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Validation of liquid chromatography – electrospray ionization ion trap mass spectrometry method for the determination of mesocarb in human plasma and urine

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Introduction

Mesocarb - (3-(1-methyl-2-phenylisopropyl)-N-(phenylcarbamoyl)sydnoneimine, Fig. 1.), also known as sydnocarb, is a stimulator of central nervous system [1] and is therefore included in the doping list of forbidden substances indicated by the Medical Commission of the International Olympic Committee [2]. Hence the methods to detect the presence of this compound or its metabolites in human urine are required.

In a previous paper [3], we have demonstrated the sensitive and specific method for the confirmation of mesocarb and its metabolites in human urine. Seven various metabolites of mesocarb: mono-, di-, three- and parent drug were detected in human urine after oral administration of 10 mg (Sydnocarb®), using the LC-ESI/MS Ion Trap system. Dihydroxymesocarb has been detected on tenth day after administration of a single oral dose. Therefore, in the case of drug abuse, the estimated detection time for mesocarb screening is 9-10 days after administration of the drug.

This paper, the metabolism of mesocarb by LC-ESI/MS Ion Trap in human plasma was investigated. The sensitive and specific method for the confirmation and quantitation of mesocarb and its metabolites in human plasma and urine was validated. The detailed analytical method validation has been based on the recommendations published by Musfeld et al. [4] and by Maurer et al. [5].

Experimental

Chemicals and reagents

Mesocarb and amphetamine were received from the Pharmacological Committee (Moscow, Russia) as pure substances. Sydnocarb® tablets, each containing 5 mg of mesocarb, were obtained from a Russian pharmacy (Manufacturer: Pharmacon®, St. Petesburg, Russia). Diphenylamine was used as an internal standard (ISTD), purchased from

Sigma (St. Louis, MO, USA). HPLC-grade methanol and acetonitrile was acquired from Merck (Darmstadt, Germany).

Instrumentation

Apparatus

All the experiments were carried out on a 1100 Series LC/MSD Trap "SL" system – Agilent Technologies (Palo Alto, CA, USA) equipped with an autosampler and an autoinjector. Chromatographic separations were performed using a Zorbax® SB-C18 (2.1 × 150 mm I.D., 80 Å, 5 μm) column connected to a guard column (cartridge 2.1 × 12.5 mm) filled with the same packing material. The column and the guard column were thermostatted at 30 °± 0.1 °C.

The mobile phase was a mixture of **A**, 0.2 *mM* ammonium acetate (pH 6.7), and **B**, methanol, in a gradient elution mode. The starting mobile phase was 80 % **A** and 20 % **B**, and the linear gradient was run over 20 min to a proportion of 40 % **A** and 60 % **B**. The flow rate was 0.2 ml/min for 20 min, and then flow rate was 0.3 ml/min.

LC-MS procedures

The Agilent Technologies "SL" ion trap mass spectrometer (LC/MSD Ion Trap "SL") with a atmospheric pressure electrospray ionization (AP-ESI) was used for quantification in a positive ionization mode. Unit mass resolution was established and maintained at ± 0.3 *m/z* (normal mass range mode). Nitrogen gas was generated from a nitrogen tank (Jun-Air, Denmark) with output pressure of 80 p.s.i. and ion source (nebulizer) inlet pressure at 40 p.s.i. A drying gas was heated to 350 °C at a flow 9 l/min. The capillary voltage was – 4000 V. The skim trap drive and capillary exit were 46.4 and 104.0 V, respectively. Compound stability was 90 %. The ion accumulation time was 300 ms with a scan range of 85 to 450 *m/z*.

Stock solutions and calibration standards

Concentrated stock solutions of mesocarb, amphetamine, and diphenylamine were prepared in methanol with concentrations of 500 μg ml^{-1}, 200 μg ml^{-1} and 2 mg ml^{-1}, respectively. Standard solutions containing an ISTD (diphenylamine, 40 μg ml^{-1}) and mesocarb in different concentrations (1, 5, 10, 50 and 100 ng ml^{-1}) were prepared in methanol by a serial dilution of the stock solution and stored at -18 °C before use and thawed in the day of the analysis.

Plasma calibration standards were prepared by adding an appropriate volume of a 100 ng ml^{-1} mesocarb working solution corresponding to the concentration of 0, 5.0, 17.0, 33.0, 51.0, and 67.0 ng ml^{-1}, into a blank plasma.

