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Review

Enantiomeric separations by nonaqueous capillary electrophoresis

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Abstract

This paper reviews the recent advances in enantioseparations by nonaqueous capillary electrophoresis (NACE) and the effect of organic solvents on mobility of enantiomers, separation selectivity and resolution. In general, the enantioseparation systems in NACE are similar to those of aqueous capillary electrophoresis (CE) except pure organic solvents are used. The influence of important parameters such as concentration and type of chiral selectors, apparent pH, ionic strength, temperature, and control of electroosmotic flow is discussed. In addition, the reported applications of NACE separations of racemates are presented.

Keywords: Enantiomer separation; Nonaqueous capillary electrophoresis; Reviews; Nonaqueous capillary electrochromatography; Buffer composition; Chiral selectors

Contents

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1. Introduction

Capillary electrophoresis (CE) has become widely popular for separation of enantiomers over the past decade. Enantioseparation by CE offers several key advantages such as high separation efficiency, feasibility of incorporating a large number of chiral selectors that greatly facilitates method development, speed, and low cost [1–6].

Most CE separations of enantiomers have been performed in aqueous media. Enantioseparation in pure organic solvents was first reported by Ye and Khaledi in 1994 [7], followed by the publication of several papers [8–16]. The present understanding of enantioseparation in nonaqueous media is limited. This is due to the lack of basic knowledge about the recognition process in purely nonaqueous media as well as the limited information that is available on the effects of organic solvents on acid–base chemistry, ion solvation, and mobility.

Separation of enantiomers by nonaqueous capillary electrophoresis (NACE) has been briefly covered in book chapters and review papers [3,17–19]. This paper focuses on the relevant theory and applications in greater detail.

2. Theory

In CE, enantiomers can be separated based on their differential interaction with a chiral selector that is dissolved in the running buffer:

$$E_1 + CS \overset{K_1}{\leftrightarrow} E_1CS \quad (1)$$

$$E_2 + CS \overset{K_2}{\leftrightarrow} E_2CS \quad (2)$$

where E_1 and E_2 are two enantiomers of a racemic mixture, CS is the chiral selector, and K_1 and K_2 are the binding constants between the enantiomers and chiral selector. The relationship between mobility and CS concentration can be expressed as [20]

$$\mu = \frac{\mu^f + \mu^c K[CS]}{1 + K[CS]} \quad (3)$$

where μ^f and μ^c are the electrophoretic mobilities at the concentrations of the chiral selector at 0 and ∞, respectively, and [CS] is the equilibrium concentration of the chiral selector.

The relationship between the mobility difference ($\Delta\mu$), or separation selectivity, and concentration of the chiral selector can be expressed by [21]

$$\Delta\mu = \frac{(\mu^f - \mu^c)\Delta K[CS]}{(1 + K_1[CS])(1 + K_2[CS])} \quad (4)$$

Eq. (4) represents a separation system where the enantiomers are charged and the chiral selector is neutral. A 1:1 complex is formed between the CS and the enantiomers. Several experimental parameters, such as type and concentration of chiral selector, pH, ionic strength, electroosmotic flow (EOF), and type of solvent, can influence the separation selectivity and/or resolution.

The nature of the chiral selector has a decisive impact on enantioseparation through its effects on the K_1, K_2, ΔK, and $\mu^f - \mu^c$ terms. As can be seen from Eq. (4), separation selectivity is directly proportional to the mobility difference of enantiomers in the free (μ^f) and totally complexed (μ^c) forms ($\mu^f - \mu^c$), the binding constant difference (ΔK), binding constants (K_1 and K_2) and CS concentration. It demonstrates that no separation can be achieved if there is no complexation between the enantiomers and the chiral selector. In addition, the two enantiomers should bind to the chiral selector to different extents in order to be separated.

