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Improvement of the liquid chromatographic separation of the enantiomers of tetracyclic eudistomins by the combination of a β-cyclodextrin stationary phase and camphorsulphonic acid as mobile phase additive

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ABSTRACT

Tetracyclic eudistomins, a class of compounds with potent antiviral and antitumour activity, contain two asymmetric centres, resulting into two (*d,l*) pairs called "*cis*" and "*trans*". The *cis*-enantiomers can be separated on the chiral stationary phase β-cyclodextrin using a mobile phase consisting of acetonitrile–triethylamine (99.5:0.5), adjusted to an apparent pH of 3.5 with trifluoroacetic acid. Separation of the *trans*-enantiomers was only achieved after the addition of camphorsulphonic acid to the mobile phase. The influence of the pH of the mobile phase, nature [D-(+) or L-(−)] and concentration of camphorsulphonic acid on retention and enantioselectivity was investigated.

INTRODUCTION

Tetracyclic eudistomins (1-amino-1,2,7,8,13,13b-hexahydro-[1,6,2]oxathiazepino[2′,3′:1,2]pyrido-[3,4-b]indole) are a class of indole alkaloids containing a tetrahydro-β-carboline fragment annulated with an oxathiazepine unit. These compounds display potent antiviral and antitumour activity [1,2]. At first, the tetracyclic eudistomins were isolated from natural sources, but recently the total synthesis of this class of compounds with interesting pharmacological activities was achieved [3]. Tetracyclic eudistomins contain two asymmetric centres, resulting into two (*d,l*) pairs called "*cis*" and "*trans*" (see Fig. 1). In order to follow the stereoselectivity of the synthesis, chiral liquid chromatographic (LC) systems were developed.

It was found that the *cis*-enantiomers can be separated on a β-cyclodextrine (β-CD) stationary phase, using as mobile phase acetonitrile–triethylamine (TEA) (99.5:0.5), adjusted to an apparent pH of 3.5 with trifluoroacetic acid (TFA). Using this system, the *trans*-enantiomers cannot be separated. A new possibility for the separation of enantiomers by using a combination of ion pairing and inclusion complexation was described by Szepesi and Gazdag [4]. They combined an achiral Nucleosil-10-CN column and a mobile phase containing a combination of α-, β and γ-CDs and D-(+)-10-camphorsulphon-

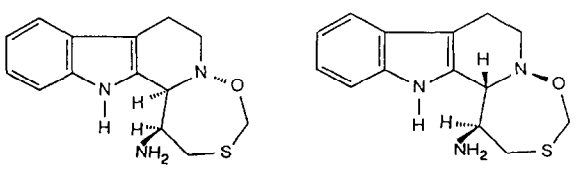

Fig. 1. Structures of *cis*-enantiomers (left) and *trans*-enantiomers (right) of tetracyclic eudistomins.

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ic acid [D-(+)-CSA] and found an improvement in selectivity for the enantiomers of Yutac and Tobanum. Pettersson and Gioeli [5] found an improved resolution for the enantiomers of naproxen by the simultaneous use of a chiral acetylquinidine-silica stationary phase and quinine as chiral mobile phase additive. Based on the results presented in both papers, we have studied the addition of the enantiomers of 10-camphorsulphonic acid to the mobile phase, which is used for the separation of the *cis*-enantiomers. We investigated the effects of nature [D-(+) or L-(−)] and concentration of camphorsulphonic acid and the pH of the mobile phase on retention and selectivity for the *cis*- and *trans*-enantiomers. Further, a comparison was made with a conventional chiral ion-pair system, using a LiChrosorb-Diol column and D-(+)-CSA as mobile phase additive [6].

EXPERIMENTAL

Apparatus

The liquid chromatographic system consisted of an HP 1050 pump (Hewlett-Packard, Waldbronn, Germany), a Model 7125 injector fitted with a 10-μl loop (Rheodyne, Cotati, CA, USA) and a variable-wavelength UV detector (Kratos Spectroflow 757, ABI, Ramsey, NJ, USA). The chromatograms were recorded on an HP 3396 integrator (Hewlett-Packard). The column temperature was controlled through a Type M57020-88-2 column oven (Chrompack, Middelburg, Netherlands).

