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Achiral and chiral analysis of camazepam and metabolites by packed-column supercritical fluid chromatography

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Abstract

Supercritical fluid chromatography, using carbon dioxide as the mobile phase and ethanol as a modifier, has been applied to the analysis of products formed in rat liver microsomal metabolism of racemic camazepam, a hypnotic/anxiolytic drug in clinical use. An achiral (amino) column and a chiral (Chiralcel OD-H) column were used. The results suggest that achiral and chiral packed-column supercritical fluid chromatography gives a shorter analysis time and higher selectivity and efficiency than achiral and chiral stationary-phase high-performance liquid chromatography in the analysis of camazepam and its derivatives.

1. Introduction

Supercritical fluid chromatography (SFC) is the application of supercritical fluids as the mobile phase for the separation of analytes on a column [1–3]. Carbon dioxide (CO_2) is often the mobile phase of choice in SFC because it is inexpensive, non-toxic, non-flammable, and transparent in the ultraviolet region [1–3]. Compared to liquid mobile phases, SFC mobile phases have lower viscosity, lower density, and higher diffusion coefficients. A high diffusion coefficient allows faster equilibrium between the stationary phase and the analytes, yielding higher separation efficiency and reduced analysis time. Furthermore, the use of modifiers (e.g. alcohols) in the mobile phase allows the analysis of a wider range of chemicals with varying structure and polarity than does a mobile phase of pure CO_2.

Packed-column SFC is very similar to normal-phase liquid chromatography (LC) in terms of the chemical interactions responsible for separation. The higher diffusion coefficient and lower viscosity of supercritical fluids enables a 3- to 10-fold faster analysis than high-performance LC (HPLC) [4,5]. Packed chiral stationary-phase (CSP) columns have been used successfully in rapid resolution of enantiomers in SFC [5–12].

Camazepam (CMZ; see Fig. 1 and Table 1 for structures and abbreviations) possesses anxiolytic, anticonvulsant, antihypermotility, mus-

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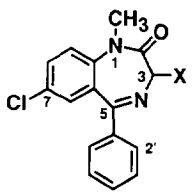
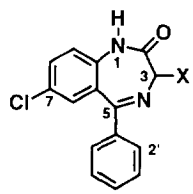


Temazepam (TMZ), X= OH
Camazepam (CMZ),
X = $OCON(CH_3)_2$



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

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

cle-relaxant and hypnotic properties in animals and shows a very low toxicity [13,14]. CMZ is clinically used as a racemate. The (*S*)-CMZ was reported to be 14-fold more potent in the in-vitro binding to benzodiazepine receptors of bovine brain than the (*R*)-CMZ [15]. Recent studies using CSP-HPLC analysis indicated that the pharmacologically less active (*R*)-CMZ was enantioselectively metabolized by rat [16] and human [17] liver microsomes, respectively. For the metabolically formed M4, M5, M7, M9, and M9′, the concentration of the (*R*)-enantiomer was always higher. A minor amount of metabolically formed M4′ (NCMZ) was enriched in the (*S*)-enantiomer, indicating that it was enantioselectively derived from *rac*-CMZ by enzymatic N-demethylation [16]. In a recent study [17], five different CSP columns were used in the HPLC separation of enantiomers of CMZ and its derivatives using various mobile phase compositions. The enantiomer of M5 (the most abundant metabolite of CMZ) [16,17] and M9′ were either poorly resolved or not resolved at all. In addition, with achiral chromatography, not all major metabolites were separated by either reversed-phase or normal-phase HPLC [16,17]. Hence, an improved achiral method is needed to quantify metabolites formed in either in-vitro or in-vivo metabolism of CMZ.

