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Enantioselective metabolism of camazepam by rat liver microsomes*

XIANG-LIN LU and SHEN K. YANG†

Department of Pharmacology, F. Edward Hébert School of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20814-4799, USA

Abstract: Camazepam [3-(*N*,*N*-dimethyl)carbamoyloxy-7-chloro-1-methyl-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one, CMZ] possesses anxiolytic, anticonvulsant, muscle relaxant and hypnotic properties. CMZ is clinically used as a racemate. The enantioselective metabolism of racemic CMZ by rat liver microsomes was studied. Major metabolites were isolated by normal-phase and reversed-phase liquid chromatography (LC) and further characterized by UV absorption, mass, and circular dichroism spectral analyses, and by chiral stationary phase LC analysis. Following an *in vitro* incubation of rac-CMZ, the unmetabolized CMZ was found to be enriched in the (S)-CMZ, indicating that the R-enantiomer was enantioselectively metabolized. Two of the most abundant metabolites, formed by hydroxylation and demethylation of a methyl group of the *N*,*N*-dimethylcarbamyloxy side chain, were found to be enriched in the R-enantiomer. The results indicated that the (R)-CMZ was metabolized at a faster rate than (S)-CMZ by rat liver microsomes.

Keywords: *Camazepam; enantioselective metabolism; rat liver microsomes; mass spectral analysis; circular dichroism spectropolarimetry; achiral and chiral stationary-phase HPLC.*

Introduction

Camazepam (CMZ; see Fig. 1 and Table 1 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 that, however, the major metabolites formed in animals and humans were derived by stepwise hydroxylation and *N*-demethylation of the methyl groups at the C(3) side chain [4–7]. Many metabolites of CMZ were reported to be 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 correspond to the S-enantiomers [10, 11]. Possible enantioselective metabolic disposition of rac-CMZ has heretofore not been reported. This study examined the enantioselective oxidative metabolism of rac-CMZ by rat liver microsomes. The results indicated that the pharmacologically less active (R)-CMZ was selectively metabolized.

Materials and Methods

Materials

Oxazepam (OX) and temazepam (TMZ; see structures in Fig. 1) were generously provided by Wyeth-Ayerst Research (Princeton, NJ) and Sandoz, Inc. (East Hanover, NJ), respectively. Racemic CMZ and norcamazepam (NCMZ) were synthesized by reaction of TMZ and OX with *N*,*N*-dimethylcarbamyl chloride (Aldrich Chemical Co., Milwaukee, WI) in pyridine, respectively [12]. Optically pure enantiomers of NCMZ were prepared by separation on a preparative chiral stationary phase (CSP) column as described [12]. 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 sulphate. Other CMZ derivatives used in this study were generously provided by Drs Akio Nakamura and Akira Morino of Nippon Shinyaku Co., Ltd (Kyoto, Japan). HPLC grade solvents

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†Author to whom correspondence should be addressed.

Table 1
Retention times of CMZ and its derivatives on reversed-phase and normal-phase HPLC

Chemical abbreviation*	Substituent*		Retention time (min)	
	N(1)	C(3)	RP-HPLC†	NP-HPLC†
M10′	H	$OCON(CH_2OH)_2$	4.3	32.9
M10	H	$OCONH(CH_2OH)$	4.4	31.1
M9″	H	$OCONH_2$	4.8	28.6
M6 (OX)	H	OH	(5.1)	(21.4)
M8	H	$OCONCH_3(CH_2OH)$	5.3	22.9
M7′	H	$OCONH(CH_3)$	5.8	20.8
M4′ (NCMZ)	H	$OCON(CH_3)_2$	7.9	19.0
M9′	CH_3	$OCON(CH_2OH)_2$	5.0	31.6
M9	CH_3	$OCONH(CH_2OH)$	5.2	29.9
M7	CH_3	$OCONH_2$	5.9	28.3
M5	CH_3	$OCONCH_3(CH_2OH)$	6.7	22.8
M3 (TMZ)	CH_3	OH	(6.7)	(20.7)
M4	CH_3	$OCONH(CH_3)$	7.5	20.8
M2 (CMZ)	CH_3	$OCON(CH_3)_2$	11.4	18.9

*See Fig. 1 for structures. Abbreviations for various CMZ derivatives are adopted from Morino and associates [4, 6].
†Chromatographic conditions are described in Materials and Methods. RP and NP abbreviate for reversed-phase and normal-phase, respectively.

