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Enantiomer resolution of camazepam and its derivatives and enantioselective metabolism of camazepam by human liver microsomes

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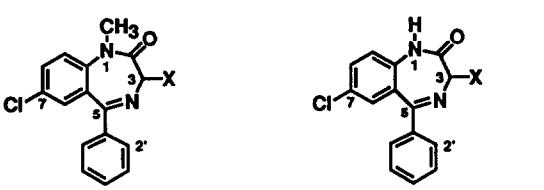
ABSTRACT

Camazepam [3-(N,N-dimethyl)carbamoyloxy-7-chloro-1-methyl-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one, CMZ] possesses anxiolytic, anticonvulsant, muscle relaxant, and hypnotic properties. CMZ is clinically used as a racemate. Enantiomer resolution of CMZ and 11 of its derivatives was studied by high-performance liquid chromatography (HPLC) using 5 different chiral stationary phase (CSP) columns. Enantiomers of 10 compounds were resolved by at least one of the 5 CSP's tested. Enantiomers of two other compounds, which have either one or two hydroxymethyl groups at the carbamoyl nitrogen, were either not resolved or resolved with very low efficiency. However, enantiomers of the hydroxymethyl derivatives were resolved via base-catalyzed dehydroxymethylation. *In vitro* metabolism of racemic CMZ by human liver microsomes was found to be enantioselective. Major metabolites were isolated by normal-phase and reversed-phase HPLC and further characterized by ultraviolet absorption and circular dichroism spectral analyses, and by chiral stationary phase HPLC analysis. Following an *in vitro* incubation of rac-CMZ, the unmetabolized CMZ was found to be enriched in (*S*)-CMZ, indicating that the *R*-enantiomer was enantioselectively metabolized. Metabolites were formed primarily by hydroxylation and demethylation of the methyl groups at the C3 side chain. All metabolites were found to be optically active, enriched in either the *S*-enantiomer or the *R*-enantiomer.

INTRODUCTION

Camazepam (CMZ; see Fig. 1 and Table I for structures and abbreviations) possesses anxiolytic, anticonvulsant, antihypermotility, muscle-relaxant and hypnotic properties in animals with very low toxicity [1,2]. CMZ is clinically used as a racemate. Early studies suggested that oxazepam (OX) and temazepam (TMZ) were the major metabolites formed *in vivo* [3]. More recent studies indicated, however, that the major metabolites formed in animals and humans were derived by stepwise hydroxylation and demethylation of the side chain at the C3 position



Diazepam (DZ), X=H
Temazepam (TMZ), X=OH
Camazepam (CMZ), X=OCON(CH_3)$_2$

Nordiazepam (NDZ), X=H
Oxazepam (OX), X=OH
Norcamazepam (NCMZ), X=OCON(CH_3)$_2$

Fig. 1. Structures of diazepam (DZ), temazepam (TMZ), camazepam (CMZ), nordiazepam (NDZ), oxazepam (OX), and norcamazepam (NCMZ). Structures and abbreviations for carbamoyloxy derivatives of OX and TMZ are indicated in Table I.

