolites of mesocarb were compared with those of the parent drug. A net change of +16, +32, +48 Da in protonated molecular ions of metabolites was observed (Table 2). These results have shown that in human urine presence monohydroxylated-, dihydroxylated- and trihydroxylated-metabolites. On the basis of the identified metabolites the following metabolic pathways could be postulated: single, double and triple aromatic hydroxylation of mesocarb (Fig. 3).

The unchanged mesocarb (**I**) was eluted at 29.9 min in the mesocarb positive urine (Fig. 2C). MS and MS–MS mass spectra unchanged mesocarb showed in Fig. 4.

Table 3
Comparison between different extraction procedures

RT (min)		Height (cm) × 10^5		
		Free fraction	Acidic fraction	Enzymatic fraction
32.8	ISTD	45.2	46.0	45.9
29.9	Mesocarb	7.1	Trace	Trace
Monohydroxy-mesocarb				
27.1	**II**	10.3	40.5	30.3
26.6	**III**	5.3	5.2	ND[a]
25.1	**IV**	12.3	124.9	80.4
Dihydroxy-mesocarb				
22.5	**V**	122.2	50.6	21.9
22.2	**VI**	74.8	29.5	15.2
Trihydroxy-mesocarb				
17.8	**VII**	9.4	34.2	ND
17.1	**IIX**	3.2	3.5	ND

[a] Not found.

The result of fragmentation was same with those of mesocarb standard. Only traces of unchanged mesocarb was found after acidic and enzymatic hydrolyses (Table 3).

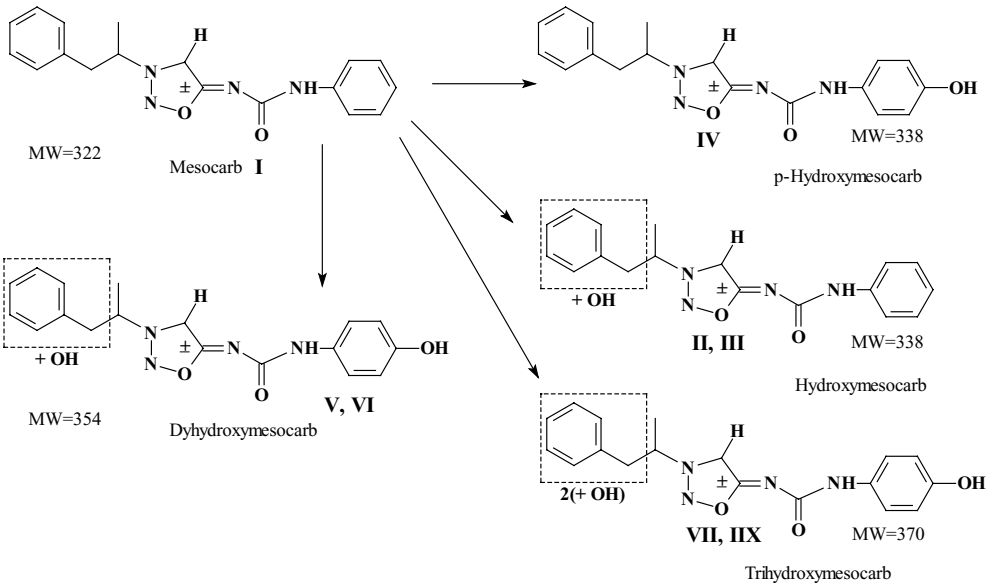

Fig. 3. Structural assignments made for mesocarb metabolites.

3.3. Monohydroxylated metabolites

Metabolites (**II**) and (**III**) had a shorter retention time than the parent drug, 27.1 and 26.6 min, respectively. MS–MS mass spectra of its metabolites showed in Fig. 4. We have studied these metabolites together because they have the same mass spectrum. The protonated molecular ion of metabolites (**II**) and (**III**) was increased by 16 Da compared to that of the unchanged mesocarb. The protonated molecular ion of metabolites is *m*/*z* 339 (323–339). The fragment ions at *m*/*z* 177 and 119 were the same as the fragment ions of the parent drug. However the ions at *m*/*z* 135 (119 + 16) and 108 (91 + 16) were not in spectrum of mesocarb. From MS–MS mass spectra of metabolites (**II**) and (**III**) can conclude that the isopropyl benzene group of the parent drug was hydroxylated. The presence of the *m*/*z* 177 and 205 ions in the mass spectrum confirms its. Comparison different extraction procedures showed that the metabolite (**III**)

