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Review

Separation selectivity in chiral capillary electrophoresis with charged selectors[1]

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Abstract

This review summarizes the separation selectivity in chiral capillary electrophoresis with charged selectors. Different parameters for a description of the separation selectivity, i.e., the absolute and the relative mobility difference between the enantiomers, the ratio of apparent mobilities and the ratio of effective mobilities as well as the intrinsic (recognition) selectivity, are compared. The electrophoretic mobility of the enantiomers and their migration with the electroosmotic flow are discussed from the viewpoint of separation selectivity. The affinity and mobility-related contributions in the separation selectivity with charged chiral selectors are separately considered. The selectivity of the separations using charged chiral selectors as carriers, in systems with a combination of chiral selectors as well as selectivity in chiral capillary electrochromatography are also briefly addressed. © 1997 Elsevier Science B.V.

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Contents

1. Introduction

Chiral separations are one of the most promising topics in the emerging field of capillary electro-

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phoresis (CE). More than 350 research papers, review articles and book chapters discuss this subject. The general merits of CE such as impressive peak efficiency, flexibility, robustness, environmental friendliness, small volumes and low costs are the most important factors contributing to the successful development of this technique. CE allows the observation of weak intermolecular interactions and even very weak stereoselective effects in these interactions. In CE it is common to observe two baseline resolved peaks for the mixture of enantiomers even in cases when the selectivity of separation does not exceed 1.01. This is difficult to achieve in alternative separation techniques such as gas chromatography (GC) or super/sub-critical fluid chromatography (SFC) and simply unimaginable in high-performance liquid chromatography (HPLC). The non-separative instrumental techniques such as optical- or NMR-spectrometry are also not ideally suitable for a study of very weak stereoselective interactions. Another important advantage of CE is that this technique operates in free solution that does not require the immobilization of the chiral selector. Thus, CE is able to model ligand–receptor interactions (among them also stereoselective ones) in a living body better than any other separation technique using immobilized selectors/receptors.

Chiral separations can be achieved in CE using various chiral selectors. Charged selectors have become increasingly important within last few years [1–7].

Questions such as comparison of the selectivity obtained with neutral and charged chiral selectors, the relevance between the selectivity of selector–selectand interactions and the selectivity of the separation, factors affecting a selectivity with charged chiral selectors, etc., are addressed in this review.

2. Basic definitions

According to the present terminology, a separation of enantiomers with neutral chiral selectors are ascribed to capillary zone electrophoresis (CZE) and charged chiral selectors to capillary electrokinetic chromatography (CEKC). The first key question one needs to resolve is if there exists any principal difference between the separation principles with charged and neutral chiral selectors.

The separation in CZE is based on a different electrophoretic mobility of charged analytes in a buffer solution under the effect of the applied electric field. Different electrophoretic mobilities of analytes are the result of their different effective charge density. The effective charge-to-mass ratio (charge density) does not differ for the enantiomers in the isotropic medium. Thus, enantioseparation is impossible in true CZE. The enantioseparations ascribed to CZE involve the addition of a chiral selector to the buffer solution. Enantioseparation is then based on the stereoselective interaction of the enantiomers with the chiral selector and not on the difference in their effective charge densities.

The principal requirement for a separation of enantiomers in CE is that one of the counterpart of separation either an analyte (selectand) or chiral selector is charged and as a consequence possesses different mobility to that of bulk solution. Both of them can also be charged. For simplicity let us to discuss two opposite cases: (a) an enantioseparation of a charged analyte with neutral chiral selector (CZE); and (b) an enantioseparation of uncharged analyte with a charged chiral selector (CEKC). The charged compounds possess a self-electrophoretic mobility and due to this reason their effective mobility differs from that of a bulk solution. Thus, the mobility of the analyte in case (a) and the mobility of the chiral selector in case (b) differ from that of the bulk solution. In contrast, the mobility of chiral selector in case (a) and the mobility of the chiral analyte in case (b) coincide with that of the bulk solution. Irrespective of these differences, the chiral separation principle is absolutely the same in cases (a) and (b) and relies on the different partition of enantiomers between the bulk solution and the chiral pseudophase. Therefore, the CE enantioseparations should be ascribed to CEKC independent of the charge of chiral selector.

This terminology-related change seems also reasonable based on the generally accepted opinion that in non-covalent intermolecular interactions (on which direct enantioseparations are based in CE) it is commonly difficult to identify which component is the selector (receptor) and which one is the selectand (ligand).

Additionally, some concepts developed for true CZE are not applicable for chiral CE separations. For instance, the concept of a virtual migration distance recently proposed by Rathore and Horváth [8] for CZE is not applicable for chiral CZE, but the same concept for CEKC is.

Selectivity is one of the important characteristics of a separation. What is the measure of selectivity in chiral CE separations? Is the definition used in pressure-driven separation techniques also useful for electrokinetically-driven separations or is a specific term required in the latter case? This question is addressed in many research papers and different parameters are proposed for the estimation of the separation selectivity.

A major goal of CE separations is to obtain resolved signals of the sample components in a form which is suitable for a precise quantitative estimation of the sample composition. Therefore, a characteristic which describes a separation in an optimal way and additionally considers the analysis time is an optimal parameter for the description of a separation. This parameter is a peak resolution (R_s) in all migration-based instrumental separation techniques and it is also suitable for CE. R_s was defined by Giddings [9] as follows:

$$R_s = \frac{\sqrt{N}}{4}\frac{\Delta\mu}{\mu_{av}} \quad (1)$$

N is the peak efficiency (plate number), $\Delta\mu$ is the mobility difference between two components (let us say $\Delta\mu_{R,S} = \mu_R - \mu_S$ for the R and S enantiomers) and μ_{av} is an averaged mobility of both enantiomers [$\mu_{av} = (\mu_R + \mu_S)/2$]. It is important to note that the analysis time does not enter the Giddings equation explicitly. However, it is mentioned implicitly in the both terms N and $\Delta\mu/\mu_{av}$.

The second term in Eq. (1) can be used as an ideal measurement of the separation selectivity.

Wren and Rowe [10] proposed an absolute mobility difference between a pair of enantiomers $\Delta\mu_{R,S}$ as a measure of a separation. A disadvantage of this term is that it does not consider the analysis time. The following example illustrates this drawback. Let us assume that in one case $\mu_R = 22$ mobility units and $\mu_S = 20$ and in another case $\mu_R = 100$ and $\mu_S = 102$. In both cases $\Delta\mu_{R,S} = \mu_R - \mu_S = 2$ but in the first case $\Delta\mu_{R,S}/\mu_{av} = 2/21 \approx 0.095$ and in the second case $\Delta\mu_{R,S}/\mu_{av} = 2/101 \approx 0.020$. Thus, an absolute mobility difference can not adequately indicate the difference between these two separation systems.

Another characteristic for the separation selectivity in CE is taken from chromatographic techniques and can be expressed as follows [11–13]:

$$\alpha_a = \mu_1/\mu_2 = (\mu_R)_a/(\mu_S)_a \quad (2)$$

In Eq. (2) $(\mu_R)_a$ and $(\mu_S)_a$ are the apparent mobilities of the R and S enantiomers of an analyte and therefore α_a is termed apparent selectivity. This parameter considers all thermodynamic and external separation parameters such as the affinity between the analytes and the selector, the effective electrophoretic mobilities of the analyte and the selector, the electroosmotic flow (EOF), nature, the pH and concentration of the buffer, etc. Additionally, as a ratio of mobilities this parameter also considers the analysis time. For instance, in the above mentioned two cases α_a would be 1.1 and 1.01, respectively. Thus, α_a describes the marked intrinsic difference between those separation systems. Additionally, α_a correlates well with the term $\Delta\mu_{R,S}/\mu_{av}$. For instance, if in one case $\mu_R = 22$ and $\mu_S = 20$ ($\alpha_a = 22/20 = 1.10$) and in the second one $\mu_R = 110$ and $\mu_S = 100$ ($\alpha_a = 110/100 = 1.10$) and $\Delta\mu_{R,S}/\mu_{av} \approx 0.095$ in both cases.

It is generally accepted that α_a between the sample components sholud be calculated so that its value always is equal or higher than the unit ($\alpha_a \geq 1.0$). However, in the case of enantioseparations it seems preferable to use values in the range $\alpha_a > 0$. The separation factor provides in this case an important information on the migration order of the enantiomers.

Several publications emphasized that α_a depends on the mobility of the EOF (μ_{EOF}) ''a phenomenon that is fundamentally non-selective'' [14]. Therefore, in order to avoid this ''non-selective'' term in some cases it is advantageous to perform a chiral separation in capillaries with eliminated EOF. Other authors attempted to eliminate μ_{EOF} from Eq. (2). This can be done by algebraic subtraction of μ_{EOF} from the apparent mobilities $(\mu_R)_a$ and $(\mu_S)_a$.

The EOF is really ''non-selective'' mobility in true electrophoretic separations which are based on a different charge density of the sample components.

Therefore, after subtraction of the EOF from an apparent mobility in pure CE separations one obtains a parameter which relates to the properties of the analyte (charge, mass, size, shape, etc.).

However, as aforementioned the enantioseparations in CE do not rely on the true electrophoretic principle. Two enantiomers do not differ from each other in an isotropic medium neither in terms of their electrophoretic mobility nor in terms of their mobility under the effect of the EOF. A chiral selector contributes to the mobility difference between the enantiomers absolutely irrespective of the fact that the enantiomers migrate by their own electrophoretic mobility or by the EOF [15]. Therefore, both μ_{el} and μ_{EOF} can be subtracted from the apparent mobility of the enantiomers with same result. However, this does not seem to be justified.

Thus, from the viewpoint of chiral separations there is absolutely no difference whether the enantiomers migrate with their electrophoretic mobility or with the EOF.

The intrinsic selectivity term (selectivity of a separating agent) was recommended recently for use as a characteristic of separation in chiral CE [14,16]. The formulation of this theory has been taken from chromatography, where a thermodynamic selectivity of a selector (stationary phase)–selectand recognition and a separation selectivity must coincide in the ideal case [17]. In contrast to chromatography, a relationship between a thermodynamic selectivity of recognition and a selectivity of separation is not straightforward in electrically-driven separation systems. This is caused by additional mobility contributions which are possible in CE. Several examples from the literature illustrate the limited value of the recognition selectivity in the determination of the separation selectivity in CE. This is especially true for charged chiral selectors.

Mechref and El Rassi [18] reported the reversal of the enantiomer migration order (EMO) of 1,1′-binaphthyl-2,2′-diyl hydrogen phosphate (BDHP) depending on the pH of the background electrolyte (Fig. 1). The affinity of the BDHP enantiomers to chiral selector does not change at least for the opposite one in the pH range (pH = 8.0–11.0) studied. The nature of this reversal of the enantiomer migration order was clearly described by the authors. The chiral selector used in this study was in-situ

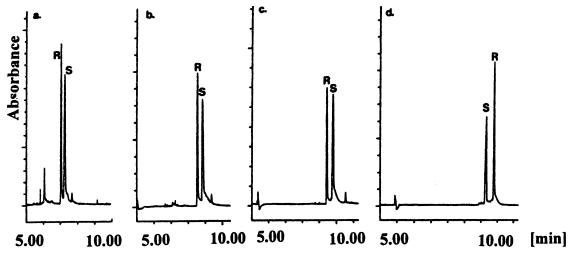

Fig. 1. Electropherograms of BDHP enantiomers obtained with Deoxy Big CHAP at various pH: 8.0 (a) 9.0 (b), 10.0 (c) and 11.0 (d). Fused-silica capillary, 57/50 cm×50 μm I.D.; running electrolytes 15.0 m*M* Deoxy Big CHAP, 50.0 m*M* borate at various pH; voltage 20.0 kV. (Reproduced from Ref. [18] with permission).

charged chiral surfactant N,N′-bis-(3-D-gluconamidopropyl)-deoxycholamide (Deoxy Big CHAP). This originally neutral surfactant becomes charged after the complexation with borate anions present in the background electrolyte. The chiral analyte BDHP is also anionic and a separation schema at pH 8.0 can be represented as in Fig. 2a. At this pH the μ_{ef} of BDHP is higher than that of the in-situ charged Deoxy Big CHAP. Therefore, the mobility of free BDHP opposes the EOF more

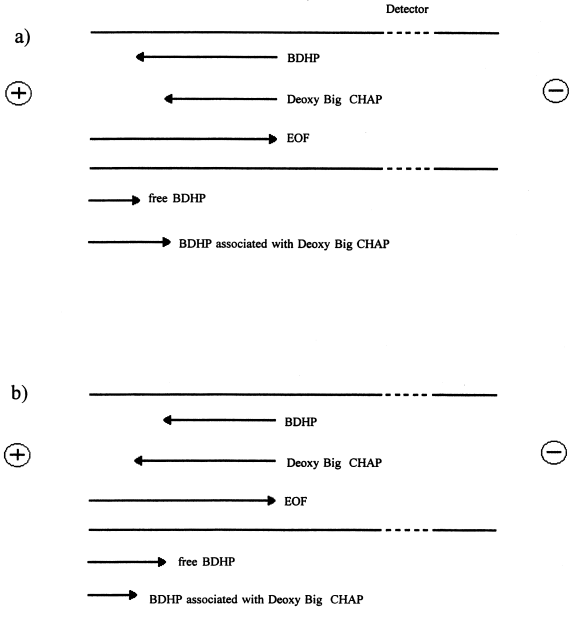

Fig. 2. Schematic representation of reversal of the enantiomer migration order of BDHP depicted in Fig. 1.

strongly than the mobility of BDHP associated with chiral surfactant. This means that the enantiomer which has higher affinity to Deoxy Big CHAP will be detected as the first peak and this is apparently *R*-BDHP. For simplistic reasons in Fig. 2 and throughout the text the mobility of the complexed analyte is considered to be equal to that of the chiral selector.

