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Comparative study on the enantiomer separation of 1,1′-binaphthyl-2,2′diyl hydrogenphosphate and 1,1′-bi-2-naphthol by liquid chromatography and capillary electrophoresis using single and combined chiral selector systems

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Abstract

The chiral recognition ability of single and dual selectors, that were used as additives, have been investigated by HPLC and CE. Native β- and γ-cyclodextrins, permethylated β-cyclodextrin, hydroxypropyl-β-cyclodextrin, cholic acid and taurodeoxycholic acid sodium salts were applied as chiral selectors, whereas the atropisomers of 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate, and 1,1′-bi-2-naphthol served as model compounds. It was found that all investigated selectors, except for γ-cyclodextrin, display the same affinity pattern for binaphthyl enantiomers, i.e., binding the *S* more strongly than the *R* enantiomer. However, the differences in the phase distribution of chiral selectors led to the opposite elution order of enantiomers: with cyclodextrins, the first eluted is *S* enantiomer, while *R* is the first eluted for bile salts. Under the conditions studied, cyclodextrins (except γ-cyclodextrin), as well as cholic acid sodium salts acting singly, enable the separation of 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate enantiomers both by HPLC and CE methods, while 1,1′-bi-2-naphthol enantiomers were resolved only under CE conditions with permethylated cyclodextrin or bile salts. In both techniques the application of dual systems could improve resolution or make it worse (or even cancel), depending on the sign of enantioselectivity of particular selectors, their concentrations and localization: mobile or stationary phase. It has been found that the mechanism of separation as well as interactions occurring between two selectors may be followed by using combined HPLC and CE methods. The obtained results proved that, as well as β-CD, TM-β-CD and γ-CD also form inclusion complexes with cholic acid sodium salts. The reversal of elution order may be realized by two procedures: changing a single selector, i.e., cyclodextrin on cholic acid sodium salt or vice versa, and by changing the proportion of selectors in the combined bile salt–cyclodextrin system.
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1. Introduction

The ability of a single selector to resolve enantiomers is not always satisfactory. Sometimes chiral recognition is poor and separation factors very close to 1.00 are achieved. For that reason many attempts

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have been undertaken to enhance the resolution. Among others, combined systems with dual selectors in HPLC have been proposed recently.

Generally the combination relies on the use of two appropriate selectors; that in the stationary phase and that in the bulk mobile phase [1–6].

The diverse behaviour of native and permethylated β-cyclodextrin (β-CD) in RP-HPLC system enabled us to design a new advantageous chromatographic system with two chiral additives working jointly but in different ways [7]. In our version the native β-CD serves as chiral selector in the mobile phase, while permethylated cyclodextrin (TM-β-CD) works as chiral selector in the dynamically generated stationary phase. As a result, a significant enhancement of enantioselectivity may be observed under appropriate conditions, namely then when the sense of enantioselectivity of two selectors is inverse. Thus, the first enantiomer is predominantly associated with the chiral additive present in the mobile phase, whereas the second enantiomer is bound more strongly by the chiral selector immobilized on the stationary phase.

However, interest in the development of HPLC dual systems appears to be moderate. In contrast to this, the use of two chiral selectors in electro-driven methods receives more and more attention [8–12].

The goal of our present study is to learn how to deal with dual component systems in RP-HPLC and CE techniques regarding the similarities and differences in their behaviour. Some regularities have been investigated:

(1) to predict the behaviour of dual selector systems with respect to enantiomer separations in passing from one technique to the another;

(2) to design conditions to reverse the order of elution for enantiomers.

It is worth noting that the latter problem, i.e., the change of elution order, is especially important in the case of non-racemic mixtures with low content of one enantiomer, which should be eluted first. Some methods to reverse the order of elution have already been described [13–15]. The simplest procedure employs a selector of opposite configuration. However, like all widely used selectors of natural origin (such as antibiotics, polysaccharides) the native cyclodextrins are available only in one configuration. Thus, with these popular selectors the reversal of elution order of enantiomers should be searched for in different ways, i.e., in combined systems.

In this study, cyclodextrins (β- and γ-cyclodextrin, permethylated and hydroxypropyl β-cyclodextrin), and bile salts (cholic acid sodium salt and taurodeoxycholic acid sodium salt) were used as chiral selectors in single and dual mode. The model tested compounds were: 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate (BNP) and 1,1′-bi-2-naphthol (BN). Atropisomers of binaphthyl and its derivatives have been widely used in organic enantioselective synthesis as building blocks in catalysts. Their chromatographic and electrophoretic separations have been the subject of many papers [16–21], the results of which were instructive for the design studies presented in this paper. It is worth mentioning that the first use of this compound (BNP) in a dual system was published by Schurig and co-workers [22].

2. Experimental

2.1. Reagents

α-Cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD), heptakis (2,3,6-tri-*O*-methyl) β-cyclodextrin (TM-β-CD) and hydroxypropyl β-cyclodextrin (HP-β-CD) were supplied by Chinoin (Budapest, Hungary), cholic acid sodium salt (CHASS), taurodeoxycholic acid sodium salt monohydrate (TDCHASS) and the enantiomers (+) and (−) of the model compounds 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate and 1,1′-bi-2-naphthol were supplied by Fluka (Buchs, Switzerland). The structural formulas of model compounds are shown in Fig. 1. All other reagents and solvents were of analytical grade and were used as received.

2.2. Apparatus and procedures

Chromatographic experiments were performed using a Waters (Vienna, Austria) Model 590 pump, a Rheodyne type injector with a 1-μl loop and a Waters UV–Vis detector Model 490 (detection: 220 and 254 nm). The column used was 250×1 mm I.D. packed with 5 μm LiChrosorb RP 18.

