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W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck
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S.A. APPOLONOVA, A.V. SHPAK, V.F. SIZOI, V.A. SEMENOV:
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Svetlana A. Appolonova, Alexey V. Shpak, Vladimir F. Sizoi and Vitaliy A. Semenov

The Investigation of Metabolism of Mesocarb by LC/MSD Trap in Human Urine

Antidoping Centre, 105005, Elizavetinzkii pr.10, Moscow, RUSSIA, antidope@rol.ru

Introduction

Mesocarb - (N-phenylcarbamoyl-3-(β-phenylisopropyl)sydnoneimine), also known as sydnocarb, is a central nervous system stimulant[1] and is therefore included in the doping list of forbidden substances indicated by the Medical Commission of the International Olympic Committee[2]. Metabolic studies of mesocarb and its major metabolites in rat urine have been published by Polgar et al[3,4] by GC/MS. These authors found that free and conjugated hydroxylated metabolites are the main metabolites. Many antidoping laboratories performed intensive investigation on 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[5-13]. A two-step analysis, with and without hydrolysis, has to be carried out in order to determine indirectly the concentration of conjugated metabolites in the sample. Using GC/MS (without drivatization)[5,6], GC/MS (as its N-fluoroacyl derivative)[7], GC/MS (as its N-trifluoroacetyl derivative)[8,9], LC-TS/MS[10], LC-PB/MS[5,11,12] and LC-ESI/MS[13] only the parent compound and sulfate conjugated para-hydroxymesocarb were detected in human urine. Other metabolites found in rat urine, such as free para-hydroxymesocarb, dihydroxymesocarb and unchanged mesocarb were not detected. All these researches demonstrated that the main metabolite of mesocarb is sulfate conjugated para-hydroxymesocarb, and the compound can be detected in human urine until 48-72 h after intake 10 mg of mesocarb. Therefore about today information concerning the human metabolism and urinary excretion of this product is limited and its analysis method mesocarb is still a question.

An aim of the present study was to investigate metabolism of mesocarb by LC-ESI/MS Ion Trap in human urine. In this work a sensitive and specific method was developed for the

confirmation and quantification of mesocarb and its metabolites in human urine and was applied to doping control analysis.

Experimental

Reagents

Mesocarb tablets each contained 5 mg of mesocarb were obtained from a Russian pharmacy. Diphenylamine used as internal standard (ISTD), was 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 purchased from Boeringer Mannheim (Germany). Distilled water for LC-MS was obtained by passing through the Milli-Q water system (Bedford, MA, USA).

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 a reversed-phase Zorbax Eclipsed SB-C18, 2.1x150 mm I.D., 5 μm, from Agilent Technologies (USA). The mobile phase was (A) a mixture of 0.2 *mM* ammonium acetate (pH 6.7) and (B) methanol with gradient elution. The gradient program was: 0-35 min: B=20-60%, flow rate: 0-20 min: 0.2 ml/min 20-35min: 0.3 ml/min. MS analysis was carried out at atmospheric pressure using an ESI source and data was acquired in positive mode. The optimized instrument setting were as follows: dry temp (350°C), capillary voltage (-4000 V), nebulizer (40 psi), dry gas (nitrogen, 9 l/min).

Sample preparation

- Administration

10 mg of mesocarb (Sydnocarb®, 2 tablets of 5 mg) were administered two healthy male volunteer as one single dose. Urine samples were collected at predose (blank sample) and up to 8 days after administration.

-Urine extraction

unhydrolyzed fraction: To 5 ml of urine samples was added 0.1 g of solid buffer ($NaHCO_3/K_2CO_3$, 2:1 mixture) to adjust the pH to 9.5. Then 100 mg of Na_2SO_4 was added and the mixture was extracted twice with 5 ml of diethyl ether. After shaken (2 min) and centrifugation (5 min, 3000 rpm), the organic layer was separated and taken to dryness at 60°C.

acidic hydrolysis: To 5 ml of urine samples 1 ml of 6 N HCl and 100 mg of cysteine were added, and mixture was heated at 100°C. After cooling, it was neutralized with 5 N NaOH and extracted twice with 5 ml of diethyl ether. Then the samples were prepare as for unhydrolyzed fraction.

enzyme hydrolyses (screening procedure for heavy volatile compounds): To 5 ml of urine samples a few drops of glacial acetic acid was added to adjust pH to 5-5.5. Then 1 ml of acetate buffer and 30 µl of β-glucuronidase are added prior to enzymatic hydrolyses. It takes 3 h at 57°C. After cooling pH was adjusted to 9.5 with a spoon of solid buffer ($Na_2CO_3/NaHCO_3$, 1:1 mixture). Vortex extraction was performed with 5 ml diethyl ether/isopropanol mixture (9:1). Approximately 500 mg of Na_2SO_4 was added during vortexing. After centrifugation organic layer was separated and taken to dryness. Isopraponol was removed in rotary evaporator.