A β-cyclodextrin column (Cyclobond I) (250 x 4.6 mm I.D.) was purchased from Advanced Separation Technologies (Whippany, NJ, USA). The Diol-bonded silica column (250 x 4.6 mm I.D., particle size 5 μm) was packed with a slurry technique using tetrachloromethane–1-propanol (80:20) as the slurry medium and LiChrosorb-Diol from Merck (Darmstadt, Germany) as packing material.

Chemicals

The tetracyclic eudistomins were synthesized by Hermkens *et al.* [3]. High-performance liquid chromatographic-grade acetonitrile, technical-grade 2-propanol, TFA and TEA were purchased from Baker (Deventer, Netherlands). Technical grade dichloromethane was obtained from Baker and purified by distillation. D-(+)-CSA was used as the sodium salt and purchased from Merck (Darmstadt, Germany), while L-(−)-CSA and racemic CSA were used as acids and obtained from Fluka (Buchs, Switzerland).

Chromatographic conditions

The samples were dissolved in the eluent to a concentration of about 0.1 mg/ml. The injection volume was 10 μl and the tetracyclic eudistomins were detected by their UV-absorption at 275 nm. All separations were performed at a temperature of 30°C.

At first, enantiomeric separations of tetracyclic eudistomins were done on a β-CD stationary phase, using a mobile phase consisting of acetonitrile–TEA (99.5:0.5), adjusted to an apparent pH of 3.5 with TFA. The flow rate was 1.0 ml/min.

In subsequent experiments different types of CSA [D-(+), L-(−) or the racemate] were added to the mobile phase. The concentration of CSA was varied between 0 and 8 m*M*. The influence of the pH on the capacity factors and selectivity was investigated by varying the pH of the mobile phase, acetonitrile–4 m*M* D-(+)-CSA, from 10 to 3.5 using 0.5% TEA–TFA buffer. In all experiments in which CSA was added, the flow-rate was increased from 1.0 to 2.0 ml/min in order to reduce the analysis time.

The conventional chiral ion-pair system consisted of a LiChrosorb-Diol column and as mobile phase 2-propanol–dichloromethane (4:96) containing 4 m*M* D-(+)-CSA. Chromatography was carried out at a flow rate of 2.0 ml/min.

RESULTS AND DISCUSSION

In Fig 2, the chromatograms of the separations of *cis*- and *trans*-enantiomers of tetracyclic eudistomins on a β-CD stationary phase are shown. As can be seen, the β-CD column exhibits high enantioselectivity towards the *cis*-enantiomers, whereas the *trans*-enantiomers are not separated. The chiral recognition mechanism of CDs is attributed to inclusion complex formation between the cavity of the CD and the hydrophobic moiety of the solute, in combination with hydrogen bonding between the polar functional groups of the solute in the vicinity of its chiral centre and the hydroxyl groups of the CD [7–10]. It is generally assumed that aqueous–organic mobile phases are required for the formation of inclusion complexes [11–13]. In our experi-

