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Journal of Chromatography A, 708 (1995) 245–251

JOURNAL OF CHROMATOGRAPHY A

Enantiomeric separation of a thiazole derivative by high-performance liquid chromatography and micellar electrokinetic chromatography

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First received 20 December 1994; revised manuscript received 27 March 1995; accepted 27 March 1995

Abstract

Enantiomeric separation of a thiazole derivative, (±)-(*RS*)-4-[1-(2-fluoro-4-biphenylyl)ethyl]-2-methylaminothiazole, can be achieved by HPLC using a β-cyclodextrin (CD)-bonded stationary phase. The effects of the type of CD, organic modifiers in the mobile phase and mobile phase pH on the retention and resolution were studied and optimum conditions were established. The enantiomers of the compound were also separated by micellar electrokinetic chromatography with β- or γ-CDs as chiral additives. The retention behaviour of some structurally related compounds was examined with the two techniques and the results were compared.

1. Introduction

A newly synthesized thiazole derivative, (±)-(*RS*)-4-[1-(2-fluoro-4-biphenylyl)ethyl]-2-methylaminothiazole (SM-8849) (Fig. 1), demonstrating immunomodulating anti-rheumatic activity [1,2], has an asymmetric carbon and has been synthesized as a racemate. Enantioselective analytical methods are essential for studying the activity and kinetics of its individual enantiomers in the pharmacological and toxicological fields.

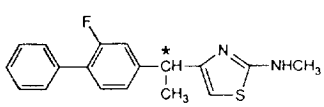

Fig. 1. Structure of SM-8849.

Cyclodextrins (CDs) are torus-shaped cyclic oligosaccharides containing six to twelve D-(+)-glucopyranose units. The interior of the CD cavities is relatively hydrophobic, thus allowing them to form inclusion complexes with a variety of molecules. Based on this ability to form stereospecific inclusion complexes, they have been successfully used for chiral separations in many chromatographic methods. Among the latter, HPLC is the most popular, with two different approaches designed for the application of CDs: (1) the use of chemically bonded CD stationary phases [3–9] and (2) the use of CDs as mobile phase components of reversed-phase (RP) systems [10–14]. Recently, the number of

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0021-9673/95/$09.50
SSDI 0021-9673(95)00403-3

papers on chiral separations by CE with CDs has also been increasing. In the CE technique, various modes have been used as in HPLC, that is, capillary zone electrophoresis (CZE) [15–19], capillary gel electrophoresis [20–22] and micellar electrokinetic chromatography (MEKC) [23–25].

We earlier reported chiral separations of agricultural chemicals by HPLC using CD-bonded stationary phases and by CD-modified MEKC (CD-MEKC) and discussed the possible mechanisms of chiral recognition [26–29].

In this investigation, we achieved the chiral separation of SM-8849 by using a β-CD-bonded stationary phase in RP-HPLC and the effects of the structure of the CD, organic modifiers in the mobile phase and mobile phase pH on retention and resolution were assessed. In addition, the enantiomers were also separated by CD-MEKC using β- or γ-CDs. The retention behaviour of SM-8849 analogues with the two methods was compared.

2. Experimental

2.1. Apparatus

HPLC separations were performed using a liquid chromatographic system which consisted of a Model L-6200 pump equipped with a Model L-4000 variable-wavelength spectrophotometric detector (Hitachi, Tokyo, Japan). The column temperature was controlled with a Shodex AO-30C oven (Showa Denko, Tokyo, Japan). Cyclobond I, II and III columns (250 mm × 4.6 mm I.D.) packed with 5-μm spherical silica gel with chemically bonded β-, γ- and α-CDs, respectively, were purchased from Advanced Separation Technologies (Whippany, NJ, USA).

CE experiments were performed with a Beckman (Palo Alto, CA, USA) P/ACE System 2100 capillary electrophoresis system. The capillary cartridge (Beckman) contained a 75-μm I.D. untreated fused-silica capillary that was 57 cm in total length and 50 cm to the detector.