Urine calibration standards were prepared accordingly by adding an appropriate volume of a mesocarb working solution into the blank urine from a healthy donor (male, 25 years, 80 kg), corresponding to the concentration 0, 0.1, 0.5, 2.0, 3.0 and 5.0 ng ml^{-1}.

Sample preparation

Administration and collection

Four healthy volunteers gave their informed consent to participate in the study. Blank urine was collected before the administration of a single oral dose of 10 mg of mesocarb (Sydnocarb®, 2 tablets of 5 mg) to four healthy volunteers. Urine samples were collected at 3-4 h interval for the first two days, and two times a day up to 10 days after the administration, and frozen at – 18 °C until analysis.

Human blood samples were collected from a healthy Caucasian male (25 age) before and after the administration of a single oral dose of 10 mg of mesocarb (Sydnocarb®). Blood samples 10 ml (blank – 25 ml) were withdrawn through the brachial vein using an indwelling catheter in: 0, 33, 71, 96, 138, 216, 278 and 336 min. Next, blood samples were transferred to tubes with heparin and centrifuged at 4000 rpm for 20 min to obtain plasma. Plasma was stored at – 36 °C before use.

Plasma

Frozen plasma samples were thawed at room temperature and mixed by inversion. An aliquots of plasma samples 150 µl were mixed with 10 µl of an aqueous solution of diphenylamine (200 µg ml^{-1}) as an internal standard (ISTD). After adding 2 ml of acetonitrile, to precipitate proteins, samples were vortex-mixed for 2 min and centrifugated at 4000 rpm for 15 min at room temperature. The resulting clear supernatant was transferred into a glass tube, and the organic phase evaporated to dryness on a rotary evaporator. The residue was dissolved in 50 µl of methanol and introduced into a HPLC vial. A volume of 1 µl of the solution was injected into the LC-MS Ion Trap system for analysis.

Urine

The urine samples were prepared by procedure which was wrote in [3].

Results and discussion

As previously described [3], a sensitive and selective method for investigation mesocarb of metabolism in human urine was proposed. Some new metabolites of mesocarb such as two isomers of hydroxymesocarb, two isomers of dihydroxymesocarb, and two isomers of threehydroxymesocarb were found in human urine.

When a wide range of samples of the human urine was analysed, three more new metabolites, hydroxymesocarb (**III**) and dihydroxymesocarb (**VII**) and amphetamine (**A**) have

been detected. Representative chromatograms of mesocarb and its metabolites in human urine collected for 1-40 h after the administration of mesocarb are shown in Fig. 1. The retention time of mesocarb was 29.9 min with a total run time of 35 min, a number of metabolites was detected at retention times shorter than those of the parent drug (i.e., most polar compounds). The typical chromatographic profiles of the human plasma from the healthy volunteer until and after the administration of mesocarb are shown in Fig. 2.

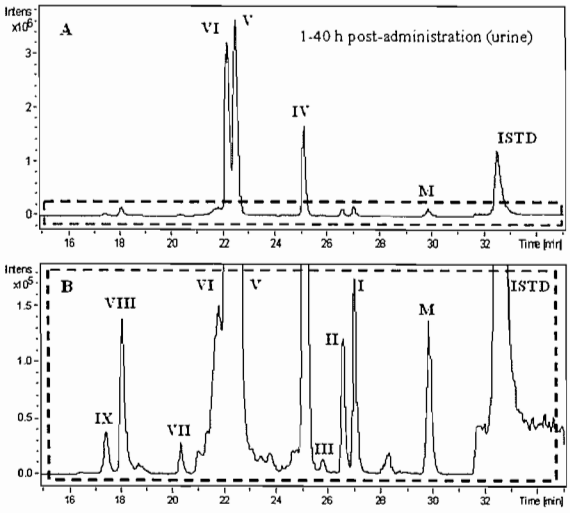

Fig. 1. Extracted-ion MS-MS chromatograms (**A**) of *m/z* 193, 177, 170 (*m/z* 170 is ISTD) of mesocarb (**M**) and its metabolites (**I-IX**) in human urine obtained at 1 - 40 h after oral administration of 10 mg mesocarb (**B** – fragment).