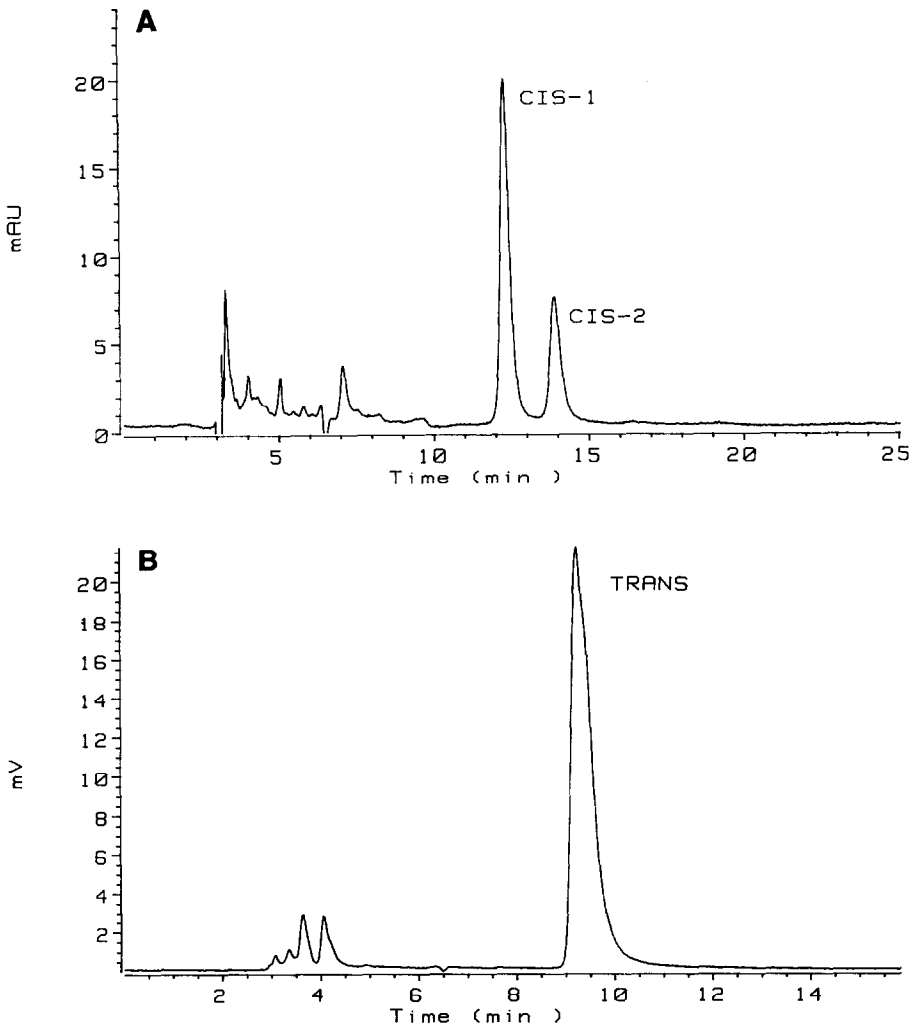

Fig. 2. Chromatograms of (A) *cis*- and (B) *trans*-enantiomers of tetracyclic eudistomins. Column, 250 × 4.6 mm I.D. β-cyclodextrin; mobile phase, acetonitrile–TEA (99.5:0.5), adjusted to pH 3.5 with TFA; flow rate, 1.0 ml/min.

ments, we succeeded in separating the *cis*-enantiomers using acetonitrile as mobile phase. Therefore, we assume that also in a non-aqueous mobile phase inclusion complexes can be formed.

The observed enantioselectivity may be attributed to the use of acetonitrile, as it is known from a study of Seeman *et al.* [13] that this modifier can cause anomalous retention behaviour on a β-CD column. They found that the retention times of the enantiomers of N'-benzylnornicotine decreased when the proportion of acetonitrile in the aqueous mobile phase was increased from 10% up to about 80%, but an increase in retention was observed when the proportion of acetonitrile was increased further up to 100%. Compared with an eluent containing 60–80% of acetonitrile, they demonstrated an improvement in enantioselectivity for acetonitrile contents above 80% and suggested that the mechanism of chiral recognition has changed. In the presence of non-polar solvents (such as hexane–2-propanol mixtures), inclusion of the solute in the cavities of CDs is probably hampered because