The resolution equation in CZE [22,23] can be applied to enantioseparation:

$$R_s = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{\Delta K[CD]}{(1 + K_1[CD])(1 + K_2[CD])}\right) \times \left(\frac{\mu^f - \mu^c}{\mu_{avg} + \mu_{eo}}\right) \quad (5)$$

where N is the number of theoretical plates, μ_{avg} is the average electrophoretic mobility of the two enantiomers and μ_{eo} is the mobility of EOF. Resolution can be improved by enhancing the separation efficiency ($\sqrt{N}$), maximizing the separation selectivity ($\Delta\mu$), optimizing migration (μ_{avg}), and controlling EOF. In order to maximize $\Delta\mu$, several parameters, such as type and concentration of chiral

selector, as well as pH (for ionizable solutes), would have to be optimized.

3. Solvent effects on enantiomer–selector binding

The type of solvent can have a significant influence on solute electrophoretic behavior as well as electroosmosis due to chemical and physical effects such as ion solvation, pK_a shifts, and viscosity [24]. In enantioseparations, solute binding to the chiral selector is greatly affected by the solvent.

Wang and Khaledi modeled the dependence of mobility on chiral selector concentration (Eq. (3)) in nonaqueous media and determined the binding constants of three hydrophobic chiral compounds, trimipramine, mianserin, and thioridazine, with β-cyclodextrin (β-CD) in water, 6 *M* urea in water, formamide, *N*-methylformamide (NMF), and *N*,*N*-dimethylformamide (DMF) [8]. Fig. 1a and b show the variation in mobility as a function of the concentration of the chiral selector for the two enantiomers of thioridazine in water and formamide. The solid lines represent the predicted behavior and the markers are experimental values. The solute–selector binding constants decrease as the polarity of the electrophoretic medium is reduced, which in turn requires a larger range of selector concentration. For example, the concentration range of β-CD at which enantiomers of thioridazine were separated was 0.04 to 0.5 m*M* in the aqueous medium (Fig. 1a), 0.25 to 2.0 m*M* in 6 *M* urea in water, and >20 m*M* in the formamide medium. No separation was achieved in NMF and DMF due to the very weak interaction between the enantiomers and β-CD in these solvents. The binding constants between thioridazine enantiomers and CD in water ($K \sim 10^4$) are around three orders of magnitude larger than those in formamide ($K \sim 10$) and around four to five orders larger than those in NME and DMF ($K \sim 10^{-2}$–10^{-3}). Even though the apparent pH (pH*) is not the same in different solvents, the analytes are fully protonated under the experimental conditions as evidenced by a lack of variation in mobility in the low pH range. Thus, one can conclude that the decrease in the binding constants was caused by the decrease in solvent polarity.

Fig. 2 shows the dependence of selectivity for thioridazine in pure aqueous buffer (curve A), 6 *M* urea in aqueous buffer (curve B), and in formamide (curve C). These curves were calculated according to Eq. (4) using the measured binding constant values. The concentration of chiral selector plays an important role in controlling separation. According to a theory by Wren and Row, for a given separation system, there exists an optimum chiral selector concentration [4,25]. The highest separation selectivity can be achieved at

$$[\mathrm{CS}]_{\mathrm{opt}} = \frac{1}{\sqrt{K_1 K_2}} \tag{6}$$

They also studied the effect of addition of a small amount of organic solvent on the separation of enantiomers. They assumed that the increase in the percentage of organic additives reduces binding constants between neutral CDs and the chiral compound. As can be seen in Fig. 2, there exists an optimum CD concentration at which separation selectivity is maximum. In the aqueous buffers, separation selectivity passes through maxima and changes rapidly with CD concentration. Therefore, it is crucial to operate at the optimum CD concentration for solutes with large binding constants. Consequently, for solutes that bind strongly to CD (such as trimipramine and thioridazine) the optimum occurs at a very small concentration (e.g., $[\mathrm{CD}]_{\mathrm{opt}} = 3.1 \cdot 10^{-5}$ for thioridazine). The occurrence of the optimum at very low concentration and the rapid changes in separation selectivity with CD concentration in the aqueous buffers would make it difficult to develop methods based on trial and error. This points out the significance of systematic method development in enantioseparations.