The residue was redissolved in 50 µl of methanol and 1 µl of this solution was injected into the LC-MS Ion Trap system.

Results and discussion

The first step in the work involved the characterisation of the mass spectrum properties of the parent drug. The mesocarb solution (1 µl, 50 ng/ml) was injected. When the optimisation of the mass spectrometer parameters was completed, MS and MS-MS measurements were made. Fragmentation of protonated mesocarb in the ion trap leads to three product ions *m/z* 91, *m/z* 119 and *m/z* 177. The ions 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 may result of ring fragmentation. Visual examination and comparison of the LC-MS-MS ion chromatograms of blank urine, mesocarb standard and sample urine showed 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 were observed. A representative ion chromatogram of *m/z* 193, 177 and *m/z* 170 (for ISTD) of mesocarb standard, blank urine in comparison with a sample urine is shown in Fig. 1.

Metabolites of Mesocarb

The LC-ITMS method was applied to the analysis and confirmation testing of mesocarb and its metabolites in human urine. For investigation of the metabolism of mesocarb, different extraction procedures ("free" fraction, enzyme hydrolysis and acid hydrolysis) and their comparison were preferred. Analysis by LC-ITMS of extracts from "free" fraction urine (Fig.

1C) 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). The results have shown that besides the unchanged parent drug (**I**), the following seven metabolites could be identified: the two isomers of hydroxymesocarb (**II, III**), p-hydroxymesocarb (**IV**), two isomers of dihydroxymesocarb (**V, VI**) and two isomers of trihydroxymesocarb (**VII, VIII**). Most of this metabolites were detected in human urine for the first time. The postulated structural assignments for metabolites of mesocarb are presented in Fig. 2.

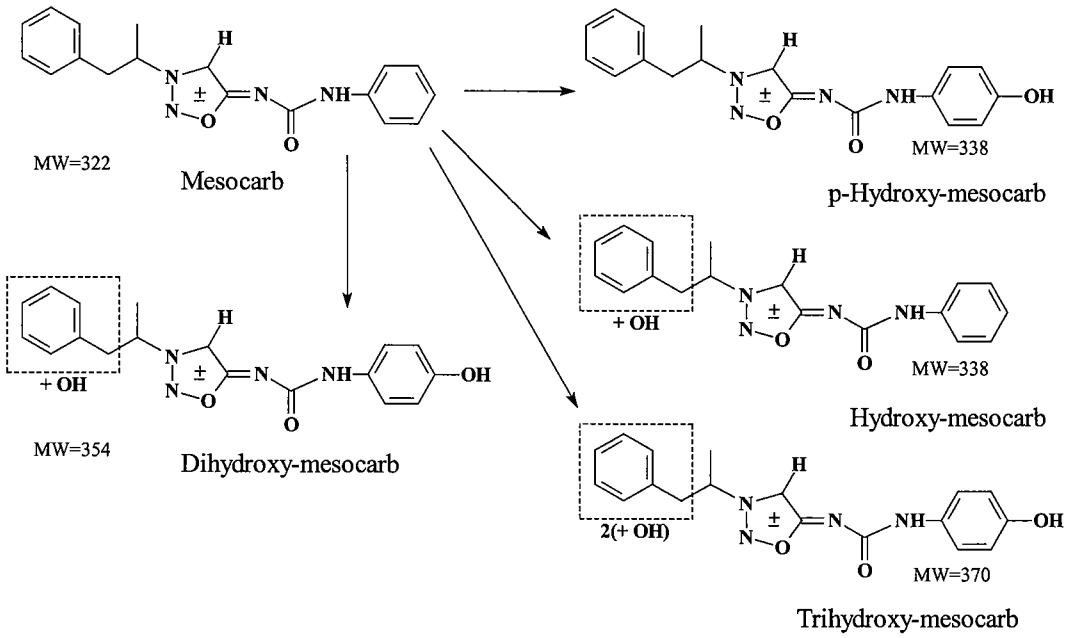

Fig. 2. Structural assignments made for mesocarb metabolites.