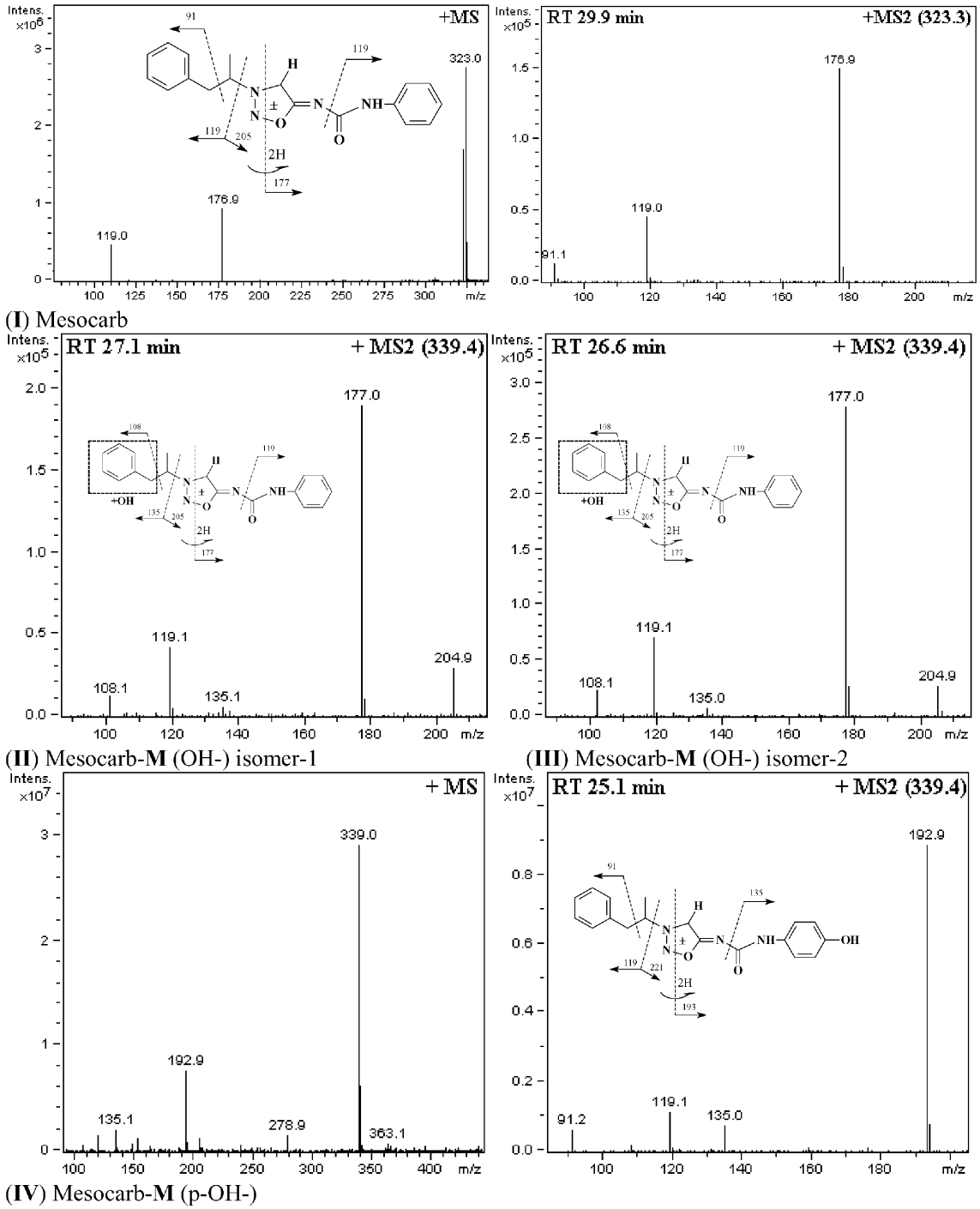

Fig. 4. Mass spectra (and MS–MS), predominant fragmentation patters, structures and retention times of mesocarb and its metabolites.