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TABLE I

CSP-HPLC RESOLUTION OF CMZ AND ITS DERIVATIVES

Chemical[a]	CSP[b]	Substituent at[c]		Mobile phase[d]	k_1' [e]	α [f]	R_s [f]
		N1	C3				
M2 (CMZ)	OA-3100	CH_3	$OCON(CH_3)_2$	D20P2	7.60 (*R*)	1.29	1.80
	Chiralcel OC			E25	9.11 (*R*)	1.13	0.50
	(*S*)-DNBL-C			D10EA2	3.56 (*R*)	1.06	0.75
	(*R*)-DNBPG-C			D10EA1	8.56	1.00	0
	(*R*)-DNBPG-I			D10EA2	6.43	1.00	0
M4′ (NCMZ)	OA-3100	H	$OCON(CH_3)_2$	D20EA6	1.76 (*R*)	1.33	2.42
	Chiralcel OC			D20EA3	2.69 (*R*)	1.23	2.08
	Chiralcel OC			E35	4.64 (*R*)	1.58	1.46
	(*S*)-DNBL-C			D20EA5	1.22 (*S*)	1.87	3.66
	(*R*)-DNBPG-C			EA7	5.91 (*R*)	1.19	2.65
	(*R*)-DNBPG-I			D20EA2	7.00 (*R*)	1.07	0.91
M4	OA-3100	CH_3	$OCONH(CH_3)$	D20EA6	2.41 (*S*)	1.51	2.65
	Chiralcel OC			E35	13.99 (*S*)	1.15	0.56
	(*S*)-DNBL-C			D10EA2	11.7 (*R*)	1.07	0.53
	(*R*)-DNBPG-C			D20EA2	3.74 (*R*)	1.17	1.52
	(*R*)-DNBPG-I			D10EA2	18.3 (*R*)	1.07	0.70
M7′	OA-3100	H	$OCONH(CH_3)$	D20EA5	4.97 (*S*)	1.15	0.95
	Chiralcel OC			E35	6.49 (*R*)	1.41	1.17
	(*S*)-DNBL-C			D20EA5	2.39 (*S*)	1.39	2.57
	(*R*)-DNBPG-C			D20EA4	5.71 (*R*)	1.25	2.56
	(*R*)-DNBPG-I			D20EA5	5.74 (*R*)	1.06	0.65
M5	OA-3100	CH_3	$OCON(CH_3)CH_2OH$	D20EA1	16.3 (*R*)	1.11	0.69
	Chiralcel OC			E25	9.60	1.00	0
	(*S*)-DNBL-C			D10EA2	9.82	1.00	0
	(*R*)-DNBPG-C			D10EA2	11.4	1.00	0
	(*R*)-DNBPG-I			D10EA2	16.5	1.00	0
M8	OA-3100	H	$OCON(CH_3)CH_2OH$	D20EA3	7.11 (*R*)	1.19	1.35
	Chiralcel OC			E35	3.28 (*R*)	1.36	1.10
	(*S*)-DNBL-C			D20EA5	2.20 (*S*)	1.65	3.23
	(*R*)-DNBPG-C			D20EA3	5.61 (*R*)	1.15	1.18
	(*R*)-DNBPG-I			D20EA5	6.08 (*R*)	1.04	0.35
M7	OA-3100	CH_3	$OCONH_2$	D20EA5	7.16 (*S*)	1.57	3.10
	Chiralcel OC			E35	18.2 (*S*)	1.11	0.38
	(*S*)-DNBL-C			D20EA5	3.75 (*R*)	1.27	2.29
	(*R*)-DNBPG-C			D20EA3	6.82 (*R*)	1.12	1.23
	(*R*)-DNBPG-I			D20EA7	2.88 (*R*)	1.06	0.68
M9″	OA-3100	H	$OCONH_2$	D20EA5	10.70 (*S*)	1.25	1.60
	Chiralcel OC			E35	10.6	1.00	0
	(*S*)-DNBL-C			D20EA8	2.70 (*S*)	1.15	1.00
	(*R*)-DNBPG-C			D20EA5	6.10 (*R*)	1.20	2.18
	(*R*)-DNBPG-I			D20EA6	6.60 (*S*)	1.06	0.60
M9	OA-3100	CH_3	$OCONH(CH_2OH)$	D20EA8	4.10 (*S*)	1.48	2.48
	Chiralcel OC			E50	8.87 (*S*)	1.48	1.14
	(*S*)-DNBL-C			D20EA5	3.95 (*R*)	1.08	0.72
	(*R*)-DNBPG-C			D20EA3	8.29 (*R*)	1.11	1.31
	(*R*)-DNBPG-I			D20EA7	5.52 (*R*)	1.08	0.85
M10	OA-3100	H	$OCONH(CH_2OH)$	D20EA5	12.71 (*S*)	1.33	1.96
	Chiralcel OC			E35	7.42 (*R*)	1.20	0.55
	(*S*)-DNBL-C			D20EA5	5.89 (*S*)	1.28	2.20

TABLE I (*continued*)

