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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Abstract

Mesocarb metabolism in humans is the target of this investigation. A high-performance liquid chromatographic (LC) method with electrospray ionization (ESI)–ion trap mass spectrometric (MS) detection ion trap "SL" for the simultaneous determination of mesocarb and its metabolites in plasma and urine is developed and validated. Ten metabolites and the parent drug are detected in human urine, and only four in human plasma, after the administration of a single oral dose of 10 mg of mesocarb (Sydnocarb, two 5-mg tablets). Seven of this metabolites have been found for the first time. The confirmation of the results and identification of all the metabolites except amphetamine is performed by LC–MS, LC–MS–MS, and LC–MS^3. In the case of doping analysis, the reliable detection time for mesocarb (long-life dihydroxymesocarb metabolites of mesocarb) is approximately 10–11 days after the administration of the drug, which is a significant increase over the existing data. The detection of amphetamine in plasma and urine is made using simple flow-injection analysis without a chromatographic separation. The addition-calibration method is used with plasma and urine. The mean recoveries from plasma are 49.2% and 57.4% for mesocarb concentrations of 33.0 and 66.0 ng/mL, respectively, whereas the recoveries from human urine are 76.9% and 81.4% for concentrations of 1 and 2 ng/mL, respectively. Calibration curves (using an internal standard method) are linear (r^2 > 0.9969) for concentrations 0.6 to 67 ng/mL and from 0.05 to 5 ng/mL in plasma and urine, respectively. Both intra- and interassay precision of plasma control samples at 3, 40, and 55 ng/mL are lower than 6.2%, and the concentrations do not deviate for more than –3.4% to 7.3% from their nominal values. In urine, intra- and interassay precision of control samples at 0.08, 1.5, and 3.0 ng/mL is lower than 14.1%, with concentrations not deviating for more than –11.3% to 13.7% from their nominal values. The plasma disappearance curve of the parent drug is obtained. The major pharmacokinetic parameters are calculated.

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Introduction

Mesocarb (*N*-phenylcarbamoyl-3-(β-phenylisopropyl)sydnoneimine (Figure 1), also known as sydnocarb, is a stimulator of the central nervous system (1) and therefore is included in the doping list of forbidden substances indicated by the Medical Commission of the International Olympic Committee (2). Therefore, the methods for the detection the presence of this compound or its metabolites in human urine are required.

Nowadays, mesocarb is a basic stimulant used in the Russian medical practice. In comparison with a phenamin (Acetedol), mesocarb is considerably less toxic and does not render expressed peripheral influence. A stimulating action develops gradually, any sharp initial activated effects are absent. The stimulating effect is not accompanied by a tachycardia, euphoria, and a sharp increase in the arterial pressure.

Today, several different biological matrices are used in clinical toxicology, forensic toxicology, and doping analysis in humans and animals for the detection of drugs and their metabolites (i.e., xenobiotics). Aside from urine and blood (whole blood, plasma, and serum) as classical matrices, hair, sweat, and saliva have become important.

Urine has traditionally been the sample of choice for the screening and identification of unknown drugs and metabolites because the concentration of drugs in urine is relatively high. Another advantage is that collecting urine does not require any special equipment, immediate centrifugation, or freezing. How-

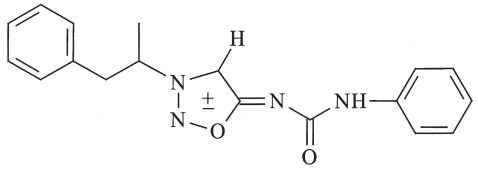

Figure 1. Chemical structure of mesocarb.

ever, clinical, forensic, and doping problems can be solved more efficiently when quantitative determination in blood can be achieved in addition to the analytical results of urine samples (3).

On the other hand, blood is the matrix that is relatively homogeneous because physiological parameters vary only within narrow limits. Another great advantage of blood as a test media is that drugs can be detected immediately after intake and prior to metabolism and elimination.

It is widely known that all drugs and new drug candidates are investigated in metabolism and pharmacokinetics studies. Metabolic studies of mesocarb in rat urine by gas chromatography (GC)–mass spectrometry (MS) were first published by Polgar et al. (4). Only traces of the unchanged drug were found in the rat urine. Three different metabolites such as hydroxymesocarb, dihydroxymesocarb, and amphetamine were established as the metabolites in rat urine.

Many antidoping laboratories performed intensive investigation on the metabolism of mesocarb in human urine. Different combined methods based on chromatographic and MS techniques have been applied to the analysis of mesocarb and its metabolites in human urine (5–13). A two-step analysis, with a nd without hydrolysis, has to be carried out in order to indirectly determine the concentration of conjugated metabolites in the sample. GC–MS (without derivatization) (5,6), GC–MS (as its *N*-fluoroacyl derivative) (7), GC–MS (as its *N*-trifluoroacetil derivative) (8,9), LC–thermospray ionization (TS)–MS (10), LC–particle beam ionization (PB)–MS (5,11,12), and LC–ESI-MS (13) can determine the parent compound and sulfate conjugated *p*-hydroxymesocarb only. All of the research reported that *p*-hydroxymesocarb can be detected in human urine in 48–72 h after an intake of 10 mg of mesocarb. Also, pharmacokinetics parameters of the mesocarb have been obtained (1).

In a previous paper (14), we have demonstrated a sensitive and specific method for the confirmation of mesocarb and its metabolites in human urine. Seven various metabolites of mesocarb: mono-, di-, trihydroxylated mesocarb, and the parent drug were detected in human urine after an oral administration of 10 mg (Sydnocarb) using an LC–ESI-MS ion trap system. Dihydroxymesocarb has been detected on 10th day after the 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 the administration of the drug.

In this paper, the metabolism of mesocarb by an LC–ESI-MS ion trap method in human plasma was investigated. A 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. (15) and by Maurer et al. (16).

The validated method has been applied to the determination of mesocarb in human plasma and urine. The concentrations of mesocarb in plasma and urine samples (collected simultaneously) were compared. The plasma disappearance curve of the parent drug was obtained. The major pharmacokinetic parameters were calculated.