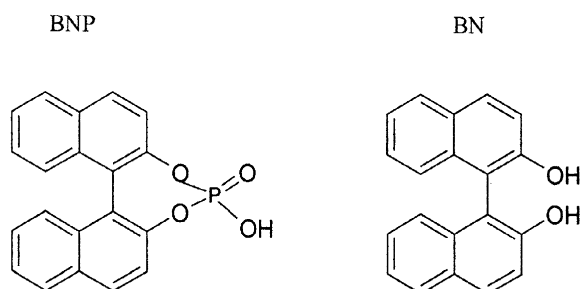

Fig. 1. The structural formulas of investigated compounds.

The mobile phases were aqueous ethanolic solutions of 18 m*M* Tris-hydroxymethyl aminomethane (Tris) adjusted to pH 6.5 with phosphoric acid, containing the appropriate chiral selectors (one or two). Flow rates were 0.04 ml/min. In the system with TM-β-CD and cholic acid salts, the measurements were made always after elution of some amounts of mobile phase to generate a chiral stationary phase and to achieve dynamic equilibrium on the column. The end of the equilibration was indicated by constant retention values and selectivity factors for the tested compounds [23]. All chromatographic measurements were performed at ambient temperature in an air-conditioned room (20 °C).

The adsorption of cholic acids sodium salts on RP 18 phase was studied on a Nova Pack C_{18}, 60 Å, 4 μm (2×150 mm) column (Waters; Milford, USA) using a Rheodyne injector equipped with a 5-μl loop and a polarimetric 'Chiralyser 1,4' detector (Knauer; Berlin, Germany)

Electrophoretic separations were performed on a Spectrophoresis Ultra apparatus (Thermo Separation Products, San Jose, CA, USA) using an uncoated 75-μm I.D. fused-silica capillary with a total length of 63 cm and an effective length of 58 cm to the detector window. The capillary was conditioned by passing 0.1 *M* NaOH solution for 20 min and then washing with distilled water for 10 min and finally equilibrating with an appropriate running buffer for 2 min. Between runs the capillary was washed with 0.1 *M* NaOH, water and run buffer for 2 min each. The buffer solution was prepared using 18 m*M* Tris solution and adjusted to pH 11 with 1 *M* NaOH.

The pH of HPLC and CE measurements differ substantially, but it seems probable, that at pH 6.5 and 11 BNP molecules are almost fully dissociated.

Samples were introduced by applying hydrodynamic pressure (0.8 p.s.i.) for 2 s. The separation temperature was 25 °C. The applied voltage was 20 kV. The EOF was determined using ethanol as the neutral marker (all samples were dissolved in ethanol).

2.3. Electrophoretic mobility

The electrophoretic mobilities of the BNP and BN enantiomers were calculated from the observed migration times according to Eq. (2):

$$\mu_{obs} = \mu_{ep} + \mu_{EOF} \quad (1)$$

$$\mu_{ep} = \mu_{obs} - \mu_{EOF} = \frac{L_D L_t}{V}\left(\frac{1}{t_m} - \frac{1}{t_{EOF}}\right) \quad (2)$$

where μ_{ep} is the electrophoretic mobility of the analyte, μ_{obs} is the observed (apparent) mobility of the analyte, μ_{EOF} is the mobility of the neutral marker (ethanol), L_t is total length of the capillary, L_D is the length of the capillary to the detector window, V is the applied voltage, t_m is the migration time of the analyte, t_{EOF} is the migration time of neutral marker (ethanol) which moves with the EOF.

2.4. Retention parameters in HPLC

The retention parameters (k) of the BNP and BN enantiomers were calculated from the observed retention times according to Eq. (3):

$$k = \frac{t - t_o}{t_o} \quad (3)$$

where t is the retention time of the analyte, t_o is the hold-up time.

3. Results and discussion

The observed chiral separation, in both techniques, may be decoupled in two basic steps: chiral recognition, which occurs on the molecular level, and transformation of the chiral recognition event to the macro phenomenon of chromatographic or electro-

phoretic chiral separation. The phenomenon responsible for enantioseparation in chromatographic and electrophoretic processes is the same; this is the enantioselective interaction between the analyte enantiomers and a chiral selector. The principal difference between these two techniques arises from different mechanisms of separation processes.

In the current study, first the chiral recognition ability of each selector separately has been investigated in HPLC and CE systems. The experimental data give crucial evidence for a proper understanding of the difference in mechanism for separation between the techniques and the use of selectors in the combined mode.

3.1. Single selectors in HPLC

The HPLC results are discussed on the basis of the behaviour of the BNP enantiomers only. Under experimental conditions, the adsorption of the BN enantiomers to the stationary phase was so strong that their broad peaks were eluted at retention times, in the best case, longer than 130 min, which does not suit elaboration.

3.1.1. Cyclodextrins

It has been stated earlier that, under experimental conditions (at concentrations of alcohol higher than 10%), β- and γ-CD do not adsorb to the RP-stationary phase [23]. Thus, for both cyclodextrins the chiral recognition is realized by complexation in the bulk mobile phase. If adsorption of the complex to the stationary phase can be neglected then the stronger complexed enantiomer is eluted first. It follows from Eq. (4) [24–28]:

$$k_1 = \frac{k_0}{1 + K_{CDm}[CD]} \quad (4)$$

where k_1 and k_0 are the retention parameters for the system with and without cyclodextrin added to the mobile phase, respectively, [CD] is the concentration of free cyclodextrin in the mobile phase and K_{CDm} is stability constant of the 1:1 stoichiometry complex.