CSP-HPLC RESOLUTION OF CMZ AND ITS DERIVATIVES

Chemical[a]	CSP[b]	Substituent at[c]		Mobile phase[d]	k_1'[e]	α[f]	R_s[f]
		N1	C3				
	(*R*)-DNBPG-C			D20EA6	4.03 (*R*)	1.17	1.64
	(*R*)-DNBPG-I			D20EA8	8.75 (*R*)	1.08	0.90
M9′	OA-3100	CH_3	$OCON(CH_2OH)_2$	D20EA2	24.3	1.00	0
	Chiralcel OC			E35	7.62	1.00	0
	(*S*)-DNBL-C			D20EA3	10.8	1.00	0
	(*R*)-DNBPG-C			D20EA4	7.38	1.00	0
	(*R*)-DNBPG-I			D20EA5	11.9	1.00	0
M10′	OA-3100	H	$OCON(CH_2OH)_2$	D20EA3	26.8	1.00	0
	Chiralcel OC			E35	3.32 (*R*)	1.25	0.56
	(*S*)-DNBL-C			D20EA5	7.69 (*S*)	1.36	2.75
	(*R*)-DNBPG-C			D20EA5	8.35 (*R*)	1.10	1.08
	(*R*)-DNBPG-I			D20EA10	5.35	1.00	0

[a] Abbreviations of various CMZ derivatives are adopted from Morino and co-workers [5,6].
[b] Various CSP's are described in the Experimental section.
[c] Substituents at N1 and C3 of NDZ are indicated (see Fig. 1).
[d] Abbreviations of mobile phases are described in Experimental.
[e] k_1' is the capacity factor for the early eluting enantiomer; absolute configuration is indicated in parentheses. Hold-up time (t_o) was determined using acetone and it was 2.34 min for OA-3100 column (flow rate 1 ml/min) and 2.43 min for other columns (flow rate 2 ml/min).
[f] α and R_s are selectivity and resolution value, respectively.

[4–7]. Several metabolites of CMZ are pharmacologically active [8].

The (+)-CMZ was reported to be 14-fold more potent in the *in vitro* binding to benzodiazepine receptors of bovine brain than the (−)-CMZ [9]. The (+)-enantiomers of 3-substituted 1,4-benzodiazepines have been found to correspond to the *S*-enantiomers [10,11]. Absolute configurations of enantiomeric 3-substituted 1,4-benzodiazepines can be assigned according to their circular dichroism (CD) spectral properties [10–13]. A recent report indicated that (*R*)-CMZ was metabolized at a faster rate than the (*S*)-CMZ by rat liver microsomes [14]. This report describes the resolution of enantiomeric pairs of CMZ and 11 of its derivatives and the enantioselective metabolism of rac-CMZ by human liver microsomes. The results indicated that the pharmacologically less active (*R*)-CMZ was selectively metabolized.

EXPERIMENTAL

Materials

OX and TMZ were generously provided by Wyeth-Ayerst Research (Princeton, NJ, USA) and Sandoz (East Hanover, NJ, USA), respectively. Racemic CMZ and NCMZ were synthesized by reaction of TMZ and OX with N,N-dimethylcarbamyl chloride (Aldrich, Milwaukee, WI, USA) in pyridine, respectively [15]. Optically pure enantiomers of NCMZ were prepared by separation on a preparative CSP column as described [15]. Optically pure CMZ enantiomers were synthesized by methylation of optically pure NCMZ enantiomers, respectively, in 0.1 *M* NaOH–ethanol (1:1, v/v) with 10% (by volume) of dimethyl sulfate. Other CMZ derivatives used in this study were generously provided by Dr. Akio Nakamura and Dr. Akira Morino of Nippon Shinyaku (Kyoto, Japan). HPLC grade

solvents were purchased from Mallinckrodt (Paris, KY, USA).

Base-catalyzed dehydroxymethylation

An N-hydroxymethyl derivative was converted to a N–H derivative by treatment with NaOH as described previously [16]. M5 was converted to M4 in *ca.* 99% yield in ethanol–0.1 *M* NaOH (1:1, v/v) at room temperature for 10 min. M9′ was converted to a mixture of M7 and M9 in a ratio of *ca.* 4:1 in ethanol–0.01 *M* NaOH (1:1, v/v) at 35°C for 10 min. The reaction mixture was neutralized, followed by extraction with ethyl acetate. M7 and M9 were separated by normal-phase HPLC as described below.

HPLC

HPLC was performed using a Waters (Milford, MA, USA) Model M45 solvent pump and a Kratos (Ramsey, NJ , USA) Spectraflow 757 UV–vis variable wavelength detector. Samples were injected via a Shimadzu (Kyoto, Japan) Model SIL-9A automatic sample injector. The detector signal was recorded via MacIntegrator (a hardware and software package from Rainin Instruments, Emerysville, CA, USA) on a Macintosh Classic II computer (Apple Computer, Cupertino, CA, USA). Sample analysis was conducted at room temperature.