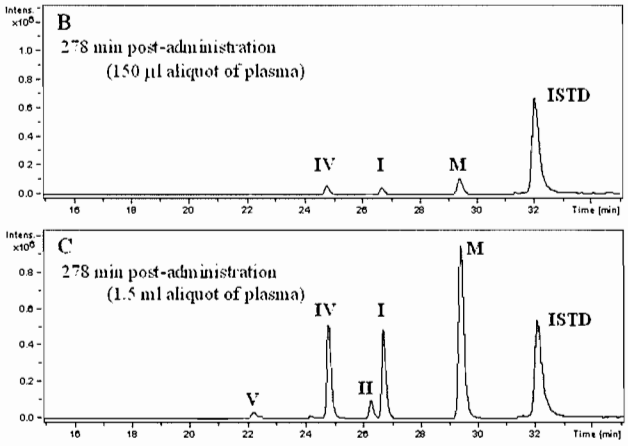

Fig. 2. Extracted-ion MS-MS chromatograms of *m/z* 193, 177, 170 (*m/z* 170 is ISTD) of mesocarb (**M**) and its metabolites in human plasma: **B** – 150 µl aliquot of plasma and **C** – 1.5 ml aliquot of plasma obtained at 336 min after administration.

The results show that apart from the unchanged parent drug (mesocarb, **M**), the following ten metabolites were detected: amphetamine (**A**), three isomers of hydroxymesocarb (**I - III**), p-hydroxymesocarb (**IV**), three isomers of dihydroxymesocarb (**V - VII**) and two isomers of trihydroxymesocarb (**VIII, IX**). Most of these metabolites were detected in human urine for the first time. A summary of retention times, 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 spectra are given in Table 1. The underlined fragmentation ions were used for quantitation: *m/z* 177 for M, I-III; *m/z* 193 for IV-IX, *m/z* 119 for A and *m/z* 170 for ISTD. The structures of metabolites could not be determined conclusively by mass spectrometry alone, because the metabolites standard is not available, but partial identification was made. The postulated structural assignments for metabolites of mesocarb are presented in Fig. 3.

LOD`s were obtained by decreasing the concentration of mesocarb in the plasma and urine samples. For standard aliquot of plasma samples - 150 µl the limit of detection of

mesocarb was found to be 0.1 ng ml^{-1}. Mesocarb LOD was estimated to be 0.012 and 0.002 ng ml^{-1} for injection of 1 and 12 µl of the plasma extract, when the aliquot 1.5 ml plasma was used (Table 2).

Table 1. Retention times, product ions, protonated molecule $[M+H]^+$, changes in observed mass for the metabolites (ΔM) and fragmentation results from MS-MS spectra. The underlined fragmentation ions were used for quantitation

Substance	Urine	Plasma	Rt, min	MW	$[M+H]^+$	ΔM	MS-MS
Mesocarb (M)	+	+	29.9	322	323		323->**177**, 119, 91
Hydroxymesocarb							
I	+	+	27.1	338	339	+16	339->205, **177**, 135, 119, 108
II	+	+	26.6	338	339	+16	339->205, **177**, 135, 119, 108
III	+	-[a]	25.6	338	339	+16	339->**177**, 135, 107
IV	+	+	25.1	338	339	+16	339->**193**, 135, 119, 91
Dihydroxymesocarb							
V	+	+	22.5	354	355	+32	355->221, **193**, 135, 108
VI	+	-[a]	22.2	354	355	+32	355->221, **193**, 135, 108
VII	+	-[a]	20.9	354	355	+32	355->135, **193**, 107
Threehydroxymesocarb							
VIII	+	-[a]	17.8	370	371	+48	371->221, **193**, 151, 135, 123
IX	+	-[a]	17.1	370	371	+48	371->221, **193**, 151, 135, 123
Amphetamine (A)	+	-[a]	-[b]	135	136	-187	136->**119**, 91

-[a] - not detected in plasma (LOD – 0.002 ng ml^{-1}); -[b] - flow injection analysis (LOD – 0.7 ng ml^{-1})