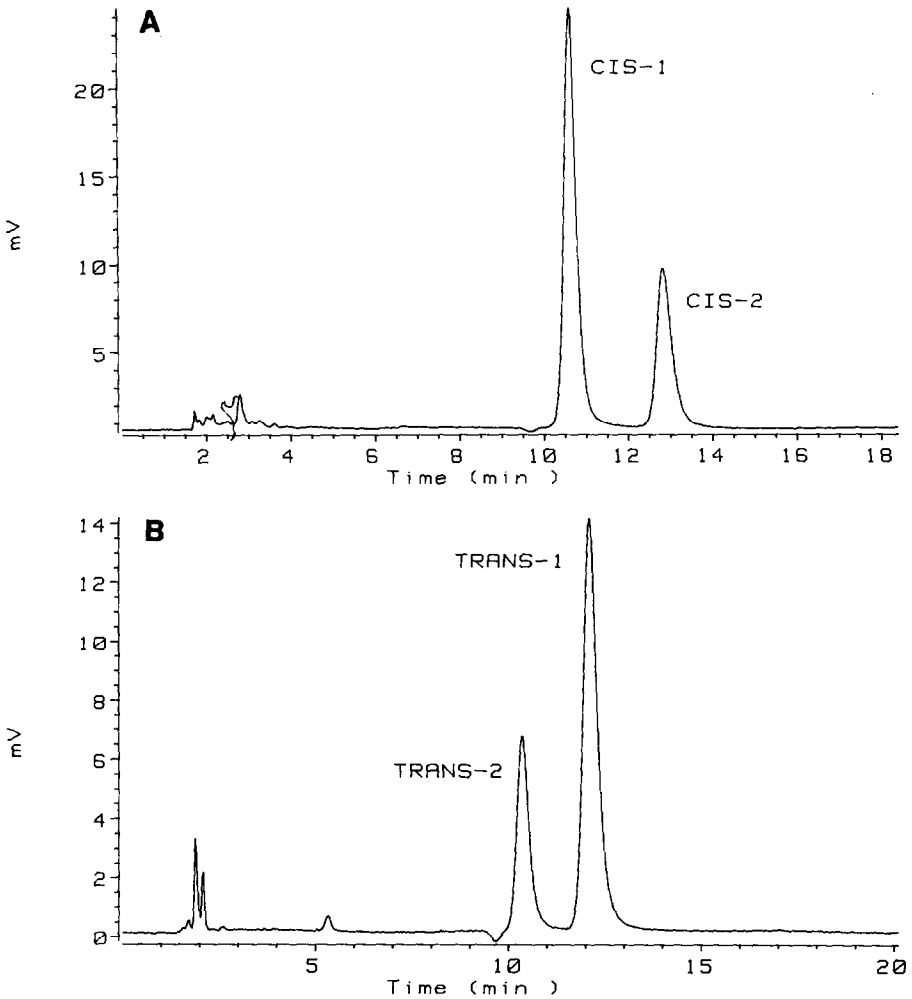

Fig. 3. Chromatograms of (A) *cis*- and (B) *trans*-enantiomers of tetracyclic eudistomins after the addition of 4 m*M* D-(+)-CSA to the mobile phase. Flow-rate, 2.0 ml/min; other chromatographic conditions as in Fig. 2.

the non-polar component of the mobile phase occupies the cavity. However, our results indicate that the modifier acetonitrile does not completely occupy the cavity of β-CD, as inclusion complexes can still be formed.

By adding D-(+)-CSA to the mobile phase, the enantioselectivity of the *trans*-enantiomers is improved significantly, and for the *cis*-enantiomers an improvement in selectivity is obtained also. The chromatograms of the enantiomeric separations after the addition of D-(+)-CSA to the mobile phase are shown in Fig. 3. It is well known that chiral amines can form diastereomeric ion pairs with CSA [6,14–17]. The gain in selectivity for the *trans*-enantiomers and, to a lesser extent, also for the *cis*-enantiomers may be ascribed to the ion-pair complexation followed by a different distribution of the ion pairs into the cavities of CD [4].

Effect of nature and concentration of CSA

The influence of nature and concentration of CSA on the capacity factors (k') and enantioselectivities (α) was studied. The results of these experiments are presented in Table I. The addition of 2

TABLE I

INFLUENCE OF NATURE AND CONCENTRATION OF CSA ON CAPACITY FACTORS (k') AND SELECTIVITIES (α) OF THE CIS- AND TRANS-ENANTIOMERS OF TETRACYCLIC EUDISTOMINS