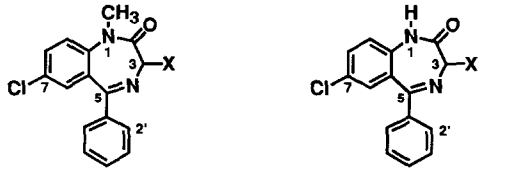

Figure 1
Structures of diazepam (DZ), temazepam (TMZ), camazepam (CMZ), nordiazepam (NDZ), oxazepam (OX) and norcamazepam (NCMZ). Structures and abbreviations for carbamoyloxy derivatives of OX and TMZ are indicated in Table 1.

were purchased from Mallinckrodt, Inc. (Paris, KY).

Base-catalysed dehydroxymethylation

A *N*-hydroxymethyl derivative was converted to a *N*-H derivative by treatment with NaOH similarly as described [13]. M5 was converted to M4 in ~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 ~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.

Chromatography

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

Reversed-phase LC. A Vydac C18 column (5 μm particles, 4.6 mm i.d. × 25 cm, catalog No. 201TP54; The Separations Group, Hesperia, CA) was used. Mobile phase was acetonitrile–0.02 M phosphate buffer (pH 7.0) (40:60, v/v) at a flow rate of 1 ml min^{-1}.

Normal-phase LC. A DuPont Zorbax SIL column (9.4 mm i.d. × 25 cm, DuPont Instruments, Wilmington, DE) was used. Mobile phase was ethanol–acetonitrile–hexane (10:5:85, v/v/v) at a flow rate of 2.5 ml min^{-1}.

Chiral stationary phase HPLC. Enantiomer compositions of CMZ, M7, and M9 were determined using a covalent (S)-*N*-(3,5-dinitrobenzoyl)leucine column (4.6 mm i.d. × 25 cm; Rexchrom Pirkle covalent L-leucine, Regis Chemical Co., Moton Grove, IL). The mobile phase for the separation of CMZ enantiomers was dioxane–ethanol–aceto-

nitrile–hexane (10:0.33:0.17:89.5, v/v/v/v) at a flow rate of 2 ml min^{-1}. The mobile phase for the separation of the enantiomers of both M7 and M9 was dioxane–ethanol–acetonitrile–hexane (10:3.33:1.67:85, v/v/v/v) at a flow rate of 2 ml min^{-1}. Enantiomer ratio of NCMZ was determined using a covalent (S)-*N*-(3,5-dinitrobenzoyl)leucine column (4.6 mm i.d. × 25 cm; Hi-Chrom Pirkle covalent leucine, Regis Chemical Co., Moton Grove, IL) with ethanol–acetonitrile–hexane (6.67:3.33:90, v/v/v) as the mobile phase at 2 ml min^{-1} [12]. Enantiomer ratio of M4 was determined using a covalent (R)-*N*-(3,5-dinitrobenzoyl)phenylglycine column (4.6 mm i.d. × 25 cm; Hi-Chrom Pirkle covalent phenylglycine, Regis Chemical Co., Moton Grove, IL) with dioxane–ethanol–acetonitrile–hexane (20:1.33:0.67:88, v/v/v/v) as the mobile phase at 2 ml min^{-1}. Enantiomer ratios of M5 and M9′ were determined as those of M4 and M7, respectively, following conversion in an alkaline solution as described above.