According to the data shown in Table 1, β-CD binds the *S*(+) enantiomer of BNP more strongly, while the *R*(−) enantiomer of BNP is more strongly associated with γ-CD. Comparing the BNP retention times for β-CD and γ-CD solutions it can be seen

Table 1
Chromatographic data of BNP enantiomers in RP-HPLC system containing single chiral selector

Basic eluent	Additives	k_1	k_2	α	First eluted enantiomer
Without chiral selector					
30% EtOH		67.9	67.9	1.00	
With cyclodextrins					
	20 m*M* β-CD	28.1	30.6	1.09	*S*
30% EtOH					
	20 m*M* γ-CD	17.3	18.0	1.04	*R*
	20 m*M* TM-β-CD	6.4	8.0	1.24	*S*
20% EtOH	20 m*M* β-CD	65.6	92.6	1.41	*S*
	0.5 m*M* TM-β-CD	57.8	70.3	1.22	
With bile salts					
30% EtOH	80 m*M* CHASS	3.0	3.4	1.13	*R*
	20 m*M* CHASS	6.7	8.6	1.28	*R*
	5 m*M* CHASS	8.7	10.3	1.19	*R*
	20 m*M* TDCHASS	4.7	5.3	1.12	*R*
	5 m*M* TDCHASS	5.4	6.5	1.20	*R*
20% EtOH	20 m*M* CHASS	16.2	22.0	1.35	*R*
	20 m*M* TDCHASS	12.2	12.2	1.00	
	5 m*M* TDCHASS	16.7	21.9	1.31	*R*

For chromatographic conditions, see Fig. 2.

that γ-CD does form more stable complexes with BNP enantiomers than β-CD. However, the chiral recognition ability of β-CD is larger ($\alpha = 1.09$) than that of γ-CD ($\alpha = 1.04$). Binding forces and selectivity appear to be the two independent phenomena.

Contrary to the native cyclodextrins, adsorption of TM-β-CD on RP phase is very strong [23]. The elution order of the BNP enantiomers for TM-β-CD was found to be the same as for β-CD. However, in 20% EtOH the TM-β-CD is strongly adsorbed and occurs on the surface of the stationary phase. This elution order agrees with that earlier observed in open capillary electrochromatography with permethylated β-CD as chiral stationary phase [12]. The concept that TM-β-CD is dealt with mainly at the surface of the RP-18 stationary phase seems to be well documented. It has been earlier stated that: (a) TM-β-CD is strongly adsorbed on the RP phase [23], (b) its ability to chirally recognize a solute appears no earlier than after elution of some volume of its dilute solution, and (c) omitting TM-β-CD from the mobile phase solution the RP column retains for some time its enantioselective properties and loses them very slowly. For comparison, enantioselectivity of β-CD complexation appears and disappears at once after changing the mobile phase composition.

In fact, the same elution order of enantiomers for β-CD and TM-β-CD indicates an opposite chiral recognition pattern for the two cyclodextrins operating in different phases. Davankov et al. [4] first formulated such a conclusion on the effect of ligand exchange studying on chromatographic systems with chiral stationary and/or chiral mobile phase.

The system with higher concentrations of TM-β-CD (20 m*M*) will be discussed later.

3.1.2. Bile salts

Using the same experimental conditions, cholic acid sodium salt and taurodeoxycholic acid sodium salt were adsorbed at the stationary phase. In separate experiments it was found that retention factors of both salts (present below cmc) on RP phase in 35% of ethanol in water (pH 6.5) are ≅ 16, while the adsorption of their micelles may be much larger. As bile salts surfactants generally form reversed micelles in polar solvents [29–31], the outside of the micelles are hydrophobic which lead to their strong adsorption on the surface of RP stationary phase. This behaviour has been confirmed by chromatographic studies [32–35].

From the experimental data shown in Table 1, one can see that for both salts the elution order of the BNP enantiomers is the reverse of that of β-CD and TM-β-CD and that is the same as for γ-CD. With the same concentration of alcohol, the retention of BNP is much shorter for bile salts than for cyclodextrins, which may suggest a relatively high coverage of the RP phase by cholic acid micelles. The mechanism of separation achieved with bile salts is very complicated and difficult to explain, because at least three phenomena play a role here, i.e., (1) complexation equilibrium, (2) micelle formation in solution and on the stationary phase and (3) mixed adsorption processes on the stationary phase. In previous work, the authors suggested that micellar bile salts are adsorbed on the stationary phase via hydrophobic interactions and that the chiral recognition occurs there [17]. The observed elution order (*R* before *S*) suggests that the partitioning of the isomers into the micelle adsorbed on the stationary phase is more favorable for the *S* than for the *R* enantiomer.

In fact, using single selectors in HPLC, the reversal of elution order for BNP enantiomers may be realized simply by changing the cyclodextrin (β-CD or TM-β-CD) to bile salts or vice versa. Relatively high separation factors achieved with β-CD or CHASS assure baseline separation. However, very long separation times for BNP enantiomers achieved with β-CD solutions remain the problem to be solved. The optimization of the retention time in the case of β-CD applied as chiral selector will be discussed below.

3.2. Dual systems in HPLC

3.2.1. Two cyclodextrins

The behaviour of the BNP enantiomers observed with the mixture of two cyclodextrins agrees well with our expectation, and the results are presented in Table 2.