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 MS, and MS-MS spectrum (Table 1, Fig. 3).

If compared the molecular ions of the metabolites with those of the parent drug, revealed a net change of +16 Da, +32 Da, +48 Da in molecular ions of metabolites (Table 1). These results confirmed the presence of monohydroxylated-, dihydroxylated- and trihydroxylated- metabolites in human urine. On the basis of the identified metabolites the following metabolic pathways, could be postulated: single, double and triple aromatic hydroxylation of mesocarb (Fig. 2).

The unchanged mesocarb (**I**) was eluted at 29.9 min in the mesocarb positive urine. MS and MS-MS mass spectrum unchanged mesocarb showed in Fig. 3(**I**). The result of fragmentation was same with those of mesocarb standard, presented early.

Table 1. Product ions for mesocarb and its metabolites, protonated molecule $[M+H]^+$, changes in observed mass for the metabolites (ΔM) and fragmentation results from MS-MS spectrum when were obtained via fragmentation of molecular ions.

	MW	$[M+H]^+$	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
(**VIII**) Mesocarb-M (tri-OH-), isomer-2	370	371	+48	**371->221, 193**, 151, 135, 123

Monohydroxy metabolites: Metabolites (**II**) and (**III**) had a shorter retention time than the parent drug, 27.1 min and 26.6 min, respectively. MS-MS mass spectra of these metabolites showed in Fig. 3(**II, III**). Metabolites (**II**) and (**III**) were considered together because they have same mass spectrum. The molecular ion (*m/z* 339) of metabolites (**II**) and (**III**) was increased by 16 Da compared to that of the unchanged mesocarb. The ions at *m/z* 177 and *m/z* 119 were the same as the fragment ions of the parent drug. However the ions at *m/z* 135 and *m/z* 108 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 *m/z* 205 ions in the mass spectrum confirm it.

The metabolite (**IV**) was eluted at 25.1 min. The MS-MS spectrum of this metabolite is shown in Fig. 3(**IV**). The ions at *m/z* 119 and *m/z* 91 were the same as the fragment ions of the parent drug. These two 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 para-substitution at the ring of phenylcarbamoyl group according to the result of Polgar et al [3] and Ventura et al [11].

Dihydroxy metabolites: The metabolite (**V**) and (**VI**) were eluted at 22.5 min and 22.2 min, respectively. These metabolites have the same mass spectra Fig. 3(**V, VI**). The molecular ion (*m/z* 355) of metabolites (**V**) and (**VI**) was increased by 32 Da compared to that of the unchanged mesocarb. The ions at *m/z* 135 and *m/z* 193 were the same as the fragment ions of the metabolite (**IV**), p-hydroxymesocarb. The ion *m/z* 193 is resulted in ring fragmentation, but the ion at *m/z* 135 may be result of cleavage as carbamoyl part ($[CONHC_6H_5]^+$) of molecule as phenylisopropyl chain ($[CONHC_6H_5]^+$ and $[C_6H_5(CH_2)_2CH_2]^+$). Fragmentaion of molecular ions of these metabolites leads to following product ions *m/z* 108, *m/z* 135, *m/z* 193 and *m/z* 221. All product

ion of these metabolites were increased 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 dihydroxy metabolites of mesocarb. Comparison different extraction procedures showed that the dihydroxy metabolites of mesocarb excreted from human urine only as "free" dihydroxymesocarb (Table 2). The long-time **V, VI** dihydroxymesocarb metabolites of mesocarb have been detected from unhydrolysed fraction for the seventh day after administration of a single oral dose.