For compounds such as thioridazine that strongly bind to a selector in water, it is difficult to control the chiral selector concentration at the optimum (~30 μ*M*). Even a small deviation from the optimum concentration will drastically reduce separation selectivity (~27%) (Fig. 2). From the practical point of view, the concentration range for observable enantioseparation for those compounds is so small that any artifact (such as errors caused by consecutive dilution of stock CD solutions, or evaporation of solvents, etc.) can be problematic. Therefore, for

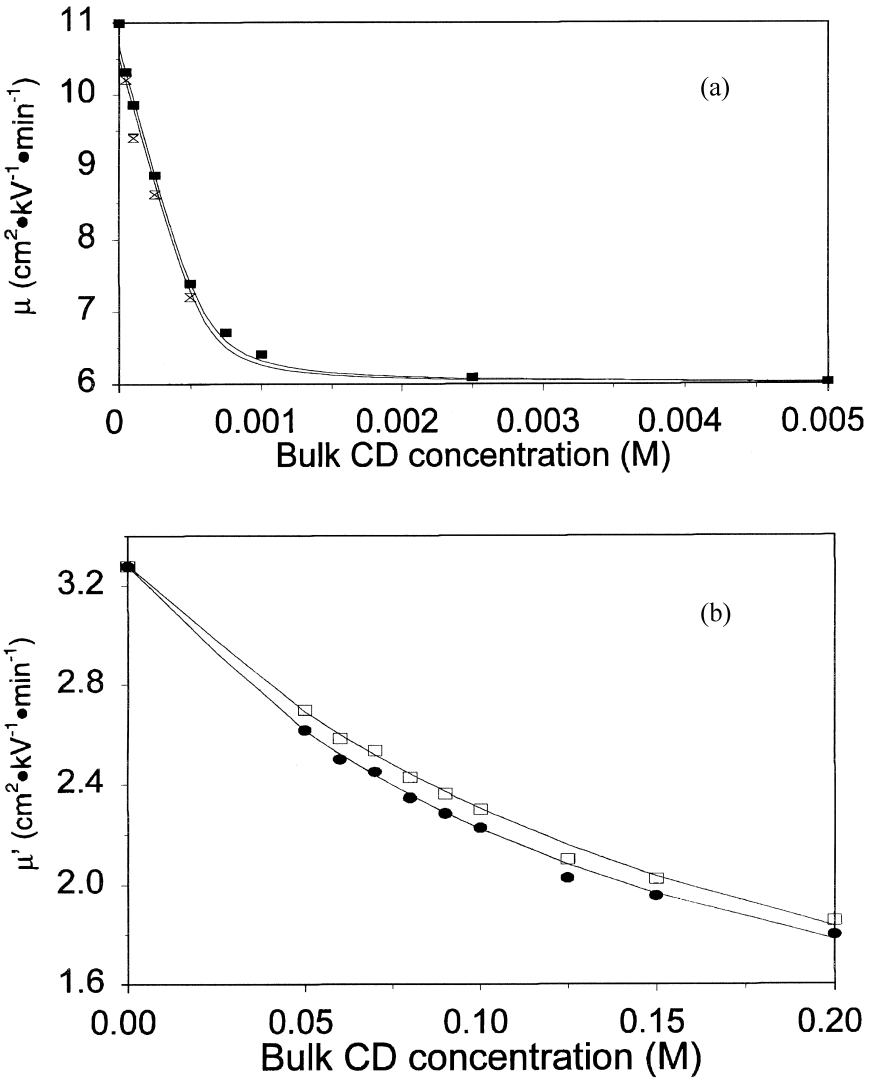

Fig. 1. Relationship between mobilities of thioridazine and β-CD concentration in different media. (a) water, pH 3.02; (b) formamide, pH* 5.4. Buffer: 50 m*M* citric acid, 25 m*M* Tris for all cases. Markers are experimental data and lines theoretical calculation from Eq. (3). From Ref. [8].