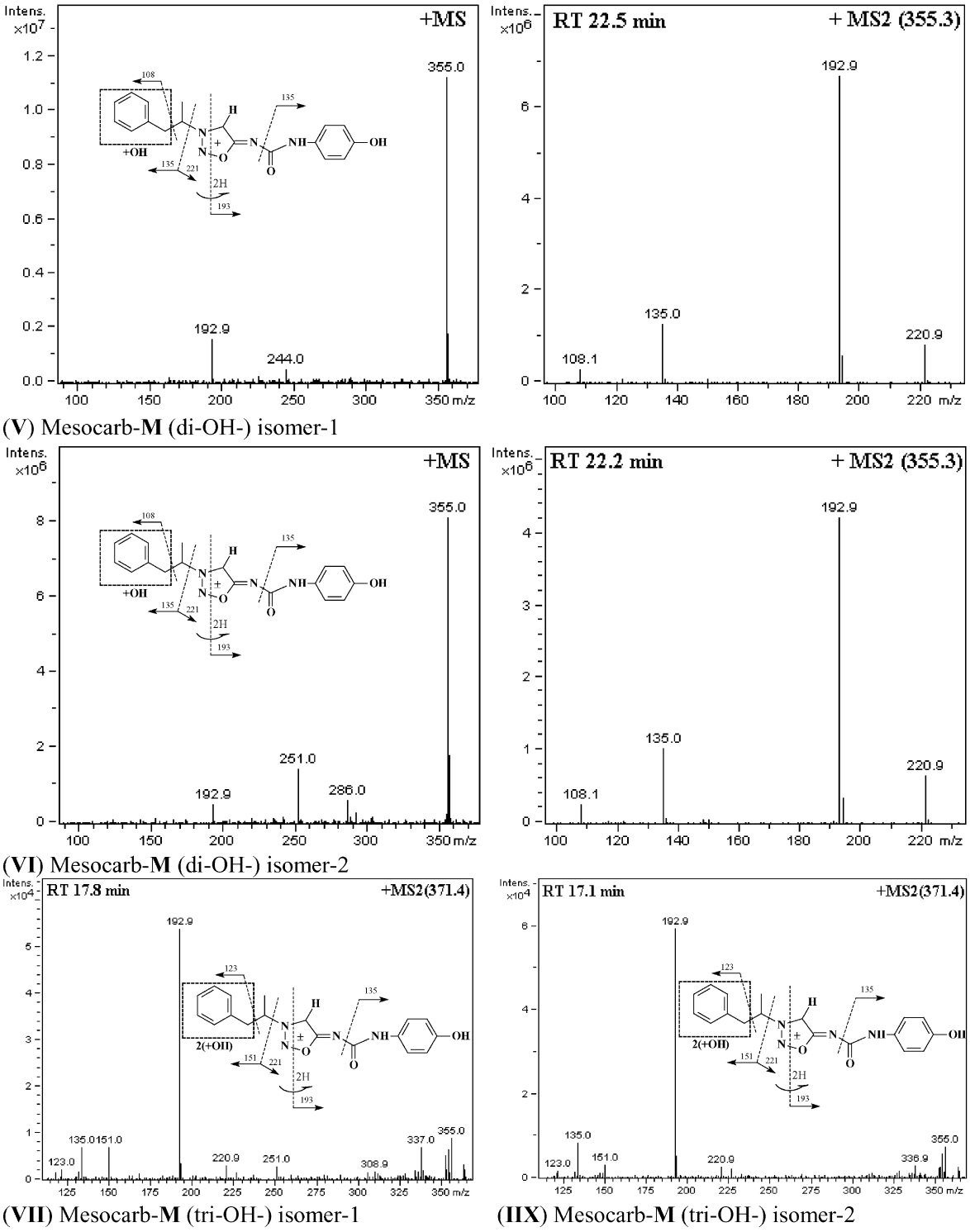

Fig. 4. (*Continued*).

excreted from human urine as the free hydroxymesocarb (isomer-1) and the metabolite (**II**) excreted as the free and sulfate conjugated hydroxymesocarb (isomer-2).