2.2. Chemicals

SM-8849, its enantiomers and structurally related compounds were supplied by the Research Laboratories of Sumitomo Pharmaceuticals (Osaka, Japan), α- and β-CDs were purchased from Kanto Chemical (Tokyo, Japan), γ-CD and HPLC-grade acetonitrile were obtained from Wako (Osaka, Japan) and Sudan IV and other organic solvents and reagents were of analytical-reagent grade from Kanto Chemical or Wako. Water was processed through an RO/NANO Pure II system (Barnstead, Dubuque, IA, USA).

2.3. HPLC separation

Experiments were carried out with Cyclobond I (β-type) unless specified otherwise. The mobile phase for the RP mode was prepared by mixing an organic solvent with 0.1% triethylammonium acetate (TEAA) buffer, filtered through a 0.45-μm membrane filter and degassed before use. That for polar-organic mode was acetonitrile containing 5 or 0% of methanol and very small amounts of glacial acetic acid and triethylamine (≤0.3%). Sample solutions were prepared by dissolving compounds in methanol or acetonitrile to give a concentration of 0.1 mg/ml. In each case, a 5-μl portion of sample solution was injected. The column temperature was kept at 20°C. The flow-rate was 0.5 ml/min for the RP mode and 1.0 ml/min for the polar-organic mode, and the detector was set at 248 nm. The void volume was determined by injecting methanol and the elution order of enantiomers was examined by injecting a sample spiked with one of the enantiomers.

2.4. CE separation

The operating conditions were as follows: applied voltage, 15 kV for MEKC or 10 kV for CZE; temperature, 25°C; detection, UV at 254 nm; and sample introduction, 1-s pressure. The injection end was the anode. The separation solution for MEKC was 100 m*M* sodium dodecyl sulfate (SDS) and 2 *M* urea in 100 m*M* borate–50 m*M* phosphate buffer (pH 9.0) containing 50 m*M* β-CD unless specified otherwise. That for CZE was 5 *M* urea in 25 m*M* phosphate buffer (pH 2.5) containing various concentrations of β-CD. Sample solutions (0.2 mg/ml) were pre-

pared by dissolving each compound in methanol followed by mixing with the separation solution in a ratio of 1:4 (v/v). For MEKC, Sudan IV was added to the separation solution to measure the migration time of the micelle. The elution order of enantiomers was examined in a similar manner to the HPLC separation.

3. Results and discussion

3.1. HPLC separation

Chiral separation of SM-8849 was investigated and achieved first with a CD-bonded phase in the RP mode. The polar-organic mode for CD-bonded phases has recently been reported by Chang et al. [30]; it can resolve enantiomers that cannot be separated in the RP or normal-phase modes. SM-8849 has functional groups which can interact with CDs through hydrogen bonding and the polar-organic mode can be considered applicable. Therefore, this mode with α-, β- and γ-CD-bonded phases was also investigated using acetonitrile containing appropriate amounts of methanol, glacial acetic acid and triethylamine as mobile phases. SM-8849 was not retained on the stationary phases, however, and demonstrated no enantiomeric separation. The following experiments were performed in the RP mode.

Chiral recognition of CDs

The effect of the type of CD on the chiral recognition of SM-8849 was investigated using α-, β- and γ-CD-bonded columns.

Chiral recognition was dependent on the type of CD, that is, the cavity diameter. The enantiomers of SM-8849 were separated only on the β-CD-bonded column (Table 1). This is reasonable because the size of the β-CD cavity is thought to match that of molecules similar to biphenyl or naphthalene [31]. The elution order of the enantiomers was first the (+)-(*S*)- and then the (−)-(R)-isomer. This result indicates that the (−)-(*R*)-enantiomer forms a more stable inclusion complex than the (+)-(*S*)-enantiomer with β-CD.

Table 1
Enantiomeric separation of SM-8849 on cyclodextrin-bonded columns

Column	k_1'[a]	α[b]	Mobile phase[c]
Cyclobond I (β-)	4.94	1.09	25:75
Cyclobond II (γ-)	4.49	1.00	22:78
Cyclobond III (α-)	5.65	1.00	22:78

[a] k_1' = Capacity factor of the first-eluted enantiomer.
[b] α = Separation factor.
[c] Acetonitrile–0.1% TEAA buffer (pH 6.0) (v/v).