At pH 11.0 (Fig. 2b) the electrophoretic mobility of Deoxy Big CHAP exceeds that of free BDHP. Therefore, in contrast to pH 8.0, at pH 11.0 the mobility of complexed analyte will oppose more strongly the EOF than that of free BDHP. This means that the enantiomer of BDHP which is preferentially associated with the chiral micellar phase will migrate as the second peak. Thus, as this example shows the intrinsic selectivity (α_{int}) which can be determined as the ratio of the affinity constants of the enantiomers towards chiral selector [2,14,16]:

$$\alpha_{\text{int}} = K_R/K_S \tag{3}$$

may remain the same even in those cases when the enantiomers show the reversed migration order. This is an unique example characteristic for electrically-driven separation systems. In pressure-driven separation systems a reversal of enantiomer migration order almost always requires the opposite affinity of the enantiomers to the chiral selectors [19]. This should be considered when the formalism of chromatographic techniques are transferred to electrically-driven separations.

The simplified mobility difference equation (Eq. (4)) derived for enantiomers by Wren and Rowe [10] clearly illustrates a difference between pressure-driven and electrically-driven separation systems in the terms of separation selectivity:

$$\Delta\mu_{R,S} = \frac{(\mu_f - \mu_c)(K_R - K_S)[C]}{1 + [C](K_R + K_S) + K_R K_S [C]^2} \tag{4}$$

In this equation $[C]$ is the equilibrium concentration of a chiral selector and μ_f and μ_c are the mobilities of the free and the complexed analyte, respectively. From Eq. (4) it is obvious that for given values of K_R and K_S (e.g., for a given intrinsic selectivity) $\Delta\mu_{R,S}$ can obtain all possible values: i.e., $\Delta\mu_{R,S}>0$; $\Delta\mu_{R,S}=0$ and $\Delta\mu_{R,S}<0$ depending on the term $(\mu_f - \mu_c)$. This means that for a given intrinsic selectivity the enantioseparation can be observed with a certain enantiomer migration order (let us assume *R* before *S*, $\Delta\mu_{R,S}>0$), no enantioseparation ($\Delta\mu_{R,S}=0$) or an enantioseparation with reversed migration order (*S* before *R*, $\Delta\mu_{R,S}<0$) may be the cases.

The experimental evidence of the phenomenon described above can be found in Refs. [2,20] (Fig. 3). ^{1}H-NMR spectroscopy was used in these studies as an independent technique in combination with CE in order to measure the binding constants of each enantiomer of BDHP with the chiral selector. As shown in Fig. 4, the affinity pattern of the BDHP enantiomers toward carboxymethyl-β-cyclodextrin (CM-β-CD) is the same at both pH 3.0 and 5.0. The calculation of apparent binding constants showed that *S*-(+)-BDHP is the more tightly bound enantiomer [2,20,21]. However, the opposite migration order of the enantiomers was observed at pH 3.0 and 5.0 and no enantioseparation was observed at pH 4.3. The effect can be explained based on Eq. (4) and using the scheme represented in Fig. 5. At pH 3.0 the electrophoretic mobility of predominantly protonated (i.e., uncharged) CM-β-CD is lower than that of the anionic BDHP. Therefore, the preferentially complexed *S* enantiomer of BDHP migrates as the

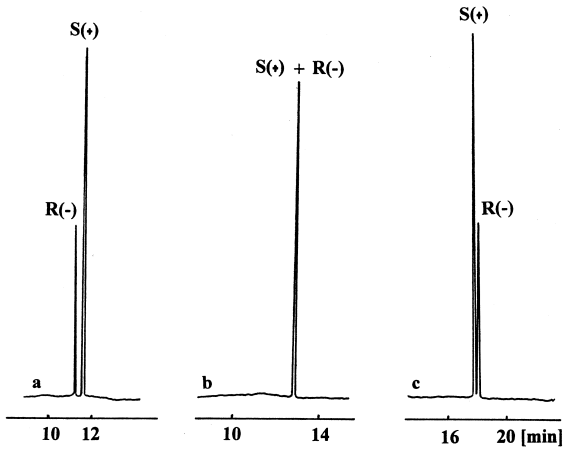

Fig. 3. pH dependent reversal of the enantiomer migration order of BDHP [nonracemic mixture of *S*-(+)/*R*-(−), 2:1] using 5.0 mg/ml CM-β-CD at various pH. Fused-silica capillary of 60/43 cm×50 μm I.D.; applied field strength 400 V/cm; (a) pH 3.0, (b) pH 4.3 and (c) pH 5.0; injection on the anode. (Reproduced from Ref. [20] with permission).

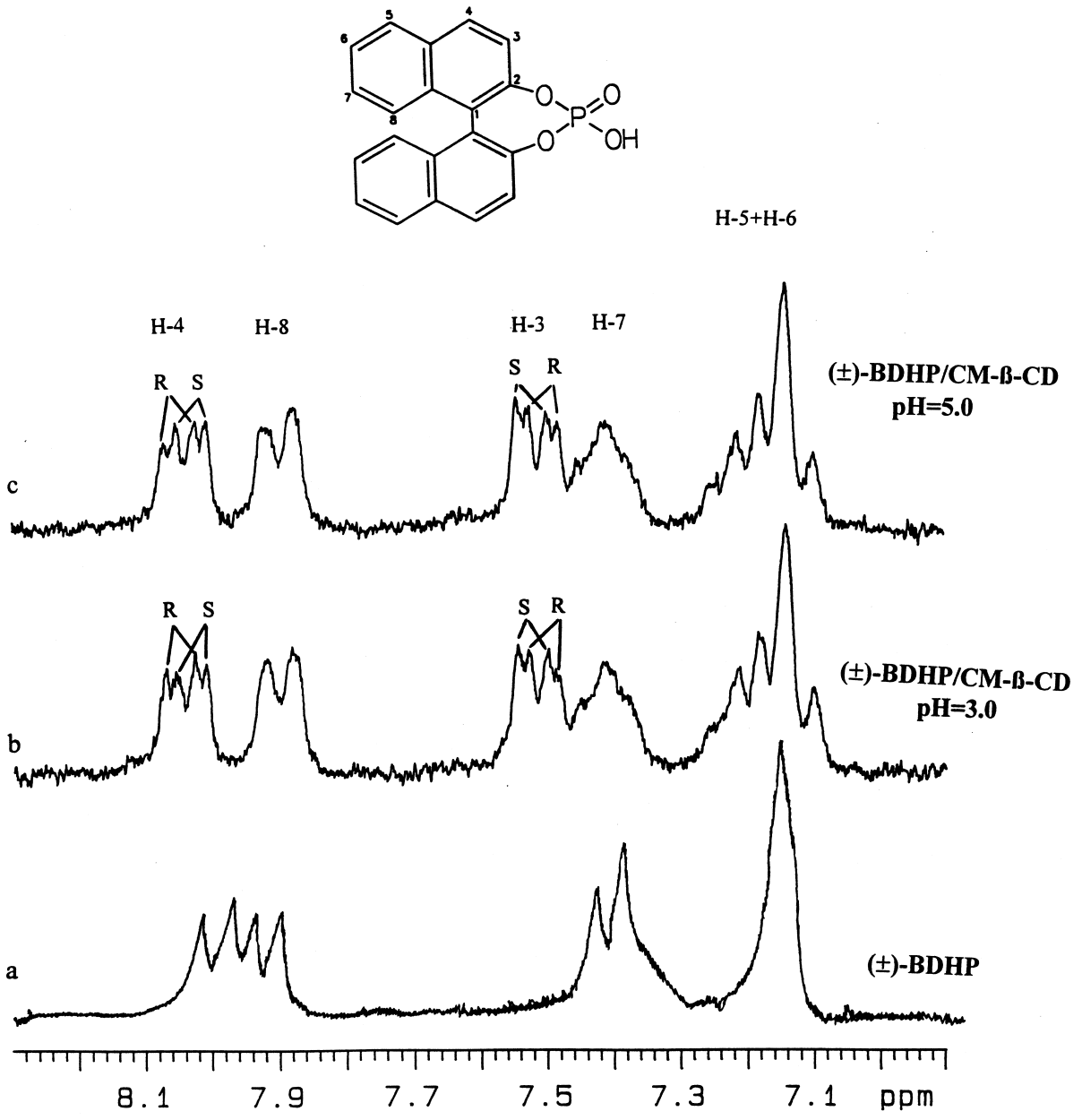

Fig. 4. ^{1}H -NMR spectra of (±)-BDHP (a) and an equimolar mixture of (±)-BDHP and CM-β-CD in $^{1}H_2O$ adjusted to p*D* equivalent to pH 3.0 (b) and pH 5.0 (c). Only aromatic range is depicted.

second peak ($\mu_f > \mu_c$ in Eq. (4)). At pH 4.3 the mobilities of BDHP and CM-β-CD are identical. Therefore, in spite of the chiral recognition no enantioseparation can be observed in CE ($\mu_f = \mu_c$ in Eq. (4)). At pH 5.0 the mobility of at least partially deprotonated CM-β-CD exceeds that of BDHP. Therefore, the preferentially bonded *S*-BDHP migrates as the first peak ($\mu_f < \mu_c$ in Eq. (4)).

The following equation has been proposed for the calculation of the intrinsic selectivity [14,16]:

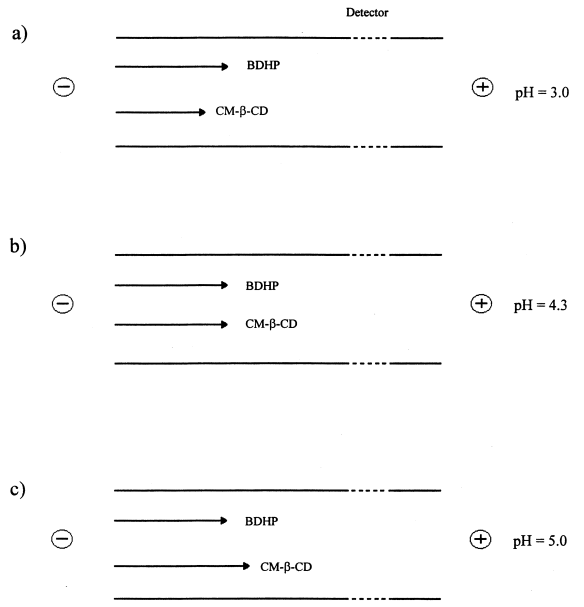

Fig. 5. Schematic representation of reversal of the enantiomer migration order of BDHP depicted in Fig. 3.

$$\alpha = \frac{k_1}{k_2} = \left(\frac{\mu_{f,1} - \mu_1}{\mu_{f,2} - \mu_2}\right)\left(\frac{\mu_2 - \mu_{c,2}}{\mu_1 - \mu_{c,1}}\right) \quad (5)$$

where k_1 and k_2 are the affinity factors of two analytes (enantiomers), $\mu_{f,1}$ and $\mu_{f,2}$ are the mobilities of the free enantiomers, $\mu_{c,1}$ and $\mu_{c,2}$ are the mobilities of complexes of the enantiomers with the chiral selectors, and μ_1 and μ_2 are the apparent mobilities of the enantiomers. To calculate an intrinsic selectivity according to Eq. (5) it is required to determine the mobilities of selector–selectand complexes. This is not always easy. Another drawback of Eq. (5) is the attempt of complete substitution of affinity terms with mobility characteristics. This may be justified in pressure-driven separation systems but is not always justified in electrically-driven separations. For instance, in the case displayed in Fig. 3b, Eq. (5) correctly predicts that α equals zero and no separation can be observed in CE. However, it does not describe the intrinsic selectivity of the selector–selectand interactions correctly. ^{1}H-NMR spectroscopy undoubtedly shows the presence of a chiral recognition in this particular case.

One important point which should be mentioned is that based on Eq. (6) [22]; an enantioseparation is possible in CE even in the case when the affinities of enantiomers to the chiral selector are the same, i.e., in a total absence of intrinsic selectivity.

$$\Delta\mu_{R,S} = \frac{\mu_f + \mu_{c,1}K_R[C]}{1 + [C]} - \frac{\mu_f + \mu_{c,2}K_S[C]}{1 + K_S[C]} \quad (6)$$

where μ_f is the mobility of the free enantiomers and $\mu_{c,1}$ and $\mu_{c,2}$ are the mobilities of complexed *R* and *S* enantiomers. Enantioseparations in CE in the absence of a chiral recognition can be based on the mobility difference between the transient diastereomeric complexes. Thus, the parallels between the intrinsic (recognition) selectivity and a separation selectivity in CE must be drawn very carefully. The particular case when the separation and recognition selectivities may coincide in CE has been discussed in [11].