For the mixture of β-CD and TM-β-CD [6,7], which have the same elution order in the single system, the resolution has been significantly improved. The enantioselectivity reaches a value of 1.41 and the retention time decreases by a factor of three in comparison with the retention times in the

Table 2
Chromatographic data of BNP enantiomers in RP-HPLC system containing dual chiral selectors

Basic eluent	Additives	k_1	k_2	α	First eluted enantiomer
Cyclodextrins					
20% EtOH	20 m*M* β-CD+ 0.5 m*M* TM-β-CD	20.9	29.6	1.41	*S*
	20 m*M* γ-CD+ 0.5 m*M* TM-β-CD	16.7	16.7	1.00	
Cyclodextrins and bile salts					
30% EtOH	20 m*M* β-CD +20 m*M* CHASS	6.6	7.6	1.16	*R*
	10 m*M* TM-β-CD +20 m*M* CHASS	4.5	4.5	1.00	
20% EtOH	20 m*M* β-CD+ 20 m*M* CHASS	17.1	20.9	1.22	*R*
	0.5 m*M* TM-β-CD+ 20 m*M* CHASS	16.1	21.5	1.33	*R*

For chromatographic conditions, see Fig. 2.

single system for comparable concentrations of alcohol. This system allows the inversion of elution order of the enantiomers with respect to that observed with cholic acid sodium salt in the single system. The chromatograms of the same non-racemic mixture of BNP presenting the reverse elution order are shown in Fig. 2.

In the dual system with TM-β-CD and γ-CD, which have inverse elution orders in the corresponding single systems, enantioselectivity is no longer observed.

3.2.2. Mixed system with cyclodextrin and bile salt

Mixing cyclodextrin with cholic acid in the HPLC system does not lead to any advantage (see Table 2) in comparison to the single systems. Mixing β-CD and cholic acid sodium salt shortens the retention time slightly in comparison with the single system with cholic acid sodium salt. However, as the single selectors have inverse elution order, the enantioselectivity overall is worse. As the bile salt is adsorbed on the stationary phase, its concentrations both at the stationary phase and in solution remains unknown. So it is difficult to evaluate the binding properties of micellar CHASS with respect to BNP and to describe the corresponding equilibrium. Anyway, from the experimental results it can be seen that, in the case of β-CD and CHASS (used at equimolar concentrations in the mobile phase), elution order specific for bile salts has been obtained. This may suggest that the concentration of bile salt in the stationary phase is higher and/or its binding ability with respect to BNP enantiomers is stronger than that of β-CD. Another explanation involves the participation of the βCD–CHASS complex [36] in the separation process.

The first couple of injections in 20% EtOH with 0.5 m*M* TM-β-CD and CHASS (20 m*M*) give unreproducible results; it seems that cholic acid displaces or binds to permethylated cyclodextrin at the stationary phase. After some time the results are perfectly reproducible and almost identical results as for the single system with cholic acid sodium salt are obtained. Increasing the TM-β-CD concentration up to 10 m*M* in 30% EtOH destroys enantioselective ability of both selectors.

Some phenomena observed under HPLC conditions cannot be explained. The difficulties in their recognition are due mainly to the strong adsorption of the selectors (TM-β-CD, CHASS, TDCHASS) on the RP stationary phase. Thus, the expectations that some of these problems could be solved with CE studies seem reasonable.

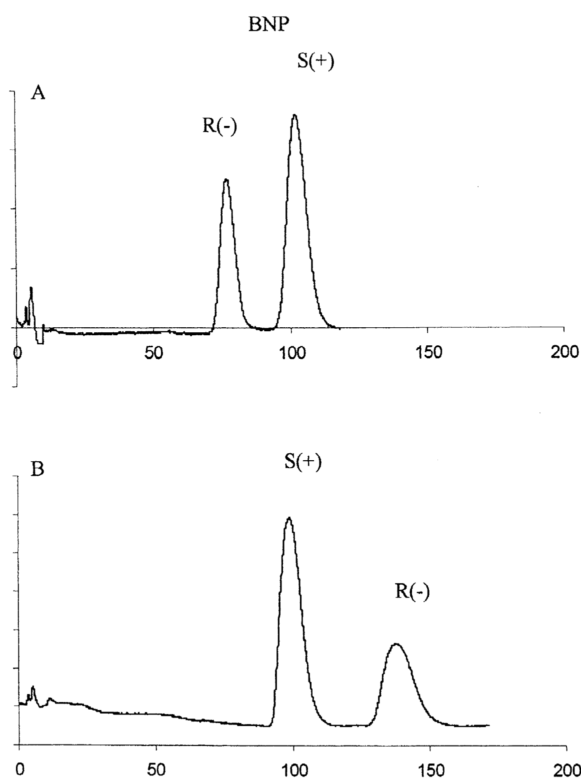

Fig. 2. Chromatograms of a non-racemic mixture of the BNP enantiomers with (A) 20 m*M* CHASS, (B) 20 m*M* βCD and 0.5 m*M* TM-βCD in 20% ethanol. Chromatographic conditions: column, 250×1 mm I.D. packed with LiChrosorb RP18, 5 μm; flow-rate, 0.04 ml/min; mobile phases were aqueous ethanolic solutions of 18 m*M* Tris, pH 6.5, containing the appropriate chiral selectors; temperature, 20 °C.

3.3. Single selector systems in CE

Data showing how single selector systems behave in CE are listed in Table 3.

The electrophoretic mobility in the background electrolyte (without chiral selector) is −14.4 and −13.2 for BNP and BN, respectively.

3.3.1. Cyclodextrins

According to our expectation, the addition of neutral CDs decreases electrophoretic mobility of BNP and BN in the direction of the anode.