The goals of the present study were: (1) to apply SFC using an achiral (amino) column to obtain improved separation of CMZ and its major metabolites and (2) to employ a CSP column (Chiralcel OD-H) in SFC for the improved resolution of enantiomers of CMZ and its major derivatives. The results indicate that achiral and chiral packed-column SFC may be used in good advantage in both the quantification of metabolite formation and the determination of enantiomer composition of metabolites formed in in-vitro and in-vivo metabolism of some chiral and prochiral drugs.

2. Experimental

2.1. Materials

Racemic CMZ and NCMZ were synthesized by reaction of temazepam (TMZ) and oxazepam (OX) with N,N-dimethylcarbamyl chloride (Aldrich, Milwaukee, WI, USA) in pyridine, respectively [18]. Optically pure enantiomers of NCMZ were prepared by separation on a preparative CSP column as described previously [18]. 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) dimethyl sulfate. The optical purity of the synthesized CMZ enantiomers was confirmed by SFC on a chiral column (see below). Other CMZ derivatives used in this study were generously provided by Drs. Akio Nakamura and Akira Morino of Nippon Shinyaku Co. (Kyoto, Japan). SFC grade CO_2 was purchased from Scott Specialty Gases (Plumsteadville, PA, USA). Ethanol was purchased from Sigma (St. Louis, MO, USA) as a HPLC grade solvent.

2.2. Incubation of CMZ with rat liver microsomes

Liver microsomes were prepared from phenobarbital-treated (80 mg/kg) male Sprague–Dawley rats (80–100 g) as previously described [19]. Microsomal protein was determined by the method of Lowry et al. [20] with bovine serum albumin as the protein standard. CMZ was incubated in a 25-ml reaction mixture. Each ml contained 0.1 mmol of Tris-HCl (pH 7.5), 3 μmol of $MgCl_2$, rat liver microsomes (equivalent

to 4 mg of protein), 0.2 unit of glucose 6-phosphate dehydrogenase (Type XIV, Sigma), 0.2 mg of $NADP^+$, 4 μmol of glucose 6-phosphate, and 60 nmol of *rac*-CMZ. The mixture was incubated at 37°C for 1 h and the reaction was stopped by the addition of 1 volume of acetone. CMZ and its metabolites were extracted by the addition of 2 volumes of chloroform. After low speed centrifugation, the organic phase was removed, dehydrated with anhydrous $MgSO_4$, and evaporated to dryness with a stream of nitrogen at ~40°C. The residue was dissolved in methanol (2 ml) and divided into two equal halves; methanol was subsequently evaporated. Half of the residue was dissolved in 0.5 ml of acetonitrile–0.02 *M* phosphate buffer pH 7 (40:60, v/v) and analyzed by reversed-phase HPLC in our laboratory in Bethesda. The other half of the residue was frozen on dry ice and shipped by express mail to our laboratory in Wilmington for analyses by achiral and chiral SFC.

CMZ and its metabolites M4, M5, and M9′ were purified by reversed-phase HPLC from a mixture of products formed by incubation of *rac*-CMZ with rat liver microsomes (4 mg protein per ml of incubation mixture). These were analyzed for enantiomer purity by CSP-HPLC [16] in Bethesda and were also shipped to Wilmington for CSP-SFC analysis.

2.3. Reversed-phase HPLC

Reversed-phase HPLC was performed as described earlier [16,17], except that a different C_{18} column, a Zorbax SB-C_{18} column (25 cm × 4.6 mm I.D.; MAC MOD Analytical, Chadds Ford, PA, USA), was used. The analytical system consisted of a Waters Associates (Milford, MA, USA) Model M45 solvent pump and a Kratos (Kratos Analytical Instruments, Ramsey, NJ, USA) Model Spectraflow 757 UV-Vis variable-wavelength detector. The mobile phase was acetonitrile–0.02 *M* phosphate buffer pH 7 (40:60, v/v) at a flow-rate of 1.2 ml/min. 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, USA) on a Macintosh Classic II computer (Apple Computer, Cupertino, CA, USA). Sample analysis was performed at room temperature.

