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Analysis of mesocarb analogues in urine and plasma of rats by high-performance liquid chromatography and thermospray liquid chromatography–mass spectrometry

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Abstract

The pharmacokinetics and metabolism of synthetic 2-hydroxymesocarb and 4-methyl-2-hydroxymesocarb analyzed by HPLC–DAD and thermospray LC–MS were studied in rats. Multistep liquid–liquid extraction (LLE) was used with diethyl ether at pH 7.0. The major metabolites of 2-hydroxymesocarb in both urine and plasma of the rat were the *p*-hydroxylated derivative of the phenylcarbamoyl group of the parent drug. The metabolites of 4-methyl-2-hydroxymesocarb in urine of rats may be the oxidized forms at the phenylcarbamoyl group of the parent drug. Absorption (k_a) and elimination (k_e) rate constants in plasma of 2-hydroxy-mesocarb were 0.0300 and 0.00485 min^{-1}, respectively; those of 4-methyl-2-hydroxy-mesocarb were 0.0546 and 0.00797 min^{-1}, respectively. The half-lives ($t_{1/2}$) of 2-hydroxymesocarb and 4-methyl-2-hydroxymesocarb in plasma were 144 and 86 min, respectively.

Keywords: Doping agents; Mesocarb; 2-Hydroxymesocarb; 4-Methyl-2-hydroxymesocarb

1. Introduction

Mesocarb [or sydnocarb; 3-(2-phenylisopropyl)-N-phenylcarbamoylsydnoneimine; Fig. 1A; 1], a central nervous system (CNS) stimulant [1], was synthesized from amphetamine as a starting material [2–5].

CNS stimulating effect and drug dependency of mesocarb were reported to be improved compared to amphetamine [6,7]. Mesocarb metabolites in urine of the rat were reported to be *p*-hydroxymesocarb (Fig. 1A; 2), dihydroxymesocarb (Fig. 1A; 3) and their conjugates by GC–MS [8,9]. Also, the major metabolite of mesocarb in the urine of human was confirmed to be *p*-hydroxymesocarb sulfate by using particle beam LC–MS [10].

Four stereoisomers of 2-hydroxymesocarb were also synthesized from DL-norephedrine as a starting material, and the L-2-hydroxymesocarb isomer showed a CNS stimulating effect [11].

With the purpose of investigating the effect of its mesoionic ring on the CNS stimulating activity of L-2-hydroxymesocarbs, we have synthesized model compounds such as C-4 substituted L-2-hydroxy-mesocarbs (Fig. 1C; 4 and 5).

Pharmacokinetics and metabolism studies of the synthetic model compounds were conducted in plasma and urine of the rat using HPLC with a diode array detector (DAD) and thermospray LC–MS (TSP-LC–MS).

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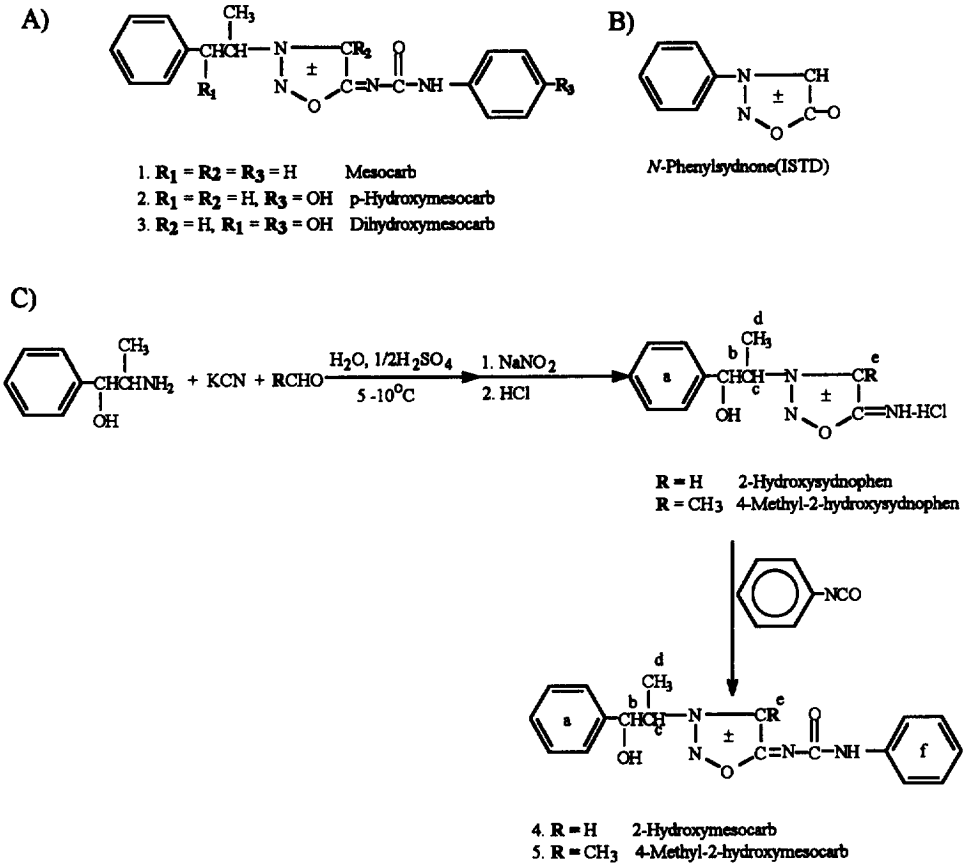