The metabolite (**IV**) was eluted at 25.1 min. The MS–MS spectrum of the *m*/*z* 339 ion is shown in Fig. 4. The protonated molecular ion of metabolite (**IV**) is *m*/*z* 339 that the same with those of the metabolites (**II**) and (**III**). The fragment ions at *m*/*z* 119 and *m*/*z* 91 were the same as the fragment ions of the parent drug. The two of ions of the metabolite (**IV**) have increased by 16 Da (*m*/*z* 119–135, 177–193). Therefore, this indicates that the phenylcarbamoyl group of parent drug was hydroxylated, and it should be a *p*-substitution at the ring of phenylcarbamoyl group according to the result of Polgar et al. [6] and Ventura et al. [14].

It should be noted that the peak area of metabolite (**IV**) was increased after acid and enzymatic hydrolysis (Table 3). Enzymatic hydrolysis gave lower yields of metabolite (**IV**) than acidic hydrolysis. Therefore, considering results received by Ventura et al. [14], *p*-hydroxymesocarb excreted from human urine as the free *p*-hydroxymesocarb, the sulfate conjugated *p*-hydroxymesocarb and traces glucuron-conjugated *p*-hydroxymesocarb.

3.4. Dihydroxylated metabolites

The metabolite (**V**) and (**VI**) were eluted at 22.5 min and 22.2 min, respectively. Its metabolites have the same mass spectrum. MS–MS mass spectra of its metabolites showed in Fig. 4. The protonated molecular ion of metabolites (**V**) and (**VI**) was increased by 32 Da compared to that of the parent drug. The protonated molecular ion of its metabolites is *m*/*z* 355. The fragment ions at *m*/*z* 135 and 193 were the same as the fragment ions of the *p*-hydroxymesocarb metabolite (**IV**). The ion *m*/*z* 193 is resulted in ring fragmentation, but the ion *m*/*z* 135 is resulted not only in cleavage of the phenylcarbamoyl moiety $[CONHC_6H_5]^+$, but also the isopropyl benzene moiety $[C_6H_5(CH_2)_2CH_2]^+$. Fragmentaion of protonated molecular ions of its metabolite leads to following product ions *m*/*z* 108, 135, 193 and 221. All product ions of its metabolites were incrised by 16 Da (91–108, 119–135, 177–193, 205–221) compared to that of the parent drug. Therefore, this indicates that both the phenylcarbamoyl group and isopropyl benzene group of parent drug were hydroxylated. Hence metabolites (**V**) and (**VI**) are dihydroxylated metabolites of mesocarb.

Comparison different extraction procedures showed that the dihydroxy metabolites of mesocarb excreted from human urine only as free dihydroxy-mesocarb (Table 3). The long-time (**V**, **VI**) dihydroxymesocarb metabolites of mesocarb have been detected from unhydrolysed fraction at the seventh day after administration of a single oral dose. Therefore, in the case of drug abuse, the estimated detection time for mesocarb by LC-ESI/MS ion trap screening is 7–8 days after administration of the drug.

3.5. Trihydroxylated metabolites

The metabolite (**VII**) and (**IIX**) were eluted at 17.8 min and 17.1 min, respectively. Its metabolites have the same mass spectrum. MS–MS mass spectra of its metabolites showed in Fig. 4. The protonated molecular ion of metabolites (**VII**) and (**IIX**) was increased by 48 Da compared to that of the unchanged mesocarb. The protonated molecular ion of its metabolites is *m*/*z* 371. The characteristic ions are *m*/*z* 123, 135, 151, 193 and 221. The ions *m*/*z* 123 and 151 of its metabolites were incrised by 32 Da (91–123, 119–151) compared to that of the parent drug. The ions *m*/*z* 135, 193 and 221 were same as the fragment ions of metabolites (**V**) and (**VI**), dihydroxymesocarb. Therefore, this indicates that the phenylcarbamoyl group of parent drug was hydroxylated and isopropyl benzene group was hydroxylated twice. Hence the metabolites (**VII**) and (**IIX**) are threehydroxylated metabolites of mesocarb.