CSA	Concentration (m*M*)	*trans*-enantiomers			*cis*-enantiomers		
		k'_1	k'_2	α[a]	k'_1	k'_2	α
D-(+)-CSA	0	2.1	2.1	1.00	2.9	3.5	1.18
	2	5.6	6.6	1.19	5.8	7.2	1.24
	4	6.8	8.1	1.19	7.0	8.9	1.27
	6	7.4	8.8	1.19	7.6	9.8	1.28
	8	8.7	10.3	1.18	8.5	11.0	1.28
L-(−)-CSA	2	4.2	4.7	−1.12	4.3	4.8	1.11
	4	9.0	10.5	−1.17	8.0	8.8	1.09
	8	8.6	9.9	−1.15	8.1	8.6	1.07
(D,L)-CSA	4	5.5	5.5	1.00	4.9	5.6	1.15

[a] The minus sign before a selectivity value indicates that the elution order of the enantiomers is reversed.

m*M* D-(+)-CSA increases the capacity factors of all the enantiomers, whereas the enantioselectivity improves markedly for the *trans*-enantiomers and, to a lesser extent, also for the *cis*-enantiomers. On further increasing the concentration of D-(+)-CSA, the capacity factors of all enantiomers increase, while no further gain in selectivity is obtained for the *trans*-enantiomers and only a minor improvement for the *cis*-enantiomers is found.

When 2 m*M* L-(−)-CSA is added to the mobile phase, for the *trans*-enantiomers a higher selectivity is obtained. However, it is striking that compared with the addition of D-(+)-CSA, the elution order of both *trans*-enantiomers is reversed, which is indicated in Table I by a minus sign before the selectivity value. This reversal could be detected as we analysed non-racemic mixtures of both (*d,l*) pairs. For the *cis*-enantiomers, the selectivity decreases after the addition of L-(−)-CSA to the eluent, whereas no reversal of the elution order of the two enantiomers is found. On increasing the concentration of L-(−)-CSA, no significant influence on the selectivity for the *trans*-enantiomers is found, whereas for the *cis*-enantiomers the selectivity seems to decrease slightly. The inversion of the elution order of the *trans*-enantiomers after the addition of L-(−)-CSA can be explained by the fact that diastereomeric ion pairs of opposite configuration are formed. These results are comparable to a situation when an optically impure chiral selector is added to the mobile phase. With a decreasing optical purity of the chiral selector, the resolution between the enantiomers decreases and a reversed selectivity is found if the antipode of the chiral selector is added [4,18].

After the addition of (D,L)-CSA to the mobile phase, no separation is obtained for the *trans*-enantiomers, whereas the enantioselectivity for the *cis*-enantiomers is comparable to that for the system in which no CSA is added. However, in contrast to the latter situation, the capacity factors of all enantiomers have increased significantly, demonstrating ion-pair formation with CSA.

Effect of pH

As can be expected for ion-pair chromatography, the pH of the mobile phase is an important factor. The results of variation of the pH of the mobile phase, acetonitrile–4 m*M* D-(+)-CSA, from 10 to 3.5 are summarized in Table II. It can be seen that at a pH of 6 or lower, a pronounced improvement in enantioselectivity is obtained for the *trans*- and *cis*-enantiomers. Moreover, the capacity factors also increase if the pH decreases. The pK_a values of all three nitrogen atoms were calculated by QSAR studies and estimated to be about 9.5 for the primary amino group, less than zero for the indolic nitrogen and about 6 for the tertiary nitrogen atom.

TABLE II

INFLUENCE OF THE pH OF THE MOBILE PHASE, WITH AND WITHOUT D-(+)-CSA, ON CAPACITY FACTORS (k') AND SELECTIVITIES (α) OF CIS- AND TRANS-ENANTIOMERS OF TETRACYCLIC EUDISTOMINS

pH	D-(+)CSA (m*M*)	*trans*-enantiomers			*cis*-enantiomers		
		k'_1	k'_2	α	k'_1	k'_2	α
10	0	0.19	0.19	1.00	2.8	3.2	1.15
	4	0.29	0.29	1.00	2.9	3.1	1.07
8	0	0.23	0.23	1.00	3.4	3.8	1.13
	4	0.40	0.40	1.00	4.3	4.9	1.12
6	0	2.4	2.4	1.00	5.1	6.0	1.17
	4	3.3	3.7	1.12	7.3	9.1	1.24
5	0	2.8	2.8	1.00	4.1	4.9	1.17
	4	8.0	9.3	1.16	8.6	10.8	1.25
3.5	0	2.9	2.9	1.00	3.6	4.2	1.17
	4	8.1	9.8	1.20	7.7	9.8	1.26