Trihydroxy metabolites: The metabolites (**VII**) and (**VIII**) were eluted at 17.8 min and 17.1 min, respectively. These metabolites have the same mass spectra Fig. 3(**VII, VIII**). The molecular ion of metabolites (**VII**) and (**VIII**) was increased by 48 Da compared to that of the unchanged mesocarb. The molecular ion of these metabolites is *m/z* 371. The characteristic ions are *m/z* 123, *m/z* 135, *m/z* 151, *m/z* 193 and *m/z* 221. The ions *m/z* 123 and *m/z* 151 of these metabolites were increased by 32 Da (91->123, 119->151) compared to that of the unchanged mesocarb. The ions *m/z* 135, *m/z* 193 and *m/z* 221 were same as the fragment ions of metabolites (**V**) and (**VI**), dihydroxy-mesocarb. Therefore, this indicates that the phenylcarbamoyl group of parent drug was hydroxylated and phenyl ring was hydroxylated twice. Hence the metabolites (**VII**) and (**VIII**) are trihydroxy metabolites of mesocarb. Comparison different extraction procedures showed that the metabolite (**VIII**) excreted from human urine as "free" trihydroxymesocarb (isomer-1) and the metabolite (**VII**) as "free" and sulfate conjugated trihydroxymesocarb (Table 2). We suppose that these metabolites are 2,4,4'- and 3,4,4'-trihydroxymesocarb.

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 2). Using LC-ESI/MS Ion Trap, the unchanged mesocarb could be detected for only 45-63 hours. The monohydroxylated metabolites (**II, III**) could be detected for up 108 hours. The para-hydroxymetabolite (**IV**) of mesocarb could be detected for up 150 hours. The dihydroxy-metabolites of mesocarb could be detected for up 190 hours. And the trihydroxymetabolites could be detected for only 45-75 hours.

para-Hydroxymesocarb (**IV**) was the most abundant metabolite on the first two days. From 3 to 5 days the excreted amount the para-hydroxymesocarb and the dihydroxymesocarb was 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.

Table 2. Comparison between different extraction procedures.

RT [min]		**"Free" fraction**	**Acidic fraction**	**Enzymatic fraction**
		Height [cnt], $x10^5$		
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	N.D.*
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	N.D.
17.1	**VIII**	3.2	3.5	N.D.

N.D.* not detected

Conclusions

For the first time, a LC-ESI/MS Ion Trap in the positive ion mode method has been developed for the analysis and confirmation testing of mesocarb and its metabolites in human urine. Seven various metabolites of mesocarb: mono-, di-, trihydroxymesocarb and parent drug was found in human urine after oral administration two tablet of Sydnocarb® (doses 10 mg). The hydroxymesocarb and the dihydroxymesocarb were identified as main metabolites. The comparison between different extraction procedures showed that the screening procedure based on acidic hydrolysis gave best responses for the mesocarb metabolites. Long time excreted metabolite (dihydroxymesocarb) has been detected from unhydrolysed fraction on seventh day after administration of a single oral dose (10 mg). 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 dihydroxy-mesocarb was very sensitive, making this a suitable metabolite for discriminating the ingestion of mesocarb longer than the parent drug or other metabolites in human urine.