Abbreviations for the names of solvents are: D = dioxane, P = 2-propanol, E = ethanol, A = acetonitrile, and EA = ethanol–acetonitrile (2:1, v/v). Examples of hexane-based solvent mixtures used as mobile phases in various HPLC analyses are (the percentage of hexane in the solvent mixture is not indicated in the abbreviation): E35, ethanol–hexane (35:65, v/v); D20EA5, dioxane–EA–hexane (20:5:75, v/v/v); D20P2, dioxane–2-propanol–hexane (20:2:88, v/v/v); EA15, EA–hexane (15:85, v/v). Compositions of other mobile phases are similarly abbreviated.

Reversed-phase HPLC

A Vydac C18 column (5 μm particles, 25 cm × 4.6 mm I.D., catalog no. 201TP54; The Separations Group, Hesperia, CA, USA) was used. The mobile phase was acetonitrile–0.02 *M* phosphate buffer pH 7 (40:60, v/v) at a flow rate of 1 ml/min.

Normal-phase HPLC

A DuPont (DuPont Instruments, Wilmington, DE, USA) Zorbax SIL column (25 cm × 9.4 mm I.D.) was used. The mobile phase was EA15 at a flow rate of 2.5 ml/min.

Chiral stationary phase HPLC

The following five CSP columns were used in this study: (1) A column packed with covalently bonded N-3,5-dinitrophenylaminocarbonyl-(*S*)-valine [Sumipax OA-3100, DNPAC-(S)-V, 15 cm × 4.6 mm I.D.; Regis, Morton Grove, IL, USA]; (2) a column packed with cellulose coated with trisphenylcarbamate (Chiralcel OC, 25 cm × 4.6 mm I.D.; Chiral Technologies, Exton, PA, USA); (3) a column packed with covalently bonded (*S*)-N-(3,5-dinitrobenzoyl)leucine (5 μm particles; 25 cm × 4.6 mm I.D.; Rexchrom Pirkle covalent L-leucine, Regis); (4) a column packed with covalently bonded (*R*)-N-(3,5-dinitrobenzoyl)leucine (5 μm particles; 25 cm × 4.6 mm I.D.; Hi-Chrom Pirkle covalent leucine, Regis); and (5) a column packed with ionically bonded (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine (5 μm particles; 25 cm × 4.6 mm I.D.; Hi-Chrom Pirkle Type 1-A, Regis). These CSP columns are referred to as OA-3100, Chiralcel OC, (*S*)-DNBL-C, (*R*)-DNBPG-C, and (*R*)-DNBPG-I, respectively. Various mobile phases were used and these are indicated in Table I. Enantiomer ratios of metabolically formed M5 and M9′ were determined as those of M4 and M7/M9, respectively, following conversion in an alkaline solution as described above.

Incubation of CMZ with human liver microsomes

A human liver microsomal preparation was generously provided by Dr. F. Peter Guengerich of Vanderbilt University (Nashville, TN) and was prepared from a 30-year-old female who died of head injury in a motor vehicle accident. Microsomal protein was determined by the method of Lowry *et al.* [17] with bovine serum albumin as the protein standard. CMZ was incubated in a 7-ml reaction mixture. Each ml contained 0.1 mmol of Tris–HCl (pH 7.5), 3 μmol of $MgCl_2$, 4 mg protein equivalent of human liver microsomes, 0.2 NADP unit of

glucose 6-phosphate dehydrogenase (Type XIV, Sigma, St. Louis, MO, USA), 0.2 mg of $NADP^+$, 4 μmol of glucose 6-phosphate, and 120 nmol of rac-CMZ. The mixture was incubated for 1 h at 37°C and incubation was stopped by the addition of acetone (7 ml). CMZ and its metabolites were extracted by the addition of chloroform (14 ml). After low-speed centrifugation, the organic phase was evaporated to dryness with a stream of nitrogen at *ca.* 40°C.