2.4. Achiral and chiral SFC

A Hewlett-Packard G1205A SFC equipped with a HP G1306A photodiode array detector, a modifier pump, a fixed-volume (5 μl) internal loop, and an automated sampler was used. Chromatographic data were collected with a Hewlett-Packard SFC 3D ChemStation. Achiral separation was performed using a LiChrosphere 5 μm amino (NH_2) column (25 cm × 4.6 mm I.D.; Alltech Associates, Deerfield, IL, USA). The mobile phase was CO_2 with ethanol as a modifier. The mobile phase gradient was 13% (v/v) ethanol for 4 min, followed by an increase of 3%/min of ethanol to 30% ethanol. Other conditions were: flow-rate, 2.5 ml/min; outlet pressure, 150 bar; oven temperature, 30°C; detection wavelength, 227 or 254 nm. Absorption spectra were routinely collected to confirm the identity of analytes. Samples were dissolved in methanol for SFC analysis.

Chiral SFC was conducted using a Chiralcel OD-H column (25 cm × 4.6 mm I.D.; Chiral Technologies, Exton, PA, USA). The mobile phase was CO_2 with ethanol as a modifier. Other conditions were: flow-rate, 2.5 ml/min; outlet pressure, 200 bar; oven temperature, 30°C; detection wavelength, 254 nm. Samples were dissolved in methanol.

3. Results and discussion

3.1. Reversed-phase HPLC

We have compared the results obtained with achiral SFC (Fig. 3) with those obtained with the previously reported reversed-phase HPLC [16] (Fig. 2) to determine which method yields the best separation. In both HPLC and achiral SFC analyses, the most abundant metabolites formed in rat liver microsomal metabolism of *rac*-CMZ

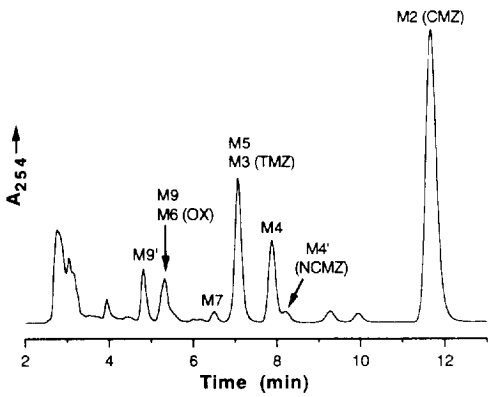


Fig. 2. Reversed-phase HPLC separation of CMZ and its rat liver microsomal metabolites. The column was a Zorbax SB-C_{18} (15 cm × 4.6 mm I.D.), and the mobile phase was acetonitrile–0.02 *M* phosphate buffer pH 7 (40:60, v/v) at a flow-rate of 1.2 ml/min.

were M4 and M5 (Figs. 2 and 3). Although M3 co-eluted with M5 (Fig. 2), normal-phase HPLC [16] and achiral SFC (Fig. 3) analysis indicated that M5 was the predominant product. In the absence of either rat liver microsomes or