Experimental

Chemicals and reagents

Mesocarb (*N*-phenylcarbamoyl-3-(β-phenylisopropyl)sydnoneimine) 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 (Pharmacon, St. Petesburg, Russia). Diphenylamine was used as an internal standard (ISTD) and was purchased from Sigma (St. Louis, MO). High-performance LC-grade methanol and acetonitrile was acquired from Merck (Darmstadt, Germany). Diethyl ether pro narcosi grade was obtained from Moscow Expzavod (Moscow, Russia). Ammonium acetate was purchased from Sigma. Potassium carbonate (K_2CO_3), sodium hydrocarbonate ($NaHCO_3$) and anhydrous sodium sulfate (Na_2SO_4) of high-purity grade were obtained from Uralskii Zavod Khimreaktivov (Pyshma, Russia). Water was purified using a Milli-Q Elix system (Millipore, Milford, MA).

Instrumentation

Apparatus

All the experiments were carried out on a 1100 Series LC–MSD Trap "SL" system from Agilent Technologies (Palo Alto, CA) 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°C ± 0.1°C.

The mobile phase was a mixture of (A) 0.2mM 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, then increased to 0.3 mL/min. The total time of analysis was 34 min.

A 1 (12)-µL sample volume was injected. An Agilent LC 3D ChemStation was used for system control, data acquisition, and postrun processing.

LC–MS procedures

An Agilent Technologies "SL" ion trap MS (LC/MSD Ion Trap SL) with an atmospheric pressure (AP)-ESI was used for the quantitation in a positive ionization mode. This study employed multiple MS, which involved repeated trapping and fragmentation of ions. 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, Tweksbury, MA) with an output pressure of 80 psi and ion source (nebulizer) inlet pressure was 40 psi. A drying gas was heated to 350°C at a flow of 9 L/min. The capillary voltage was –4000 V. The skim trap drive and capillary exit were 46.4 and 104.0 V, respectively. The ion accumulation time was 300 ms with a scan range from 85 to 450 *m/z*.

Stock solutions and calibration standards

Concentrated stock solutions of mesocarb, amphetamine, and diphenilamine were prepared in methanol with concentrations of 500, 200, and 2 µg/mL, respectively. The stock meso-

carb solution was diluted with methanol to achieve working solutions with concentrations of 100 and 1 ng/mL, which were used for the preparation of calibration and control samples of plasma and urine. The amphetamine calibration solutions of 1, 10, and 200 ng/mL in urine were prepared by adding an appropriate amount of stock and 10-µg/mL solutions. A 2-mg/mL diphenilamine solution was diluted with methanole down to 200 µg/mL for the preparation of the ISTD solution used for analysis.

Standard solutions containing an ISTD (diphenilamine, 40 µg/mL) and mesocarb in different concentrations (1, 5, 10, 50, and 100 ng/mL) 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 mesocarb working solution corresponding to the concentration of 0, 5.0, 17.0, 33.0, 51.0, and 67.0 ng/mL, 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.

Plasma and urine calibration samples were immediately processed as described below and analyzed or stored at –18°C before use.

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 4 healthy volunteers (males, 25 years, 80 kg, 61 years, 73 kg; females, 21 years, 55 kg, 32 years, 59 kg). Urine samples were collected at 3–4 h interval for the first 2 days, and 2 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.

Blank urine and urine samples were also collected from the same volunteer. Urine and plasma samples were collected in 71 and 278 min after an oral administration of 10 mg of mesocarb, simultaneously. It made it possible to compare the concentrations of mesocarb and its metabolites in blood plasma and urine of the same human.

Plasma

Frozen plasma samples were thawed at room temperature and mixed by inversion. An aliquots of plasma samples 150 µL were mixed in a 13- × 52-mm polypropylene tube (Centricon, Millipore) with 10 µL of an aqueous solution of diphenylamine (200 µg/mL) as an ISTD. After adding 2 mL of acetonitrile to precipitate proteins, samples were vortex-mixed for 2 min and centrifugated at 4000 rpm (Univervsal 32 R, Hettich, centrifuge) 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 an HPLC vial. A volume of 1 µL of the solution was injected into the LC–MS ion trap system for analysis.

To decrease the limits of detection of mesocarb and its metabolites found in urine, the aliquots 1.5 mL of plasma samples were used. The proteins in plasma samples were precipitated with 15 mL of acetonitrile. Finally, the residue was dissolved in 50 µL of methanol. The volumes of 1 and 12 µL were injected into the LC–MS system.

Urine

Ten microliters of diphenilamine (ISTD, 200 µg/mL) was added to 5 mL of urine samples and approximately a 100-mg amount of a solid buffer ($NaHCO_3$–K_2CO_3, 2:1 mixture) was added to adjust the pH to 9.5. Next, 100 mg of anhydrous sodium sulfate was added, and the mixture was extracted twice with 5 mL of diethyl ether. After shaking (2 min) and centrifugation (5 min, 3000 rpm), the organic layer was separated and taken to dryness at 60°C.

After cooling, the residue was dissolved with 50 µL of methanol and introduced into an HPLC vial, and 1 (12) µL of this solution was injected into the LC–MS ion trap.

The detection of amphetamine in plasma and urine was made using simple flow injection analysis without a chromatographic separation. The 10-µL aliquots of plasma and urine sample extracts prepared as previously mentioned and were directly injected in the ion trap MS by LC autosampler.

Method validation

The detailed analytical method validation was based on the criteria established by Musfeld et al. (15) and by Maurer et al. (16). It is necessary to note that the flow injection method for analysis of the amphetamine was not validated.

Selectivity

Selectivity is the ability of a method to determine accurately and specifically the analyte of interest in the presence of other components in a sample matrix under the stated conditions of the test (17). To demonstrate the selectivity of the analytical procedure, four different blank plasma and eight different blank urine samples obtained from various healthy volunteers were analyzed for the peaks interfering with the detection of the analytes or internal standard.