Incubation of CMZ with rat liver microsomes

Liver microsomes were prepared from phenobarbital-treated (80 mg kg^{-1}) male Sprague-Dawley rats (80–100 g) as previously described [14]. Microsomal protein was determined by the method of Lowry *et al.* [15] with bovine serum albumin as the protein standard. CMZ was incubated in a 25-ml reaction mixture. Each millilitre contained 0.1 mmol of Tris–HCl (pH 7.5), 3 μmol of $MgCl_2$, either 1 or 4 mg of protein equivalent of rat liver microsomes, 0.2 NADP unit of glucose 6-phosphate dehydrogenase (Type XIV, Sigma Chemical Co., St. Louis, MO), 0.2 mg of $NADP^+$, 4 μmol of glucose 6-phosphate, and either 200 nmol (for mixture containing 1 mg protein ml^{-1} or 120 nmol (for mixture containing 4 mg protein ml^{-1}) of rac-CMZ. The mixture was incubated at 37°C for either 40 min (for mixture containing 1 mg protein ml^{-1}) or 1 h (for mixture containing 4 mg protein ml^{-1}) and was stopped by the addition of 1 vol of acetone. CMZ and its metabolites were extracted by the addition of 2 vol of chloroform. After low speed centrifugation, the organic phase was removed and evaporated to dryness with a stream of nitrogen at ~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^{-1}; SLM Instruments, Inc., Urbana, IL).

Mass spectral analysis was performed on a JEOL Model JMS-SX102 mass spectrometer (JEOL Ltd, Tokyo, Japan). Fast atom bombardment (FAB) spectra were generated by a 8-keV xenon atom beam. Samples were dissolved in methanol–glycerol (1:3, v/v). Each sample (1 μl) was applied to the probe tip. Ten scans at 10 s decade were accumulated to obtain a spectrum. The resolution of the instrument was set to 1000. Between 10 and 20 scans were stored and accumulated in each analysis.

CD spectra of samples in acetonitrile 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 Co., Tokyo, Japan). The concentration of the sample is indicated by $A_{\lambda 2}$ ml^{-1} (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). Under conditions of measurements indicated above, the molar ellipticity ($[\theta]_{\lambda 1}$, in deg cm^2 $dmol^{-1}$) and ellipticity ($\Phi_{\lambda 1}/A_{\lambda 2}$, in millidegrees) are related to the molar extinction coefficient ($\epsilon_{\lambda 2}$, in cm^{-1} M^{-1}) as follows:

$$[\theta]_{\lambda 1} = 0.1\, \epsilon_{\lambda 2}\, (\Phi_{\lambda 1}/A_{\lambda 2}). \qquad (1)$$

Results and Discussion

Quantification and characterization of CMZ and its metabolites

Retention times of CMZ, TMZ, NCMZ, OX and the C(3) side chain derivatives on reversed-phase and normal-phase LC are shown in Table 1. In order to minimize potential confusion, we have adopted the abbreviations (Table 1) used by Morino and colleagues [4, 6] for CMZ and its derivatives. The retention times were determined on the same day using a single column and an identical mobile phase. Retention times varied slightly among chromatographic runs that were carried out on different days due to nonidentical column performance and mobile

phase. However, the relative elution order of analytes was unchanged. The results in Table 1 indicated that, when OX and TMZ were excluded for comparison, the relative polarity of CMZ derivatives with different substituents on the carbamoyl nitrogen was: CH_2OH > H > CH_3. This trend was the same among compounds with either a hydrogen or a methyl group at N(1). The elution order of compounds with an identical substituent on N(1) on reversed-phase LC was reversed from that on normal-phase LC.

The relative abundance of metabolites formed in the *in vitro* incubation of rac-CMZ with rat liver microsomes was determined by reversed-phase LC [Figs 2(A) and 2(B)]. The percentages of AUCs of CMZ and its metabolites are indicated in Table 2. The metabolite profiles shown in Figs 2(A) and 2(B) were obtained from samples using low (1 mg protein ml^{-1}) and high (4 mg protein ml^{-1}) concentration of rat liver microsomes, respectively, in the *in vitro* incubations. Both incubation conditions produced M4 and M5 as the most abundant metabolites. M9′ was less abundant than those of M4 and M5. M4′ (NCMZ), M7 and M9 were relatively minor products. Comparison of the relative AUCs of M4, M5 and M9′ in Figs 2(A) and 2(B) suggested that M4 and M9′ were derived from further metabolism of M5. OX and TMZ cochromatographed with M9′ and M5, respectively, in the reversed-phase LC analysis (Table 1). Normal-phase HPLC analysis (see below) of M9′ and M5 collected from reversed-phase LC (Fig. 2) indicated that both OX and TMZ were minor products. The results indicated that the N(1)-desmethylation of CMZ derivatives was a minor metabolic pathway.