Spectral analysis

UV–visible absorption spectra of samples in acetonitrile were determined using a 1-cm path length quartz cuvette on a DW2000 UV–vis scanning spectrophotometer (slit 2 nm and scan rate 2 nm/s; SLM Instruments, Urbana, IL, USA). CD spectra of samples in acetonitrile contained in a quartz cell of 1 cm path length at room temperature (23 ± 1°C) were measured using a Jasco Model 500A spectropolarimeter equipped with a Model DP500 data processor (Japan Scientific, Tokyo, Japan). The concentration of the sample is indicated by $A_{\lambda 2}$/ml (absorbance units at wavelength λ2 per ml of solvent). CD spectra are expressed by ellipticity ($\Phi_{\lambda 1}/A_{\lambda 2}$, in millidegrees) for solutions that have an absorbance of $A_{\lambda 2}$ unit per ml of solvent at wavelength λ2 (usually the wavelength of maximal absorption).

RESULTS AND DISCUSSION

CSP-HPLC resolution of enantiomers

Enantiomer separation of CMZ and 11 of its derivatives was tested on five CSP columns using a variety of mobile phase compositions (Table I). The mobile phase composition that gave the best combination of k' and R_s values (*i.e.*, small k' and large R_s) for each compound is listed in Table I. The performance of CSP columns deteriorated gradually due to frequent use. The R_s values in Table I are the highest numbers obtained during the course of this investigation using the indicated mobile phase compositions.

The major observations are summarized as follows: (1) The OA-3100, (*S*)-DNBL-C, and (*R*)-DNBPG-C columns provided enantiomeric separation for most, but not all, compounds tested. Chiralcel OC and (*R*)-DNBPG-I columns did not afford efficient enantiomer separation for the majority of compounds tested. (2) Relative to other compounds, enantiomers of M4′ (NCMZ) were efficiently resolved by all five CSP's tested. (3) The enantiomers of M5 were very poorly resolved on the OA-3100 column and were not resolved at all on the other CSP columns. When the hydroxymethyl group of M5 was removed by treatment with NaOH, enantiomers of the resulting M4 could be efficiently resolved on either OA-3100 or (*R*)-DNBPG-C column. (4) The enantiomers of M9′ were not resolved by any of the 5 CSP columns. The hydroxymethyl group of M9′ could be removed by treatment with NaOH to form both M7 and M9. Enantiomers of M7 were efficiently resolved on either the OA-3100 or the (*S*)-DNBL-C column. Enantiomers of M9 were efficiently resolved on the OA-3100 column. (5) The enantiomeric pairs of compounds with a hydrogen at the N1 position were resolved on either the (*S*)-DNBL-C or the (*R*)-DNBPG column (the Pirkle columns). The role of N1 hydrogen of 3-substituted 1,4-benzodiazepines in enantiomer separation was reported by Pirkle and Tsipouras [11]. Enantiomers of some compounds that have a methyl group at N1 position (such as M2, M4, M7, and M9) were also resolved on the Pirkle columns with varying efficiencies.

Metabolism of racemic CMZ by human liver microsomes

The metabolites formed in the *in vitro* incubation of rac-CMZ with human liver microsomes were analyzed by reversed-phase HPLC (Fig. 2). The incubation conditions were chosen such that sufficient amounts of metabolites were formed to allow stereochemical characterization. Metabolites contained in chromatographic peaks marked in Fig. 2 were identical to the corresponding authentic compounds with respect to their retention times on reversed-phase HPLC and ultraviolet absorption spectral properties. The relative areas under the chromatographic peaks (expressed in percentages of the total) of CMZ and its metabolites, detected at 254 nm, were: M9′ (6.5), M9 (1.7), M7 (0.6), NCMZ (1.0), M5 (33.6), M4 (15.1), and CMZ (41.5). The dis-

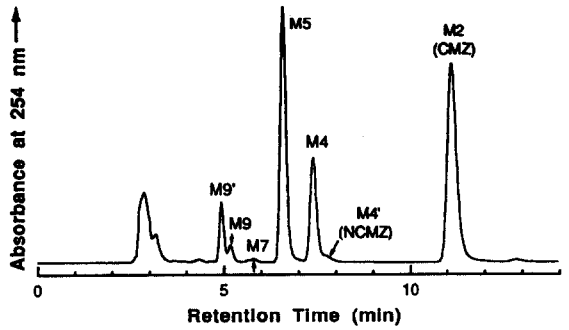

Fig. 2. Reversed-phase HPLC separation of CMZ and its human liver microsomal metabolites. Identities of metabolites contained in each chromatographic peak are indicated. OX and TMZ were coeluted with M9 and M5, respectively. Unmarked peaks were primarily derived from organic solvents and/or human liver microsomes. Chromatographic conditions are described in Experimental.