Limits of detection

The limit of detection (LOD) of an individual analytical procedure is the lowest concentration of analyte in a sample that can be reliably distinguished from the background noise. The LOD [signal-to-noise ratio (s/n) higher than 3:1] of mesocarb in plasma and urine was determined by analyzing a set of plasma and urine blank samples, which were prepared by adding an appropriate volume of a 1-ng/mL mesocarb solution into the blank plasma aliquot.

Limits of quantitation

The quantitation limit (LOQ) is the lowest level of analyte that can be accurately and precisely measured. The LOQ is determined as a sample of plasma or urine providing s/n higher than 20:1 and measurements with a precision and accuracy within the recommended ± 20% from the nominal values; it was found by (*i*) analyzing a set of plasma and urine samples with a known concentrations of mesocarb or (*ii*) adding a mesocarb standard to a blank.

Linearity of calibration curves

The linearity range of a calibration is a concentration range of mesocarb lying between the LOQ and the point where a plot of response versus concentration starts deviating from a straight line. For assay methods, this study is generally performed by preparing standard solutions for five concentration levels, from 50% to 150% of the target analyte concentration (18).

Three calibration curves using methanol, plasma, and urine as matrices were prepared. In the presence of a mesocarb-free biological matrix, quantitation of mesocarb was performed using the addition-calibration method. Calibration curves for plasma and urine were obtained by unweighted least-squares linear regression analysis of the peak-area ratio (mesocarb–ISTD) versus the amount of mesocarb added to each standard sample.

Calibration standards with concentrations of 5.0, 17.0, 33.0, 51.0, and 67.0 ng/mL of mesocarb in plasma (methanol, 1, 5, 10, 50, and 100, ng/mL and urine, 0.1, 0.5, 2.0, 3.0, and 5.0 ng/mL) were prepared and analyzed in duplicate.

Repeatability

Repeatability is related to a single homogeneous sample and is measured as a relative standard deviation (RSD%) for this sample when developing a new method. Control plasma samples in the low (3.0 ng/mL, low P) and high concentration ranges (55.0 ng/mL, high P) of mesocarb and control urine samples in the low (0.2 ng/mL, low U) and high concentration ranges (4.0 ng/mL, high U) were prepared according to the previously mentioned method using blank plasma and urine. Each sample was injected five times within a single sequence and during the course of five consecutive sequences, alternately (sequence order: low P, high P, low P, high P, low P, high P, low P, high P, low P, high P, and similar for control urine samples).

Precision and accuracy

The control samples were used for the determination of the precision and accuracy of the method. The precision was calculated as the coefficient of variation (CV%) within a single run (intra-assay) and between different runs (interassay). Accuracy was determined as the percentage of deviation between nominal and measured concentrations obtained from the calibration curves.

Three control samples with low, medium, and high levels of mesocarb (3.0, 40.0, and 55.0 ng/mL and 0.08, 1.5, and 3.0 ng/mL for plasma and urine, respectively) were analyzed six times within the same run (intra-day) and on six times (9 for urine) in 2 days (interdays). It should be noted that a set of control samples was obtained from different aliquots of the spiked blank plasma (urine) under the same operation conditions.

Extraction recovery

Extraction recovery is a measure of extraction efficiency of the analyte from the sample matrix. It is expressed as the ratio of the response obtained when the analyte is submitted to the extraction procedure to that measured when it is determined without an extraction step (17).

A 50- and 100-µL portion of the methanol solution of mesocarb (100 ng/mL) and 10 µL of ISTD (200 µg/mL) were added to 150 µL of blank plasma and 5 mL of blank urine for obtaining the spiked samples. Next, the spiked samples (33.0 and 66.0 ng/mL, and 1.0 and 2.0 ng/mL of mesocarb for plasma and urine, respectively) were prepared according to the standard sample preparation procedures described previously (Plasma and Urine subsections).

For comparison (control samples), 50 and 100 µL of the mentioned solution of mesocarb and 10 µL of the ISTD methanol solution were mixed and evaporated carefully at room temperature. The residue was dissolved in 50 µL of methanol and introduced into an HPLC vial. A 1-µL portion of the control solution was injected into the LC–MS ion trap system for analysis.

The extraction recovery of mesocarb was studied by the analysis of four replicates of control and spiked samples. Extraction recovery for each concentration was calculated by comparing the peak areas of spiked plasma and urine samples with control samples.

Stability studies

It is often essential for the solutions to be stable enough to allow delays such as instrument breakdowns or overnight and weekend analyses using autosamplers or for routine testing, in which many samples are prepared and analyzed each day. At this point, the limits of stability were tested.

The stability of mesocarb and its metabolites in biological samples and the samples prepared for analysis was assessed by subjecting control, calibration standard solutions, and extracts to various storage conditions: at room temperature for 8, 24, and 48 h, and kept frozen at –36°C.

Quantitation of mesocarb was determined by comparing them with freshly prepared standards.

Application of the method

The proposed analytical method was used in the doping control analysis for the determination of mesocarb and its metabolites in human urine samples. The determination of the mesocarb and its metabolites in human plasma and urine samples after the administration of the mesocarb was used to study the metabolism. The plasma disappearance curve of the parent drug was obtained. The major pharmacokinetic parameters were calculated.

Results and Discussion

Mesocarb and its metabolites

Urine

As previously described (14), 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 analyzed, three more new metabolites—hydroxymesocarb (III), 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 (female, 21 years, 55 kg) are shown in Figures 2 and 3. 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).