Fig. 1. Chemical structures of mesocarb analogues (A), sydnone (B) and the synthetic scheme of model compounds (C).

2. Experimental

2.1. Reagents

Acetonitrile and methanol were purchased from Burdick and Jackson (Muskegon, MI, USA) or E. Merck (Darmstadt, Germany). Polypropyleneglycol for standard tuning mass range, was purchased from Hewlett-Packard (Palo Alto, CA, USA). 1*R*,2*S*-(−)-Phenylpropanolamine was purchased from Sigma (St. Louis, MO, USA). β-Glucuronidase (from *E. Coli*) was purchased from Boeringer Mannheim (Mannheim, Germany). Distilled water for HPLC was obtained by passing through the Milli-RO and Milli-Q water system. Peroxide-free diethyl ether was obtained by distillation over calcium hydride and all other reagents were of analytical grade.

2.2. Synthesis

The internal standard, sydnone, was obtained by the reaction of aniline, sodium nitrite and monochloroacetic acid with hydrogen chloride [12].

The synthetic scheme of the model compounds is shown in Fig. 1C. The closing of the sydnone imine ring was obtained by the action of hydrogen chloride on N-nitroso-α-aminoacetonitrile, which was itself obtained by nitrosation of the condensation product of norephedrine with potassium cyanide and formaldehyde (or methylaldehyde). 2-Hydoxymesocarb (or 4-methyl-2-hydroxymesocarb) was synthesized by the reaction of sydnone imine at the imino group with phenylisocyanate in weakly alkaline media.

^{1}H NMR data of target compounds were as follows. 2-Hydroxymesocarb (Fig. 1C; 4, D_2O): a

and f (m, 6.98–7.50 ppm, 10H); b and c (m, 5.02–5.12 ppm, 2H); d (d, 1.61 ppm, J=6.63 Hz, 3H); e (s, 7.90 ppm, 1H). 4-Methyl-2-hydroxymesocarb (Fig. 1C; 5, CD_3OD): a and f (m, 6.89–7.52 ppm, 10H); b (d, 4.93 ppm, J=6.3 Hz, 1H); c (m, 4.68 ppm, 1H); d (d, 1.66 ppm, J=6.6 Hz, 3H); e (s, 1.91 ppm, 3H). Varian Gemini 300 NMR spectrometer (300 MHz; s, singlet; d, doublet; m, multiplet; J, coupling constant).

2.3. High-performance liquid chromatography

The liquid chromatographic analysis was carried out with a Hewlett-Packard Model 1090M HPLC equipped with a diode array detector, autosampler and autoinjector. The HP 9000-300 computer with HP 9133 disc drive was used for data processing. The analytical column was a Hypersil-ODS (100× 4.6 mm I.D., 5 μm particles) and it was maintained at 40°C. Gradient elution was carried out with a solvent composed of phosphate buffer (pH 6.8, 0.01 M K_3PO_4 and 0.02 M KH_2PO_4) and acetonitrile for the analysis of urine samples. The gradient program was as follows: initial acetonitrile was 10%; it was then increased to 30% in 10 min, to 50% for 5 min and held for 5 min. Plasma samples were also analyzed by elution with a solvent composed of water and acetonitrile. The gradient program was as follows: initial acetonitrile was 30%; it was then increased to 70% in 15 min and held for 5 min.

The flow-rate was 1.0 ml/min. The diode array detector was set to monitor the signal at 254 and 330 nm.

2.4. Thermospray LC–MS

The LC–MS system was a Hewlett-Packard Model 1090A HPLC linked via capillary tubing and thermospray vaporizer probe to a HP 5988A mass spectrometer. The data system was a HP 9000-300 computer and a HP 7946 disc drive. The MS was tuned daily using polypropyleneglycol.

The analytical column was the Shiseido (Tokyo, Japan) Capcell PAK C_{18} (60×4.6 mm I.D., 3 μm particles) and the mobile phase was ionization solution (0.15 M ammonium acetate) and methanol at the flow-rate of 1.0 ml/min. The solvent gradient program was as follows: initial methanol was 20%, increased to 60% in 20 min, and held for 5 min. The optimal temperature of the vaporizer probe was programmed according to the composition of the mobile phase. The typical probe temperature program was as follows: initial stem temperature was 120°C, decreased to 108°C at the rate of 0.6°C/min, and held for 5 min.