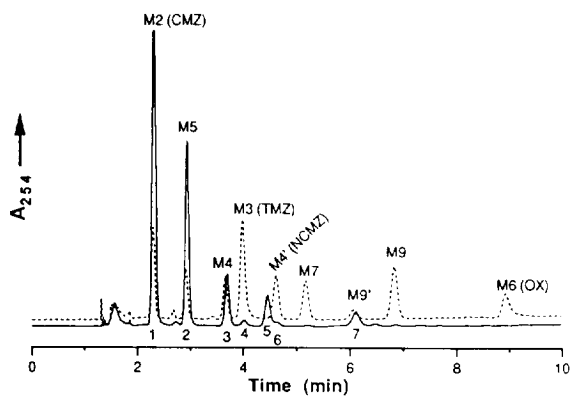


Fig. 3. Achiral SFC separation of CMZ and its derivatives. The dotted line represents a mixture of authentic compounds and the identities are indicated at the top of each chromatographic peak. The solid line represents a metabolite sample, identical to that analyzed in Fig. 2 following shipment from the Bethesda laboratory to the Wilmington laboratory. The metabolites are numbered at the base of each chromatographic peak. The column was a LiChrosphere NH_2 (25 cm × 4.6 mm I.D.). The column was eluted with 13% ethanol in CO_2 for 4 min, followed with a linear gradient from 13% to 30% ethanol at a rate of 3%/min. The flow-rate was 2.5 ml/min. The outlet pressure was 150 bar and the oven temperature was 30°C.

NADPH, metabolites were not formed. M5 is a metabolic precursor of M4 [16,17,21,22]. Prolonged incubation with a high protein concentration of rat liver microsomes lead to the formation of a higher amount of M4 relative to that of M5 [16]. M5 and M9′ are thermolabile [22,23] and they may be non-enzymatically converted to M4 and M7, respectively. M9′, M9, M7, M6 (OX), M4′ (NCMZ), and M3 (TMZ) were all minor products formed in rat liver microsomal metabolism of *rac*-CMZ [16,17,21,22].

3.2. Achiral SFC

Although reversed-phase HPLC analysis (Fig. 2) revealed the major products formed in rat liver microsomal metabolism of *rac*-CMZ, the analytical system was not ideal for the separation of all major metabolites that are formed in vivo [14,21,24,25]. In this study, we explored the feasibility of applying SFC to the separation of CMZ and its derivatives using a variety of achiral packed columns (including silica, cyano, amino, and diol). The amino column was found to be the most useful. The broken line curve in Fig. 3 illustrates the separation of CMZ and some of its authentic derivatives by SFC using an amino column. The metabolite sample which was analyzed by reversed-phase HPLC (Fig. 2) was also analyzed by achiral SFC (solid line curve in Fig. 3). The achiral SFC method showed a considerable improvement with respect to analysis time, selectivity, and efficiency. The chromatographic peaks of CMZ and its metabolites are numbered from 1 to 7 in Fig. 3.

In Fig. 3, metabolite peaks 1, 2, 3, and 7 were identical to M2 (CMZ), M5, M4, and M9′ with respect to their retention times and ultraviolet absorption spectra. Reproducible SFC retention times (Fig. 3) and ultraviolet absorption spectral (Fig. 4) analyses established that the minor metabolite peaks 4 and 6 were M3 (TMZ) and M4′ (NCMZ), respectively. M6 (OX) was not detectable, consistent with earlier findings [16]. Ultraviolet absorption spectra of metabolite peak 5 indicated that this compound did not have absorption characteristics similar to those

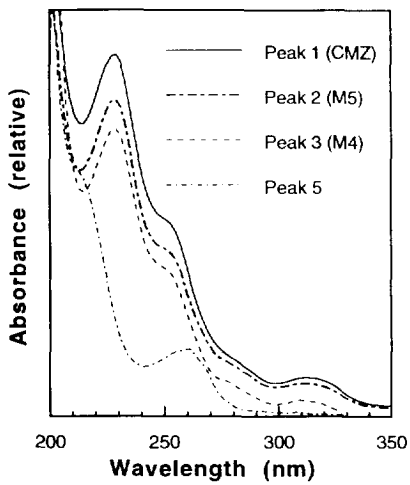


Fig. 4. UV absorption spectra of the metabolites contained in chromatographic peaks 1, 2, 3, and 5 shown in Fig. 3. See text for discussion.

of CMZ (Fig. 4). This peak might represent an impurity from microsomes. The relative peak areas of M2 (CMZ), M5, M4, and M9′ in reversed-phase HPLC (Fig. 2) and achiral SFC (Fig. 3) analyses were consistent.