these compounds, separation methods are not rugged. However, in NACE enantioseparation, the drastic reduction in binding constants shifts the optimum CD concentration to much higher values (160 m*M*) and separation selectivity deviation from maximum is small (~4%) over a wide concentration range ($\Delta C = 50$ m*M*). Therefore, the selection of the optimum concentration is not as critical as compared to aqueous CE separation of racemates. Similar behavior is observed for charged cyclodextrins in nonaqueous media. Fig. 3 shows the effect of the concentration of a cationic quaternary ammonium β-CD (QA-β-CD) on separation selectivity of solutes with acidic functional groups. With increase in the concentration of QA-β-CD, the mobility difference increases and reaches a plateau. No maximum selectivity was observed in the QA-β-CD concentration range [12].

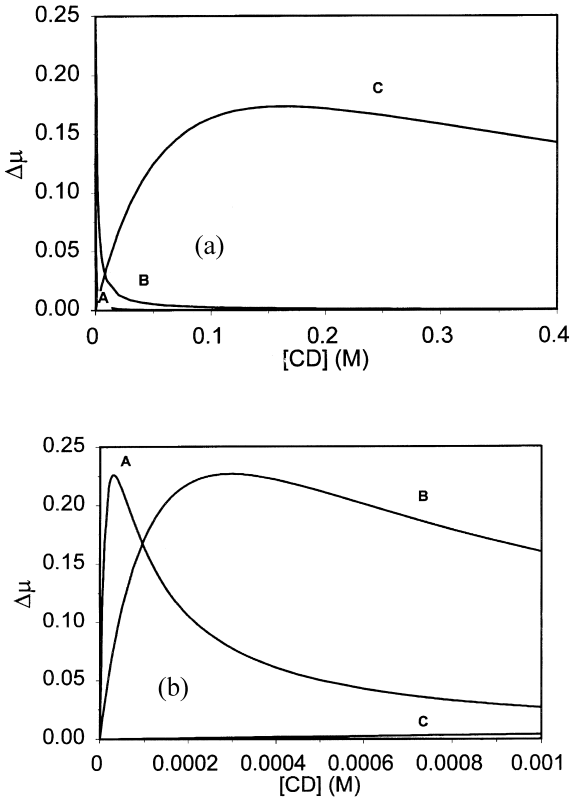

Fig. 2. Relationship between $\Delta\mu$ and β-CD concentration for thioridazine in different solvent systems. Curves were calculated from Eq. (4). (A) Aqueous buffer, (B) 6 *M* urea in aqueous buffer, and (C) formamide. From Ref. [8].

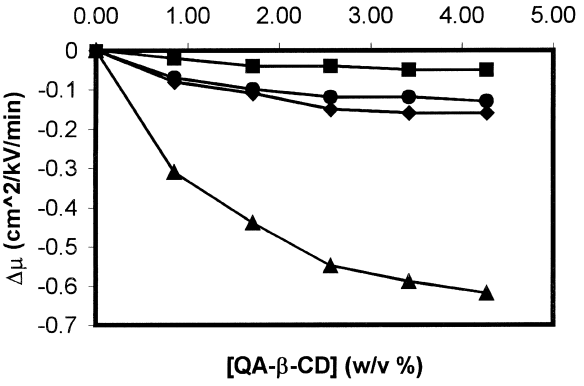

Fig. 3. Effect of the concentration of QA-β-CD on the mobility difference of some acidic solutes in formamide. Test solutes: dansyl-valine (triangle), dansyl-tryptophan (diamond), fenoprofen (circle), and ketoprofen (square). From Ref. [12].

4. Type of chiral selector

Similar to enantioseparation in aqueous CE, the type of chiral selector plays the most important role in NACE enantioseparations as it determines the effectiveness of the recognition mechanism. Generally, the chiral selectors used in aqueous CE can be directly applied in NACE enantioseparations. An obvious exception is proteins due to their limited solubility in most organic solvents.