Other MS parameters were as follows: ion source temperature, 276°C; emission current, 150 μA; electron energy, 955 eV; mode, positive ion and filament on.

2.5. Samples

2.5.1. Urine

Samples were collected for 24 h using a metabolic cage before (blank) and after oral administration of 20 mg/kg of L-2-hydroxymesocarb or L-4-methyl-2-hydroxymesocarb (1% suspension of 1% carboxymethylcellulose solution) to the male Sprague–Dawley rat.

2.5.2. Plasma

Rat blood samples (n=3) were collected in heparinized containers at 0, 1, 5, 10, 30, 60, 120, 240, 420, 600 and 1440 min after oral administration of 20 mg/kg of L-2-hydroxymesocarb or L-4-methyl-2-hydroxymesocarb to the rat and immediately centrifuged for 10 min at 2000 g for plasma separation. Plasma was stored at −20°C until analyzed.

Pharmacokinetic parameters were calculated using the PKCALC program (R.C. Shumaker, Merrel Dow Research Institute).

2.6. Extractions

2.6.1. Urine

Extraction procedure was carried out as follows: unconjugated fraction, enzyme hydrolysis and acid hydrolysis.

First, 5 ml of urine sample were transferred to a 15-ml centrifuge tube with a glass stopper. A 0.1-g amount of solid buffer (K_3PO_4–KH_2PO_4, 1:1 mixture) was added to adjust the pH to 7.0. After addition of 0.5 g of anhydrous sodium sulfate (salting out reagent), the mixture was extracted twice with 5 ml of diethyl ether. The mixture was shaken

mechanically for 10 min and then centrifuged at 1000 *g* for 5 min. The organic layer was transferred to another tube and evaporated to dryness in a vacuum rotary evaporator. The residue was dissolved with 200 μl of methanol and 10 μl of this solution was injected into the HPLC and TSP-LC–MS (unconjugated fraction). Secondly, to the aqueous layer (separated urine layer pH 7.0), 25 μl of β-glucuronidase was added and the mixture was heated at 50°C for 1 h. After cooling, it was extracted twice with 5 ml of diethyl ether. The organic layer was transferred to another tube and evaporated to dryness. The residue was dissolved in 200 μl of methanol and 10 μl of this solution was injected into the HPLC (glucuronide conjugated fraction). Finally, 1 ml of 6 *M* hydrochloric acid and 100 mg of L-cysteine (anti-oxidant) were added to the remaining aqueous layer, and the mixture was heated at 105°C for 30 min. After cooling, it was neutralized with potassium hydroxide and extracted twice with 5 ml of diethyl ether.

The organic layer was transferred to another tube and evaporated to dryness, and the residue was dissolved in 200 μl of methanol and 10 μl of this solution was injected into the HPLC (another conjugated fraction).

2.6.2. Plasma

An aliquot (200 μl) of plasma was transferred to a 15-ml centrifuge tube and 4 ml of acetonitrile containing 1 μg of sydnone as an internal standard were added. The sample was vortex mixed for 30 s and then centrifuged for 10 min at 2000 *g* to remove the protein. The supernatant was evaporated to dryness. The residue was dissolved in 200 μl of methanol and 20 μl of this solution was injected into the HPLC.

2.7. Calibrations

All standards of 2-hydroxymesocarb and 4-methyl-2-hydroxymesocarb at concentrations of 0.1, 0.5, 1, 2, 3, 4 and 5 μg/ml were spiked to 200 μl of plasma with 5 μg/ml of sydnone (internal standard; Fig. 1B). A 20-μl aliquot of plasma extracts was analyzed by HPLC.

3. Results and discussion

3.1. Metabolites in urine

3.1.1. Metabolites of 2-hydroxymesocarb

2-Hydroxymesocarb was eluted at 14.3 min (Fig. 2I; A) and showed two characteristic UV maximal wavelength at 254 and 333 nm (Fig. 2I; UV2). By comparison of the two chromatograms of the unconjugated fraction extracts in urine before (Fig. 2I; B) and after (Fig. 2I; C) oral administration of 2-hydroxymesocarb to the rat, parent drug and a metabolite (Met-1) were forced to elute at 14.3 and 10.6 min, respectively. UV maxima of Met-1 (Fig. 2I; UV1, 254 and 333 nm) were the same as those of the parent drug.

Met-1 had a shorter retention time than the parent drug. Thus, we could expect that the parent drug and Met-1 had similar structures and thus the polarity of Met-1 increased.