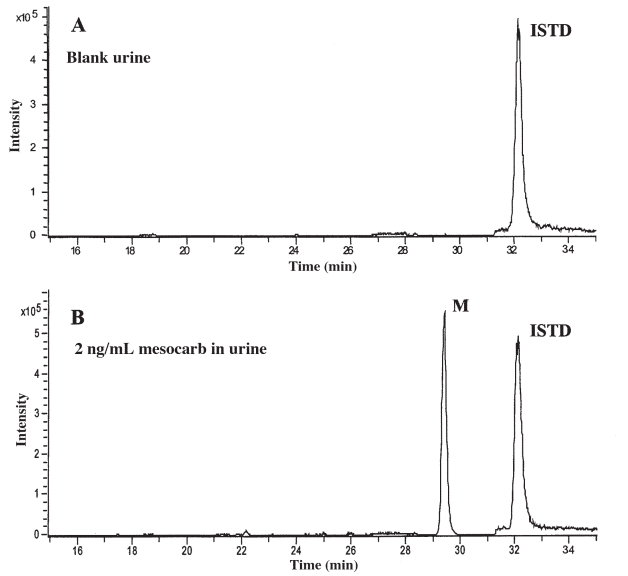

Figure 2. Extracted-ion MS–MS chromatograms of *m/z* 193, 177, and 170 (*m/z* 170 is ISTD) of mesocarb in urine: (A) blank and (B) 2 ng/mL mesocarb (M). The retention time of mesocarb is 29.9 min.

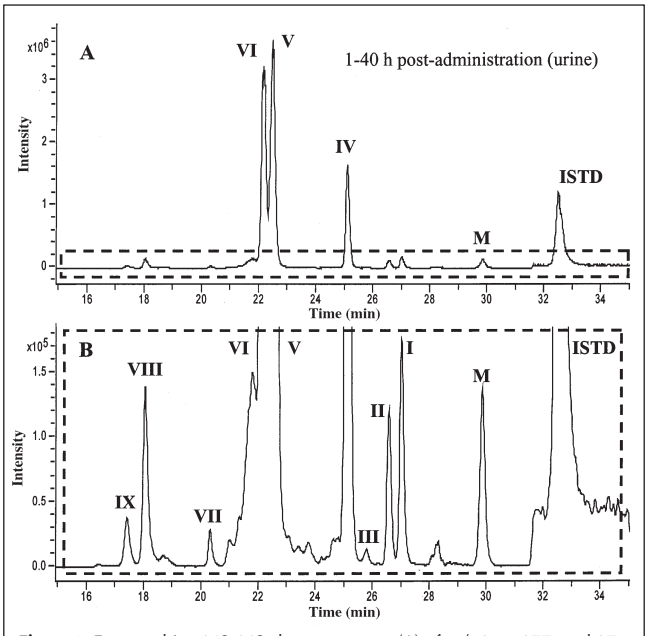

Figure 3. Extracted-ion MS–MS chromatograms (A) of *m/z* 193, 177, and 170 (*m/z* 170 is ISTD) of mesocarb (M) and its metabolites (I–IX) in human urine obtained at 1–40 h after an oral administration of 10 mg of mesocarb, and (B) is the fragment.

The identification of the metabolites of mesocarb is based on MS, MS–MS, and MS^3 spectra (Figures 4 and 5). LC–MS–MS and LC–MS^3 mass spectra were obtained througth the fragmentation of protonated molecular ions for more precise structural identification of metabolites. This study employed multiple MS, which involved repeated trapping and fragmentation of ions. The molecular ions of the metabolites were compared with those of the parent drug. Net changes of +16, +32, and +48 Da in molecular ions of metabolites were observed.

The results show that apart from the unchanged parent drug (mesocarb, M), the following 10 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).

Although the unresolved isomers of dihydroxymesocarb (V and VI) gave absolutely similar MS and MS–MS spectra, the isopropyl benzene group of the parent drug was hydroxylated in different positions of the ring. The two metabolites could be resolved using chromatography if a slower gradient had been used, namely 20% ammonium acetate, 0.2mM (pH 6.7) + 80% methanol changing to 50% ammonium acetate 0.2mM (pH 6.7)– 50% methanol in 40 min. Next, this composition was kept for 10 min. The analysis time was increased significantly.

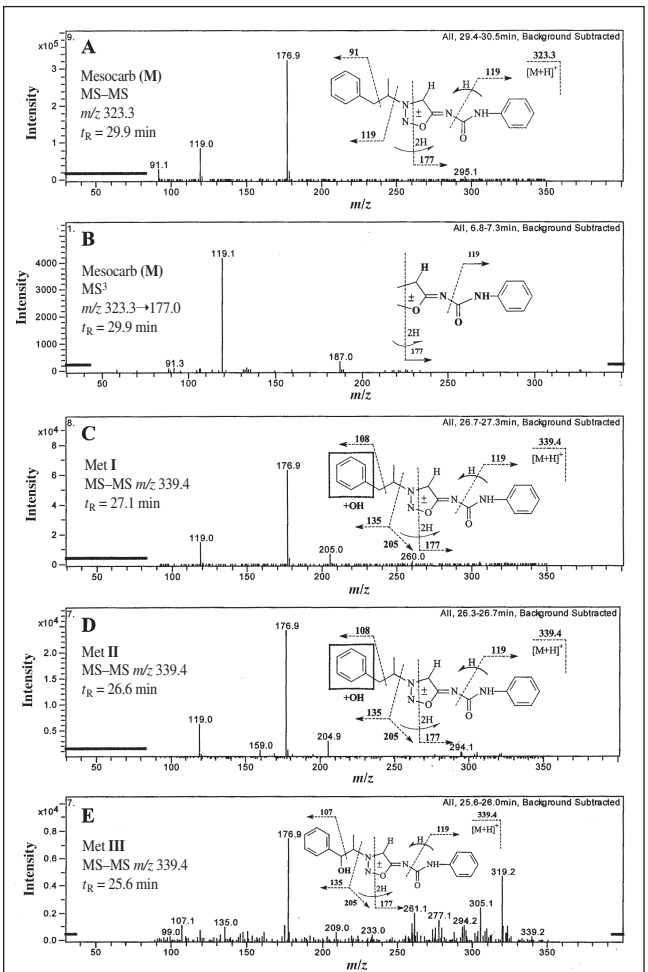

Figure 4. LC–MS–MS and LC–MS^3 mass spectra of mesocarb and its metabolites (urine): (A) MS–MS of mesocarb, (B) MS^3 of mesocarb, (C) MS–MS of Met I, (D) MS–MS of Met II, and (E) MS–MS of Met III.