3.3. Chiral SFC

Several chiral columns were screened for their ability to separate the enantiomers of CMZ and its derivatives. A Chiralcel OD-H column with ethanol-modified carbon dioxide as the mobile phase was found to be the most effective. No single concentration of ethanol was found suitable to resolve all enantiomeric pairs in one chromatographic run because of the large ranges of elution times of the compounds and the possible differences in chiral recognition of the column for the different enantiomer pairs. For example, a poorly-resolved early-eluting compound would require a lower ethanol concentration so that it could be retained long enough by the column to be enantiomerically resolved; however, this would lead to excessively long elution times for other compounds that are more easily resolved. Therefore, isocratic conditions were determined for optimal resolution of each enantiomeric pair, with the goal of achieving baseline resolution of the enantiomers in the shortest possible time. The results are shown in Table 1.

The results in Table 1 indicate that the enantiomers of CMZ and some of its derivatives were readily resolved. By adjusting the ethanol concentration, the trade-off between resolution, analysis time, and selectivity could be made. However, enantiomer resolutions of M9′ and M5 were marginal (Fig. 5) and required a relatively low concentration of ethanol as a modifier and longer run times. In comparison, enantiomers of M5 and M9′ were either very poorly resolved or not resolved at all by CSP-HPLC [16,17]. Compared to the results achieved with CSP-HPLC [16,17], CSP-SFC generally gave better resolution with a considerably shorter analysis time. Representative examples of the influence of ethanol concentration on the resolution of CMZ enantiomers are shown in Figs. 6 and 7. A higher concentration of ethanol in the mobile phase may often be used to good advantage because of the considerable reduction in analysis time.

The enantiomers of compounds with a hydrogen at the N1 position, such as NCMZ and OX, were more efficiently resolved than those of compounds with a methyl group at the N1 position, such as CMZ and TMZ (Table 1). Substitution on the nitrogen of the C3 side chain also significantly influenced enantiomer resolution. If there are two hydroxymethyl groups present, such as with M9′, resolution was poor (Table 1). However, enantiomers of M9′ could not be resolved at all by CSP-HPLC [16,17]. When one of the hydroxymethyl group was replaced by a hydrogen (e.g. conversion of M9′ to M9), the enantiomer pair was more efficiently resolved. Similarly, enantiomers of M4 were considerably more resolved than those of M5. In CSP-HPLC, the enantiomers of M5 and M9′ were either very poorly resolved or not resolved at all [16,17].

For optimal resolution of each enantiomer pair and because some enantiomers co-eluted with some of the compounds (Table 1), it was not possible to resolve all enantiomeric pairs of CMZ and its derivatives in a single chromatographic run. We have tried to use either a cyano

Table 1
Reversed-phase HPLC and CSP-SFC separation of enantiomers of CMZ and some of its derivatives

Chemical abbreviation[a]	Substituent at		$t_{1/2}^{b}$ (min)	CSP-SFC[c]				
	N1	C3		EtOH (%)	k_1'	k_2'	α	R_s
M10′	H	$OCON(CH_2OH)_2$	2.73	ND	ND	ND	ND	ND
M10	H	$OCONH(CH_2OH)$	2.94	ND	ND	ND	ND	ND
M9″	H	$OCONH_2$	3.45	ND	ND	ND	ND	ND
M6 (OX)	H	OH	3.70	8	6.6 (*S*)	7.2 (*R*)	1.09	6.3
M8	H	$OCONCH_3(CH_2OH)$		ND	ND	ND	ND	ND
M9′	CH_3	$OCON(CH_2OH)_2$	3.73	8	6.3 (*S*)	6.6 (*R*)	1.04	0.52
M9	CH_3	$OCONH(CH_2OH)$	4.04	15	4.0 (*S*)	5.4 (*R*)	1.37	4.5
M7′	H	$OCONH(CH_3)$	4.77	ND	ND	ND	ND	ND
M7	CH_3	$OCONH_2$	4.95	12	6.2 (*S*)	7.1 (*R*)	1.14	2.1
M3 (TMZ)	CH_3	OH	6.00	15	3.1 (*R*)	3.8 (*S*)	1.22	1.6
M5	CH_3	$OCONCH_3(CH_2OH)$	6.05	2.2	22.8 (*R*)	24.5 (*S*)	1.07	0.94
M4	CH_3	$OCONHCH_3$	7.20	6	15.6 (*R*)	17.2 (*S*)	1.10	1.5
M4′ (NCMZ)	H	$OCON(CH_3)_2$	7.61	25	2.5 (*R*)	4.0 (*S*)	1.58	3.0
M2 (CMZ)	CH_3	$OCON(CH_3)_2$	12.30	8	3.2 (*R*)	3.6 (*S*)	1.13	2.2