Glucuronide conjugates of the parent drug are not found in Fig. 2II. It was, however, observed that there was conjugated Met-1 from the results of enzyme hydrolysis (Fig. 2II; A and B). Compared with Fig. 2II-B and Fig. 2II-D, it was evident that the peak area of Met-1 was increased after acid hydrolysis. This indicated the possible presence of other conjugates as well as the glucuronide conjugate of Met-1.

The TSP-LC–MS spectrum of the parent drug (Fig. 3I) showed a protonated molecular ion $[MH^+]$ at *m/z* 339. The ions at *m/z* 220 and *m/z* 237 (base) may result from the loss of a phenylisocyanate group of $[MH^+]$ and ammonium adducted molecular ion $[MNH_4^+]$, respectively.

The TSP-LC–MS spectrum of Met-1 (Fig. 3II) showed a protonated molecular ion $[MH^+]$ at *m/z* 355. The ions at *m/z* 220 and *m/z* 237 were the same as the fragment ions (sydnophen, Fig. 1C) of the parent drug. The base ion at *m/z* 249 may result from the loss of a benzaldehyde group of $[MH^+]$.

The molecular mass of Met-1 was increased by 16 (oxygen) compared to that of the parent drug and Met-1 maintained the form of mesoionic ring. Because Met-1 maintained the structure of sydnophen and its polarity was increased, this indicates that the phenylcarbamoyl group of parent drug was oxidized. Compared with the results of Polgar et al. [8,9] and

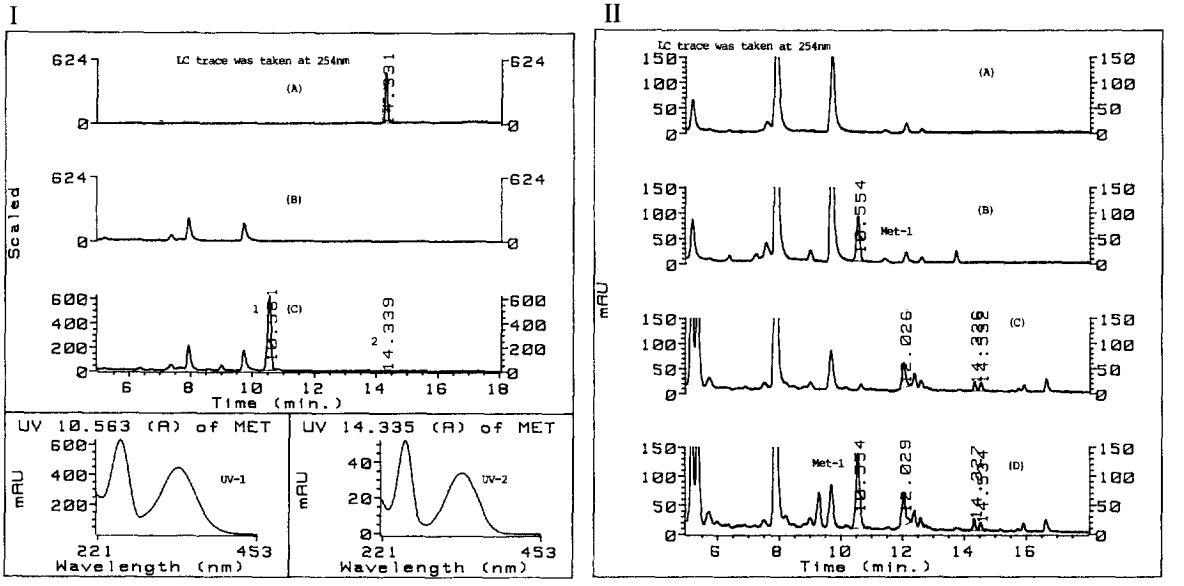

Fig. 2. HPLC of unconjugated (I) and conjugated (II) fraction extracts of urine before and after oral administration of 2-hydroxymesocarb to rat. (I) A, authentic 2-hydroxymesocarb; B, before (blank); C, after (sample: 1, Met-1; 2, parent drug); UV-1 (254, 333 nm); UV-2 (254, 333 nm). (II) Enzyme hydrolysis: before (A) and after (B). Acid hydrolysis: before (C) and after (D)

Ventura et al. [10], the structure of Met-1 was expected to be the hydroxylated mesocarb at the *para*-position with respect to the phenylcarbamoyl group. Metabolites of 2-hydroxymesocarb in urine of the rat may be Met-1 (dihydroxymesocarb, Fig. 1A; 3), its glucuronide and other conjugates.