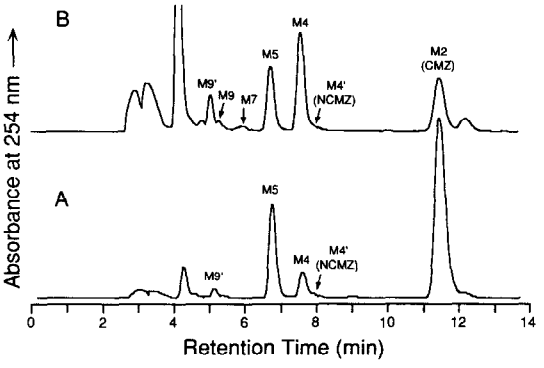

Figure 2
Reversed-phase HPLC separation of CMZ and its rat liver microsomal metabolites. Chromatogram A shows a metabolite profile derived from an incubation of rac-CMZ using a lower microsomal protein (1 mg ml^{-1}) concentration; ~31% of CMZ was metabolized. Chromatogram B shows a metabolite profile derived from an incubation of rac-CMZ using a higher microsomal protein (4 mg ml^{-1}) concentration; ~72% of CMZ was metabolized. Unmarked peaks were primarily derived from organic solvents and/or rat liver microsomes. Chromatographic conditions are described in Materials and Methods.

Because the hydroxymethyl derivatives such as M5 and M9′ are known to be thermolabile [6, 7] and because of the difficulty in isolating thermolabile metabolites from aqueous solutions, we have approached the isolation of major CMZ metabolites by normal-phase HPLC for further characterization. Figure 3 shows the normal-phase LC separation of CMZ and its major metabolites. The sample used in Fig. 3 was identical to that used in reversed-phase LC analysis [Fig. 2(B)]. The relative AUCs of CMZ and its metabolites

Table 2
Relative abundance and enantiomeric ratios of the major metabolites formed in the metabolism of rac-CMZ by rat liver microsomes

	Experiment 1*		Experiment 2†	
Abbreviation of metabolite	AUC (%)‡	R/S ratio§	AUC (%)‡	R/S ratio§
M2 (CMZ)	68.8	44.8:55.2	28.1	25.5:74.5
M4′ (NCMZ)	0.8	39.2:60.8	1.6	36.7:63.3
M4	6.58	61.0:39.0	36.4	56.7:43.3
M5	21.8	62.0:38.0	22.8	69.9:30.1
M7	0.14	58.4:41.6	1.22	53.1:46.9
M9	0.31	61.4:38.6	1.74	74.3:25.7
M9′	1.47	60.1:39.9	7.94	59.7:40.3

* In experiment 1, incubation was carried out using 1 mg protein equivalent of rat liver microsomes per ml of incubation mixture and the incubation time was 40 min.
† In experiment 2, incubation was carried out using 4 mg protein equivalent of rat liver microsomes per ml of incubation mixture and the incubation time was 60 min.
‡ Percentage of AUC (detected at 254 nm) relative to the total AUC of CMZ and all identifiable metabolites.
§ Enantiomer ratio determined by CD and/or CSP-LC.