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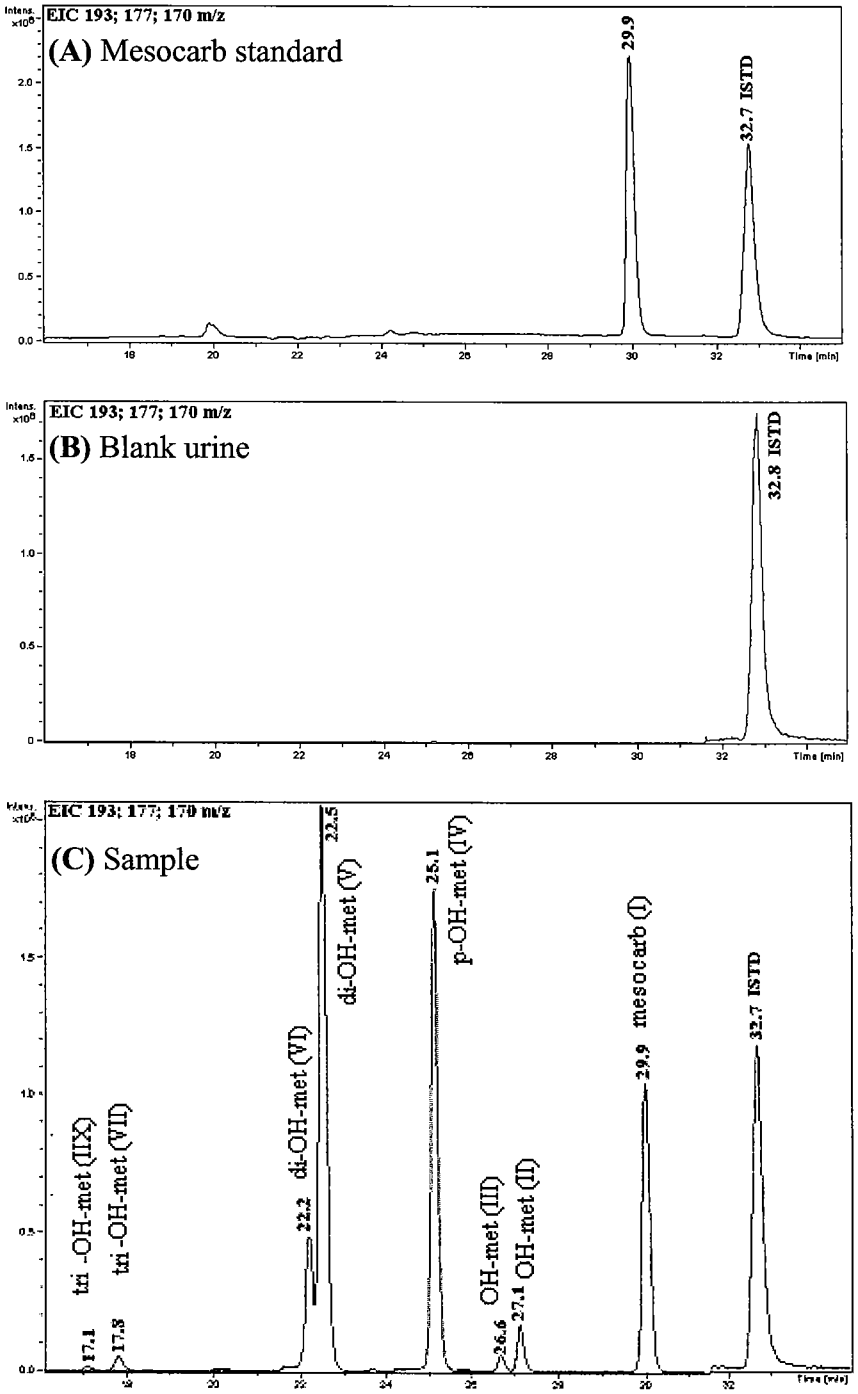


Fig. 1. Extracted-ion chromatograms of *m/z* 193, 177, 170 (*m/z* 170 is ISTD, "free" fraction): **A** - mesocarb standard (50 ng/ml), **B** - blank urine; **C** - 8 hours after administration of a single oral dose of mesocarb (10 mg).