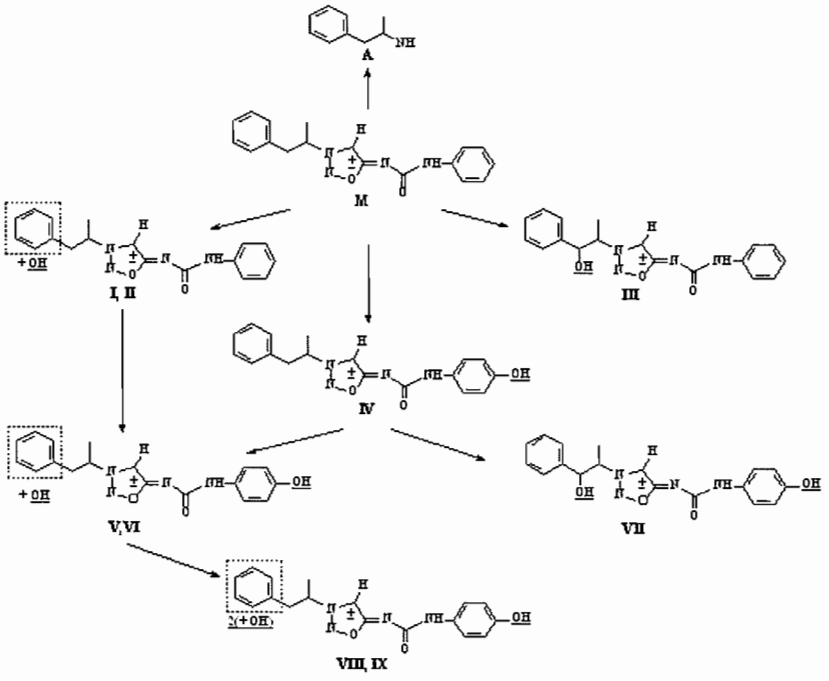

Fig. 3. The metabolism pathways of mesocarb.

The mean recoveries from plasma were 49.2 and 57.4% for mesocarb concentrations of 33.0 and 66.0 ng ml^{-1}, respectively, whereas the recoveries from human urine were 76.9 and 81.4% for concentrations of 1 and 2 ng ml^{-1}, respectively. Calibration curves (using an internal standard method) are linear ($r^2 > 0.9969$) for concentrations 0.6 to 67 ng ml^{-1} and from 0.05 to 5 ng ml^{-1} in plasma and urine, respectively. Both intra- and inter-assay precision

of plasma control samples at 3, 40 and 55 ng ml^{-1} were lower than 6.2 % and concentrations did not deviate for more than –3.4 to +7.3 % from their nominal values. In urine intra- and inter-assay precision of control samples at 0.08, 1.5 and 3.0 ng ml^{-1} is lower than 14.1 %, with concentrations not deviating for more than –11.3 to 13.7 % from their nominal values.

Conclusions

The metabolism of mesocarb by LC-ESI/MS Ion Trap in human was investigated. After the administration of a single oral dose 10 mg of mesocarb (Sydnocarb®, 2 tablets of 5 mg) ten metabolites and the parent drug were detected in human urine, and only four in human plasma. Seven of this metabolites have been detected for the first time.

A sensitive and specific method for the confirmation and quantitation of mesocarb and its metabolites in human plasma and urine was validated. The applicability of the method was demonstrated by analyzing mesocarb and its metabolites in plasma and urine from healthy volunteers. It was shown that in the case of dope analysis, the estimated detection time for mesocarb (long-life dihydroxymesocarb metabolites of mesocarb) was about 10-11 days.

References

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Table 2. Linearity results, detection and quantitation limits for mesocarb in plasma and urine

Sample matrix	Sample aliquot, µl	Inject volume, µl	K[a]	LOD[b], ng ml^{-1}	LOQ[c], ng ml^{-1}	Linearity range, ng ml^{-1}	Calibration curve[d]	r^2
Methanol	-	1	1	0.05	-	0.4 – 2000	y = 0.0641x + 0.0197	0.9992
Plasma	150	1	3	0.1	0.6	0.6 – 67	y = 5.4955x + 0.0014	0.9973
	1500	1	30	0.012	-	-	-	-
	1500	12	360	0.002	-	-	-	-
Urine	5000	1	100	0.001	0.05	0.05 – 5	y = 394.07x – 0.0022	0.9969
	5000	12	1200	0.0001	-	-	-	-

[a]K, factor of concentration = inject volume*(sample aliquot/extract volume)
[b]LOD, limit of detection
[c]LOQ, limit of quantitation
[d]$y = S_{Mes}/S_{ISTD}$; x – concentration ng ml^{-1} of mesocarb in the sample