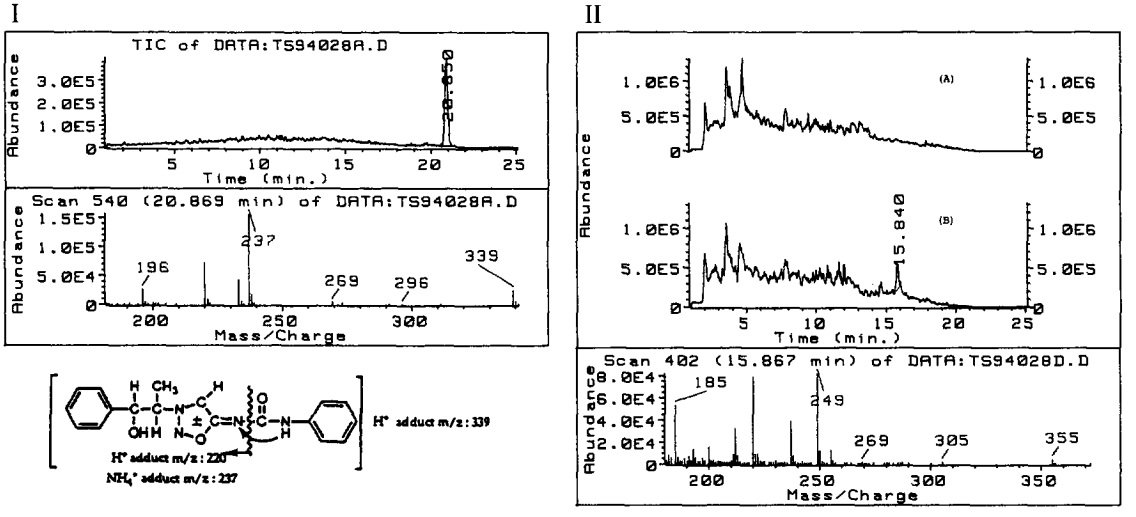

Fig. 3. TSP-LC–MS total ion chromatograms and mass spectra of authentic 2-hydroxymesocarb (I), and Met-1 (II) in unconjugated fraction extracts of urine before (II-A) and after (II-B) oral administration of 2-hydroxymesocarb.