The theoretical possibility of an enantioseparation even in the case of the same affinity of the enantiomers towards a chiral selector is a conceptual difference between chromatographic techniques with chiral stationary phases (CSPs) and CE. A similar effect could be principally observed in HPLC with chiral mobile phase additives (CMPA). It occurs than when the transient selector–selectand diastereomeric complexes which are characterized by the same equilibrium constants exhibit a different affinity towards an achiral stationary phase. To the author's knowledge no enantioseparation has been described so far in chiral CE based purely on the different mobility of transient diastereomeric complexes of two enantiomers with the same chiral selector but this phenomenon has been documented and apparently plays an important part in various separation effects observed in several studies [23–26].

In summary, the term $\Delta\mu/\mu_{av}$ which enters the peak resolution Eq. (1) proposed by Giddings [9] is recommended to characterize the separation selectivity in chiral CE. The modified version of this term proposed by Gebauer and Boček [27] can be advantageous in some special cases [28]. The ratio of the migration times (t_2/t_1) or the apparent mobilities (μ_1/μ_2) can describe chiral CE separations satisfactorily. On the other hand a subtraction of the EOF from the apparent mobility of the enantiomers in order to calculate the effective selectivity of the separation is not absolutely justified in chiral CE.

The intrinsic selectivity of selector–selectand interactions (selectivity of chiral recognition) which correlates well with the separation selectivity in pressure-driven separation systems is not always useful in electrically-driven systems. Therefore, this characteristic should be used with care in chiral CE.

3. Selectivity in chiral CE vs. selectivity in other techniques

The separation mechanism in CE differs conceptually from that in other separation techniques. This implies some additional requirements in order to observe a separation in CE. Some of them were mentioned above. For instance, a difference in the mobilities of the free and the complexed analyte is required to observe a separation in CE. This requirement is obviously fulfilled in chromatographic techniques. The separation selectivity in chromatographic techniques in ideal case approaches to the thermodynamic selectivity of recognition but never exceeds it. However, this is in principle possible in CE.

From non-separation techniques the selectivity in NMR spectroscopy can correlate to some extent to the separation selectivity observed in CE. NMR spectroscopy can mimic a recognition process in chiral CE better than any other instrumental technique. Both CE and NMR spectroscopy are liquid-phase techniques. If required a NMR-spectrum can be taken exactly in the same buffer in which a CE separation can be performed. Additionally, NMR spectroscopy can provide important data on the stoichiometry, the apparent equilibrium constants and structure of the selector–selectand complex. The latter can not be derived from CE data. The apparent binding constants determined in CE may be useful for the determination of the intrinsic (recognition) selectivity ($\alpha = K_R/K_S$) [2,21,29]. However, a chiral recognition observed in a NMR spectrum for a given selector–selectand pair does not a priori mean a chiral separation in CE. First, a chiral recognition caused by a different affinity of the enantiomers to a chiral selector does not necessarily result in a separation in CE [2,29]. Additionally, a signal splitting observed for the enantiomers due to the complexation-induced chemical shift non-equivalence is not always caused by a different affinity toward the chiral selector in NMR spectroscopy. Another reason for chiral recognition in NMR spectroscopy can be the intrinsically different NMR characteristics of the selector–selectand transient diastereomeric complexes in analogy to the above mentioned different mobilities of these complexes in CE. As a rule a chiral recognition of this origin observed in NMR spectroscopy will not correlate with chiral separations in CE. This may be the case for instance, in the pair of chiral arylpropionic acid derivative fenoprofen and β-CD. The ^{13}C-NMR spectrum of racemic fenoprofen (Ga^{2+} salt) in $^{2}H_2O$ in the presence of β-CD shows a chiral recognition (Fig. 6). However, Rawjee et al. [12] reported the equal binding constant for the anionic form of the fenoprofen enantiomers with β-CD based on CE measurements. A chiral recognition in the same selector–selectand pair was also confirmed by X-ray analysis of crystals isolated from the aqueous solution of the racemic fenoprofen and β-CD [30]. In both ^{13}C-NMR and X-ray experiments fenoprofen was apparently present in the predominantly anionic form. As this example shows chiral recognition data from other techniques must be transferred with care to CE separations.

4. Separation selectivity with charged chiral selectors vs. separation selectivity with neutral chiral selectors

As mentioned above there is no conceptual difference between chiral separations using neutral and charged chiral selectors. However, the self-electrophoretic mobility of a charged chiral selector may result in some effects which cannot be observed with neutral chiral selectors [2].

In the case of neutral chiral selectors the mobility of a complexed analyte (a separation window) may change in the finite range which for the normal polarity mode can be determined as follows:

$$\mu_{EOF} < \mu_c < \mu_f \quad (7)$$

In capillaries without EOF Eq. (7) transforms to:

$$0 < \mu_c < \mu_f \quad (8)$$

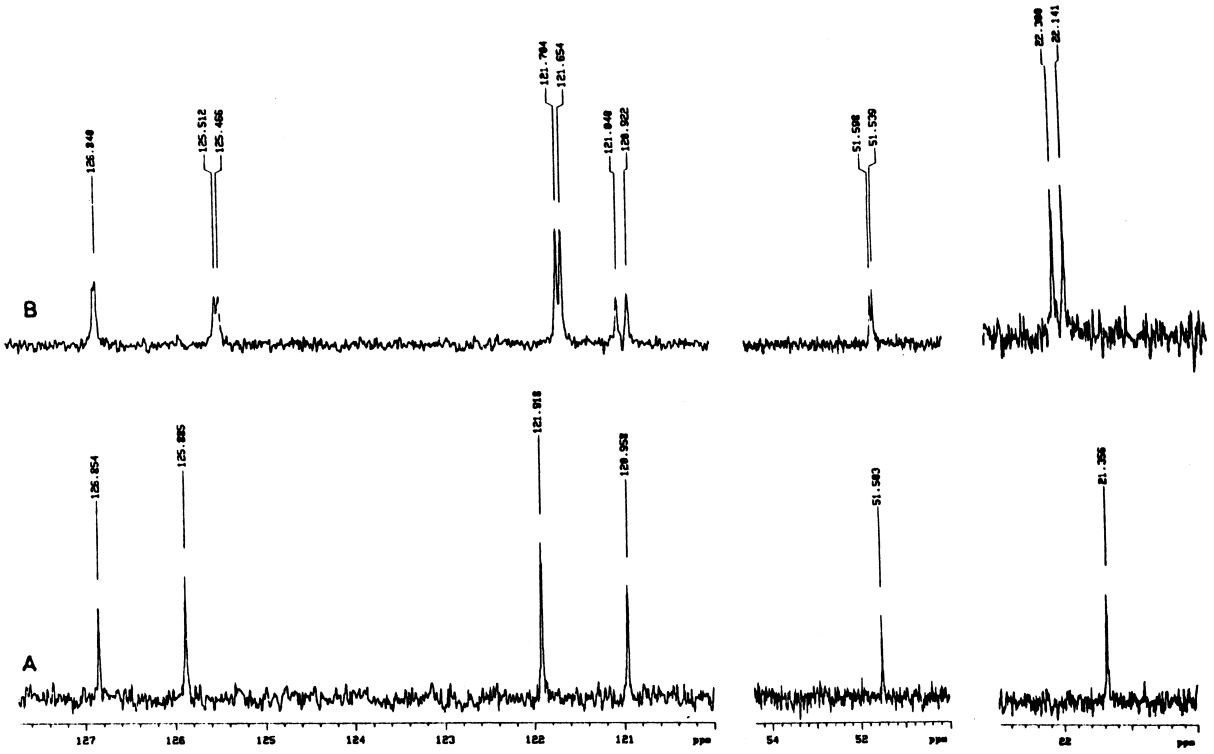

Fig. 6. Partial ^{13}C-NMR spectra of (±)-fenoprofen (A) and (±)-fenoprofen–β-CD (molar ratio 1:2) (B) in $^{2}H_2O$.

In the case of a charged chiral selector Eq. (7) can be written as:

$$\mu_{sel} < \mu_c < \mu_f \quad (9)$$

From Eqs. (7) and (9) it is clear that the separation selectivity with charged chiral selectors may exceed the thermodynamic selectivity of the chiral recognition. This is in principle feasible with neutral chiral selectors too but only with a designed EOF.

From Eqs. (1), (4), (9) it is obvious that for obtaining a high separation selectivity it is desirable that an analyte and a chiral selector are oppositely charged. This has been also noted in several studies [2,6,7,22,31].

5. Factors affecting the separation selectivity with charged chiral selectors

Before discussing the external factors which may affect the separation selectivity with charged chiral selectors it seems important to note that the mobility of a chiral selector does not explicitly enter the separation equation (Eq. (1)). However, it affects the separation implicitly.

5.1. Effect of a charge of chiral selector on selector–selectand interactions

Together with hydrophobic interactions, hydrogen bonding, etc., electrostatic forces also play an important part in the formation of intermolecular complexes [32–34]. In aqueous media where the role of hydrogen bonding seems to be somewhat limited, electrostatic interactions are one of the critical forces together with hydrophobic interactions. CE is mainly dedicated to the analysis of charged compounds and when the chiral selector is also charged the role of electrostatic interactions becomes evident. However, these forces may be important even in separations of

neutral analytes (because dipole-induced–dipole interactions may be involved in this case).

Several examples are described in the literature when enantioseparations which were virtually impossible with neutral CDs became possible with charged chiral selectors. The neutral chiral compounds are not mentioned there which are conceptually impossible to resolve using neutral chiral selectors in CE.

Nardi et al. [35] reported a higher separation ability of 6-monomethylamino-6-deoxy-β-CD and 6A,6D-dimethylamino-6A,6D-dideoxy-β-CD towards aromatic hydroxy acids compared to neutral CDs such as native β-CD, heptakis-(2,6-di-O-methyl)-β-CD (DM-β-CD) and 2-hydroxypropyl-β-CD (HP-β-CD). For example, 6A,6D-dimethylamino-β-CD resolved almost baseline racemic *meta*-methylmandelic acid and 3,4-dimethylmandelic acid at a concentration as low as 1 m*M* while none of the neutral CD derivatives exhibited a chiral resolving ability even at concentration of up to 80 m*M*. This improvement of the chiral separation was explained with the stabilization of the inclusion complexes and the enhanced enantioselectivity of binding due to additional electrostatic interactions between the amino groups (chiral selector) and the carboxy groups (analyte). Perhaps an effect of the self-mobility of cationic chiral selector in the opposite direction of the anionic analytes could also positively contribute the enhanced separation in this case.

The higher chiral separation ability of 6-monoamino-6-deoxy-β-CD toward the mandelic acid compared with native β-CD has been also reported [2]. 2 m*M* of the former chiral selector resulted in a baseline separation of the mandelic acid enantiomers whereas the latter was ineffective even at concentration of 18 m*M*.

Brown et al. [36] reported that 6-monoaminopropylamino-6-deoxy-β-CD discriminates the enantiomers of sodium 2-phenylpropanoate much better than unmodified β-CD. Furthermore, the chiral recognition of this selector is enhanced dramatically by its complexation with Ni^{2+}.

Many examples of better chiral separation were reported in CE with anionic CD derivatives compared to neutral analogues [2–7,24,29,31,37,38]. However, when only a quantitative improvement of the selectivity occurs by changing from a neutral chiral selector to a charged one it is quite difficult to attribute the observed effect definitely either to the affinity or the mobility factors based on the CE data alone. Additional techniques such as NMR spectroscopy can be helpful in these cases.

As mentioned above charged chiral selectors are more effective compared with their neutral analogues for the separation of oppositely charged analytes [2,5–7,21,37–46]. However, a decrease of the enantioseparation is observed when a chiral selector and an analyte carry a net charge of the same sign. A general trend is to ascribe this effect to the electrostatic selector–selectand repulsion [42,44]. It seems worth noting that the effect of electrostatic repulsion can be minor compared to the negative effect of the migration of the chiral selector and the analyte in the same direction. This negative effect is as common as the positive effect in the case of an oppositely charged analyte–selector pair [2,5–7,22,31].

In several studies combining CE and NMR spectroscopy a higher chiral recognition ability of charged selectors toward oppositely charged analytes has been confirmed [29,45,46]. A ^{13}C-NMR study showed that CM-β-CD and sulfobutyl β-CD (SBE-β-CD) possess a higher chiral recognition ability compared to native β-CD toward the cationic antihistaminic drug mianserine [45]. This result correlates well with the separation selectivity observed with comparable amounts of these chiral selectors in CE. The binding constant measurements of the pairs of anionic BDHP with CM-β-CD and with native β-CD indicated that the selector–selectand affinity is lower for the pair where the both counterparts possess a negative charge. However, the enantioselectivity of the binding was higher for this pair (Table 1) [21].