Effect of organic modifier

Because CDs form inclusion complexes with various hydrophobic compounds, organic solvent molecules in the mobile phase compete with the solute for occupation of the CD cavity. The effect of the hydrophobicity or the bulkiness of the organic modifier in the mobile phase on the separation of SM-8849 was investigated using primary and secondary alcohols and acetonitrile. Each organic solvent concentration was adjusted so that the capacity factor of the first-eluted enantiomer was about 5. 2-Propanol, 1-butanol and 2-methyl-1-propanol could not be used owing to the high column pressure or low miscibility for adjusting the capacity factor.

As shown in Table 2, smaller alcohols tend to give better separation factors and resolutions. This can be explained as follows: an increase in the bulkiness and hydrophobicity of the alcohol would increase its interaction with the CD cavi-

Table 2
Effects of organic modifiers on the separation factor, α, and the resolution, R_s

Organic modifier[a]	α	R_s	log K_a[b]
Acetonitrile (25:75)	1.09	0.94	–
Methanol (55:45)	1.06	0.45	−0.49
Ethanol (37:63)	1.07	0.47	−0.03
1-Propanol (20:80)	1.05	0.32	0.57
2-Butanol (15:85)	1.00	0.00	1.19
2-Methyl-2-propanol (20:80)	1.00	0.00	1.68

Column, Cyclobond I.
[a] Mobile phase, organic modifier–0.1% TEAA buffer (pH 6.0) (v/v).
[b] Logarithm of the association constant for the β-CD–alcohol complex [32].

ty, reflected in the association constant, log K_a [32], in Table 2, and thus its ability to compete with the solute. Acetonitrile was found to provide much better resolution than alcohol systems, and was therefore chosen as the mobile phase in subsequent experiments.

The (+)-(*S*)-enantiomer always eluted first with any of the organic solvents used.

Effect of pH

Because SM-8849 is a secondary amine, an influence of pH on its retention behaviour was predicted. Therefore, the effects of changing the pH of the mobile phase were investigated.

The capacity factor and the resolution significantly increased with increase in pH up to 5.0 and the best resolution was obtained at pH 6.0, as shown in Table 3. At pH 7.0, the peak broadened and the resolution decreased, although the reason was not clear.

The dissociation constant of SM-8849 was established to be between 4 and 6 in 50% methanol solution in a preliminary experiment. In general, the binding strength of the charged species to the CD cavity is smaller than that of the corresponding neutral species, presumably owing to diminished hydrophobic interactions between the charged guest molecule and the non-polar cyclodextrin cavity. This is in line with the change in retention behaviour mentioned above.

In spite of the fact that the acetonitrile content in the mobile phase and the column used were the same as those used in the previous section, the retention time at pH 6.0 obtained in this experiment was almost doubled (see Tables 1 and 3). The acetonitrile content was therefore adjusted so that the retention time of the first-eluted peak was about 40 min in the subsequent experiments.

Although better resolution is generally expected at lower temperature, as confirmed in a previous experiment [26], temperature effects were not investigated in this work. Fig. 2 shows the chiral separation of SM-8849 under the optimum conditions. The elution order of the enantiomers did not change throughout the investigation.

Table 3
Effects of pH on the capacity factor, k'_1, the separation factor, α, and the resolution, R_s

pH	k'_1[a]	α	R_s
3.5	2.06	1.06	0.26
4.0	4.04	1.07	0.64
5.0	9.99	1.08	1.02
6.0	9.93	1.08	1.05
7.0	9.74	1.08	0.91

Column, Cyclobond I; mobile phase, acetonitrile–0.1% TEAA buffer (25:75, v/v).
[a] k'_1 = Capacity factor of the first-eluted enantiomer.