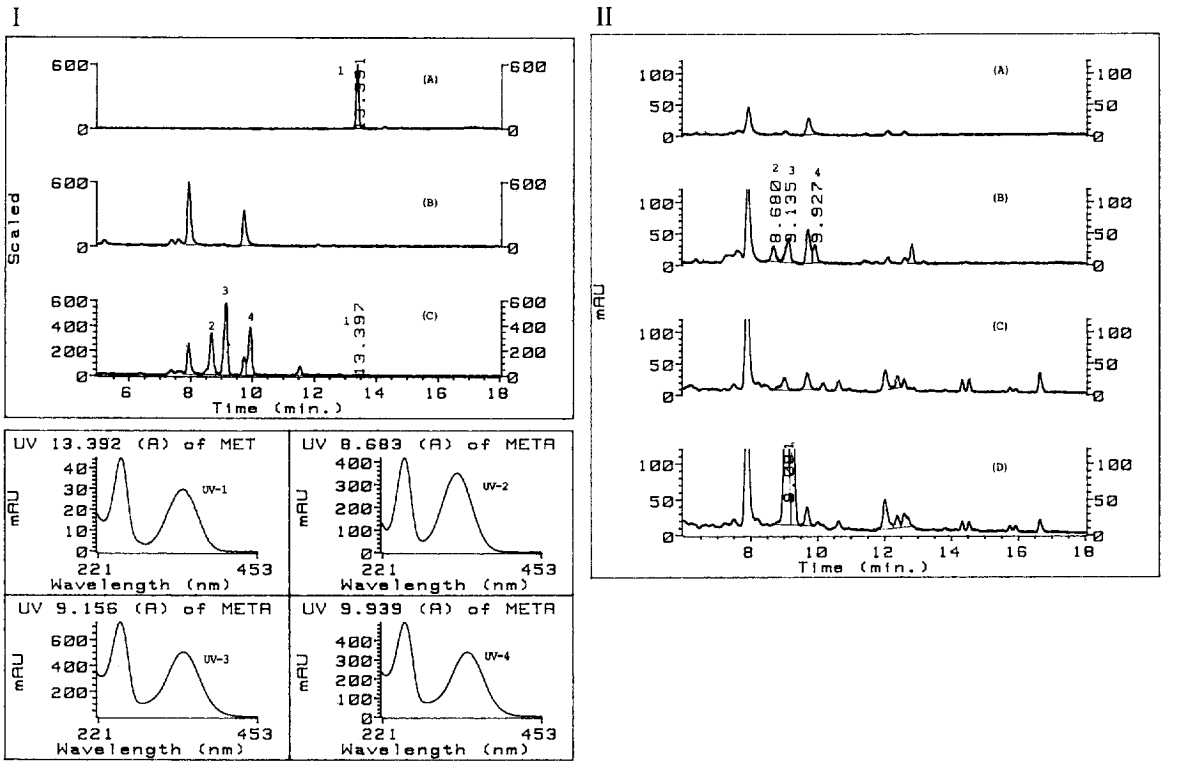

Fig. 4. HPLC of unconjugated (I) and conjugated (II) fraction extracts of urine before and after oral administration of 4-methyl-2-hydroxymesocarb to rat. (I) A, authentic 4-methyl-2-hydroxymesocarb; B, before; C, after; 1, parent drug (254, 342 nm); 2, Met-2; 3, Met-3; 4, Met-4. (II) Enzyme hydrolysis: before (A) and after (B). Acid hydrolysis: before (C) and after (D)

3.1.2. Metabolites of 4-methyl-2-hydroxymesocarb

4-Methyl-2-hydroxymesocarb was eluted at 13.4 min (Fig. 4I; A) and showed two characteristic UV maxima at the wavelength of 254 nm and 342 nm (Fig. 4I; UV1). When the chromatogram (Fig. 4I; C) obtained from the urine treated with 4-methyl-2-hydroxymesocarb compared to that (Fig. 4I; B) of the unconjugated fraction of the blank, several metabolites (Fig. 4I-C; 2, 3 and 4) were eluted at 8.7 min (Met-2), 9.2 min (Met-3), 9.9 min (Met-4). A trace amount of the parent drug (Fig. 4I-C, 1) was detected. The UV absorption spectra (Fig. 4I; UV2, UV3 and UV4) of the three metabolites (Met-2, Met-3 and Met-4) were similar to that of the parent drug. The UV spectrum of Met-2 (UV2, 254 and 342 nm) was the same as that of the parent drug. These metabolites have shorter retention times than that of the parent drug. Thus, the glucuronide conjugate forms of the three metabolites and other conjugate forms of Met-3 were detected by HPLC after enzyme hydrolysis (Fig. 4II; B) and acid hydrolysis (Fig. 4II; D), respectively. However, conjugated forms of the parent drug are not shown in Fig. 4II.

The TSP-LC–MS spectrum of the parent drug (Fig. 5I) showed a protonated molecular ion [MH^+] at m/z 353; the ions m/z 234 (protonated 4-methyl-2-hydroxysydnophen) and m/z 247 ion may result from the loss of phenylisocyanate and benzaldehyde groups in [MH^+].

The TSP-LC–MS spectra of metabolites (Fig. 5II; 1, 2 and 3) did not show protonated molecular ions, but showed the base ion at m/z 234 or m/z 233. The m/z 234 ion suggested that these metabolites had common fragment ions (protonated 4-methyl-2-hydroxysydnophen). Considering the UV spectra and mass fragments of the metabolites, three metabolites