Plasma

Typical chromatographic profiles of the human plasma from a healthy volunteer prior and after the administration of mesocarb are shown in Figure 6. There were no interfering peaks at the retention time of mesocarb and its metabolites in blank plasma. Mesocarb and its metabolites contained in plasma extracts in much lower concentrations than in urine extracts because the aliquots were 5 mL for urine, but 150 µL for plasma. In order to decrease the limits of detection of metabolites that have been found in urine, the aliquots of 1.5 mL of plasma samples and 1-µL

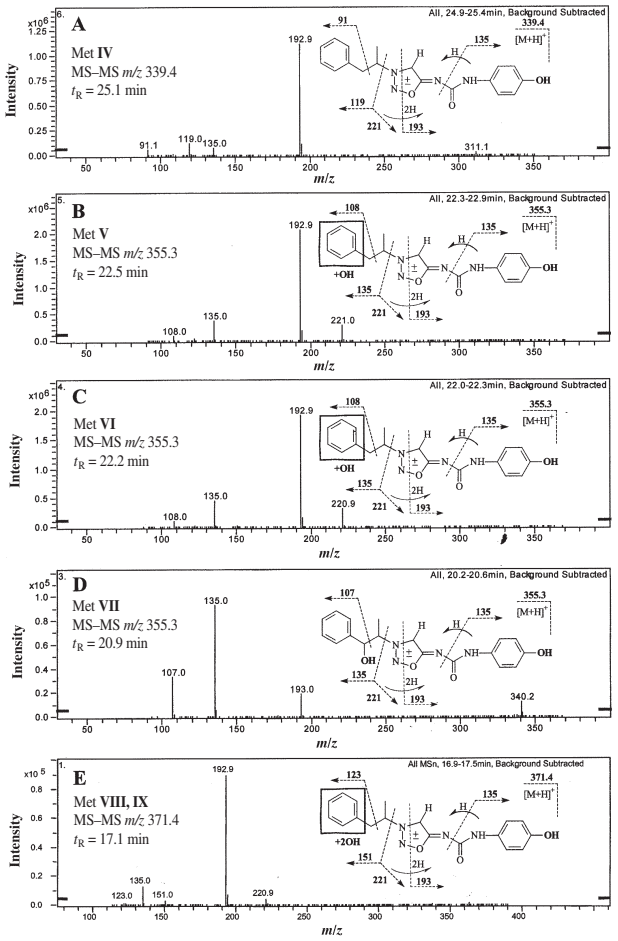

Figure 5. LC–MS–MS mass spectra of mesocarb metabolites (urine): (A) Met IV, (B) Met V, (C) Met VI, (D) Met VII, and (E) Met IX.

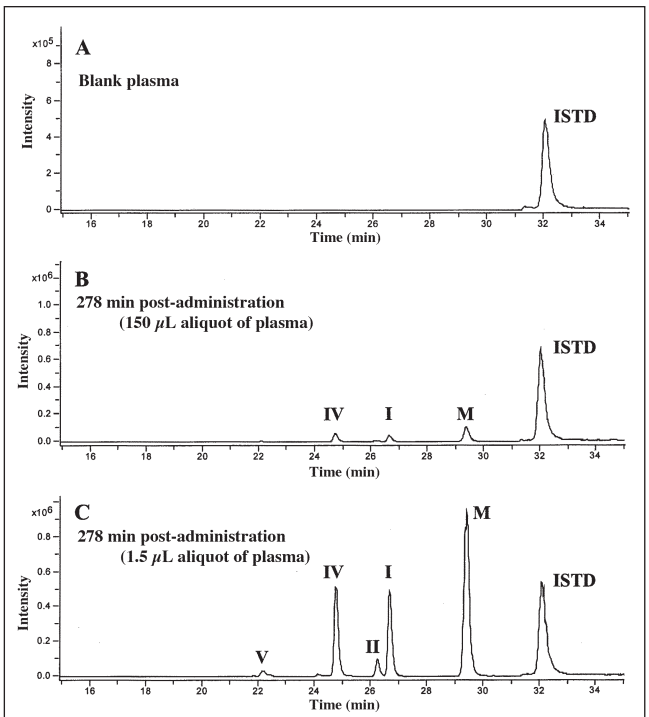

Figure 6. Extracted-ion MS–MS chromatograms of *m/z* 193, 177, and 170 (*m/z* 170 is ISTD) of mesocarb (M) and its metabolites in human plasma: (A) blank, (B) 150-µL aliquot of plasma, and (C) 1.5-mL aliquot of plasma obtained at 336 min after administration. The injected volume was 1 µL.

Table I. Retention Times, Product Ions, Protonated Molecule $[M+H]^+$, Changes in Observed Mass for the Metabolites (ΔM), and Fragmentation Results from MS–MS Spectra*

Substance	Urine	Plasma	t_R (min)	MW	$[M+H]^+$	DM	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	+	–†	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	+	–†	22.5	354	355	+32	355 → 221, 193, 135, 108
VII	+	–†	20.9	354	355	+32	355 → 135, 193, 107
Threehydroxymesocarb							
VIII	+	–†	17.8	370	371	+48	371 → 221, 193, 135, 123
IX	+	–†	17.1	370	371	+48	371 → 221, 193, 135, 123
Amphetamine (A)	+	–†	–‡	135	136	–187	136 → 119, 91

* The underlined fragmentation ions were used for quantitation.
† Not detected in plasma (LOD is 0.002 ng/mL).
‡ Flow injection analysis (LOD is 0.7 ng/mL).

injected volumes were used (Figure 6C**)**. Only 4 of 10 metabolites such as: **I**, **II**, **IV**, **V**, and the parent drug (**M**) were detected in human plasma (Table I). The LOD was approximately 0.002 ng/mL.

The identification of metabolites in plasma was based on the retention time and MS, MS–MS, and MS^3 mass spectra. The results of MS–MS and MS^3 analyses of mesocarb and its metabolites in plasma showed characteristic peaks similar to the peaks of the metabolites found in urine. Relative abundances between the ions of mass spectra fit with these metabolites.