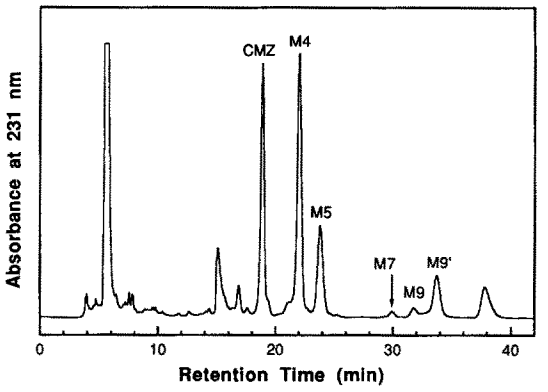

Figure 3
Normal-phase HPLC separation of CMZ and its rat liver microsomal metabolites. The sample was identical to that shown in Fig. 2(B). Unmarked peaks were primarily derived from organic solvents and/or rat liver microsomes. Chromatographic conditions are described in Materials and Methods.

(Table 2) obtained from Figs 2(B) and 3 were consistent. Unmetabolized CMZ and the major metabolites were isolated by repetitive chromatography for further characterization. In order to preserve the stability of thermolabile metabolites, the solvent in the collected fractions was evaporated without heating by blowing with a gentle stream of nitrogen.

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 and normal-phase HPLC and UV absorption spectral properties. Metabolite M5 was reported to be thermolabile and decomposed to form M4 at room temperature [6]. The characterization of metabolites M4 and M5 by mass spectrometry has been reported previously [7].

The metabolically formed M7, M9 and M9′ were characterized by FAB mass spectrometry. Metabolite M7 had characteristic mass ions at *m/z* 344 (M + H), 366 (M + Na) and 436 (M + glycerol). Metabolite M9 had characteristic mass ions at *m/z* 374 (M + H), 356 (M − H_2O + H), and 466 (M + glycerol). A mass ion at *m/z* 344, which was the molecular ion (M + H) of M7, was also observed in the mass spectrum of M7. Metabolite M9′ had the expected characteristic mass ion at *m/z* 404 (M + H). In addition, metabolite M9′ had mass ions at *m/z* 374 and 356 which were characteristic mass ions of M9, and *m/z* 344 and 436 which were characteristic mass ions of M7. These results consistently indicated that the hydroxymethyl groups at carbamoyl nitrogen in various CMZ derivatives were thermolabile [6, 7].

Stereochemical characterization of metabolites

The remaining CMZ and metabolites formed in the incubations of rac-CMZ by low and high concentrations of rat liver microsomes (see Fig. 2 and Table 2) were isolated by normal-phase HPLC (Fig. 3) for CD spectral and CSP HPLC analyses. CD spectral analysis indicated that the unmetabolized CMZ remaining after incubations with high and low concentrations of rat liver microsomes were both enriched in the S-enantiomer [Fig. 4(A)]. The S/R enantiomer ratios (Table 2) increased as the percentage of CMZ metabolized increased [Fig. 4(A)]. CSP HPLC analysis of the unmetabolized CMZs (not shown) gave con-

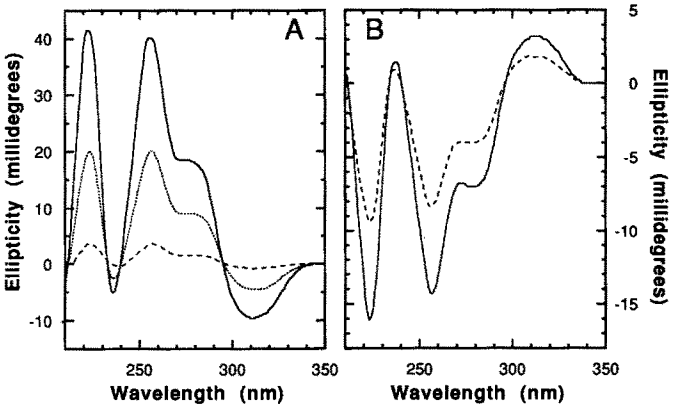

Figure 4
(A) CD spectra (Φ/A_{231}, in millidegrees) of optically pure (S)-CMZ (——) and the unmetabolized CMZ isolated from experiments 1 (---) and 2 (·····) shown in Table 2, respectively. (B) CD spectra (Φ/A_{231}, in millidegrees) of metabolically formed M5 isolated from experiments 1 (——) and 2 (---), respectively (see Fig. 2 and Table 2). See Fig. 2 for experimental conditions in experiments 1 and 2.