tribution of metabolites was similar to that observed in the metabolism of rac-CMZ by rat liver microsomes [14]. OX and TMZ cochromatographed with M9 and M5, respectively. However, normal-phase HPLC analysis (Fig. 3) of M9 and M5, collected from reversed-phase HPLC, indicated that both OX and TMZ were minor products. The results indicated that N1-desmethylation of CMZ derivatives was a minor metabolic pathway.

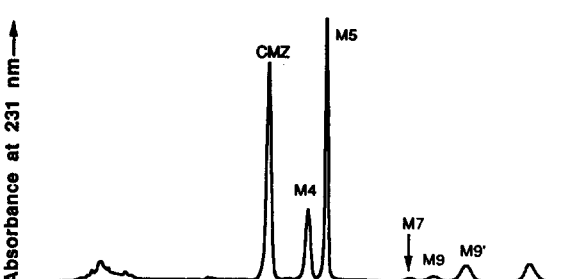

Fig. 3. Normal-phase HPLC separation of CMZ and its human liver microsomal metabolites. The sample was identical to that shown in Fig. 2. TMZ was eluted slightly (*ca.* 0.1 min) ahead of M4. OX was eluted between M4 and M5. Unmarked peaks were primarily derived from organic solvents and/or human liver microsomes. Chromatographic conditions are described in Experimental.

Stereochemical characterization of metabolites

The unmetabolized CMZ and metabolites formed in the incubation of rac-CMZ with human liver microsomes were isolated by normal-phase HPLC (Fig. 3) for mass spectral, CD spectral, and CSP-HPLC analyses. Isolation of metabolites by normal-phase HPLC was necessary due to thermolability of some metabolites [6,7,14]. The solvents in fractions collected in normal-phase HPLC operation could be readily evaporated, thus avoiding procedures that could cause decomposition of thermolabile metabolites. M4, M5, M7, M9, and M9′ formed metabolically *in vivo* and *in vitro* have been characterized by mass spectral analysis [6,7,14].

CD spectral analysis of the unmetabolized CMZ following an *in vitro* incubation of rac-CMZ with human liver microsomes indicated that it was enriched in the *S*-enantiomer (Fig. 4A); the *R*:*S* enantiomer ratio was 24.1:75.9. CSP-HPLC analysis of the unmetabolized CMZ (chromatogram not shown) gave an *R*:*S* enantiomer ratio of 23.8:76.2. These results are interesting in view of an earlier finding indicating that the (+)-(*S*)-CMZ is 14-fold more potent than

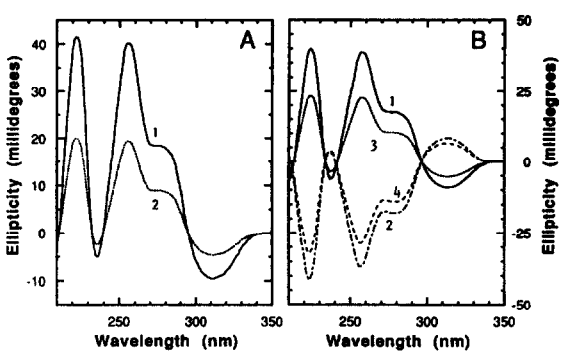

Fig. 4. (A) CD spectra (Φ/A_{231}, in millidegrees) of optically pure (S)-CMZ (1, $\Phi_{224}/A_{231} = 39.9$ millidegrees) and the unmetabolized CMZ (2) isolated from a mixture of products formed by incubation of rac-CMZ with human liver microsomes. (B) CD spectra (Φ/A_{231}, in millidegrees) of optically pure (S)-M4 (1), optically pure (R)-M5 (2, $\Phi_{223}/A_{231} = 40.5$ millidegrees), and metabolically formed M4 (3) and M5 (4) isolated from a mixture of products formed by incubation of rac-CMZ with human liver microsomes. The CD spectrum of an optically pure (*S*)-M5 was derived from an M5 with known optical purity.

the (−)-(*R*)-CMZ in binding to benzodiazepine receptors [9].