[a] See Fig. 1 for structures. Abbreviations for various CMZ derivatives are adopted from Morino and associates [24,25].
[b] Retention time on reversed-phase HPLC. See Experimental section for chromatographic conditions.
[c] A Chiralcel OD-H column (25 cm × 4.6 mm I.D.) was used. See Experimental section for chromatographic conditions.
ND = not determined.

or an amino column in series with a Chiralcel OD-H column to adjust the relative retention. Although some improvement was accomplished, none of these trials was completely successful.

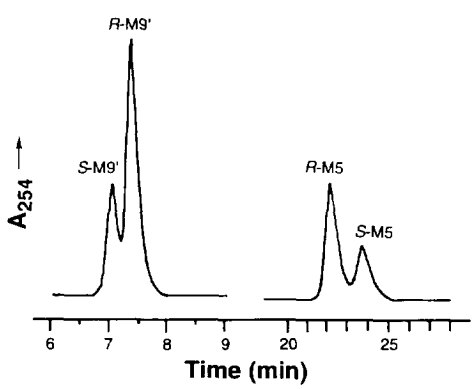


Fig. 5. CSP-SFC resolution of enantiomers of M9′ and M5 formed in the in-vitro incubation of *rac*-CMZ by rat liver microsomes. The mobile phases for the analysis of M9′ and M5 contained 10% and 2.5% ethanol in CO_2, respectively. A Chiralcel OD-H column (25 cm × 4.6 mm I.D.) was used. The flow-rate was 2.5 ml/min. The outlet pressure was 150 bar and the oven temperature was 30°C.

By using a Chiralcel OD-H alone, the enantiomers of CMZ, NCMZ, and M7 could be efficiently resolved in a single chromatographic run

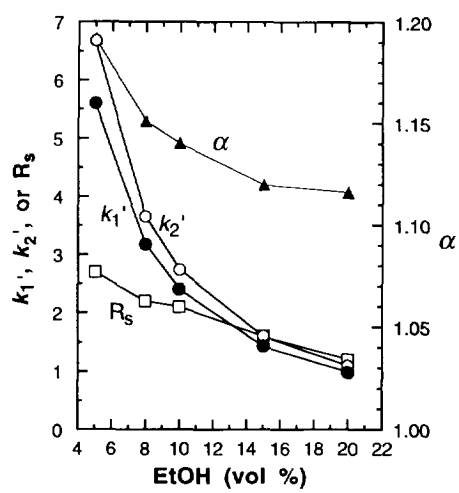


Fig. 6. Effects of ethanol concentration in the mobile phase (CO_2) on the chromatographic parameters (k_1', k_2', α, and R_s) in CSP-SFC resolution of CMZ enantiomers. A Chiralcel OD-H column (25 cm × 4.6 mm I.D.) was used. The flow-rate was 2.5 ml/min. The outlet pressure was 150 bar and the oven temperature was 30°C.