4.1. Cyclodextrins

Cyclodextrins have been the most popular chiral selectors in aqueous CE. Among the natural CDs, β-CD is the most popular. However, the solubility of β-CD in aqueous solution is only about 16 m*M*. This causes limitations in the optimization of separation selectivity for enantiomers that bind weakly to the CD since the optimum CD concentration can be beyond the solubility limit. Chemical modification of β-CD increases its solubility, and may improve selectivity. In certain organic solvents, such as amides, solubility is not an issue; for example, solutions of β-CD in NMF in excess of 400 m*M* have been reported [3,26].

Fig. 4 shows the separation of trimipramine enantiomers with native β-CD in water (Fig. 4a) and in formamide (Fig. 4b) as well as using a sulfated β-CD (S-β-CD) in formamide (Fig. 4c). In the aqueous media, enantiomers could be separated in the β-CD concentration range of 0.08–1 m*M* (Fig. 4a). No separation was observed as the concentration of β-CD was increased to >1 m*M*. Previously, Quang and Khaledi reported the separation of trimipramine enantiomers in aqueous media using 20 m*M* HP-β-CD at pH 2.50 with 50 m*M* tetramethylammonium ion, TMA^+ [27]. The baseline separation of the optical isomers was achieved at the expense of a long separation time (~1 h) in a counter-EOF setup. A baseline separation of the enantiomers was achieved in the formamide system at 100 m*M* β-CD (Fig. 4b). According to the binding constant values for trimipramine, the $[CD]_{opt}$ values are 0.17 m*M* in aqueous buffer and 106 m*M* in formamide. Note that the $[CD]_{opt}$ value represents the optimum equilibrium CD concentration (rather than the analytical concentration). For compounds with large binding