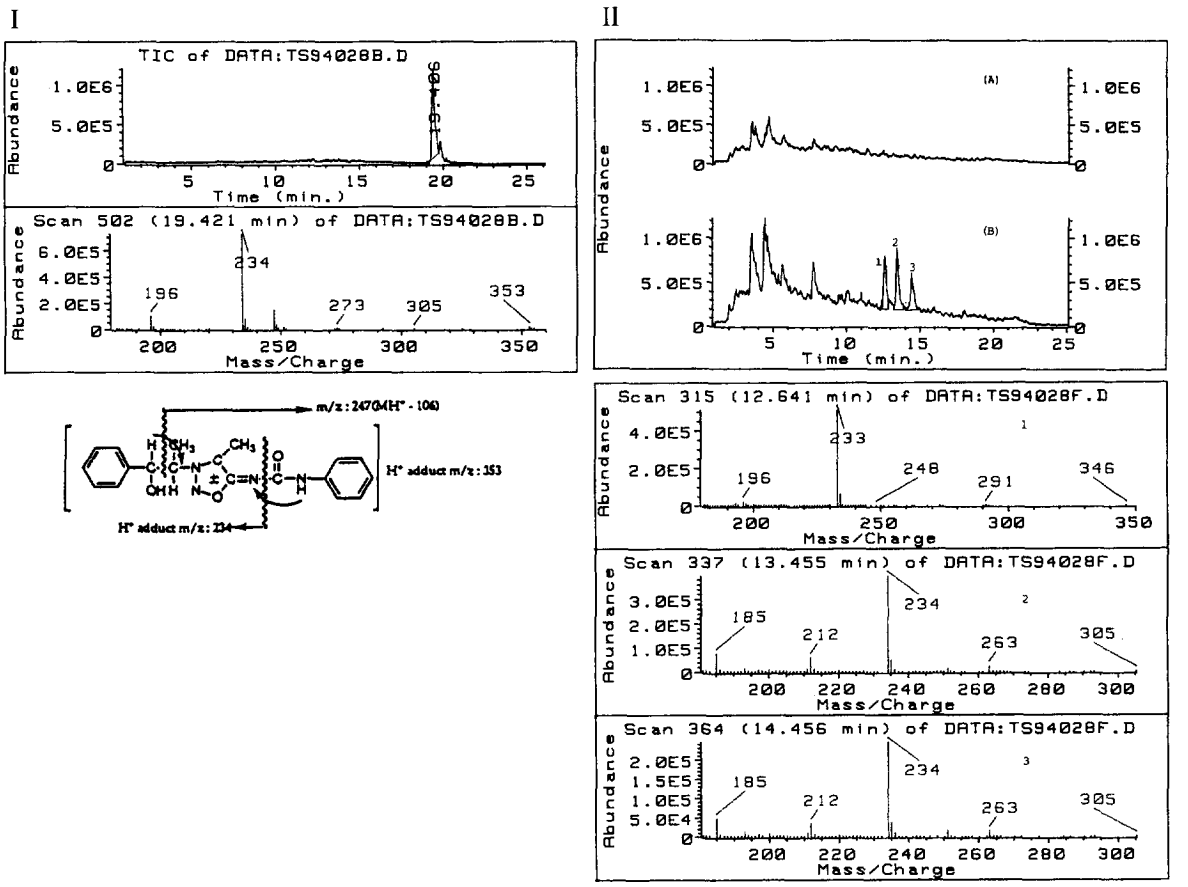

Fig. 5. TSP-LC–MS total ion chromatograms and mass spectra of authentic 4-methyl-2-hydroxymesocarb (I), and unconjugated fraction extracts of urine before (II-A) and after (II-B) oral administration of 4-methyl-2-hydroxy-mesocarb to rat. In panel II, mass spectra 1, 2 and 3 indicate Met-2, Met-3 and Met-4, respectively.

(Met-2, Met-3 and Met-4) were considered to be derived from the oxidized compounds in the phenylcarbamoyl group of the parent drug.

3.2. Calibrations

Calibration curves were obtained by the internal standard method. Each calibration curve (at 254 nm) was as follows: 2-hydroxymesocarb, $y=1.7646x-0.0371$ ($r=0.999$), detection limit=0.1 μg/ml; 4-methyl-2-hydroxymesocarb, $y=1.1165x+0.0273$ ($r=0.999$), detection limit=0.1 μg/ml.

Met-1 and Met-2 concentrations in the plasma were determined using the calibration curves of their parent drugs since UV absorbance of Met-1 and Met-2 was the almost same as that of their parent drugs, respectively.

3.3. Pharmacokinetics in plasma

2-Hydroxymesocarb was detected from 5 min (0.52 μg/ml) to 600 min (0.24 μg/ml) after oral administration to rats. The metabolite of 2-hydroxy-mesocarb in plasma was the same as the urinary metabolite (Met-1), and it was detected between 30 min (0.18 μg/ml) and 600 min (0.17 μg/ml). The estimated total amount of Met-1 in plasma was 13.4% of the parent drug.

4-Methyl-2-hydroxymesocarb was detected from 5 min (0.20 μg/ml) to 240 min (0.11 μg/ml) after

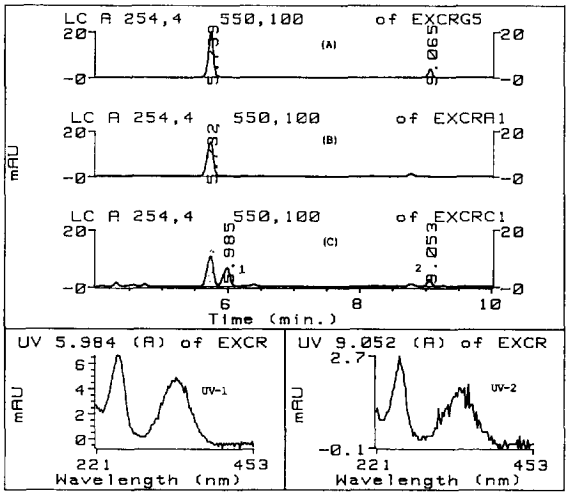

Fig. 6. HPLC and UV spectra of plasma extracts before and after oral dosing of 4-methyl-2-hydroxymesocarb to rat. A, Authentic 4-methyl-2-hydroxymesocarb; B, before (blank); C, after (sample) 1, Met-2; 2, parent drug.

oral administration to rats. Only one metabolite (Met-2) of 4-methyl-2-hydroxymesocarb was detected in plasma (Fig. 6). Met-2 was detected from 5 min (0.48 μg/ml) to 240 min (2.22 μg/ml). The estimated total amount of Met-2 in plasma was about 550% that of the parent drug.