Kano et al. [46] performed a combined CE, ^{1}H-NMR spectroscopy and molecular modeling study on the chiral recognition of anionic binaphthyl derivatives with β-CD and 6-monoamino-6-deoxy-β-CD. The latter selector allowed a chiral separation of BDHP and 1,1′-binaphthyl-2,2′-dicarboxylic acid in a 33 m*M* phosphate buffer at pH 7.21. At this pH the self electrophoretic mobilities of these anionic analytes are lower than the oppositely directed EOF resulting in the drag of the analytes towards the cathode by the EOF. The shorter migration time of BDHP is considered as an indication of its stronger

Table 1
Average apparent binding constants (K_a) and selectivity of binding (α_i) for the complexes of *S*-(+)-BDHP and *R*-(−)-BDHP with β-CD and CM-β-CD

CD	$K_{a[R\text{-}(-)\text{-BDHP}]}$ (M^{-1})		$K_{a[S\text{-}(+)\text{-BDHP}]}$ (M^{-1})		$(K_a)_{av[R\text{-}(-)\text{-BDHP}]}$ (M^{-1})	$(K_a)_{av[S\text{-}(+)\text{-BDHP}]}$ (M^{-1})	α_i [a]
	H-3	H-8	H-3	H-8			
β-CD	312	210	413	263	261	338	1.30
CM-β-CD	106	68	175	119	87	147	1.70

[a] $\alpha_i = (K_a)_{av[S\text{-}(+)\text{-BDHP}]} / (K_a)_{av[R\text{-}(-)\text{-BDHP}]}$.
Reproduced from Ref. [21] with permission.

binding to the chiral selector and this is evidently confirmed by ^{1}H-NMR spectroscopy. However, when the migration times are used as a measure of binding interactions an attention must be paid to the net charge of the ions that are compared. For instance, BDHP in the above mentioned separation conditions is present as a monoanion, whereas 1,1′-binaphthyl-2,2′-dicarboxylic acid probably as a dianion. This can also contribute to the longer migration time of the latter compound. In this study the electrostatic interaction is assumed to be a major binding force contributing to the chiral separation. This seems to be also well illustrated. However, it is somewhat difficult to understand why this interaction is so much higher between of two single oppositely charged ions (BDHP and the chiral selector) compared to an apparently double charged anion (1,1′-binaphthyl-2,2′-dicarboxylic acid) and single charged cation (chiral selector).

More evident is an effect of the introduction of a charge in the structure of the chiral selector in those examples in which the migration order of the enantiomers reverts due to this manipulation. It is obvious that enantiomers possess opposite affinities to chiral selectors with opposite chirality. However, the optical antipodes are not available for some chiral selectors of natural origin (CDs, bile salts, macrocyclic antibiotics, etc.).

A classical example when an achiral modification of the CD led to the reversal of its enantioselective effect was described by Breslow and Czarnik [47]. The use of β-CD derivatized with a pyridoxamine moiety at its secondary hydroxy group as a catalyst resulted the amino acids with opposite chirality to that produced from the corresponding 6-O-modified β-CD in transamination reaction. The thermodynamic and kinetic effects may overlap in this experiment.

Several examples are described in the literature [19,48–52] when the introduction of a charge in the CD structure caused the reversal of the enantiomer migration order. Schmitt and Engelhardt [48] reported the opposite migration order of the ephedrine enantiomers with DM-β-CD [(1*R*,2*S*) before 1*S*,2*R*] and carboxymethyl-β-CD (CM-β-CD) [(1*S*,2*R*) before (1*R*,2*S*)]. The reversed migration order of the enantiomers of chiral antihistaminic drug dimethindene was observed by alternative use of β-CD (*S* before *R*) and CM-β-CD (*R* before *S*) [19]. The recognition selectivity of heptakis (tri-O-methyl)-β-CD (TM-β-CD) and CM-β-CD are also opposite toward chiral calcium antagonist drug verapamil and its major metabolite norverapamil [50,51]. *R*-(+) enantiomers of both compounds migrate as the first peak with the neutral CD and as the second peak with the charged CD.

It is also worth noting that even in those cases when an introduction of chargeable group in the CD structure leads to a reversal of recognition selectivity it is difficult to attribute this effect to pure electrostatic interactions. For instance, CM-β-CD exhibits an opposite recognition compared with neutral CDs toward ephedrine and dimethindene even at pH 2. Apparently, CM-β-CD is predominantly undissociated at this pH. The carboxylic group may also serve as a hydrogen-bonding site. As several studies claim this results in a change of the affinity of the chiral selector even in an aqueous medium. Additionally, the effect of substituent on the chiral recognition ability can be of pure geometric origin. Several examples are described in the literature when the introduction of neutral substituents also led to the

reversal of the recognition selectivity of CDs [37,53–55].

In some cases the reversed migration order of enantiomers with neutral and charged CDs is not caused by an opposite affinity of the chiral selectors. For example, the opposite migration order of the BDHP enantiomers has been observed by alternative use of permethylated β-CD (PM-β-CD) and sulfopropyl-β-CD (SPE-β-CD) as chiral selectors [49]. This reversal of the enantiomer migration order does not seem to be the result of opposite affinities of the chiral selectors. A possible explanation is illustrated in Fig. 7. The separation conditions in this study were similar to those described in Ref. [46]. Therefore, the preferentially bonded enantiomer will migrate as the first peak with a neutral CD (Fig. 7a). In contrast to the neutral PM-β-CD the anionic SPE-β-CD also migrates in the same direction as the chiral analyte. Apparently, the mobility of SPE-β-CD exceeds that of BDHP. Due to this reason the more tightly complexed enantiomer will migrate as the second peak (Fig. 7b). As shown by this example in case of charged CDs the reversal of the enantiomer migration order must be explained with the reversal of the recognition pattern with some care.

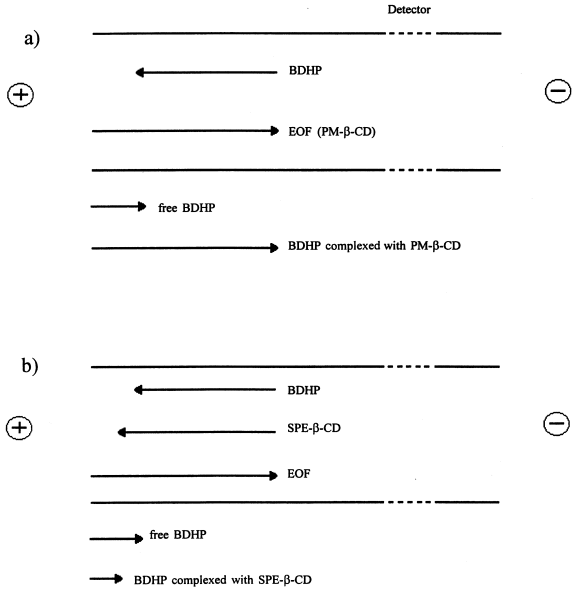

Fig. 7. Schematic representation of reversal of the enantiomer migration order of (±)-BDHP with PM-β-CD (a) and SPE-β-CD (b).

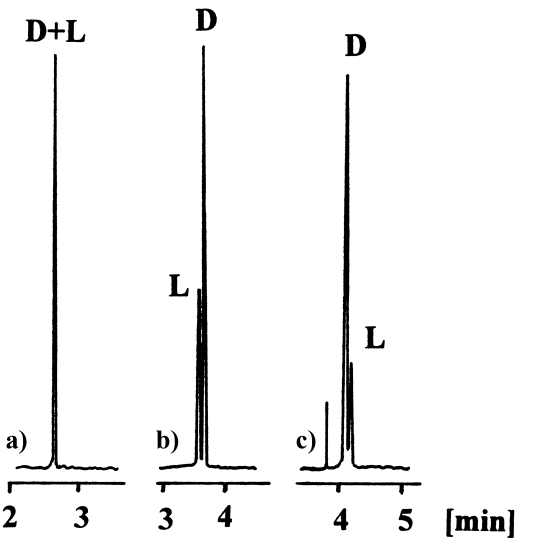

Fig. 8. Enantiomer separation of dansyl-*tert*.-leucine by CE using a 30 m*M* phosphate buffer (pH 7) containing 20 m*M* γ-CD and 50 m*M* SDS (a), 10 m*M* SBE-γ-CD (b) or 10 m*M* SBE-β-CD (c). Fused-silica capillary. Injection at the anode. (Reproduced from Ref. [42] with permission).

Recently, Jung and Francotte [42] described an opposite migration order of the enantiomers of dansyl-*tert*.-leucine (Dns-*tert*.-Leu) by alternative use of SBE-β-CD or sulfobutyl-γ-CD (SBE-γ-CD) as chiral selectors (Fig. 8). The reversal of the EMO in this case also seems to be related rather to the mobility than to the affinity. Apparently one of these chiral selectors migrates with a higher self-mobility than the analyte while the other one migrates with a lower self-mobility compared to the analyte (Fig. 9). This may result in an opposite migration order of the

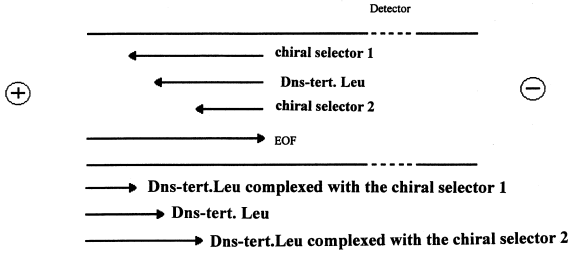

Fig. 9. Schematic representation of possible origin of reversal of the enantiomer migration order of dansyl-*tert*.-leucine with SBE-γ-CD or SBE-β-CD depicted in Fig. 8.

enantiomers despite of the same affinity pattern to both chiral selectors. The different self-mobilities of SBE-β-CD and SBE-γ-CD may be caused by different degrees of substitution (D.S.) and/or by the different molecular masses of native β- and γ-CD.

5.2. Effect of a charge of chiral selector on migration phenomena involved in separation

As above mentioned examples indicate the role of the various mobilities (analyte, EOF, chiral selector) is as important factor in the separation selectivity in CE as is the affinity. The most important external parameters which may affect a mobility difference of enantiomers with charged chiral selectors will be discussed shortly below.

An applied voltage (field strength) affects the mobilities in CE in a non-selective way. This means that it affects the mobility of all charged components of a separation system as well as the EOF to the same extent. Certainly, this does not mean that the applied voltage does not affect the separation at all. For instance, when a chiral analyte and a chiral selector are oppositely charged and the EOF does not markedly contribute to the mobilities an increase of the applied voltage will accelerate the analyte and the chiral selector in the opposite directions. This way an increase of the applied voltage can result in an increase of the absolute difference between the mobility of a free and complexed analyte. The increase of the EOF with increasing applied field strength can positively or negatively contribute to a separation selectivity depending on the relationship between the direction of migration of the EOF, the analyte, and the chiral selector. The change of the migration times as well as other general effects of the applied field strength on CE separations must be also considered when optimizing the applied voltage with charged chiral selectors.

The pH of the background electrolyte is another important external parameter which may affect a separation selectivity in CE significantly.

If a charged chiral selector contains weak acidic (for instance carboxylic acid) or basic functionalities then a pH variation allows the adjustment of the self-mobility of the chiral selector. This will affect the mobility of a complexed analyte and, moreover, a mobility difference between a free and a complexed analyte, e.g., the separation selectivity.

If a charged chiral selector contains the substituents such as sulfonic, phosphoric, quaternary ammonium, etc., which are ionized in the entire pH range available in CE then the pH does not markedly affect the self-mobility of chiral selector but allows to adjust the EOF and the mobility of chiral analyte. This will again affect the mobility difference between a free and bonded analyte and thus the separation selectivity.

The pH dependent switch between the charged and neutral forms of CM-β-CD was employed to observe a reversal of the separation selectivity of the ephedrine enantiomers in a polyacrylamide-coated capillary [52]. A similar effect for the neutral chiral analyte benzoin was observed by Mazzeo et al. (Fig. 10) [56]. The mechanism of this effect is the following: at pH 5.5 the EOF is sufficiently high to transport the analyte to the opposite direction of the anionic micellar phase used as a chiral selector. This allows the detection of the analyte at the cathodic side of the capillary. The enantiomer with the higher affinity to the micellar phase will migrate as the second peak. At pH 4.0, however, the mobility of the anionic micellar phase is higher than that of the EOF. The affinity of benzoin to the micellar phase is apparently also high. A combination of these factors allows to use a chiral micellar phase as a carrier to transport the neutral compound benzoin to the anode. Certainly the enantiomer which has a higher affinity to the micellar phase will migrate with higher mobility and it appears as the first peak in the

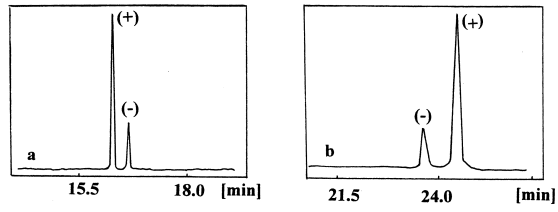

Fig. 10. Reversal of the migration order of benzoin enantiomers [(+)/(−)=1:3] at pH 5.5 (a) and pH 4.0 (b). Fused-silica capillary of 60/50 cm×50 μm I.D.; buffer: 25 m*M* (*S*)-2-[(dodecoxycarbonyl)amino]3(*S*)-methyl-1-sulfoxypentane, 25 m*M* sodium acetate–25 m*M* monosodium phosphate; applied voltage 15 kV; injection at the cathode (a) and at the anode (b). (Reproduced from Ref. [56] with permission).

electropherogram. It must be noted that similar effect may also be obtained with neutral chiral selectors [20].

The effect of the concentration of the charged chiral selector on the selectivity of enantioseparation can be more pronounced compared with neutral chiral selectors. This is caused by the fact that the mobility of a chiral selector also becomes involved in the separation parallel to the affinity. Only the latter acts in separations with a neutral chiral selector.