sistent results. CD spectral analysis of the most abundant metabolite M5 (Fig. 4B) indicated that it was enriched in the R-enantiomer and the R/S enantiomer ratio increased with increasing metabolism of CMZ. The enantiomeric composition of metabolically formed M5 (R > S) was consistent with the enantiomer composition of the remaining CMZ (S > R). The enantiomeric ratios of metabolically formed M5 were determined by CSP HPLC (see below). CD spectral analysis (not shown) also indicated that the metabolically formed M4 was enriched in the R-enantiomer. The enantiomer composition of metabolically formed M4 was determined by CSP HPLC (see below). The results described above indicated that the (R)-CMZ was metabolized at a faster rate than the (S)-CMZ by rat liver microsomes.

Enantiomeric pairs of M2 (CMZ), M4, M4′ (NCMZ), M7 and M9 could all be resolved on a CSP column. Enantiomers of M4, M4′ and M7 were resolved more efficiently than those of M2 and M9. Enantiomers of M5 and M9′ could not be resolved by any of the CSP columns employed in this study. Enantiomer compositions of metabolically formed M5 and M9′, both of which were major rat liver microsomal metabolites of rac-CMZ (Figs 2 and 3), were determined by an indirect method. The method involved base-catalysed conversion of M5 to M4 and of M9′ to M9. Since the enantiomeric pairs of both M4 and M9 could both be efficiently resolved, the enantiomer compositions of metabolically formed M5 and M9′ could therefore be determined. Examples in the determination of enantiomer compositions of metabolically formed M4, M5 and M9′ by CSP HPLC are shown in Fig. 5. Enantiomer ratio of metabolically formed M4 was directly determined (Fig. 5A). Enantiomer ratios of metabolically formed M5 and M9′ were determined as M4 [Fig. 5(B)] and M7 [Fig. 5(C)], respectively. Results on the enantiomer compositions of remaining CMZ and its major metabolites are summarized in Table 2.

Metabolic pathways

Pathways in the enantioselective metabolism of rac-CMZ by liver microsomes prepared from phenobarbital-treated Sprague-Dawley rats are shown in Fig. 6. The stepwise hydroxylation and *N*-demethylation pathways depicted in Fig. 6 are similar to those reported by Morino and colleagues [4–6]. The results of this study indicated that *N*-demethylation of the methyl group at the N(1) position was a minor pathway in the metabolism of rac-CMZ by rat liver microsomes. In mice, rats, monkeys and dogs, significant amounts of N(1)-desmethylated metabolites were formed following either i.v. 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* N(1)-desmethylated metabolites observed earlier [4, 6] were likely formed from the N(1)-desmethylated derivatives of M4, M5, M7, M9 and M9′.

Based on the enantiomer ratios of metabolites and the stepwise pathways of metabolism, the enantioselectivity in each of the pathway is indicated in Fig. 6. The initial and predominate hydroxylation reaction (CMZ → M5) was R-enantioselective, whereas the de-

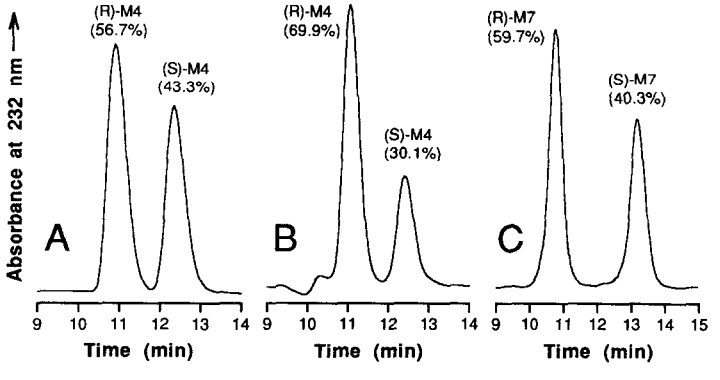

Figure 5
CSP HPLC analysis of metabolically formed M4 (panel A), M4 derived by NaOH treatment of metabolically formed M5 (panel B), and M7 derived by NaOH treatment of metabolically formed M9′ (panel C), respectively. Metabolites were derived from experiment 2 (see Table 2) and were isolated by NP-HPLC as shown in Fig. 3. Chromatographic conditions for CSP HPLC and base-catalysed conversions of M5 to M4 and M9′ to M7, respectively, are described in Materials and Methods.