Under the conditions used, the chiral separation of BNP enantiomers was achieved with TM-β-CD and HP-β-CD. In contrast to HPLC behaviour, the separation achieved with β-CD was poor and perceptible only after addition of ethanol (to decrease the EOF), while separation was not observed with -γ-CD. In the midst of three CDs only TM-β-CD enabled the separation of the BN enantiomers.

The elution order of BNP enantiomers with β-CD, HP-β-CD and TM-β-CD is the same: *S* before *R*. Thus, the affinity pattern for enantiomers seems to be identical for these cyclodextrins acting in the bulk buffer solution.

The above results seem to contradict with the results observed with HPLC, which indicate inverse affinity patterns for β-CD and TM-β-CD with respect to BNP enantiomers. Such results were obtained for 5×10^{-4} TM-β-CD in 20% EtOH, assuming that TM-β-CD is active mainly in the dynamically generated stationary phase and its action in solution is negligible.

In order to be able to explain the discrepancies, we have used higher concentration of TM-β-CD in the HPLC system (2×10^{-2} *M* in 30% EtOH), hoping to observe some loss of enantioselectivity as a result of the competitive inverse direction complexation in solution. However, in contrast to our expectations, the elution order was the same (with a much shorter retention time) as for β-CD and the selectivity slightly improved when compared to that achieved at lower concentrations of TM-β-CD. These results suggest that TM-β-CD molecules strongly adsorbed at the stationary phase, forming aggregates that reverse the selectivity when compared to the monomer in solution. The fact that TM-β-CD molecules do not form aggregates in water has been well documented by Coleman et al. [37,38]. Another possibility may be different stoichiometry of complexes formed at the stationary phase and in the bulk mobile phase, which are endowed with inverse sign of enantioselectivity. Another explanation could be the interaction of the sorption complex with the hydrophobic surface, which often dramatically changes the extent of chiral recognition or even the sign of enantioselectivity. This was observed and discussed by Davankov et al. [39,40]. However all hypotheses should be studied by the other methods.

The system with 20 m*M* TM-β-CD seems to be very similar to the dual system in which we have combined β-CD and TM-β-CD. A significant loss of

Table 3
Electrophoretic data of BNP and BN enantiomers for a single selector system

Buffer	BNP						BN					
	t_{EOF}	t_1/t_2	μ_1	μ_2	$\alpha=\mu_2/\mu_1$	First eluted enantiomer	t_{EOF}	t_1/t_2	μ_1	μ_2	$\alpha=\mu_2/\mu_1$	First eluted enantiomer
Without chiral selector												
	3.8	5.4/5.4	−14.4	−14.4	1.00		3.8	5.1/5.1	−13.2	−13.2	1.00	
Cyclodextrins												
20 m*M* β-CD	4.8	6.0/6.0	−7.3	−7.3	1.00		4.9	6.0/6.0	−6.7	−6.7	1.00	
20 m*M* β-CD+5%EtOH	6.1	7.77/7.82	−6.5	−6.7	1.02	*S*	6.1	7.67/7.67	−6.0	−6.0	1.00	
20 m*M* γ-CD	5.4	6.89/6.89	−7.0	−7.0	1.00		5.4	6.49/6.49	−6.4	−6.4	1.00	
20 m*M* HP-β-CD	5.3	6.41/6.46	−6.3	−6.6	1.04	*S*	5.3	6.2/6.2	−5.3	−5.3	1.00	
20 m*M* TM-β-CD	4.6	5.39/5.51	−6.0	−6.7	1.12	*S*	4.7	5.24/5.28	−4.1	−4.4	1.06	*S*
5 m*M* TM-β-CD	4.5	5.50/5.72	−7.9	−9.2	1.16	*S*	4.5	5.49/5.59	−7.3	−7.9	1.08	*S*
1 m*M* TM-β-CD	4.4	5.75/5.93	−10.0	−11.0	1.10	*S*						
Bile salts												
20 m*M* CHASS	5.5	9.48/9.87	−14.3	−15.1	1.05	*R*	5.7	8.58/8.75	−10.9	−11.4	1.03[a]	*R*
30 m*M* CHASS	5.5	9.71/9.92	−14.8	−15.2	1.03	*R*	5.5	9.14/9.23	−13.6	−13.8	1.01	*R*
50 m*M* CHASS	6.9	17.41/18.03	−16.0	−16.4	1.02	*R*	6.5	13.7/13.7	−14.7	−14.7	1.00	
80 m*M* CHASS	7.4	18.62/19.0	−15.1	−15.3	1.01	*R*	7.7	19.3/19.3	−14.3	−14.3	1.00	
20 m*M* TDCHASS	5.0	9.05/9.61	−16.5	−17.7	1.07	*R*	5.0	9.41/9.6	−17.0	−17.4	1.02	*R*
80 m*M* TDCHASS	6.4	16.46/16.97	−17.7	−18.1	1.02	*R*	6.8	19.5/19.5	−17.7	−17.7	1.00	

t_{EOF} is the migration time of neutral marker; t_1/t_2 is the migration time (min) of the first and second eluting enantiomer; μ_1, μ_2 are electrophoretic mobilities (cm^2 kV^{-1} min^{-1}) of the first and second eluting enantiomer.
For CE conditions, see Fig. 3

[a] Bad peak shapes.

retention of BNP enantiomers in both systems is due to coverage of the RP phase by TM-β-CD. The great advantage of both systems consists in the decrease of retention without losing selectivity, which is not possible using higher concentrations of alcohol.