At a pH of 6 or lower, the tertiary amine function is (partly) protonated resulting into the formation of ion pairs and a separation of the *trans*-enantiomers. Therefore, it can be concluded that for ion pairing, electrostatic interaction of the protonated tertiary nitrogen with the sulphonic group of CSA is essential. As the *cis*-enantiomers are separated over the whole pH range investigated, it can be concluded that ion pairing is no prerequisite for the chiral recognition of the *cis*-enantiomers. In the system without CSA, the capacity factors of the *trans*-enantiomers increase with decreasing pH, whereas for the *cis*-enantiomers an increase in the capacity factors is observed in the pH range from 10 to 6, followed by a decrease at lower pH values. The observation that very low capacity factors for the *trans*-enantiomers are found at high pH may be explained by the fact that TEA will bind to the hydroxyl groups of CD and thereby compete with hydrogen bonding of the solute. Therefore, it can be concluded that especially for the *trans*-enantiomers, interaction with the external hydroxyl groups at the mouth of the CD cavity play an essential role in the retention.

Comparison with a conventional chiral ion-pair system

For the LiChrosorb-Diol stationary phase, it has been reported that a large enantioselectivity is obtained under conditions that favour a high degree of ion-pair formation [6]. Water, even in very low concentrations, can have an adverse influence on the enantioseparation. In our experiments, this condition is fulfilled as we used a mobile phase of 100% acetonitrile. Further, it is known that for good complexation with CSA, a hydrogen-donating function at a distance of two carbon atoms from the hydrogen-accepting amine function is essential for the stereoselectivity [17]. In the present system, tetracyclic eudistomins contain a protonated amino group two carbon atoms from the tertiary amine function. This amino group can interact by hydrogen bonding with the oxo group of CSA. The electrostatic interaction of the tertiary amine nitrogen with the sulphonic group of CSA in combination with the hydrophobic interaction between the ring systems of CSA and tetracyclic eudistomins provide for three interaction points (Dalgliesh's "three-point" rule [19]), so in principle efficient enantioselective ion-pair formation can occur.

Therefore, the results described above, obtained with a combination of a chiral column and a chiral mobile phase additive, were compared with those obtained on a conventional chiral ion-pair system using a LiChrosorb-Diol column. From the chromatogram shown in Fig. 4, it can be concluded that separation of *cis*- and *trans*-enantiomers is also possible on a LiChrosorb-Diol column with D-(+)-CSA in the mobile phase, although the selectivity [α(*cis*-enantiomers) = 1.08; α(*trans*-enantiomers) = 1.10] and efficiency of this system are lower. For this system, the plate number is about 2900 (*trans*-