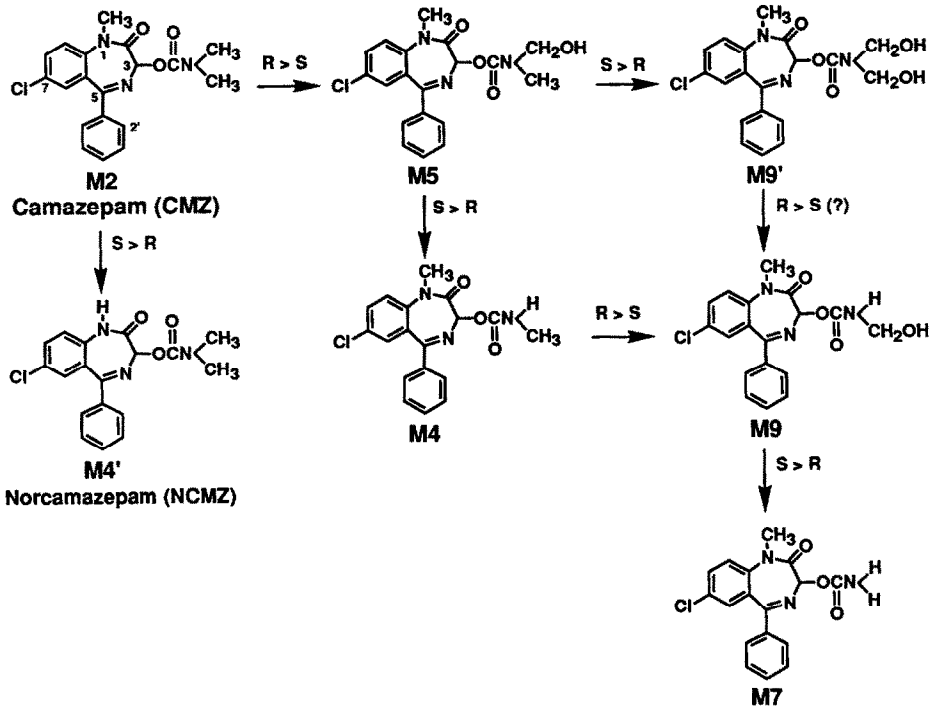

Figure 6
The major pathways in the enantioselective and stepwise metabolism of rac-CMZ by rat liver microsomes. The exact enantioselectivity in M9′ → M9 pathway could not definitively established. M9 was likely to be primarily derived from M4, the latter was one of the most abundant metabolites. See text for discussion.

hydroxymethylation of M5 (to form M4) appeared to be S-enantioselective. However, further hydroxylation at the carbamoyl *N*-methyl group of M5 appeared to be slightly S-enantioselective. The N(1)-desmethylation of CMZ was S-enantioselective. The stepwise enantioselecive metabolism of rac-CMZ resulted in unmetabolized CMZ highly enriched in the S-enantiomer, M5 highly enriched in the R-enantiomer, M4 slightly enriched in the R-enantiomer, and M9′ highly enriched in the R-enantiomer (Table 2).

Conclusions

The R-enantiomer of CMZ is enantioselectively metabolized by rat liver microsomes. The major metabolic reactions are enantioselective hydroxylation and the subsequent dehydroxymethylation reactions occurred at the *N*-methyl groups of the C(3) carbamoyloxy side chain of CMZ. The pharmacologically more active (S)-CMZ is metabolized at a slower rate than the pharmacologically less active (R)-CMZ.

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