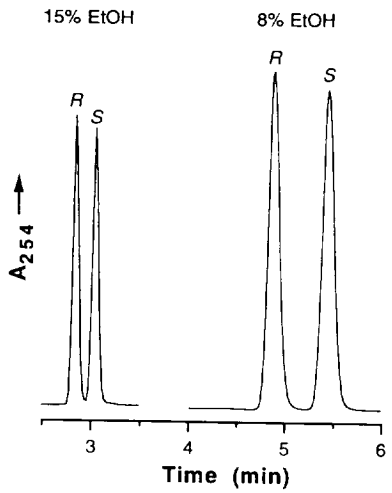


Fig. 7. Examples of chromatograms in CSP-SFC resolution of CMZ enantiomers. The volume percentages of ethanol in the mobile phase (CO_2) are indicated above the chromatogram. Other chromatographic parameters are indicated in Fig. 6.

using a linear gradient of 10% ethanol to 18% ethanol in CO_2 (flow-rate 2 ml/min; outlet pressure 200 bar; oven temperature 30°C) at a rate of 0.7%/min, followed by elution with 18% ethanol for 3 min (chromatogram not shown). However, this simultaneous resolution of three pairs of enantiomers proved to be of limited practical use in the analysis of metabolite samples due to the presence of interfering substances and other metabolites.

The results in Figs. 2 and 3 suggest that it may be possible to use a column-switching technique to effect both achiral and chiral analyses of samples derived from the metabolism of *rac*-CMZ in vitro and in vivo. This possibility is being investigated in our laboratories.

3.4. Enantioselective metabolism of rac-CMZ

The remaining CMZ and its major metabolites, formed in an in-vitro incubation of *rac*-CMZ with rat liver microsomes, were purified by a combination of reversed-phase and normal-phase HPLC [16]. The enantiomeric compositions of the metabolically formed compounds were analyzed by CSP-SFC and were compared to those determined by CSP-HPLC. Enantiomers of CMZ, M4, M5, and M9′ can all be resolved by CSP-SFC (Table 1). The enantiomeric compositions of metabolically formed M5 and M9′ could not be resolved directly by CSP-HPLC and were determined following base-catalyzed dehydroxymethylation to M4 and M7, respectively [16,17]. The enantiomeric compositions of CMZ, M4, and M5 (Table 2) obtained by CSP-SFC were in good agreement with those obtained by CSP-HPLC; the *R*/*S* enantiomer ratio of M9′ determined by CSP-SFC was higher than that determined by CSP-HPLC. The base-catalyzed conversion of the metabolically formed M9″ to M7 employed in the earlier study [16] might have caused a change in the enantiomer ratio of M9′. Overall, the results confirmed the results of the earlier study [16]; the (*R*)-CMZ was selectively metabolized at the methyl groups of the substituent at the C3 position to form M4 and M5 as the most abundant metabolites. Both the metabolically formed M4 and M5 were enriched in the (*R*)-enantiomer.

Table 2
Comparison of enantiomeric compositions of CMZ metabolites determined by CSP-HPLC and CSP-SFC

Metabolite[a]	Enantiomer ratio (*R*/*S*)	
	CSP-HPLC[b]	CSP-SFC[c]
CMZ	25.5:74.5	23.0:77.0
M4	56.7:43.3	54.4:45.6
M5	69.9:30.1	66.5:33.5
M9′	59.7:40.3	78.1:21.9

[a] Remaining CMZ and metabolites, formed in an in-vitro incubation of *rac*-CMZ by rat liver microsomes, were purified by a combination of reversed-phase and normal-phase HPLC [16]. Samples used in both CSP-HPLC and CSP-SFC were identical.
[b] Results taken from Ref. [16] for comparison.
[c] A Chiralcel OD-H column (25 cm × 4.6 mm I.D.) was used.

4. Conclusions

Packed-column SFC is a normal-phase chromatographic technique with short analysis time and high chromatographic selectivity and res-

olution. As such, SFC is a very useful technique complementary to HPLC, especially in the direct separation of enantiomers. Both the achiral and chiral separations of CMZ and eight of its derivatives were accomplished by SFC, and provided information that the HPLC method could not.

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