An example for a separation selectivity reversal using different concentrations of the positively charged chiral selector 2-hydroxypropyltrimethylammonium salt of β-CD (TMA-β-CD) is shown in Fig. 11 [57]. The anionic analyte BDHP and the cationic chiral selector TMA-β-CD possess an opposite self-electrophoretic mobilities. At low concentrations of TMA-β-CD the chiral analyte migrates preferentially in its free form to the anode (Fig. 11a). At high concentrations of TMA-β-CD a chiral analyte is predominantly complexed with the cationic chiral selector and will be transported with it to the cathode (Fig. 11b). The more tightly complexed *S* enantiomer of BDHP will migrate as the first peak in the latter case. A similar effect was previously described by Aturki and Fanali [58] for the cationic chiral analyte propranolol as well as by Mazzeo et al. [56] for the neutral chiral analyte benzoin.

By optimization of the concentration of a charged chiral selector one needs to consider higher current and consequently unfavorable Joule heating problems. Additionally, peak dispersion can be higher with charged chiral selectors as a result of a conductivity mismatch between the sample and the background electrolyte zones as well as due to multicomponent composition of commercially available charged CD derivatives.

The effect of the EOF is somewhat specific in chiral CE separations with charged chiral selectors compared to neutral ones. In general, when a chiral selector possesses an electric charge of the opposite sign compared to that of the capillary inner wall then the chiral selector and the EOF have the same direction. An effect of the EOF on a chiral separation can be optimized based on the charge of chiral selector, the analyte and the capillary inner wall.

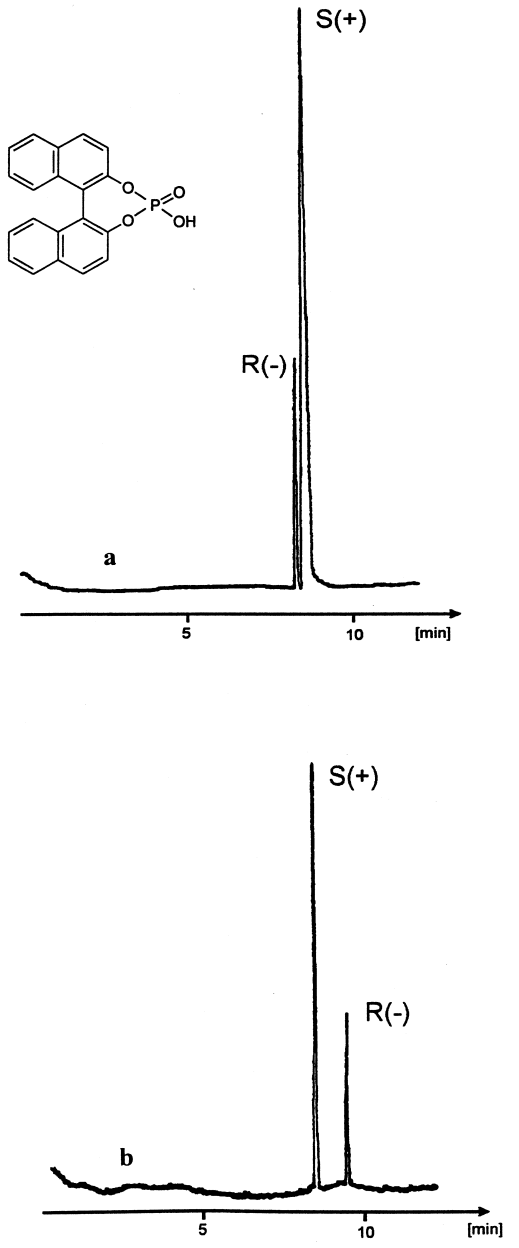

Fig. 11. Enantioseparation of BDHP [(*R*-(−)/*S*-(+)=1:3] with 0.05 mg/ml (a) and 10 mg/ml (b) TMA-β-CD. Polyacrylamide-coated capillary of 60/43 cm×50 μm I.D.; 50 m*M* phosphate buffer; applied field strength 400 V/cm; injection at the cathode (a) and at the anode (b). (Reproduced from Ref. [57] with permission).

A tend of a charged chiral selector to interact with a capillary wall having the opposite charge is obviously higher compared to neutral chiral selectors. The selector–wall interactions may significantly affect the EOF as well as the peak form and the separation efficiency. The decrease and reversal of the EOF as a consequence of the adsorption of TMA-β-CD on a fused-silica capillary wall is shown in Fig. 12 [57]. A similar effect was also reported by Bunke and Jira [59].

The effect of the charge-induced chiral selector–capillary wall interactions are especially significant for peptide and protein type chiral selectors [α_1-acid glycoprotein (α_1-AGP), ovomucoid, bovine serum albumin (BSA), human serum albumin (HSA), etc.]. Yang and Hage [60] performed a study in order to estimate the contribution of wall-adsorbed HSA on the chiral separation of D,L-tryptophan and *R*,*S*-warfarin. The frontal analysis technique was used to estimate the amount of HSA adsorbed on the capillary wall. The value was estimated to be $2.9 \cdot 10^{-8}$ M/m^2 which corresponds a coverage of about 0.72 monolayer for a totally smooth capillary and an apparent concentration of adsorbed HSA in the capillary of 2.7 μ*M*. The applied voltage causes a migration and this way also desorption of the adsorbed HSA from the capillary wall. Therefore minor amounts of HSA are required in background electrolyte to maintain a constant amount of HSA adsorbed on the capillary wall for a relatively long time. The enantioseparation of D,L-tryptophan was possible with a HSA-containing buffer in relatively wide concentration range. The importance in this separation of HSA adsorbed onto the capillary wall was studied by injecting the sample onto a capillary pretreated with HSA but using only 25 m*M* phosphate buffer (pH 7.4) as the running buffer. Under these conditions, no enantioseparation was observed. This indicated that HSA in the buffer, and not adsorbed HSA, was responsible for separating D- and L-tryptophan.

In contrast to the D,L-tryptophan case, no resolution of the warfarin enantiomers was observed at any HSA buffer concentration or electric field strength (Fig. 13a). Since *R*- and *S*-warfarin could not be separated when moderate amounts of HSA were added to the background electrolyte, the use of the HSA coated capillary was considered next. These results are shown in Fig. 13b. With only pH 7.4 phosphate buffer in the system, it was found that baseline resolution of *R*- and *S*-warfarin could easily be obtained. At an applied voltage of 23 kV, the

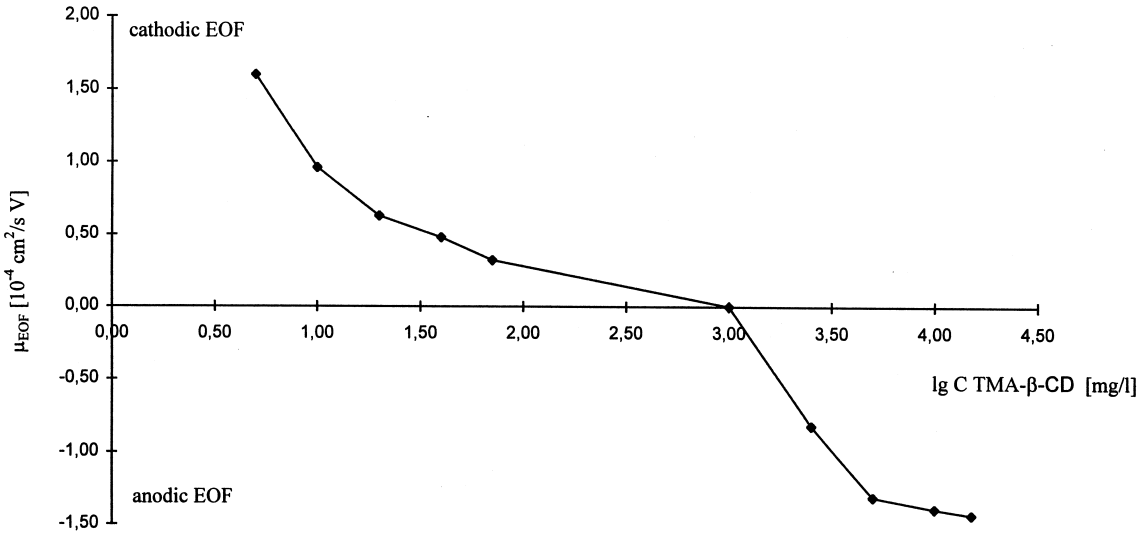

Fig. 12. Dependence of the EOF (mesityl oxide as the marker) on the concentration of TMA-β-CD (mg/l) in the background electrolyte in a fused-silica capillary. Capillary of 60/43 cm×50 μm I.D.; 50 m*M* phosphate buffer pH 6.0; applied field strength 400 V/cm; injection at the anode in the concentration range of TMA-β-CD 0.005–0.100 mg/ml and at the cathode at higher TMA-β-CD concentrations. (Reproduced from Ref. [57] with permission).

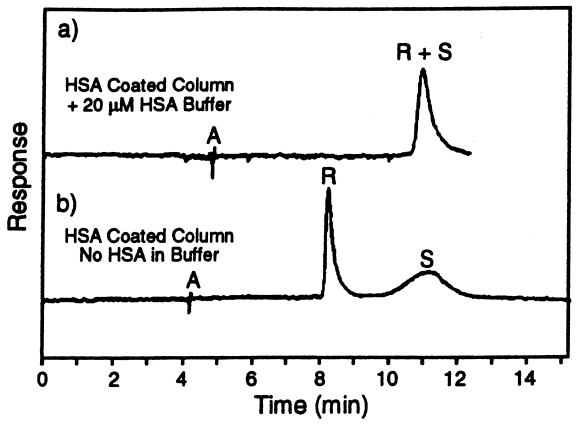

Fig. 13. Electropherograms of (*R,S*)-warfarin and acetone in the freshly coated HSA capillary with and without HSA present in the running buffer. The applied voltage 23 kV. (Reproduced from Ref. [60] with permission).

enantiomers were resolved in approximately 12 min using a freshly coated HSA capillary.

The addition of small amount of HSA (0.05 μM) to the background electrolyte was required to maintain the sufficient amount of HSA in the adsorbed state on the capillary wall for the chiral separations. However, at slightly higher HSA concentrations in the buffer (0.4 μM), no observable separation was obtained [60].

The substitution degree (content of ionic groups) is another important characteristic of charged chiral selectors which contributes to the separation selectivity significantly. It is evident that the number of ionic groups will affect the charge density, e.g.. the electrophoretic mobility of a chiral selector. The latter affects the mobility difference between enantiomers and consequently a separation selectivity by a similar way as discussed above.

CDs contain a wide range of derivatizable hydroxy groups which may in principle be substituted with ionic groups in a regio- and site-specific way [61–64]. However, the preparation of these derivatives involves multistep syntheses and yields are usually very low. Several examples of CE enantioseparations have been reported with selectively substituted charged CD derivatives [1,2,14,16,35,46]. However, to the authors knowledge no selectively substituted charged CD derivative is commercially available at the present time. Therefore, a discussion below focuses on randomly substituted charged CDs.

The effect of the substitution degree on the separation selectivity of SBE-β-CD was clearly demonstrated by Rickard et al. [37]. The randomly substituted SBE-β-CD mixture with an average D.S. of 4 but containing a components with a substitution degrees ranging from 1 up to 10 was fractionized on Bio-Rex 5 Resin weak anion exchange column. The components with the same D.S. (but probably containing regio- and site-isomers) were collected and used as chiral selectors for the enantioseparation of duloxetine hydrochloride (Fig. 14). As shown in this figure the separation selectivity increases significantly with an increasing degree of substitution. This effect is most likely caused by an increasing mobility of chiral selector due to the introduction of increasing number of charged groups. Increased electrostatic interaction can also contribute to longer migration times and higher separation selectivity but this is less likely. As shown, the highly charged chiral selector although being advantageous from the viewpoint of the separation selectivity causes a drastic deterioration of the peak form. Therefore, it can not always be superior from the viewpoint of the peak resolution (R_s) which is defined by a combination of both the separation selectivity (α) and the peak efficiency (N) (see Eq. (1)).

The reversal of enantiomer migration order is also feasible depending on the substitution degree of charged chiral selectors. The example shown in Fig. 15 may be explained by the following scheme (Fig. 16). The chiral analyte 1,1′-binaphthyl-2,2′-diamine (BNDA) and the chiral selector (TMA-β-CD) both are positively charged. TMA-β-CD with a D.S. of approx. 1 possesses a lower self-mobility than the chiral analyte. This causes the migration of this preferentially bonded enantiomer as the second peak (Figs. 15a and 16a). In contrast, TMA-β-CD with average D.S. of 3.5 possesses a higher self-mobility than the chiral analyte. Thus, the preferentially bonded enantiomer migrates as the first peak with this chiral selector (Figs. 15b and 16b).