Comparison different extraction procedures showed that the metabolite (**IIX**) excreted from human urine as free trihydroxymesocarb (isomer-1) and the metabolite (**VII**) as free and sulfate conjugated trihydroxymesocarb (Table 3).

Ventura et al. [14,15] reported that unchanged mesocarb and dihydroxymesocarb were not detected in human urine, and the main metabolite of mesocarb was *p*-hydroxymesocarb and they could detect it 47–72 h. In our study both unchanged mesocarb and dihydroxymesocarb by LC-ESI/MS ion trap method were detected. *p*-Hydroxymesocarb was detected at very low level even in the 150 h urine samples. This discrepancy in detection time may be due to the differences in the detection limits. The *p*-hydroxymesocarb and dihydroxymesocarb were identified as the main metabolites of mesocarb in human urine.

Comparison between different extraction procedures showed that the screening procedure based on free fraction and acidic fraction gave good responses for the mesocarb metabolite (Table 3). The time of excretion of mesocarb and its metabolites is shown in Fig. 5. Using LC-ESI/MS ion trap, the unchanged mesocarb could be detected for only 45–63 h. The monohydroxylated metabolites (**II**, **III**) could be detected for up 108 h. The *p*-hydroxymetabolite

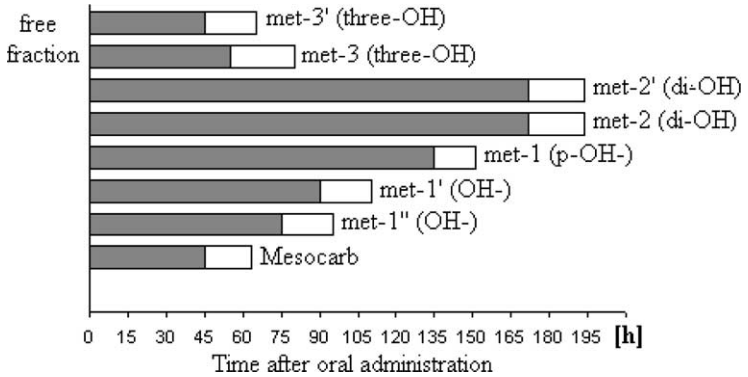

Fig. 5. Excretion of detectability of mesocarb and its metabolites by LC-ESI/MSD ion trap using free fraction and acidic fraction in human urine after oral administration of 10 mg of mesocarb ($n = 2$). The white portion of the bar represents different between the two volunteers.

(**IV**) of mesocarb could be detected for up 150 h. The dihydroxy-metabolites of mesocarb could be detected for up 190 h. And the trihydroxymetabolites could be detected for only 45–75 h.

p-Hydroxymesocarb (**IV**) was the most abundant metabolite on the first 2 days. From 3 to 5 days the excreted amount the *p*-hydroxymesocarb and the dihydroxymesocarb was the same and largest. After 5 days the excreted amount the dihydroxymesocarb metabolites (**V**, **VI**) was largest among the all excreted metabolites. Only dihydroxymesocarb metabolites (**V**, **VI**) were detected at 7–8 days after oral administration single dose—10 mg of mesocarb.

4. Conclusions

For the first time, the method using a LC-MS/MS ion trap with atmospheric pressure electrospray ionization in the positive ion mode has been developed for the analysis and confirmation testing of mesocarb and its metabolites in human urine. Seven various metabolites of mesocarb: mono-, di-, tri-hydroxymesocarb and parent drug were detected in human urine after oral administration of 10 mg (Sydnocarb®). Hidroxymesocarb and dihydroxymesocarb were identified as main metabolites. Comparison between different extraction procedures showed that the screening procedure based on acidic hydrolysis gave best responses for the mesocarb metabolites. The long-time dihydroxylated metabolites of mesocarb have been detected from unhydrolysed fraction for the seventh day after administration of a single oral dose. Therefore, in the case of drug abuse, the estimated detection time for mesocarb by LC-ESI/MS ion trap screening is 7–8 days after administration of the drug. This analytical method for dihydroxymesocarb was very sensitive for discriminating the ingestion of mesocarb longer than the parent drug or other metabolites in human. In addition, the results indicated that this method is suitable for determination of mesocarb and its metabolites in urine samples.

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