3.3.2. Bile salts

The addition of the bile salts increases the electrophoretic mobility of BNP and BN in the direction of the positive electrode. The elution order of BNP enantiomers using the same selector is identical to HPLC. The first eluted peak corresponds to the enantiomer with the *R* configuration. These data agree with the earlier published paper of Szoko et al. [21]. The authors have found that the recognition is the result of complexation by tetramers of TDCHASS and that the *S* enantiomer of BNP is complexed twice as strongly by these tetramers than the *R* enantiomer

The influence of the bile salt concentration on the mobilities of the BNP and BN enantiomers was studied in the range of 20–80 m*M* (all concentrations above cmc [41]). The shortest time of analysis and the best selectivity were obtained at low concentrations of chiral selectors.

3.4. Dual system in EC

The influence of the bile salts concentration on the BNP and BN mobility has been studied at constant concentration of cyclodextrin (β-CD, TM-β-CD and γ-CD) equal to 20 m*M*. The concentration of CHASS changed in the range of 0–80 m*M*. The results are shown in Table 4. In Fig. 3 the electrophoretic mobilities of BNP enantiomers in the CHASS system and in the dual systems with cyclodextrins are compared. The results are dependent on the nature of cyclodextrin used.

It is interesting to note that by using a mixture of 20 m*M* β-CD and 20 m*M* CHASS, the elution order of BNP enantiomers was found equally specific compared to β-CD. Considering this result, it is worth noting that with β-CD alone only weak separation was observed. When, taking into account the fact that β-CD and CHASS form a very stable complex [36], one may suggest that this complex plays an important role in the resolution process and doing so imposes its own sense of selectivity, which is similar to that of β-CD. This explanation supposes the formation of ternary complex (BNP-β-CD–CHASS). With increasing CHASS concentration (higher than of β-CD) the elution order for the BNP enantiomers slowly changes from *S* first to *R* first, which is characteristic for free CHASS. So by changing the cholic acid concentration from 30 to 50 m*M* it is possible to switch the elution order of the BNP enantiomers, as shown in the electropherograms in Fig. 4. The elution order of enantiomers of charged selectands in dual systems with chiral selectors dealing independently in the stationary phase and mobile phase, have been discussed in detail by Schurig et al. [12].

For BNP and BN with CHASS and TM-β-CD the elution order for all concentrations of cholic acid at constant concentration of cyclodextrin appeared to be specific for cyclodextrin: *S* first.

For γ-CD and CHASS separation of the BNP enantiomers was obtained only at 50 and 80 m*M* of CHASS, while for the BN enantiomers separation was only observed at 50 m*M* CHASS, with elution order specific for CHASS.

From the single system in CE we learned that β-CD, TM-β-CD and CHASS have the same sense of enantioselectivity in respect to BNP enantiomers. If we consider information from HPLC experiments, γ-CD and CHASS exhibit, for BNP enantiomers, opposite enantioselectivity. Because of the various effects of their mechanisms of actions, cyclodextrin and CHASS influence the electrophoretic mobility of the enantiomers in opposite directions. Negatively charged bile salts exhibit electrophoretic movement opposite to the electroosmotic flow and behave like a pseudo-stationary phase. Neutral cyclodextrins moving with the electroosmotic flow can be considered as the chiral mobile phase. The situation is similar to the HPLC system, with the selectors residing in the mobile and stationary phase. If the cyclodextrins and CHASS act independently in the dual system, one may expect suppression of selectivity in the dual system for β-CD or TM-β-CD with CHASS and increase of selectivity for γ-CD and CHASS [12].

However, such effects have not been observed. Thus, our electrophoretic results confirm not only the literature data on the formation of mixed complexes of β-CD and CHASS [36], but also indicate that