Mayer et al. [49] postulated that the oppositely charged chiral selectors with the same affinity will result the opposite migration order of the neutral enantiomers. This is illustrated in Fig. 17 [57]. The origin of this reversal of EMO is that the negatively

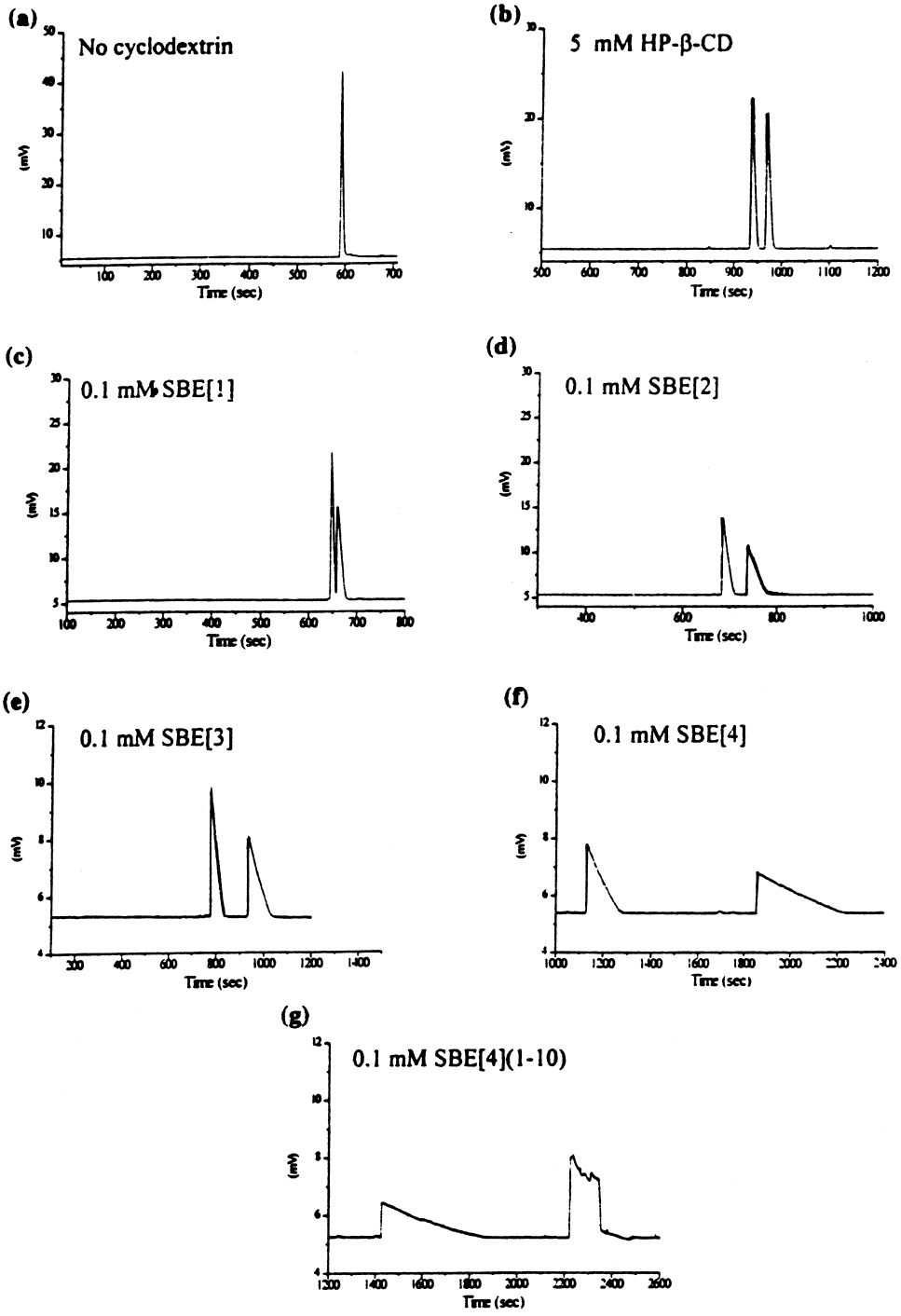

Fig. 14. Chiral separations of duloxetine enantiomers with SBE-β-CDs. Fused-silica capillary of 75/50 cm×50 μm I.D.; 50 m*M* phosphate buffer pH 2.5; applied voltage 25 kV. SBE-β-CDs with a concentration of 0.1 m*M* and hydroxypropyl β-CDs of 5.0 m*M* were used. (Reproduced from Ref. [37] with permission).

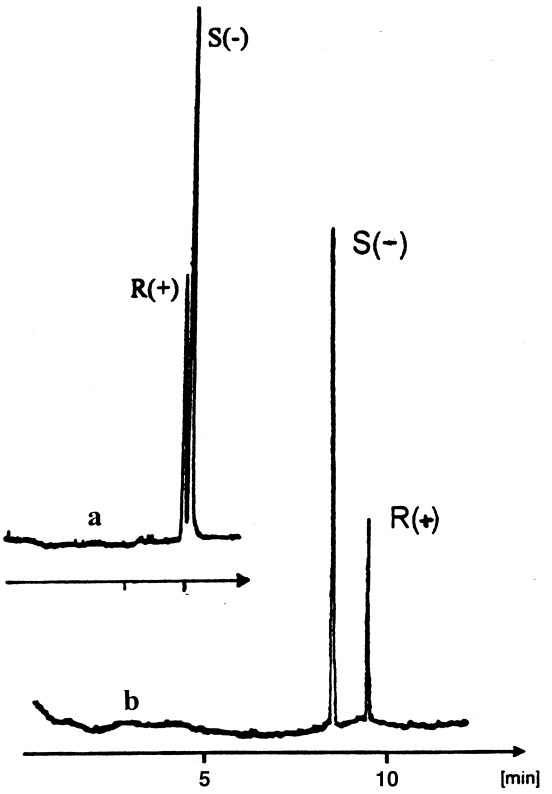

Fig. 15. Electropherograms of nonracemic mixture [(+)/(−)= 1:3] of BNDA enantiomers with TMA-β-CDs of D.S.=1.0 (a) and D.S.=3.5 (b). Polyacrylamide-coated capillary of 60/43 cm×50 μm I.D.; 50 m*M* phosphate buffer pH 3.0.; applied field strength 400 V/cm; injection at the anode. (Reproduced from Ref. [57] with permission).

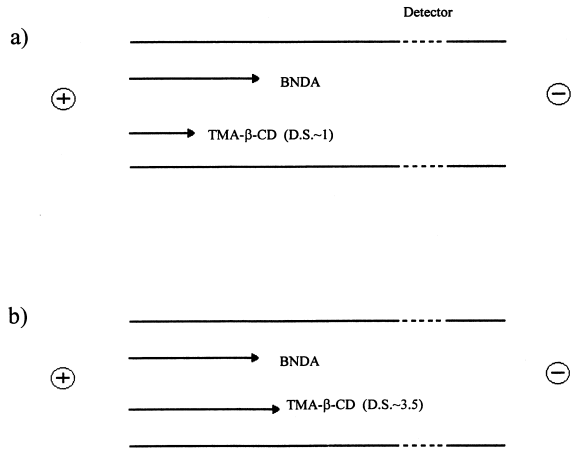

Fig. 16. Schematic representation of reversal of the enantiomer migration order of BNDA depicted in Fig. 15.

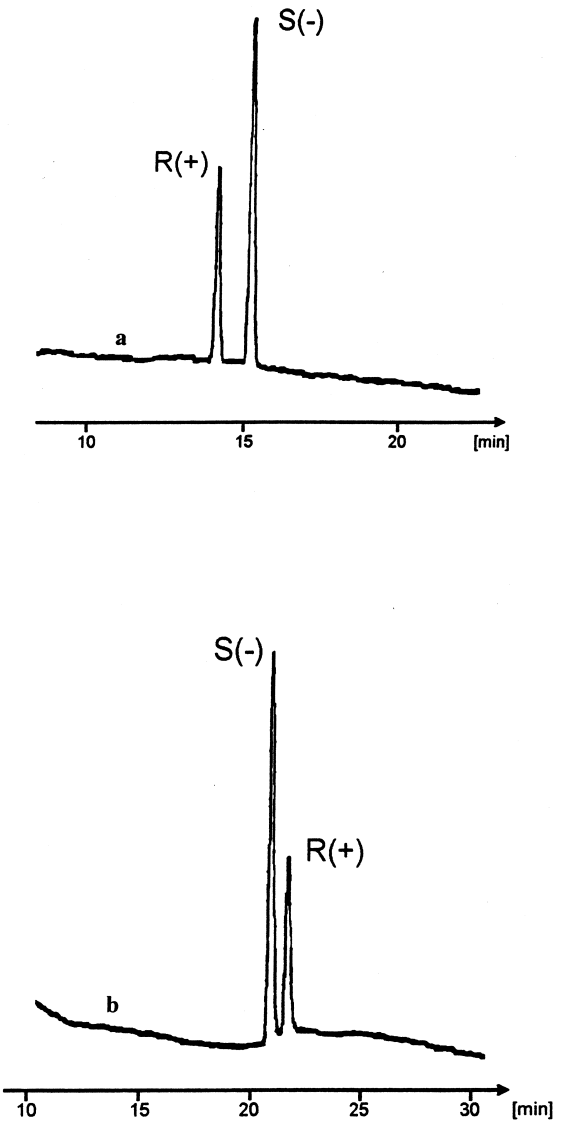

Fig. 17. Enantioseparation of thalidomide (*R*/*S* ratio equal to 1:2) with 20 mg/ml CM-β-CD (a) and 20 mg/ml TMA-β-CD (b). Fused-silica (a) and polyacrylamide-coated (b) capillary of 60/43 cm×50 μm I.D.; 50 m*M* phosphate buffer pH 6.0 (a) and pH 3.0 (b); applied field strength 400 V/cm; injection at the anode. (Reproduced from Ref. [57] with permission).

charged CM-β-CD decelerates the preferentially bonded *S*(−)-thalidomide (Fig. 17a) whereas the positively charged TMA-β-CD accelerates it (Fig. 17b).

6. Separation selectivity with a charged chiral selector as a carrier

The potential of the charged CD derivatives as a carrier for the transport of neutral analytes to the detector was first noted by Terabe [1]. Schmitt and Engelhardt [52] reported the application of the ability of charged CDs for the reversal of the EMO of charged analytes as well as for the transport of neutral analytes to the detector. Later, the carrier abilities of charged CDs [50,51,65–70] as well as anionic surfactants [56] were used by several groups.

Stalcup and Gham [65,66] demonstrated the potential of this technique. The basic and neutral chiral analytes were successfully enantioseparated using the polyanionic chiral selector, β-CD sulfate. The basic analytes were injected at the cathodic end of the capillary and were transported with a chiral selector to the anode in the opposite direction to their own electrophoretic mobility. The same is true for neutral analytes only with a difference that they do not migrate with a self-electrophoretic mobility. Neutral analytes migrate in the uncomplexed form with the EOF. In bare silica capillaries this is also opposite to the electrophoretic mobility of anionic chiral selector. The authors also noted an important advantage of this technique for analytes which posses a low affinity to the selector. Usually a higher concentration of chiral selector is required for the enantioseparation of weekly bonded analytes [10]. However, peak broadening due to longer migration times combined with the high current somewhat limits the application of high concentrations of polyionic chiral selectors. Peak broadening can be at least partially eliminated using the carrier ability of charged chiral selector because the analysis time shortens with increasing chiral selector concentration in this case [65,66]. Several attempts were made in these studies to characterize the affinity of the chiral analyte to the chiral carrier based on the migration times. This is certainly justified in general but needs to be done carefully taking into account the migration direction and the magnitude of the uncomplexed analyte. For instance, the migration times and separation selectivity of two atropisomeric compounds, 1,1′-binaphthyl-2,2′-diol (BD) and BDHP with β-CD sulfate as a chiral carrier were compared [66]. The shorter migration time of BDHP was considered as the indication of its stronger interaction with β-CD sulfate compared to BD. However, the selectivity of the enantioseparation was lower in the case of BDHP which was attributed to the rigidity of the phosphate group. This has been also confirmed with an energy minimization calculations. However, the most likely reason for the observed shorter migration time of BDHP as well as for the lower separation factor of the enantiomers evidently is the self-electrophoretic mobility of this analyte (which is the single charged anion) in the same direction as the mobility of the chiral selector. This is not the case for BD which does not possess a self-electrophoretic mobility and migrates to the detector only in the complexed form.

Janini et al. [67] reported the enantioseparation of dansyl amino acids (Dns-DL-AA) using SBE-β-CD as a chiral carrier in polyacrylamide coated capillary. It has been definitely shown that the separation of Dns-DL-AAs from each other as well as their enantioseparation is superior at pH 3.1 where the analytes are uncharged or very weakly negatively charged compared to pH 6.5 where they are negatively charged (Fig. 18). The electrostatic repulsion between the negatively charged chiral selector and the analytes as well as the electrophoretic migration of the analytes to the detector which shortens the period of time for interactions with the chiral selector have been considered as the two main reasons for a lower separation selectivity at pH 6.5. However, it seems again worthwhile to note that the migration of an analyte in the same direction as the chiral selector will significantly decrease the separation selectivity even in the absence of an electrostatic repulsion and shortening the analysis time. Even the best chiral recognition will be obviously canceled when the selector and the selectand migrate in the same direction with the same mobilities (see Fig. 3 and Eq. (4)). Thus, a lower mobility difference between the free and complexed analyte appears to be the major reason for the decrease of selectivity at pH 6.5 shown in Fig. 18.