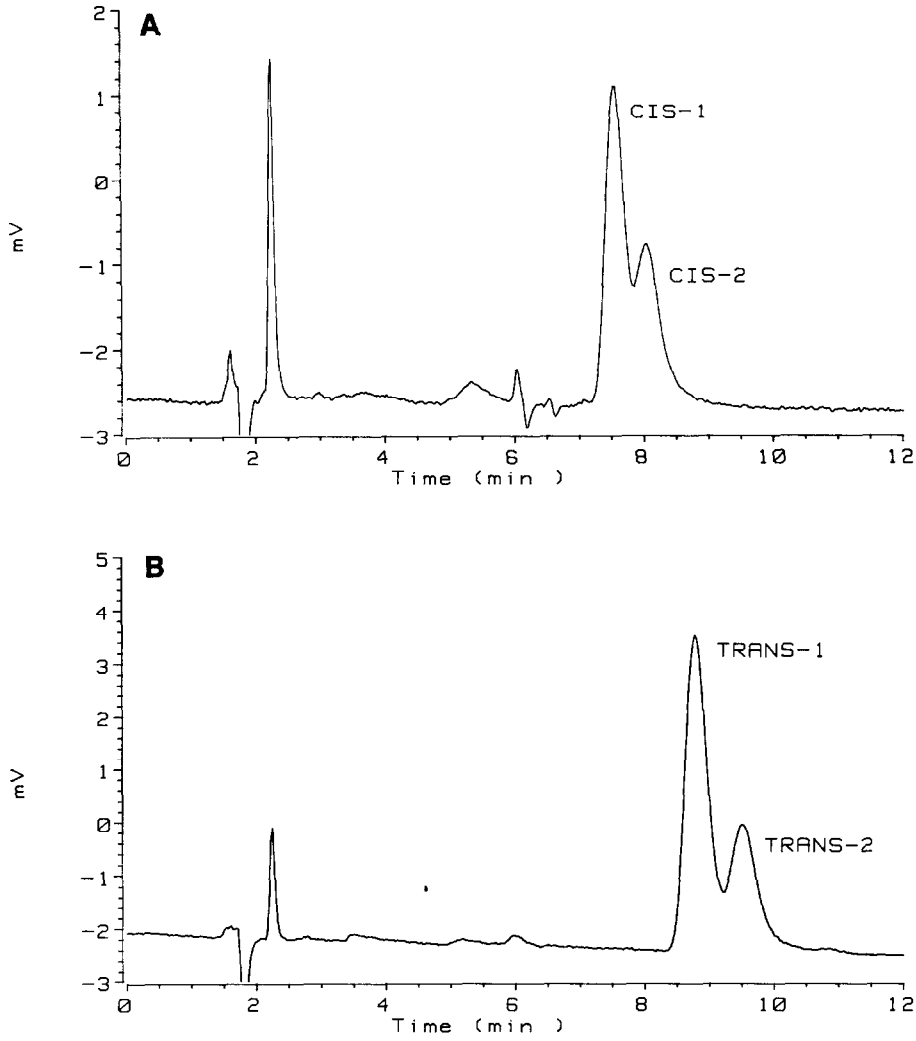

Fig. 4. Chromatograms of (A) *cis*- and (B) *trans*-enantiomers of tetracyclic eudistomins. Column, 250 × 4.6 mm I.D. LiChrosorb-Diol; mobile phase, 2-propanol–dichloromethane (4:96) containing 4 m*M* D-(+)-CSA; flow-rate, 2.0 ml/min.

enantiomers) or 2600 (*cis*-enantiomers) against 4500 (*trans*-enantiomers) or 5600 (*cis*-enantiomers) in the CD system. It should be noted that in comparison with the chromatogram presented in Fig. 3, a reversal in elution order for the *trans*-enantiomers occurs, whereas for the *cis*-enantiomers the elution order is identical in both chromatographic systems. The reversal of elution order for the *trans*-enantiomers suggests that the stationary phases LiChrosorb-Diol and β-CD have a different separation mechanism, as in both LC systems the same amount and nature of CSA were added to the mobile phase. For a LiChrosorb-Diol column, the stereoselectivity is obtained by a different distribution of the diastereomeric ion pairs between the mobile and the stationary phases. Evidently, for a β-CD column the different inclusion complexation of the diastereomeric ion pairs may cause a change in retention behaviour for the *trans*-enantiomers in comparison with the LiChrosorb-Diol column.

CONCLUSIONS

In contrast to what is commonly assumed, our results indicate that inclusion complex formation can occur on a β-CD bonded phase using a non-aqueous mobile phase. Moreover, by choosing the proper conditions for ion-pair chromatography, using camphorsulphonic acid as ion-pair reagent, an increase in stereoselectivity can be obtained, probably as a consequence of a more effective discrimination of β-CD for the diastereomeric ion pairs. The pH of the mobile phase and the nature and concentration of CSA added are important factors in the chiral selectivity.

We have frequently applied the combination of a β-CD stationary phase and D-(+)-CSA in the mobile phase to separate the enantiomers of various new chemical entities with similar structural features to the tetracyclic eudistomins. For those compounds which can form ion pairs, this combination was found to offer an excellent possibility of improving enantioselectivity. Further studies are in progress to investigate if this approach can be exploited to improve enantioselectivity by adding other chiral counter ions, *e.g.* N-benzoxycarbonyl-glycyl-L-proline (ZGP), to the mobile phase.

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