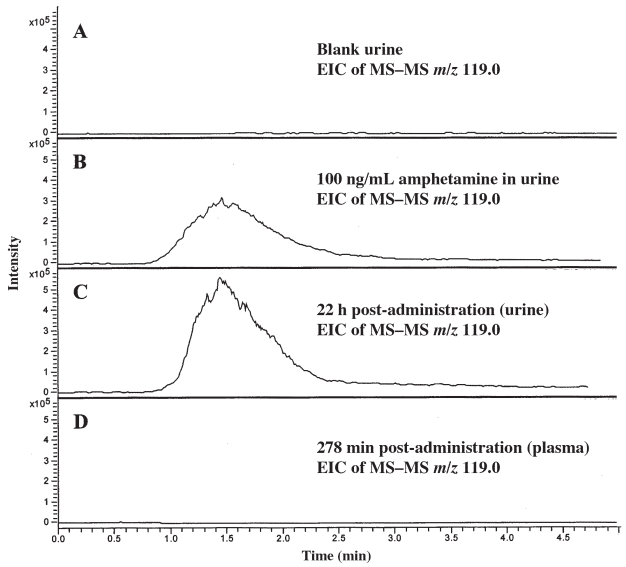

Figure 7. Extracted-ion MS–MS chromatograms of *m/z* 119 of amphetamine: (A) blank urine, (B) 100 ng/mL amphetamine in urine, (C) urine obtained at 22 h after administration of mesocarb, and (D) plasma obtained 278 min after administration. The flow injection analysis without a chromatographic separation was used.

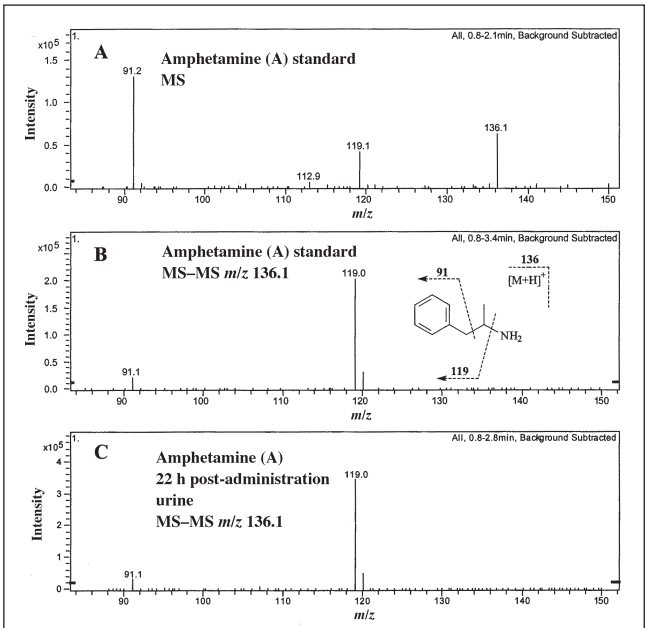

Figure 8. MS and MS–MS (*m/z* 136 →) mass spectra of amphetamine: (A) MS, (B) MS–MS mass spectra of amphetamine standard, and (C) MS–MS mass spectrum of urine obtained at 22 h after the administration of mesocarb.

The peak of amphetamine was eluted with the retention time of mesocarb with a very poor efficiency. The identification and quantitation of the amphetamine in such complex matrices as plasma and urine was achieved using simple flow injection analysis without a chromatographic separation. The extracted-ion MS–MS chromatograms of *m/z* 136 → 119 of a blank urine with added amphetamine and 22 h urine sample are presented in Figure 7.

The simple flow injection analysis has much lower selectivity than a LC–MS mode, but the ions with *m/z* 136 were not found in four different plasma and urine samples (Figures 7A and 7D). Figure 8 shows a comparison of the product ion spectra obtained from MS–MS flow injection analysis following the transition *m/z* 136 → 119 (Figure 8C) done on the 22-h urine sample, as well as for a standard sample of pure amphetamine (Figure 8B.) MS–MS spectra of pure amphetamine shows a unique "fingerprint-type" pattern that is rich in structurally specific product ions.

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 I. 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 unambiguously by MS alone because a metabolite standard sample was not available, but a partial identification only was made. The proposed metabolism pathways of mesocarb are presented in Figure 9. In the case of drug abuse, the free fraction treatment was selected for analysis (14).

Validation of the method

Selectivity

Although the superior selectivity of the MS–MS detection has been well recognized, the sufficient chromatographic separation of an analyte from its metabolites or the parent drug (or both) is always recommended in drug-metabolism studies. Because of the structural similarity of mesocarb and its metabolites, these compounds may share some common MS-detecting channels (in our

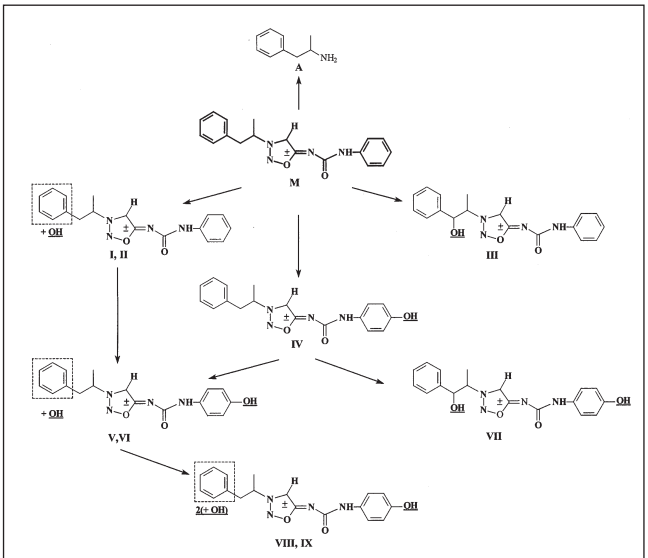

Figure 9. The metabolism pathways of mesocarb.

case, all the ions with *m/z* 91, 119, and 177 are present in the mass spectra of mesocarb and its metabolites). For this reason, the chromatographic conditions were optimized and the optimum time of analysis was 34 min.