The plasma disappearance curves of parent drugs and their metabolites are shown in Fig. 7. Table 1 shows pharmacokinetic parameters of mesocarb [5], 2-hydroxy-mesocarb and 4-methyl-2-hydroxymesocarb.

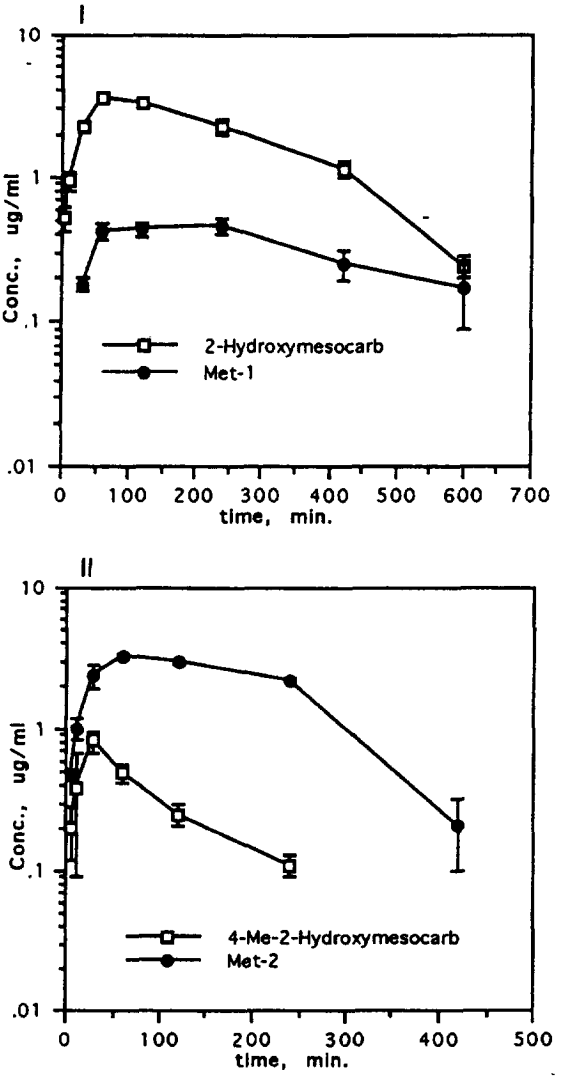

Fig. 7. Plasma disappearance curves of 2-hydroxymesocarb (I, 20 mg/kg) and 4-methyl-2-hydroxymesocarb (II, 20 mg/kg) in rats.

4. Conclusions

The metabolites of 2-hydroxymesocarb in the urine of rat were identified to be hydroxylated at the *para*-position of the phenylcarbamoyl group of the parent drug, such as Met-1 (dihydroxymesocarb, Figs. 1–3) in the unconjugated fraction. We could detect the glucuronide conjugate of Met-1 after enzyme hydrolysis using β-glucuronidase. The parent drug was detected only in the unconjugated fraction by the HPLC–DAD and TSP-LC–MS.

Several metabolites of 4-methyl-2-hydroxymesocarb excreted in the urine of rat were characterized as oxidized forms at the phenylcarbamoyl group of the parent drug. A trace amount of parent drug was detected in the unconjugated fraction.

Pharmacokinetic parameters of synthetic mesocarb analogues with respected to those of mesocarb, such as absorption (k_a) and elimination (k_e) rate constants of 2-hydroxymesocarb were two times faster than those of mesocarb. The constants (k_a and k_e) of 4-methyl-2-hydroxymesocarb were three and half times faster than those of mesocarb. The estimated

Table 1
Pharmacokinetic parameters of mesocarb [5], 2-hydroxy-mesocarb and 4-methyl-2-hydroxymesocarb ($n=3$)

Parameters	Unit	Compound		
		Mesocarb [5]	2-Hydroxymesocarb	4-Me-2-hydroxymesocarb
k_a	10^{-2} min^{-1}	1.49	2.30±0.375	5.46±0.750
T_{max}	min	115	76.7±5.13	27.7±6.18
C_{max}	μg/ml	7.47	3.65±0.729	0.655±0.134
$t_{1/2}$	min	295	144±12.0	86.3±16.5
V_d/F	ml	734	1180±294	5220±1160
Clt/F	ml/min	2.08	5.34±1.43	48.8±6.88
AUC	μg·min/ml	4802	1020±181	91.3±13.3
MRT	min	353	204±29.8	106±14.2

V_d/F: Volume of distribution, Clt/F: Total clearance, AUC: Area under concentration–time curve, MRT: Mean residence time.

half-lives ($t_{1/2}$) of mesocarb, 2-hydroxymesocarb and 4-methyl-2-hydroxymesocarb were 295, 144 and 86.3 min, respectively.

Acknowledgments

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