CD spectral analysis (Fig. 4B) of the most abundant metabolite M5 indicated that it was enriched in the *R*-enantiomer (*R*:*S* = 89.5:10.5). The metabolically formed M5 was converted to M4 in an alkaline solution; the resulting M4 was found to have an *R*:*S* enantiomer ratio of 91.2:8.8 by CSP-HPLC analysis (Fig. 5B). The finding that the metabolically formed M5 was highly enriched in the *R*-enantiomer was consistent with the enantiomeric composition of the unmetabolized CMZ described above. The pharmacological activities of rac-M5 was reported to be similar to those of rac-CMZ [4,5]. The implication of (*R*)-enantioselective metabolism of rac-CMZ to form M5 enriched in the *R*-enantiomer is unknown because the relative pharmacological activities of M5 enantiomers have not been studied to date.

CD spectral analysis (Fig. 4B) of the second most abundant metabolite M4 indicated that it was enriched in the *S*-enantiomer (*R*:*S* = 20.7:79.3). CSP-HPLC analysis of the metabolically formed M4 (Fig. 5A; *R*:*S* = 19.2:80.8) gave a consistent result. M4 was formed from M5 by dehydroxymethylation [5,6]. Since the metabolically formed M5 was enriched in the *R*-enantiomer, it appeared that the dehydroxymethylation

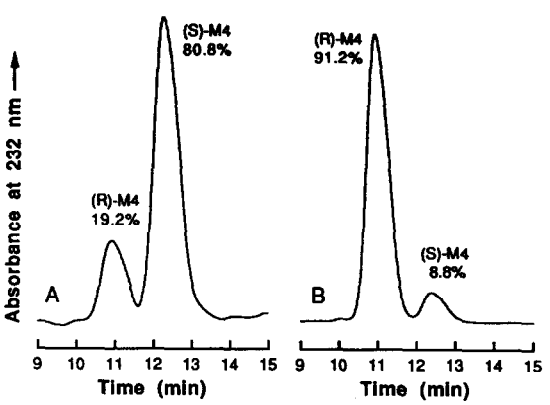

Fig. 5. CSP-HPLC analysis of metabolically formed M4 (A) and M4 derived by NaOH treatment of metabolically formed M5 (B). Metabolites were isolated by NP-HPLC as shown in Fig. 3. Chromatographic condition for CSP-HPLC and base-catalyzed conversions of M5 to M4 are described in Experimental.

of M5 was highly selective toward the *S*-enantiomer.

The *R*:*S* enantiomer ratios of the metabolically formed M7 and M9 were 62.9:37.1 and 34.6:65.4, respectively, by CSP-HPLC analysis (chromatograms not shown). The enantiomer ratio of the metabolically formed M9′ was determined by an indirect method, similar to that described for M5 above. The metabolically formed M9′ was first converted to a mixture of M7 and M9 in an alkaline solution. Enantiomeric pairs of the resulting M7 and M9 were then determined by CSP-HPLC (chromatograms not shown). These data established the *R*:*S* ratio of 88.7:11.3 for the metabolically formed M9′.

Metabolic pathways

Pathways in the enantioselective metabolism of rac-CMZ by human liver microsomes are proposed in Fig. 6. The stepwise hydroxylation and N-desmethylation pathways depicted in Fig. 6 are similar to those reported by Morino and colleagues [4–6]. The results of this study were similar to those of a study using rat liver microsomes [14] and indicated that N-desmethylation at the N1 position was a minor pathway in the metabolism of rac-CMZ. In mice, rats, monkeys, and dogs, significant amounts of N1-desmethylated metabolites were formed following either intravenous or oral administration of rac-CMZ [4,6]. NCMZ (M4′) was a minor metabolite in all animal species examined [4,6]. Thus the *in vivo* N1-desmethylated metabolites observed earlier [4,6] were likely formed from the N1-methylated metabolites such as M4, M5, M7, M9, and M9′.