To evaluate the selectivity of method, four different blank plasma and eight different blank urine samples obtained from different healthy volunteers were tested. No peaks were found near the retention times for mesocarb and its metabolites and the ISTD, indicating no interference from endogenous compounds. Representative chromatograms of plasma and urine spiked with analytes and blank samples are shown in Figures 2 and 6.

Linearity

Three calibration curves using methanol, blood blank plasma, and blank urine as matrices were prepared. The results presented in Table II indicate that all the calibration curves were linear but there was a significant difference between the parameters of regression equations because the extraction recoveries for mesocarb were not 100% and a matrix effect was observed. Therefore, calibration curves using blank plasma and blank urine as matrices was selected and used as calibration curves for the determination of mesocarb and its metabolites in human blood plasma and urine, respectively.

Calibration curves based on the addition–calibration method were linear (regression coefficients r^2 – 0.9973 and 0.9969 for plasma and urine, respectively) up to 67 ng/mL in plasma and to 5 ng/mL in urine (i.e., up to the highest mesocarb levels observed in our experiments).

A standard sample of urine having the concentration 10.0 ng/mL of mesocarb in urine does not belong to the linear range when the standard technique of the sample preparation is used. For mesocarb concentrations higher than 2 µg/mL in the injected solution (corresponding to 20 ng/mL of mesocarb in urine); both peak area and peak height MS responses were saturable. If necessary, the determination of such high concentrations could be achieved by reducing the aliquot of urine samples to 0.5 mL or by increasing the final reconstitution volume to 500 µL (or both).

Repeatability

Repeatability was examined as described previously (n = 10, 10 replicate injections of the 1 sample). The relative standard deviations (RSD) values for mesocarb were 1.5% and 2.2% for low and high control plasma samples, and 3.1 and 1.8% for low and high urine control samples.

Precision and accuracy

Six replicates at three different concentrations of mesocarb were spiked in blank plasma and urine for the determination of intra-assay precision and accuracy. The interassay precision and accuracy were determined for 3 different assays (3 replicates per day for 2 days for plasma; and 5 replicates per day for 2 days for urine).

The mean intra-assay (n = 6) and interassay (n = 12) precision (CV%) of the mesocarb-added control plasma samples were lower than 6.2 % with concentration values not deviating for (accuracy) more than –3.4% to + 7.3% from their nominal values.

Slightly lower precision and accuracy were obtained for urine samples compared with those achieved for plasma. The mean intra-assay and interassay CV% for the 0.08, 1.5, and 3.0 ng/mL urine control samples were lower than 14.1%. Similarly, the intra-assay (n = 6) and interassay (n = 15) deviation (accuracy) from nominal values was also satisfactory, with values within the –11.3 to +13.7 range. The results are shown in Tables III and IV.

LOD

LODs were obtained by decreasing the concentration of mesocarb in the plasma and urine samples. For a standard aliquot of plasma samples of 150 µL the limit of detection of mesocarb was found to be 0.1 ng/mL. Mesocarb LODs were estimated to be 0.012 and 0.002 ng/mL for an injection of 1 and 12 µL of the plasma extract, when a 1.5-mL aliquot plasma was used (Table II).

The LOD of mesocarb in urine was found to be 0.001 and 0.0001 ng/mL for the injection of 1 and 12 µL of the urine sample extract, respectively. It should be noted that we aspired to lower the LOD of mesocarb metabolites only in plasma. The purpose was the detection of 10 mesocarb metabolites in the blood plasma found previously in human urine.

Limits of quantitation

The lower LOQ of mesocarb was found to be 0.6 ng/mL (s/n = 45:1) and 0.05 ng/mL (s/n = 150:1) in plasma and urine, respectively. It was stated that at concentrations below this level in urine, for example 0.01 ng/mL, the precision was still acceptable (CV13%) but the deviation (accuracy) of +27% from the theoretical level exceeded the recommended value of ± 20%.

Table II. Linearity Results, Detection, and Quantitation Limits for Mesocarb in Plasma and Urine

Sample matrix	Sample aliquot (µL)	Injected volume (µL)	K*	LOD (ng/mL)	LOQ (ng/mL)	Linearity range (ng/mL)	Calibration curve†	r^2
Methanol	–	1	1	0.05	–	00.4–2000	$y = 0.0641x + 0.0197$	0.9992
Plasma	150	1	3	0.1	0.6	0.6–67	$y = 5.4955x + 0.0014$	0.9973
	1500	1	30	0.12	–	–	–	–
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	–	–	–	–

* Factor of concentration = injected volume × (sample aliquot/extract volume).
† $y = S_{Mes}/S_{ISTD}$; x is the concentration of mesocarb in the sample in ng/mL.

Extraction recovery

The mean recoveries (n = 4) from plasma were 49.2% (CV 10.8%) and 57.4 (CV 9.2%) for mesocarb concentrations of 33.0 and 66.0 ng/mL, respectively, whereas the recoveries from human urine were 76.9% (CV 12.6%) and 81.4% (CV 13.3%) for concentrations of 1 and 2 ng/mL, respectively.

Stability of samples

The variation of the mesocarb levels in plasma and urine left at room temperature for 8 h over time were from 3.2% and 5.8% in plasma and urine, respectively. It indicates that no significant decomposition of mesocarb can be observed at room temperature. On the other hand, a 100 ng/mL solution of mesocarb in methanol is not stable at room temperature. The concentration of mesocarb decreased approximately in two-fold (46% and 51%) for 48 h at room temperature.

The stability of plasma and urine extracts was checked with control samples (33.0 and 66.0 ng/mL; and 1.0 and 2.0 ng/mL of mesocarb for plasma and urine, respectively) placed in HPLC vials stored at room temperature for 24 h at + 4°C for 72 h. The stability was calculated by comparing the peak areas and the peak-area ratios (mesocarb–ISTD) obtained when analyzed freshly prepared control samples and the stored samples. The variations were from 2.6% to 6.7%. The results indicate that the plasma and urine extracts prepared for analysis are stable over time, which can satisfy the requirement of large analyses, weekend, or confirmatory analyses. Nevertheless, the time in which plasma, urine samples, and, finally, extracts, are stored at room temperature should be kept to a minimum to reduce the probability of anything changing.