Table 4
Electrophoretic data of BNP and BN enantiomers for dual selector system

Buffer solution	BNP						BN					
	t_{EOF}	t_1/t_2	μ_1	μ_2	$\alpha=\mu_2/\mu_1$	First eluted enantiomer	t_{EOF}	t_1/t_2	μ_1	μ_2	$\alpha=\mu_2/\mu_1$	First eluted enantiomer
Cyclodextrins and bile salts												
20 m*M* β-CD +20 m*M* CHASS	6.7	9.25/9.41	−7.5	−7.8	1.05	*S*						
20 m*M* β-CD +30 m*M* CHASS	6.5	9.23/9.32	−8.3	−8.5	1.02	*S*	6.5	7.72/7.77	−4.4	−4.6	1.03[a]	*R*
20 m*M* β-CD +50 m*M* CHASS	8.3	17.86/18.61	−12.0	−12.4	1.03	*R*	8.4	17.95/18.55	−11.8	−12.1	1.03	*R*
20 m*M* β-CD +80 m*M* CHASS	8.3	20.47/21.21	−13.3	−13.6	1.02	*R*	8.5	19.89/20.15	−12.3	−12.4	1.01	*R*
20 m*M* β-CD +20 m*M* TDCHASS	6.0	7.96/7.96	−7.9	−7.9	1.00		6.0	7.93/7.93	−7.3	−7.3	1.00	
20 m*M* β-CD +80 m*M* TDCHASS	7.3	16.10/16.74	−13.8	−14.2	1.03	*R*	7.5	18.38/18.82	−14.6	−14.9	1.02	*R*
20 m*M* TM-β-CD +20 m*M* CHASS	6.5	8.38/8.77	−6.3	−7.2	1.15	*S*	6.6	7.51/7.59	−3.4	−3.7	1.08[a]	*S*
20 m*M* TM-β-CD +30 m*M* CHASS	6.3	8.48/8.77	−7.7	−8.4	1.09	*S*	6.4	7.95/8.05	−5.7	−6.0	1.05	*S*
20 m*M* TM-β-CD +50 m*M* CHASS	8.0	14.05/14.37	−9.9	−10.2	1.03	*S*	8.1	12.81/13.08	−8.4	−8.7	1.04	*S*
20 m*M* TM-β-CD +80 m*M* CHASS	8.4	17.4/17.4	−11.3	−11.3	1.00		8.5	16.83/16.83	−10.3	−10.3	1.00	
20 m*M* TM-β-CD +20 m*M* TDCHASS	6.6	9.21/9.45	−8.1	−8.6	1.06	*S*	6.7	8.89/9.04	−6.8	−7.2	1.05	*S*
20 m*M* TM-β-CD +80 m*M* TDCHASS	9.7	26.94/27.62	−12.2	−12.3	1.01	*S*	10.0	28.9/28.9	−11.9	−11.9	1.00	
20 m*M* γ-CD +20 m*M* CHASS	6.0	7.89/7.89	−7.5	−7.5	1.00							
20 m*M* γ-CD +30 m*M* CHASS	6.7	9.87/9.87	−8.7	−8.7	1.00		6.4	8.25/8.25	−6.4	−6.4	1.00	
20 m*M* γ-CD +50 m*M* CHASS	6.9	13.40/14.05	−12.9	−13.6	1.05	*R*	6.9	12.40/12.69	−11.9	−12.3	1.03	*R*
20 m*M* γ-CD +80 m*M* CHASS	7.0	16.66/17.33	−15.1	−15.6	1.03	*R*	7.1	14.95/14.95	−13.7	−13.7	1.00	
20 m*M* HP-β-CD +20 m*M* CHASS	7.1	9.58/9.89	−6.9	−7.5	1.09	*S*	7.3	9.07/9.07	−5.1	−5.1	1.00	
20 m*M* HP-β-CD +80 m*M* CHASS	8.8	21.44/22.35	−12.3	−12.7	1.03	*R*	8.9	20.21/20.51	−11.6	−11.7	1.01	
20 m*M* HP-β-CD +20 m*M* TDCHASS	7.1	9.58/9.58	−6.9	−6.9	1.00		7.3	9.15/9.31	−5.0	−5.4	1.07[a]	*S*
20 m*M* HP-β-CD +80 m*M* TDCHASS	9.0	26.69/28.21	−13.5	−13.8	1.03	*R*	9.4	29.57/30.68	−13.4	−13.7	1.02	*R*

t_{EOF} is the migration time of neutral marker; $t_1 t_2$ is the migration time (min) of the first and second eluting enantiomer; μ_1, μ_2 are electrophoretic mobilities ($cm^2\ kV^{-1}\ min^{-1}$) of the first and second eluting enantiomer.
For CE conditions, see Fig. 3.
[a] Bad peak shapes.

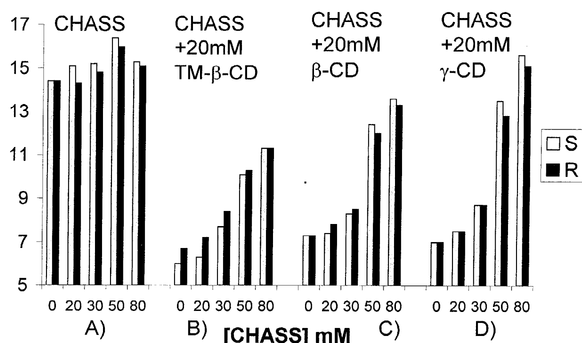

Fig. 3. Electrophoretic mobilities of the BNP enantiomers in the direction of the positive electrode (A) with single CHASS, (B) in its dual system with 20 m*M* TM-β-CD, (C) with 20 m*M* β-CD and (D) with 20 m*M* γ-CD, the concentration of CHASS changed in the range of 0–80 m*M*. Experimental conditions: capillary, 63 (58) cm×75 μm I.D. fused-silica; BGE, aqueous solution of 18 m*M* Tris, pH 11, containing the appropriate chiral selectors; applied voltage, 20 kV; temperature, 25 °C.

TM-β-CD and γ-CD form relatively stable complexes with bile salts.

4. Conclusion

It has been found that all investigated selectors, except for γ-CD, have the same affinity pattern in respect to BNP enantiomers in the bulk solution, i.e., they bind *S* enantiomer more strongly than *R*.

However various mechanisms of separation lead to opposite elution order for enantiomers: with cyclodextrins the *S* enantiomer is first eluted, while for bile salts *R* is the first eluted. Similar elution orders have been obtained both with HPLC and CE.

Considering results from an analytical point of view it is worth noting that, under the conditions used, the cyclodextrins (except γ-CD) as well as cholic acid sodium salts acting alone enable the separation of the BNP enantiomers both by HPLC and CE, while the BN enantiomers were resolved only under CE conditions with TM-β-CD or bile salts. In both techniques the application of dual systems either improves the resolution or makes it worse (or even destroys it), depending on the sign of selectivities of the particular selectors, their concentrations, localization: mobile or stationary phase and eventual interaction between selectors.