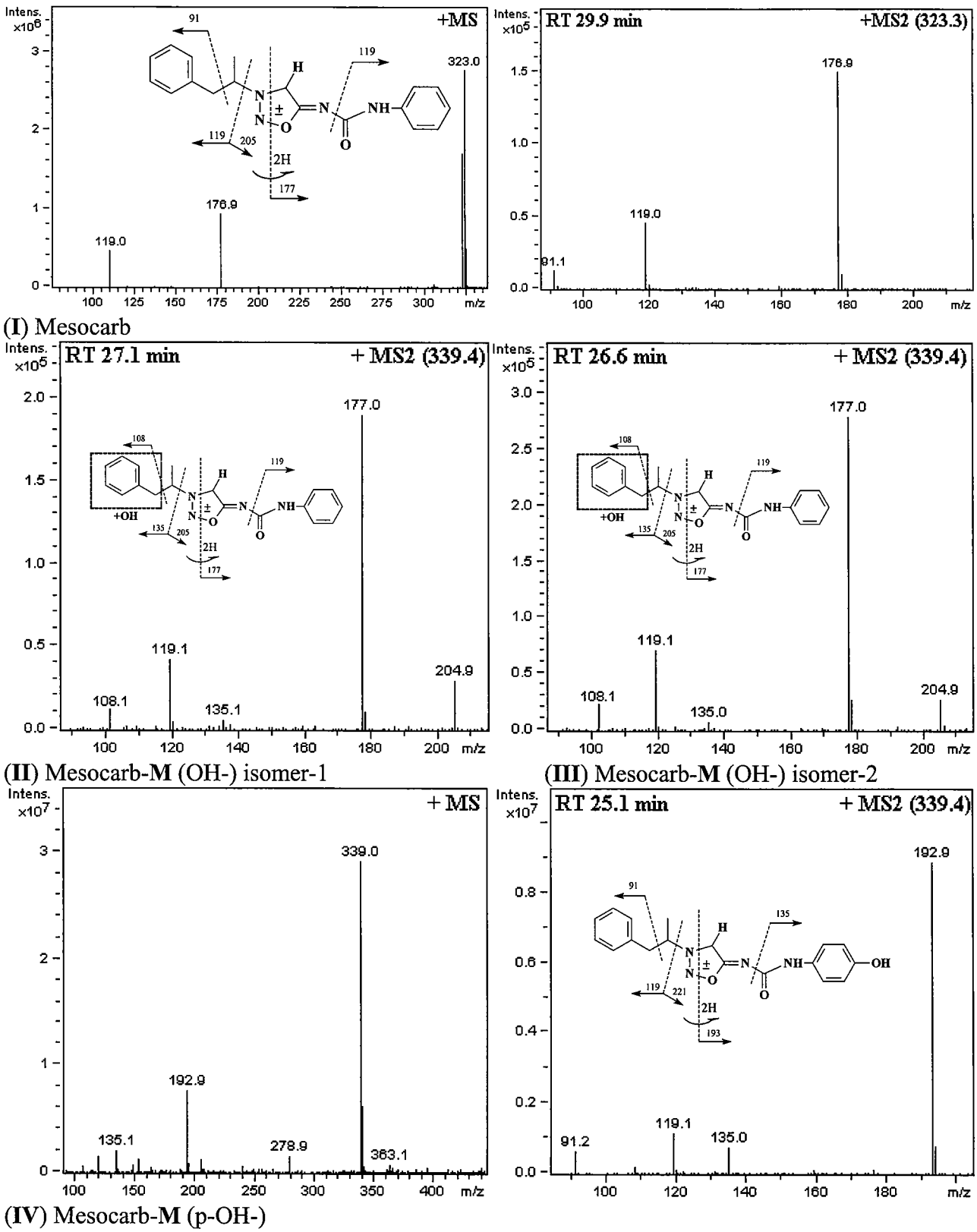
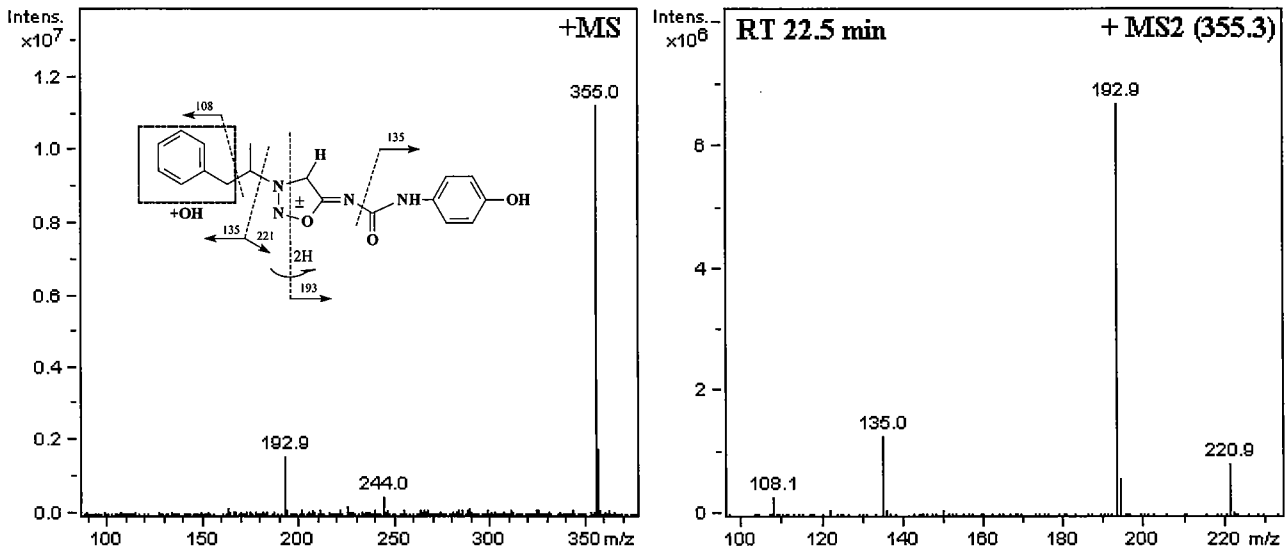
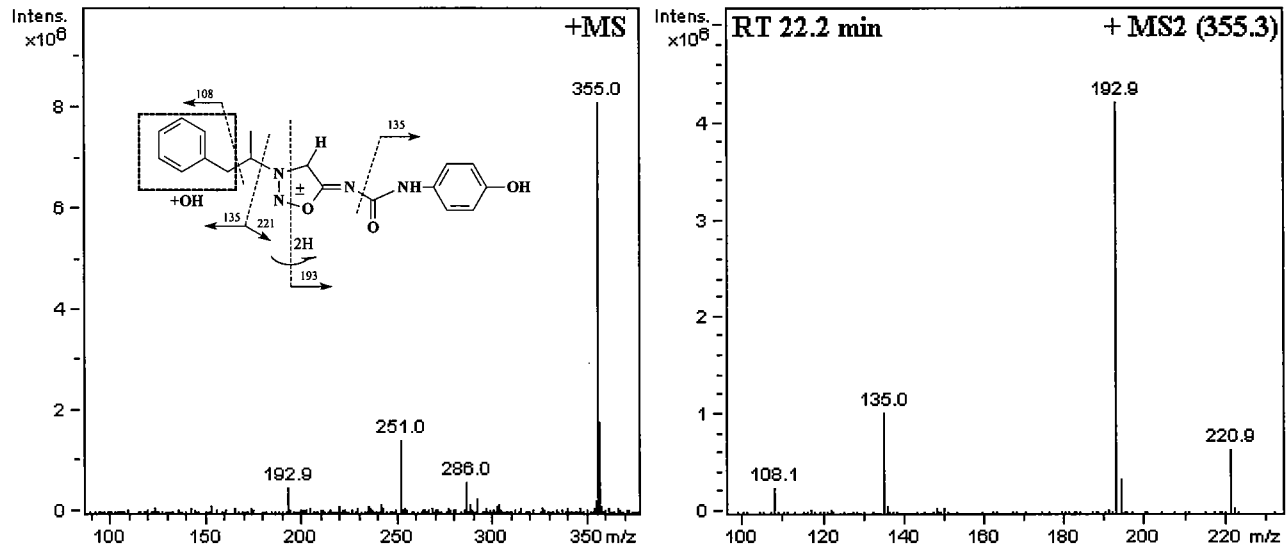