The selector–selectand affinity has been discussed in [67]. However, it does not seem absolutely correct to compare multicomponent mixtures of SBE-β-CD and β-CD sulfate with different substitution degrees without an electrophoretic characterization of their composition as well as the mobility of the different fractions. Indirect [71,72] as well as direct [40]

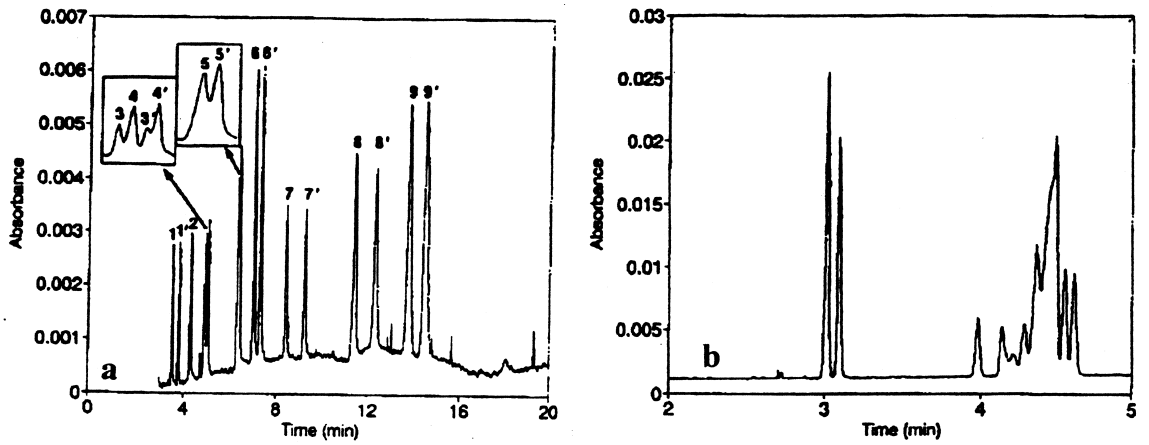

Fig. 18. Enantioseparation of a racemic mixture of dansylated amino acids at pH 3.1 (a) and pH 6.5 (b). Polyacrylamide-coated capillary of 27/20 cm×50 μm I.D.; 10 m*M* phosphate buffer; applied voltage 10 kV; injection at the cathode; solutes: (1) Trp; (2) Phe; (3) nor-Leu; (4) Leu; (5) Met; (6) nor-Val; (7) Val; (8) Glu and (9) Asp. (Reproduced from Ref. [67] with permission).

techniques are described to perform such a characterization. The positive effect of the alkyl spacer between the charged group and the CD rim on the chiral recognition ability observed in this study seems to be reasonable and is also confirmed in the independent study by Zia et al. [73].

Multiple positively charged TMA-β-CD has been also used as a chiral carrier for the enantioseparation of anionic and neutral chiral analytes (Fig. 19) [57]. Some complementary properties of this mode of separation were also emphasized in the comparison to normal-mode CE. One difference is a decrease of the migration times with increasing concentrations of the chiral selector as previously mentioned in [65,66] (Fig. 20) [57]. These two separation modes are also markedly distinct from the viewpoint of a separation selectivity. A neutral analyte does not migrate in its free form but migrates only when it is complexed, i.e., when chiral recognition occurs. The suppression of any non-selective migration of the analyte certainly contributes positively to an increase of the separation selectivity (for a given migration distance) [8]. Another important point is that the peak efficiency (N) as a rule decreases with increasing chiral selector concentration in normal-mode CE but increases when a chiral selector is used as a carrier (Fig. 20). The separation factor (α) and peak resolution (R_s) may depend formally in a similar manner on the selector concentration in both modes. However, the inherent mechanisms of these dependencies may be completely different. The above discussion concerning normal-mode CE mainly considers neutral and cationic analytes. However it is also true for anionic analytes when these are detected at the anodic side of the separation capillary and chiral selector does not posses the same charge as the analyte.

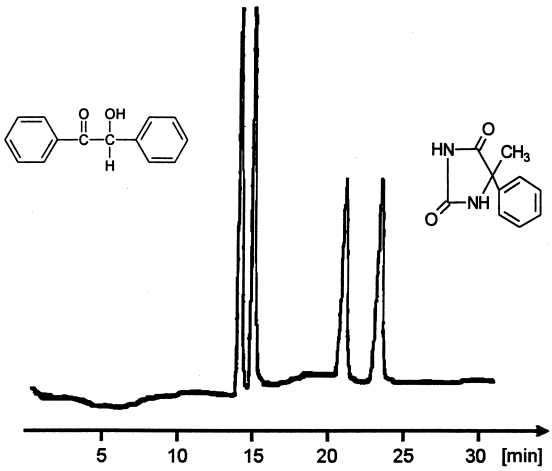

Fig. 19. Enantioseparation of (±)-benzoin and (±)-5-methyl-5-phenylhydantoin using 25 mg/ml TMA-β-CD as a chiral selector. Polyacrylamide-coated capillary of 60/43 cm×50 μm I.D.; 50 m*M* phosphate buffer pH 3.0.; applied field strength 400 V/cm; injection at the anode. (Reproduced from Ref. [57] with permission).

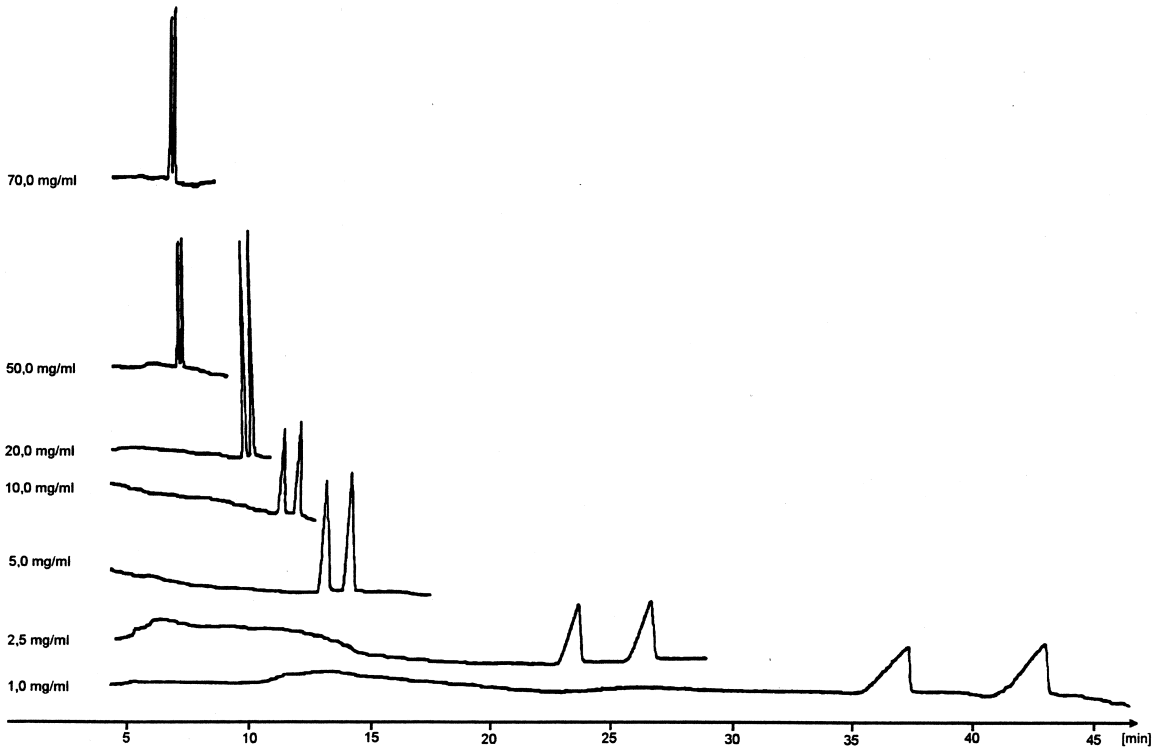

Fig. 20. Enantioseparation of (±)-hexobarbital using various concentrations of TMA-β-CD. Conditions as in Fig. 19. (Reproduced from Ref. [57] with permission).

Thus, the use of charged chiral selectors for a transport of analytes to the detector seems to be a valuable alternative for normal-mode CE from the viewpoint of the adjustment of a desirable selectivity and significantly expanding the application potential of chiral CE.

7. Selectivity in CE separations with combinations of chiral selectors

Chiral CE separations using a combination of chiral selectors is becoming increasingly important [16,43,68,69,74–80]. The extended treatment of this subject by Lurie can be found in this volume [81]. Therefore, only those examples in which at least one chiral selector is charged will be discussed in this paper. Some of these studies were well-designed in order to achieve a cooperation of the chiral selectors whereas in other studies the chiral selectors are simply mixed together in order to create a superior separation system solely based on a trial and error approach.

Lin et al. [80] described micellar electrokinetic chromatography (MEKC) enantioseparations of Dns-DL-AAs with a combination of β-CD and sodium taurodeoxycholate (STDC) (Fig. 21). The advantage of a combination of chiral selectors is evident in this case.

An excellent example of a designed combination of two chiral selectors for the enhancement of a separation has been described by Terabe et al. [75]. The authors noted that STDC incorporates Dns-L-AAs more strongly [82] whereas CDs include the D-isomers preferentially [75]. At the same time in γ-CD-MEKC these chiral selectors migrate in the opposite direction. Therefore, their combination is expected to result in a superior separation selectivity which has indeed been observed experimentally [75]. Apparently, this study explains also the effect observed by Lin et al. (Fig. 21) [80]. Later, Wang and Warner [79] studied enantioseparations in CD-

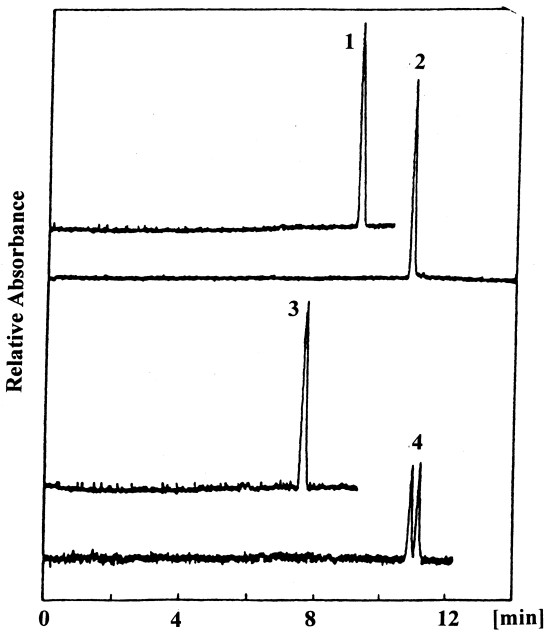

Fig. 21. Enantioseparation of Dns-DL-norvaline in (1) 40 m*M* β-CD–50 m*M* SDS solution; (2) 3% butanol–80 m*M* STDC solution (3) 10 m*M* β-CD and (4) 10 m*M* β-CD–80 m*M* STDC solution. Fused-silica capillary of 75/50 cm length; 10 m*M* borate–phosphoric acid buffer pH 7.8; applied voltage 15 kV. (Reproduced from Ref. [80] with permission).

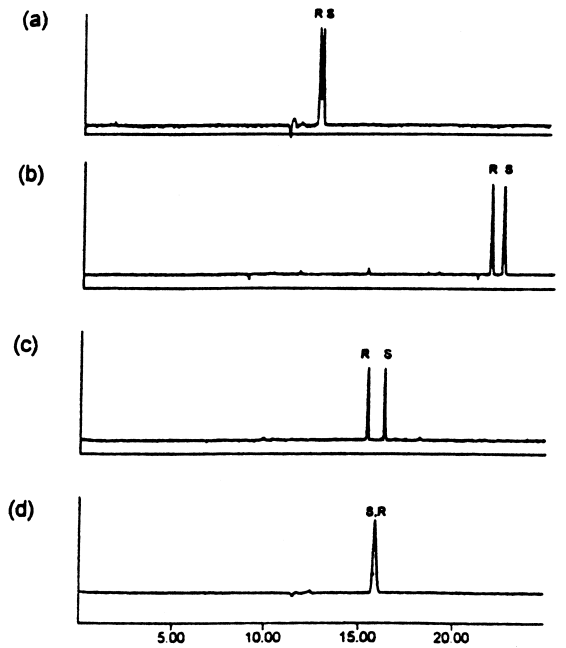

Fig. 22. Chiral separation of (±)-1,1′-binaphthyl-2,2′-diol by use of poly(D- or L-SUV) and γ-CD. 25 m*M* borate buffer pH 9.0; applied voltage 12 kV. (a) 10 m*M* γ-CD, (b) 0.5% poly(D-SUV), (c) 10 m*M* γ-CD and 0.5% poly(D-SUV), (d) 10 m*M* γ-CD and 0.5% poly(L-SUV). (Reproduced from Ref. [79] with permission).

MEKC using a combination of γ-CD and the polymerized chiral micelles poly(sodium N-undecylenyl-L-valinate) [poly(L-SUV)] and its optical antipode poly(sodium N-undecylenyl-D-valinate) [poly(D-SUV)]. The expression proposed for the selectivity in this study indicated that the combination of the chiral selectors to which the enantiomers exhibit the opposite affinity will superior for their separation. This was clearly illustrated by combination of γ-CD alternatively with the polymerized chiral micelles of the opposite configuration (Fig. 22) [79]. The same conclusion about the opposite chiral recognition of chiral selectors used in a combination was recently made by Lellievre et al. [16].