Table III. Precision and Accuracy of the Measurements of Mesocarb in Plasma

Nominal concentration (ng/mL)	Average concentration (ng/mL)	SD* (ng/mL)	Precision† (CV%)	Accuracy‡ (deviation %)
Intra-assay (n = 6)				
3.00	3.14	0.18	5.6	4.7
40.00	39.05	0.92	2.5	–2.4
55.00	53.12	3.13	5.9	–3.4
Interassay (n = 12)				
3.00	3.22	0.20	6.2	7.3
40.00	40.97	1.88	4.6	2.4
55.00	54.72	1.94	3.6	–0.5

* SD = standard deviation.
† Precision = 100% × (SD/average concentration).
‡ Accuracy = 100% × (average concentration – nominal concentration)/nominal concentration.

Table IV. Precision and Accuracy of the Measurements of Mesocarb in Urine

Nominal concentration (ng/mL)	Average concentration (ng/mL)	SD* (ng/mL)	Precision† (CV%)	Accuracy‡ (deviation %)
(n = 5)				
0.08	0.074	0.008	10.8	–7.5
1.50	1.40	0.11	7.9	–6.7
3.00	3.11	0.27	8.7	3.7
(n = 15)				
0.08	0.071	0.010	14.1	–11.3
1.50	1.42	0.07	5.0	–5.3
3.00	3.41	0.32	9.4	13.7

* Precision = 100% x (SD/average concentration).
† Accuracy = 100% x (average concentration—nominal concentration)/nominal concentration.

Application of the method

This method was applied to the analysis of urine samples from four male and female healthy volunteers participating in the mentioned study. Urine and plasma samples from a male volunteer were collected simultaneously to compare the concentrations of mesocarb and amphetamine in plasma and urine. Results are presented in Table V. The quantitation of the amphetamine (**A**) was made using simple flow-injection analysis without a chromatographic separation.

The long-life dihydroxymesocarb metabolites of mesocarb **V** and **VII** have been detected from the unhydrolyzed fraction for the 10th day after the 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 9–10 days after the administration of the drug.

The concentration in urine ranged between 0.004 and 0.17 ng/mL for unconjugated mesocarb (**M**) and 0.7 and 186 ng/mL (22 h post-administration) for amphetamine (**A**). Plasma concentration ranged between 64.5 and 21.1 ng/mL for mesocarb (Table V).

p-Hydroxymesocarb (**IV**) was the most abundant metabolite in the first hours. After 2 days, the excreted amount the dihydroxymesocarb metabolites (**V** and **VI**) was largest among all the excreted metabolites. Only **V** and **VI** were detected in 6–11 days after an oral administration of a single dose of 10 mg of mesocarb. In the case of doping analysis, the estimated detection time for mesocarb (long-life dihydroxymesocarb metabolites of mesocarb) was approximately 10–11 days.

Table V. Plasma and Urine Concentrations of Mesocarb after an Administration of 10 mg of Mesocarbe (n = 4)

Sample	Amphetamine	Mesocarb
1.2 h		
Plasma	n/f*	61 ± 5
Urine	n/d†	0.8 ± 0.01
4.6 h		
Plasma	n/f	30 ± 1
Urine	0.8 ± 0.6	0.17 ± 0.02
31 h		
Urine	39 ± 11	0.020 ± 0.002

* n/f = not found in human plasma (LOD is 0.002 ng/mL).
† n/d = not detected at this time (LODs are 0.1 and 0.001 ng/mL for plasma and urine, respectively).

Pharmacokinetica of plasma

The plasma disappearance curve of the parent drug from a healthy volunteer after an oral administration of 10 mg of mesocarb are shown in Figure 10. The mesocarb was detected from 33 min (30.8 ng/mL) to 336 min (21.1 ng/mL) after the administration.

Pharmacokinetic parameters were calculated using noncomparmental analysis, using Excel 97 (Microsoft, Redmond, WA) and Origin 6.0 (OriginLab, Northampton, MA) spreadsheet processors. Table VI shows pharmacokinetic parameters for mesocarb.

Conclusion

The metabolism of mesocarb by LC–ESI-MS ion trap in humans was investigated. After the administration of a single oral dose 10 mg of mesocarb (Sydnocarb, 2 tablets of 5 mg) 10 metabolites and the parent drug were detected in human urine, and only 4 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 approximately 10–11 days.

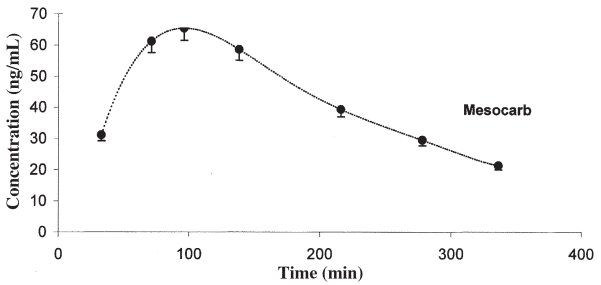

Figure 10. The plasma disappearance curve of the parent drug from a healthy volunteer after an oral administration of 10 mg of mesocarb.

Table VI. Pharmacokinetic Parameters (*n* = 4) of Mesocarb after an Oral Administration of 10 mg (0.12 mg/kg)*

Parameter	Mean	Unit
c_{max}*	64.5 ± 3.7	ng/mL
t_{max}†	1.61 ± 0.01	h
$t_{1/2}$‡	2.73 ± 0.19	h
k_e§	0.25 ± 0.02	1/h
$C1_T/F$**	8.88 ± 1.21	(mL/h)/kg
V_d/F††	35.004.56	mL/kg
AUC‡‡	255.6 ± 22.3	ng × h/mL
MRT§§	3.94 ± 0.63	h

* Maximum concentration.
† Time of maximum concentration.
‡ Half-life during the terminal slope.
§ Rate constant of the terminal slope.
** Total clearance.
†† Aparent volume of distribuation.
‡‡ Area under concentration-time curve extrapolated to infinity.
§§ Mean residence time.

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