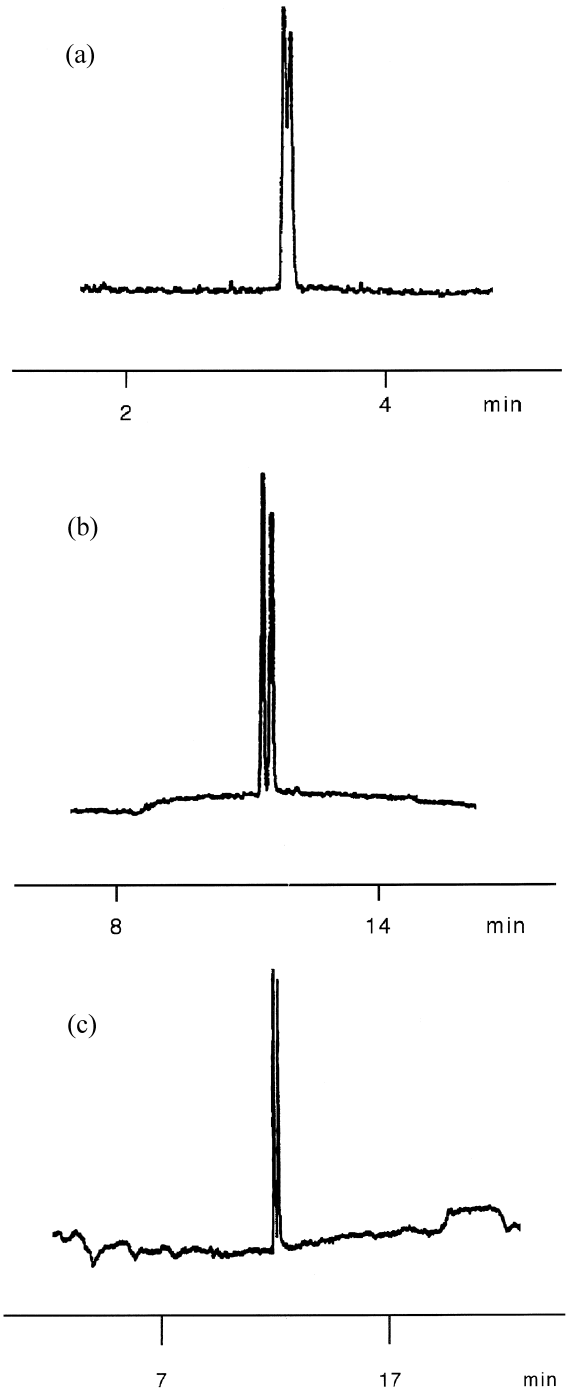

Fig. 4. Enantioseparation of trimipramine: (a) aqueous, 0.2 m*M* β-CD in 50 m*M* citric acid, 25 m*M* Tris, pH 3.02; (b) formamide, 100 m*M* β-CD in 150 m*M* citric acid, 100 m*M* Tris, pH* 5.1; (c) formamide, 1.54% (~10 m*M*) S-β-CD (DS = 4) in 150 m*M* citric acid, 100 m*M* Tris, pH* 5.1. From Ref. [8].

constants (such as trimipramine in aqueous buffer), the optimum concentration value is small and is comparable to the solute concentration in the sample zone. Therefore, the difference between the equilibrium and analytical concentrations is significant. In such a case, the equilibrium CD concentration would have to be calculated from the analytical CD concentration and analyte concentration. On the other hand, for cases where the binding constants are not too large (such as formamide systems), the optimum CD concentration is significantly larger than the solute concentration; thus, the analytical CD concentration is nearly identical to the equilibrium CD concentration.

As shown in Fig. 4c, trimipramine was successfully separated using a much lower concentration of a charged CD than that of native β-CD. In nonaqueous media, the positively charged amines interact more strongly with the negatively charged S-β-CD as compared to the neutral β-CD. As a result of the increased binding of chiral solutes, the optimum CD concentration would shift to lower values.

Recent application of charged CDs in aqueous CE has broadened the applicability of this type of chiral selector due to the fact that they can enhance not only separation selectivity, but also resolution by establishing a counter-EOF setup [28–30]. Anionic CDs have been successfully applied to separate a large number of basic compounds [31–33]. However, an anionic CD might not be suitable for hydrophobic charged amines that already have a strong interaction with uncharged CD. The additional electrostatic interaction would further strengthen the binding. This results in an optimum selector concentration that is quite small and difficult to control. In this case, even if higher separation selectivity is achieved by the charged CD, the very small optimum CD concentration would not be desirable.

Another problem with charged CDs in aqueous CE is that severe peak tailing can be observed due to the mobility mismatch between the buffer co-ions and CD-analyte ions. Fig. 5A shows the enantioseparation for thioridazine with aqueous CE using a sulfated β-CD (S-β-CD) at a concentration of about $1.54 \cdot 10^{-4}$% (w/v) (~1.0 μ*M*) in 50 m*M* Tris–phosphate aqueous buffer (pH 2.50). As can be seen, the peaks are severely tailed and the number of theoretical plates for these peaks is around 1000. In