In all aforementioned examples of combined chiral selectors the opposite chiral recognition of a chiral selector was required in order to obtain an enhancement of the separation selectivity. However, this statement can not be generalized. In examples above the chiral selectors used in combination tend to migrate in the opposite direction (either with a self-electrophoretic mobility or with the EOF). Therefore, the association of an analyte with one chiral selector accelerates the analyte while the association with another chiral selector decelerates it. If the chiral selectors which display the opposite effect on the mobility of the analyte also exhibit an opposite chiral recognition pattern they will cooperate (both of them will accelerate or decelerate the same enantiomer of a chiral analyte) and an enhancement of the separation selectivity occurs.

However, in other examples a complexation with both chiral selectors used in a combination may have the same effect on the mobility of the analyte, i.e., an association with each of the chiral selectors may accelerate or decelerate the analyte. In contrast to the examples described in [16,75,79,80], the same chiral recognition pattern of selectors will be favorable in this case in order to obtain an enhancement of the separation selectivity.

Lurie et al. [77] described the enantioseparation of cationic chiral drugs with a combination of neutral

DM-β-CD and anionic SBE-β-CD (Table 2). In this case both chiral selector decelerate a complexed analyte. Therefore, a gain of the separation selectivity will be observed only for those analytes which possess the same affinity pattern to both chiral selectors. Indeed, as can be seen from Table 2, the combination of DM-β-CD–SBE-β-CD is favorable for the enantioseparation of (±)-pseudoephedrine toward which both β-CD derivative possess the same chiral recognition pattern [(+)-pseudoephedrine is preferentially complexed with both of them]. Apparently, the separation selectivity could also be higher for (±)-norpseudoephedrine, (±)-amphetamine and (±)-methamphetamine but unfortunately only the resolution (R_s) (and not the selectivity) data are given in Table 2. In contrast to these compounds, the same combination of chiral selectors (DM-β-CD–SBE-β-CD) was unfavorable for the enantioseparation of those racemates for which these chiral selectors exhibit an opposite recognition pattern. In Table 2 (±)-cathinone, (±)-methcathinone and (±)-ephedrine belong to this group.

Table 2
Migration times, t_m (min) and chiral resolution (R_s) for drugs of forensic interest using different combinations of neutral and charged cyclodextrins

Chiral compound	System a		System b		System c		System d	
	t_m	R_s	t_m	R_s	t_m	R_s	t_m	R_s
(+)-Cathinone	8.25	1.9	9.59	1.9	10.32	0.5	8.92	0.5
(−)-Cathinone	8.38		9.42		10.28		8.96	
(+)-Norpseudoephedrine	9.18	3.6	10.87	6.8	11.89	6.4	10.08	5.0
(−)-Norpseudoephedrine	8.87		9.92		10.81		9.43	
(+)-Norephedrine	9.00	1.5	10.17	0.0	11.12	1.0	9.66	1.3
(−)-Norephedrine	8.87		10.17		10.97		9.52	
(+)-Amphetamine	9.34	0.7	12.24	3.0	13.28	2.2	10.76	1.3
(−)-Amphetamine	9.28		11.82		12.92		10.59	
(+)-Methcathinone	8.70	1.0	10.23	3.6	11.07	2.2	9.45	0.8
(−)-Methcathinone	8.78		9.80		10.83		9.39	
(+)-Pseudoephedrine	9.65	5.2	11.50	6.2	12.60	8.7	10.59	6.5
(−)-Pseudoephedrine	9.23		10.39		11.38		9.87	
(+)-Ephedrine	9.43	1.2	10.93	1.5	11.93	1.0	10.20	0.0
(−)-Ephedrine	9.33		11.16		12.10		10.20	
(+)-Methamphetamine	9.82	1.2	13.50	3.1	14.40	2.9	11.39	1.6
(−)-Methamphetamine	9.72		12.82		13.88		11.17	
(+)-Cocaine	15.15	1.9	[a]		32.00	3.8	22.00	2.2
(−)-Cocaine	14.80		[a]		36.90		23.30	
(+)-Propoxyphene	17.20	0.0	[a]		23.80	3.4	20.30	2.0
(−)-Propoxyphene	17.20		[a]		25.50		21.10	

[a] No peak detected within 60 min.
System a: 1.2% MeOH, 98.8% 25 m*M* Tris–phosphate, pH 2.4 containing 5 m*M* DM-β-CD.
System b: 1.2% MeOH, 98.8% 25 m*M* Tris–phosphate, pH 2.4 containing 1 m*M* SBE-β-CD.
System c: 1.2% MeOH, 98.8% 25 m*M* Tris–phosphate, pH 2.4 containing 5 m*M* DM-β-CD and 1 m*M* SBE-β-CD.
System d: 1.2% MeOH, 98.8% 25 m*M* Tris–phosphate, pH 2.4 containing of 5 m*M* DM-β-CD and 0.5 m*M* SBE-β-CD.
Reproduced from Ref. [77] with permission.

Another interesting approach has been described by Anigbogu et al. [78]. The chiral drug aminoglutethimide (AGT) studied in this work is uncharged at pH 9. The enantioseparation of this compound could not be achieved with CM-β-CD at this pH. However, it was possible after the addition of 1 m*M* β-CD and improved further by using of 50% (v/v) methanol (Fig. 23). The most likely explanation of this effect could be that β-CD recognizes the enantiomers of AGT stereoselectively at pH 9 but this does not result the enantioseparation in CE because no difference between the mobilities of the complexed and the free analyte exists at this pH. CM-β-CD in this system plays the role similar to that of an achiral micelle and allows to observe the enantioseparation in CE. Thus, the neutral β-CD is responsible for the stereoselectivity and the charged CM-β-CD for the mobility difference between the free and complexed analyte in this system. In contrast to pH 9, no separation of the positively charged AGT enantiomers was observed using β-CD at pH 3.0. This is the reason why the authors proposed an alternative scheme for the chiral recognition i.e., a non-stereoselective complexation between β-CD and the AGT enantiomers and a separation of these transient diastereomeric complexes with CM-β-CD at pH 9.0. This explanation seems to be quite sophisticated and needs additional experimental verification.

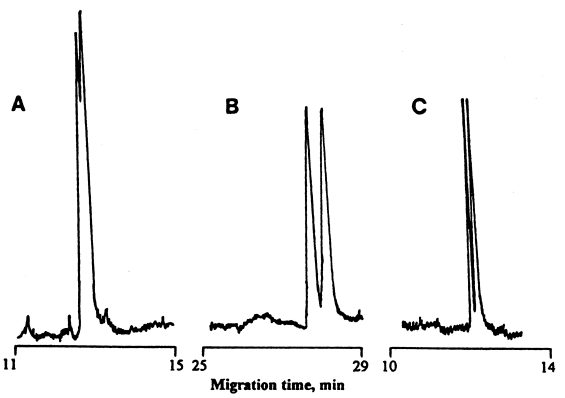

Fig. 23. Dual CD-phase CE enantioseparation of AGT. Running buffer: NaH_2PO_4–$Na_2B_4O_7$ (pH 9.0) with 5 m*M* CM-β-CD and 1 m*M* β-CD; (A) 0% methanol and 10 kV applied voltage; (B) 50% (v/v) methanol at 10 kV; and (c) 50% (v/v) methanol at 20 kV. (Reproduced from Ref. [78] with permission).

Gahm et al. [68] and Fillet and coworkers [69,70] used a combination of neutral and anionic CDs for the enantioseparation of neutral analytes [68] or chargeable analytes in their neutral form [69,70]. The same approach was described by Lelievre et al. [16] with cationic 6-monoamino-6-deoxy-β-CD as the charged component. Charged CDs are used in these studies also as carriers for the uncharged analytes. Therefore, the chiral selectors act in an opposite manner on the mobility of an analyte similar to the examples described in [75,79,80]. Thus, the opposite chiral recognition of chiral selectors will result in an enhancement of the selectivity. This is also noted in [16]. Extremely high selectivities and resolution factors ranging between 10 and 30 were reported in these studies [69]. In general, an equilibrium in separation system with multiple chiral selectors is extremely complex and some additional effects together with above mentioned are possible depending on the internal and external factors.

8. Separation selectivity in chiral capillary electrochromatography (CEC)

CEC is currently undergoing rapid development. This hybrid technique is based on electrophoretic migration in combination with a chromatographic separation mechanism and offers several important advantages over pressure-driven separations (for instance, a plug-like flow-profile) as well as over CE (stationary beds). The coupling of CEC with other techniques seems to be easier due to absence of separation agent in the mobile phase. Chiral CEC with coated capillaries was introduced by Mayer and Schurig [83] and with packed capillaries by Li and Lloyd [84]. Both techniques are rapidly developing and several interesting applications have been reported [85–92].

Since its appearance [93] chiral CE has been powerfully influenced by the experience from chiral HPLC. The CSPs and chiral mobile phase additives (CMPAs) from HPLC were mostly successfully used as buffer additives in CE. Chiral CE is more flexible (it does not require an immobilization of chiral selector) and less expensive technique (only μg amount of chiral selector is required) for testing new chiral selectors. Due to its high peak efficiency CE

makes feasible to prevail the stereoselective recognition ability of those chiral selectors which cannot be screened in HPLC. Additionally, CEC allows to study chiral selectors which are insoluble in common buffers or possess a detector response and therefore are not suitable buffer additives. Thus, the experience from both techniques HPLC and CE will be transferred to CEC in the near future. Therefore, selectivity related aspects in CEC will be shortly discussed below.

Obviously a separation selectivity in CEC can be described by a similar way as in chromatography. One important aspect seems to be the reversal of the EMO. As it is well-documented by Davankov et al. [94,95] in pressure-driven separation system (HPLC) a given chiral selector as a mobile phase additive (CMPA) and the same chiral selector as a stationary phase (CSP) will result the opposite EMO. This is true when the adsorption of transient diastereomeric complexes on the achiral stationary phase does not critically change (reverse) the migration order which is defined by the equilibrium in the liquid phase. The reversal of EMO in HPLC is caused by the fact that the complexation with a chiral selector in the mobile phase accelerates an analyte and a complexation with a chiral selector in the stationary phase decelerates it. This is not always the case in electrically-driven systems. In CE (where the chiral selector is presented in buffer) the complexation of the analyte can either accelerate or decelerate the analyte in contrast to HPLC with CMPA where only acceleration occurs. In CEC the complexation of the analyte will always decelerates it similar to HPLC with CSPs. Therefore, the EMO by the transfer of a given chiral selector from CE to CEC will be defined with the interplay of above mentioned variations as described in Fig. 24. Taking into account Fig. 24 and the migration order of the BDHP enantiomers with PM-β-CD in CE [*S*-(+)-BDHP before *R*-(−)-BDHP] also explains the origin of the effect which has been described by Mayer et al. [49] in a CE–CEC combination.

9. Conclusions

Chiral separations in CE which are conceptually impossible without the interaction of an analyte with a chiral selector must be ascribed to capillary electrokinetic chromatography (CEKC) independent of the charge of a chiral selector. The migration of enantiomers by their own electrophoretic mobility or under the effect of EOF does not differ from each other from the viewpoint of separation selectivity. The number of variables which affect the selectivity of a separation is significantly higher in CE than in pressure-driven separation systems. Charged chiral

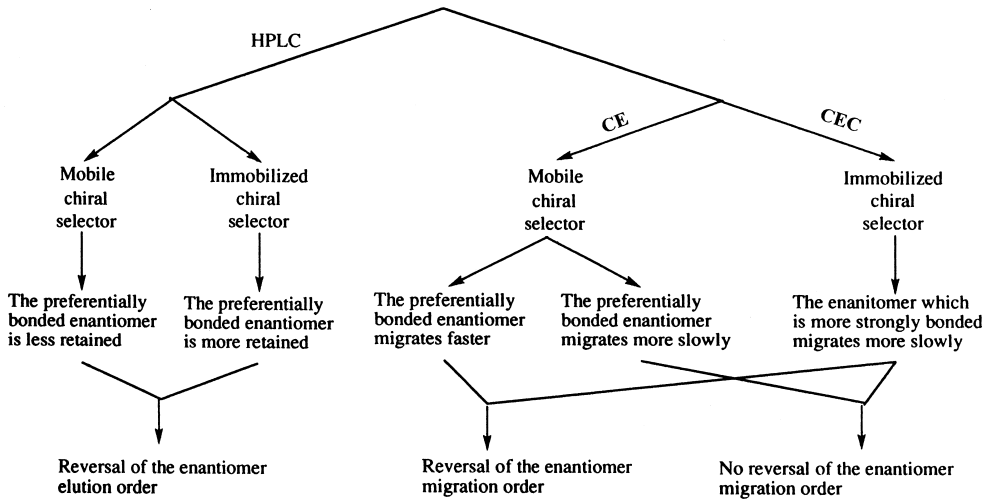

Fig. 24. Schematic representation of the effect of immobilization on the enantiomer migration order in HPLC and electrokinetic techniques (CE and CEC).

selectors offer additional possibilities for an adjustment of a desirable separation selectivity compared to neutral selectors. The contribution of the mobility of charged chiral selector for a separation selectivity must be especially considered for separation systems using a combination of chiral selectors and in CEC.

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