The enantiomer ratios of the remaining CMZ and the metabolites formed following an *in vitro* incubation of rac-CMZ with human liver microsomes are shown in Fig. 6. The (*R*)-CMZ was apparently metabolized at a faster rate than the (*S*)-CMZ. M5 was formed by hydroxylation of a methyl group at the carbamoyl nitrogen of CMZ and was highly enriched in the *R*-enantiomer. M5 underwent both a hydroxylation reaction at the second methyl group to form M9′ and a dehydroxymethylation reaction (probably via an aldehyde intermediate) to form M4. The enantiomer ratio of M9′ (*R*:*S* = 89:11) was similar

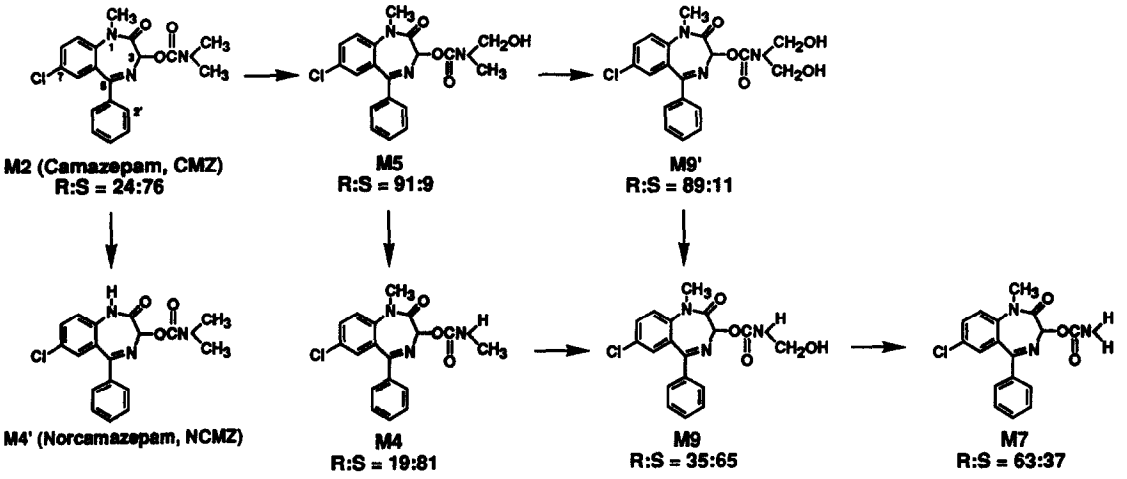

Fig. 6. The proposed major pathways in the enantioselective metabolism of rac-CMZ by human liver microsomes. The *in vitro* incubation condition and the methods in the determination of *R*:*S* enantiomer ratios are described in Experimental. The *R*:*S* enantiomer ratios of unmetabolized CMZ and the various metabolites formed are dependent on the percentage of substrate (CMZ) metabolized. See text for discussion.

to that of M5 (*R*:*S* = 91:9). In contrast, M4 was highly enriched in the *S*-enantiomer (*R*:*S* = 19:81). The results suggested that the dehydroxymethylation reaction of M5 was selective toward the *S*-enantiomer. The dehydroxymethylation reaction of M5, catalyzed by rat liver microsomes, was also found to be selective toward the *S*-enantiomer [14]. Although M9 was found to be enriched in the *S*-enantiomer, it was not possible to conclude the exact enantioselectivity of its formation because M9 could be derived by either dehydroxymethylation of M9′ or hydroxylation of the carbamoyl methyl group of M4. The M9 to M7 pathway appeared to be *R*-enantioselective. Additional insights may be obtained using individual enantiomers as the substrate. The enantioselective pathways indicated in Fig. 6 may be altered in the presence of phase II enzymes and cofactors for conjugation.

CONCLUSIONS

Enantiomeric pairs of CMZ and 11 of its derivatives were resolved, either directly or indirectly by prior treatment of the analyte in an alkaline solution, by CSP-HPLC. CSP-HPLC and spectropolarimetry were employed to characterize the enantiomer ratios of the major products formed in the metabolism of rac-CMZ by human liver microsomes. The pharmacologically more active (*S*)-CMZ was metabolized at a slower rate than the pharmacologically less active (*R*)-CMZ. The most abundant metabolite (M5) was formed by hydroxylation at a methyl group of the C3 carbamoyloxy side chain of CMZ and was highly enriched in the *R*-enantiomer. The second most abundant metabolite (M4) was formed by dehydroxymethylation of M5 and was highly enriched in the *S*-enantiomer. Other metabolites were also found to be optically active. The clinical implications of these enantioselective metabolic pathways remain to be established.

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