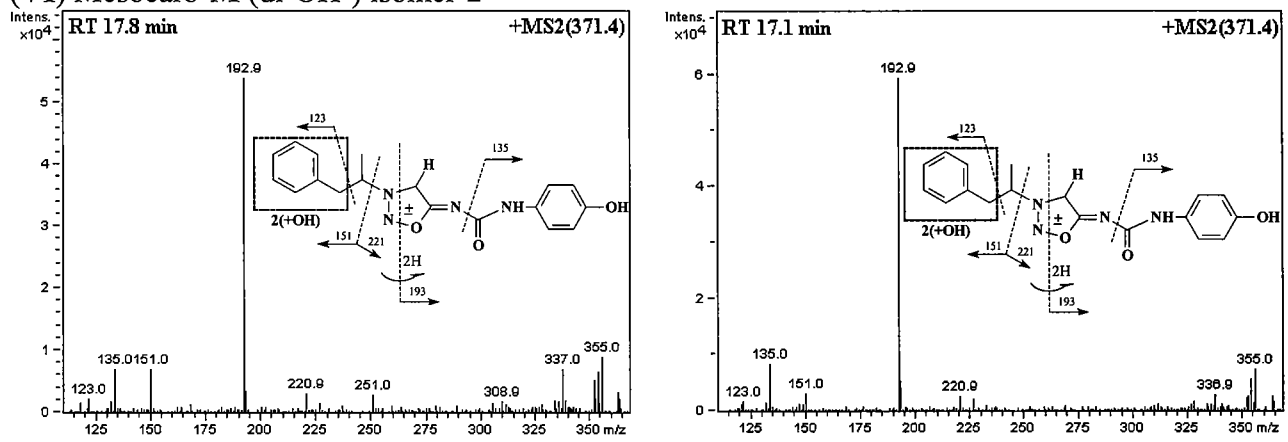
Fig. 3. Mass spectra (MS and MS-MS) proposed, predominant fragmentation patters, structures and retention times (RT) of mesocarb and Its metabolites.



(V) Mesocarb-**M** (di-OH-) isomer-1



(VI) Mesocarb-**M** (di-OH-) isomer-2



(VII) Mesocarb-**M** (tri-OH-) isomer-1

(VIII) Mesocarb-**M** (tri-OH-) isomer-2

Fig. 3. *(continued)*