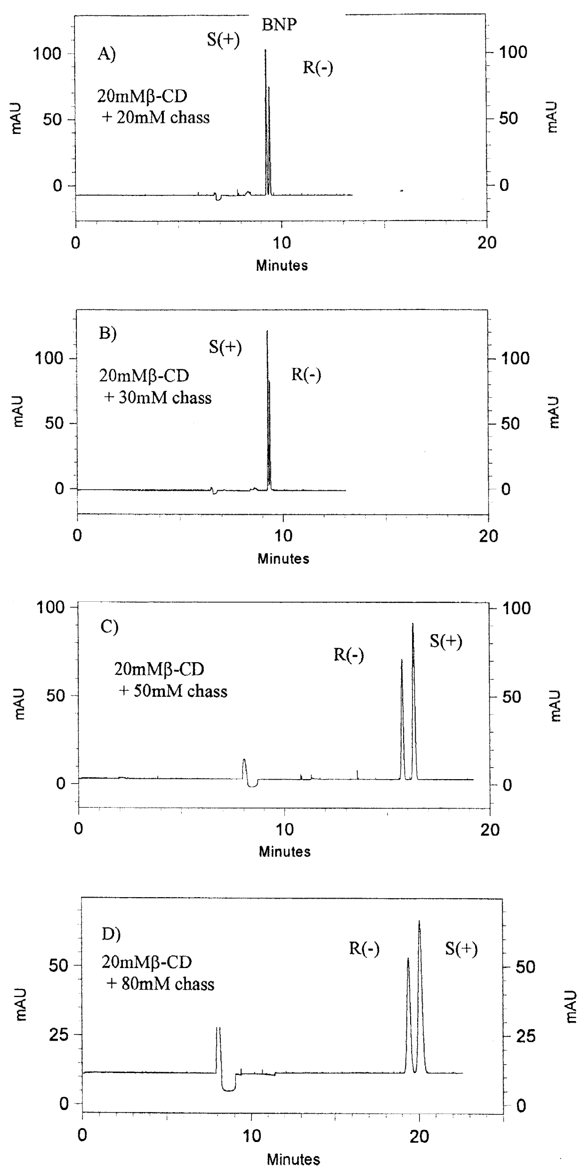

Fig. 4. Electropherograms of non-racemic mixture of the BNP enantiomers obtained with mixtures of 20 m*M* β-CD and CHASS: (A) 20 m*M*, (B) 30 m*M*, (C) 50 m*M*, (D) 80 m*M*. Conditions as in Fig. 2.

The reversal of elution order may be realized by two procedures: changing the single selector, i.e., cyclodextrin on cholic acid sodium salt or vice versa, or by changing the proportion of selectors in the bile salt–cyclodextrin combined system

For the determination of small amount of the *S*

enantiomer in non-racemic mixtures of BNP, different solutions are recommended depending on the method: in CE the dual system of 20 m*M* TM-β-CD with 20 m*M* CHASS, in HPLC the dual system of 20 m*M* β-CD with 0.5 m*M* TM-β-CD in 20% EtOH. For the *R* enantiomer the single system with 20 m*M* of CHASS gives the best separation for both techniques. For the BN enantiomers in CE (depending on non-racemic composition) two dual systems are recommended, for trace amounts of the *S* enantiomer 20 m*M* TM-β-CD with 30 m*M* CHASS and for trace amounts of the *R* enantiomer 20 m*M* β-CD with 50 m*M* CHASS (see electropherograms in Fig. 5).

Considering the physicochemical aspects of the current study it is worth underlining that the joint use of HPLC and CE methods to follow chiral solute behaviour in single and dual selector systems may provide interesting information. They can illuminate the mechanism of separations as well as give evidence on the interactions between analyte and selector or between two selectors.

Chromatographic and electrophoretic studies confirm not only the literature data on the formation of mixed complexes of β-CD and CHASS, but also indicate that TM-β-CD and γ-CD are also able to form relatively stable associates with bile salts. The behaviour of BNP enantiomers in RP-HPLC and CE systems modified with TM-β-CD has only been partially solved. The opposite recognition patterns of TM-β-CD in respect to BNP enantiomers observed in mobile solution (CE) and at the stationary phase (RP-HPLC) may be due to the various phenomena.

Change of recognition pattern may arise from different stoichiometry of complexes, formation of the aggregates at the stationary phase endowed with opposite optical activity or different adsorption of diastereomeric complexes. Chromatographic and electrophoresis physicochemical studies give evidence of some phenomena occurring in solution and on the stationary phase. However, these data have to be confirmed by more specific methods.

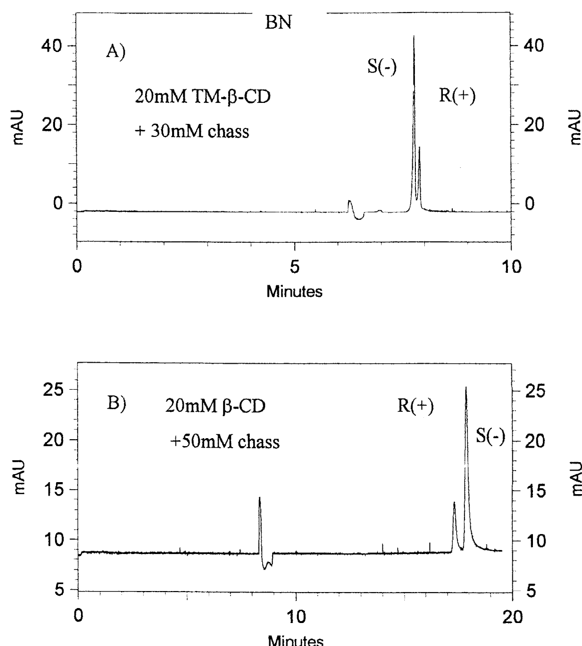

Fig. 5. Electropherograms of non-racemic mixture of the BN enantiomers obtained with the mixtures: (A) 20 m*M* TM-β-CD and 30 m*M* chass and (B) 20 m*M* β-CD and 50 m*M* chass. Conditions as in Fig. 2.

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