3.2. CE separation

High-resolution chiral separation of SM-8849 enantiomers was achieved by MEKC. In this case β- and γ-CDs could be successfully used as chiral additives with better resolution obtained with β-CD (Table 4), differing from the HPLC case. When α-CD was used, SM-8849 did not interact because the size of the CD cavity is too small, and therefore it migrated with the micelle. The (−)-(*R*)-enantiomer eluted first when either β- or γ-CD was used. In MEKC, CDs are not solubilized by the micelle and migrate with the same velocity as the electroosmotic flow. Consequently, stable inclusion-complex formation of the solute with CDs provides a faster migration

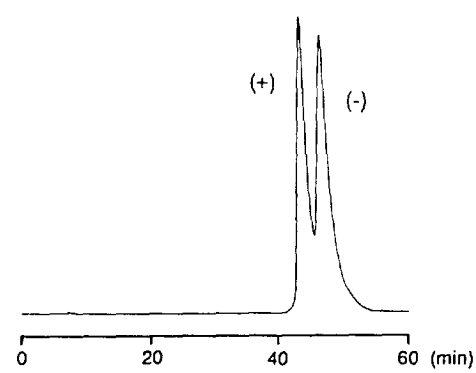


Fig. 2. Enantiomeric separation of SM-8849 by HPLC with Cyclobond I. Mobile phase, acetonitrile–0.1% TEAA buffer (pH 6.0) (25:75, v/v); for other conditions, see text.

Table 4
Enantiomeric separation of SM-8849 by MEKC

CD	$\tilde{k}'_1$[a]	α[b]	R_s[c]
α-	∞	1.00	0.00
β-	15.3	1.25	4.31
γ-	10.2	1.08	1.99

Separation solution, 100 m*M* SDS and 2 *M* urea in 100 m*M* borate–50 m*M* phosphate buffer (pH 9.0) containing 50 m*M* CD.
[a] $\tilde{k}'_1$ = Capacity factor of the first-eluted enantiomer calculated according to the equation derived by Terabe et al. [33].
[b] α = Separation factor.
[c] R_s = Resolution.

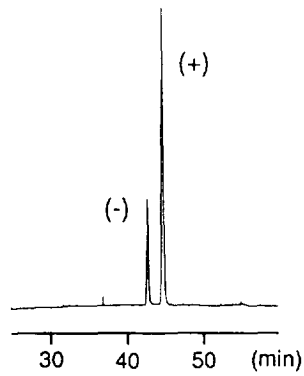


Fig. 3. Enantiomeric separation of SM-8849 spiked with the (+)-(*S*)-enantiomer by MEKC with β-CD. Separation solution, 100 m*M* SDS and 2 *M* urea in 100 m*M* borate–50 m*M* phosphate buffer (pH 9.0) containing 50 m*M* β-CD; for other conditions, see text.

under experimental conditions where the electroosmotic flow is stronger than the electrophoretic mobility of the micelle [24]. Namely, the (−)-(*R*)-enantiomer of SM-8849 forms a more stable inclusion complex with CDs than the (+)-(*S*)-enantiomer as well as on the β-CD-bonded stationary phase.

Because SM-8849 is positively charged at low pH, CZE with a chiral additive can be considered applicable. Consequently, CZE separation with various concentrations of β-CD at low pH was tried. A 5 *M* concentration of urea was added to increase the solubility of the β-CD in this experiment. However, enantiomeric separation could not be achieved, even when 50 m*M* β-CD was added. The lowest concentration of β-CD allowing effective separation of the SM-8849 enantiomers in MEKC was 20 m*M*, although baseline separation was not achieved at this concentration (R_s = 1.18).

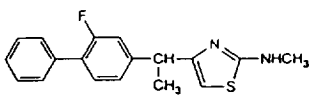
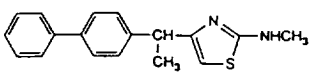
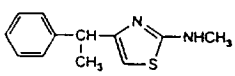
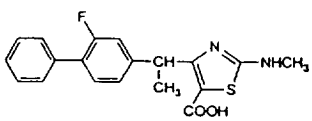
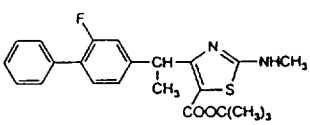
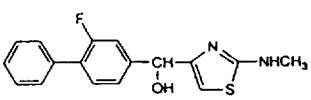
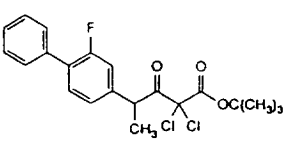
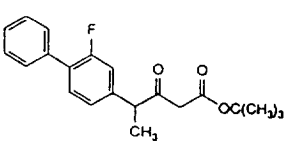
An electropherogram of SM-8849 spiked with the (+)-(*S*)-enantiomer in MEKC using β-CD as a chiral additive is shown in Fig. 3.

3.3. Retention behaviour of structurally related compounds

The retention behaviour of SM-8849 analogues was investigated using the HPLC and MEKC methods with β-CD established in this study, to obtain comparative information on chiral recognition by CD. Table 5 gives the results obtained for the capacity factors of the first-eluted enantiomers and the separation factors and resolutions of the enantiomers, showing that the enantioselectivity differs between the two methods.

The biphenyl group of SM-8849 is probably included in the CD cavity when an inclusion complex with β-CD is formed [31], although NMR spectroscopic studies are needed to confirm this. The data for compounds **1** (SM-8849) to **3** indicate that the biphenyl group plays an important role in chiral recognition with these methods. From the comparison of compounds **1** and **2**, a biphenyl group without a fluoro substituent is preferable for chiral recognition by β-CD in both methods. Compound **3**, which has a phenyl group that is too small for the β-CD cavity, instead of a biphenyl group, did not show an enantiomeric separation. This illustrates that the relative sizes of the CD cavity and the enantiomer to be complexed are of critical importance for chiral recognition: a tighter fit of molecules is preferable. With the HPLC method, compound **3** was less retained on the stationary phase than compounds **1** and **2**, that is, it demonstrated less interaction with the CD. With the MEKC method, however, the capacity factor of **3** was found to be about the same as that of **2**, although less than that of **1**. In this case, inter-

Table 5
Retention behaviour of structurally related compounds of SM-8849 using HPLC and MEKC methods with β-CD

No.	Compound	HPLC			MEKC		
		k_1'[a]	α[b]	R_s[c]	$\tilde{k}_1'$[d]	α	R_s
1[e]		6.25	1.08	0.72	14.48	1.25	4.33
2		5.75	1.09	0.88	8.95	1.33	7.70
3		1.63	1.00	0.00	7.95	1.00	0.00
4		8.97	1.08	1.03	0.37	1.00	0.00
5		7.08	1.05	0.26	150.60	1.00	0.00
6		4.27	1.00	0.00	8.07	1.11	3.11
7		2.78	1.00	0.00	10.29	1.03	0.66
8		4.24	1.00	0.00	67.67	1.00	0.00

HPLC conditions as in Fig. 2, except the mobile phase was acetonitrile–0.1% TEAA buffer (pH 6.0) (27:73); MEKC conditions as in Fig. 3.

[a] k_1' = Capacity factor of the first-eluted enantiomer.

[b] α = Separation factor.

[c] R_s = Resolution.

[d] $\tilde{k}_1'$ = Capacity factor of the first-eluted enantiomer [33].

[e] SM-8849.

pretation is more complicated because a solute distributes among the micelles, aqueous phase and CDs.

The carbonyl group on the thiazole ring influences the retention and resolution differently with the two methods. Compound **4**, having a 5-carboxy-2-methylaminothiazole ring, showed the best resolution among the HPLC data, while no resolution was apparent with MEKC. The carboxyl group exerts a positive effect, probably by hydrogen bonding with the secondary hydroxyl group of the CD in HPLC, but it has a negative effect in MEKC, because the solute mainly distributes to the aqueous phase and does not interact with the CD. Although compound **5** interacted strongly with the micelle owing to its high hydrophobicity and it showed no resolution in the MEKC case, slight enantiomeric separation was evident with HPLC.

Compound **6**, which has a hydroxyl group attached to the chiral centre, was separated only with the MEKC method. Both methods exhibited low enantioselectivity for compounds **7** and **8**.

4. Conclusions

The enantiomers of SM-8849 can be successfully separated by HPLC and MEKC using CDs. With both methods, chiral recognition is dependent on the type of CD, and inclusion complex formation between the biphenyl group of the solute and the CD cavity is considered to be an important factor in the chiral recognition. These HPLC and MEKC methods can also be applied to structurally related compounds, and will be used complementarily for studies of biological action and activity, dynamics and kinetics, in addition to quality control.

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