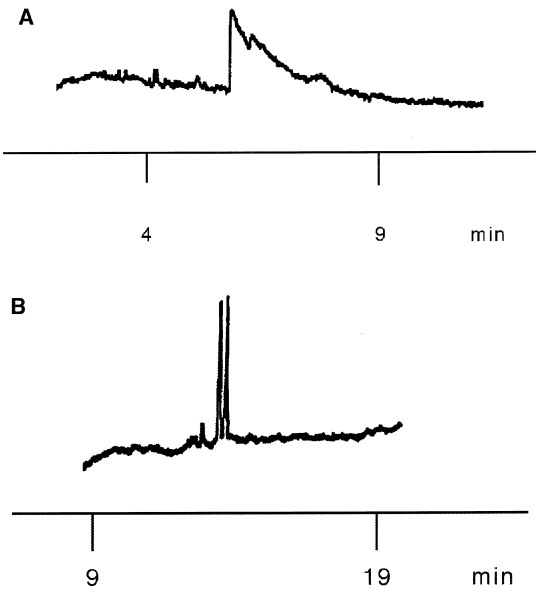

Fig. 5. Comparison of separation of thioridazine enantiomers with S-β-CD (DS=4) in aqueous and nonaqueous media. Buffer: (A) $1.54 \cdot 10^{-4}$% (w/v) (~1 μ*M*) S-β-CD (DS=4) in 50 m*M* Tris–phosphate (pH 2.50) aqueous media; (B) 1.54% (w/v) (~10 m*M*) S-β-CD in 150 m*M* citric acid–100 m*M* Tris (pH* 5.1) in formamide. Field strength: (A) 357 V/cm, (B) 595 V/cm. From Ref. [13].

aqueous CE, due to the strong binding between the enantiomers and the chiral selector ($K \sim 30\,000$), the optimum concentration of native β-CD for separation of thioridazine enantiomers is about ~0.05 m*M* at pH 2.50. Therefore, it is expected that the optimum concentration of the anionic CD for this compound be lower, at micromolar levels, due to the additional electrostatic attractions. The electrodispersion (not Joule heating) resulting from the mobility mismatch between S-β-CD-analyte complexes and the co-ions in the running buffer is probably the cause of poor peak shape [34]. A considerably better separation is achieved by NACE in formamide at a higher concentration of 1.54% (w/v) (~10 m*M*) S-β-CD in 100 m*M* Tris–150 m*M* citric acid (pH* 5.1) (Fig. 5B). In formamide media, higher voltages can be applied because of the overall lower electrical current than in aqueous buffer. Note that a higher electrolyte concentration was also used in formamide. The better separation efficiency (around 150 000 plates) in formamide can also be attributed to the stacking effect in formamide with higher ionic strength [35]. The combination of higher ionic strength and weaker interactions between S-β-CD and analytes reduced the electrodispersion (Fig. 5B).

Fig. 6 shows another example, the NACE enantioseparation of labetalol, a β-blocker which has two chiral centers, with S-β-CD in Tris–citrate buffer at apparent pH (pH*) 5.1. Under this condition, the analyte is positively charged. At 0.77% (w/v) (~5 m*M*) S-β-CD, separation of the diastereomers with two different stereogenic centers was achieved (Fig. 6A). A further increase in the CD concentration to 1.54% (w/v) (~10 m*M*) significantly increased the resolution of the first pair of enantiomers and slightly improved the enantioseparation of the second pair (data not shown). The migration times of the analyte increased with the CD concentration. At CD concentrations higher than 1.54% (w/v) (~10 m*M*), separations of both pairs of enantiomers of labetalol were achieved in NACE (Fig. 6B).

Twenty-four basic racemates have been separated using anionic CD in formamide [13,26]. As compared with neutral CDs, lower concentrations of S-β-CD were needed to resolve these racemates in formamide. Some analytes, such as doxylamine, labetalol and propranolol (which were not resolved by neutral CDs in formamide), were separated by S-β-CD due to the additional electrostatic interactions. For polar compounds such as acebutolol, metanephrine, and normetanephrine, that weakly bind to neutral CD, charged chiral selectors in aqueous media would be more effective.

An important issue in using charged CD is the degree of substitution (DS) for both aqueous and nonaqueous CE separations of enantiomers. Fig. 7 shows the separation of trimeprazine racemate using two types of S-β-CDs that have different numbers of sulfate substituents, S-β-CD (DS=4) (Fig. 7a) and S-β-CD (DS=7–11) (Fig. 7b), at the same concentration. Note that a higher electrical field strength can be used for the CD with a lower DS as the ionic strength and consequently the electrical current were smaller. The reason that different DS of CDs causes different separation selectivity is due to the changes in the interaction sites and/or the steric effects on the rims of the CD, which can change binding constants of the isomers and separation selectivity.

Vigh's group synthesized single-isomer anionic β-CDs and successfully used them for separations of

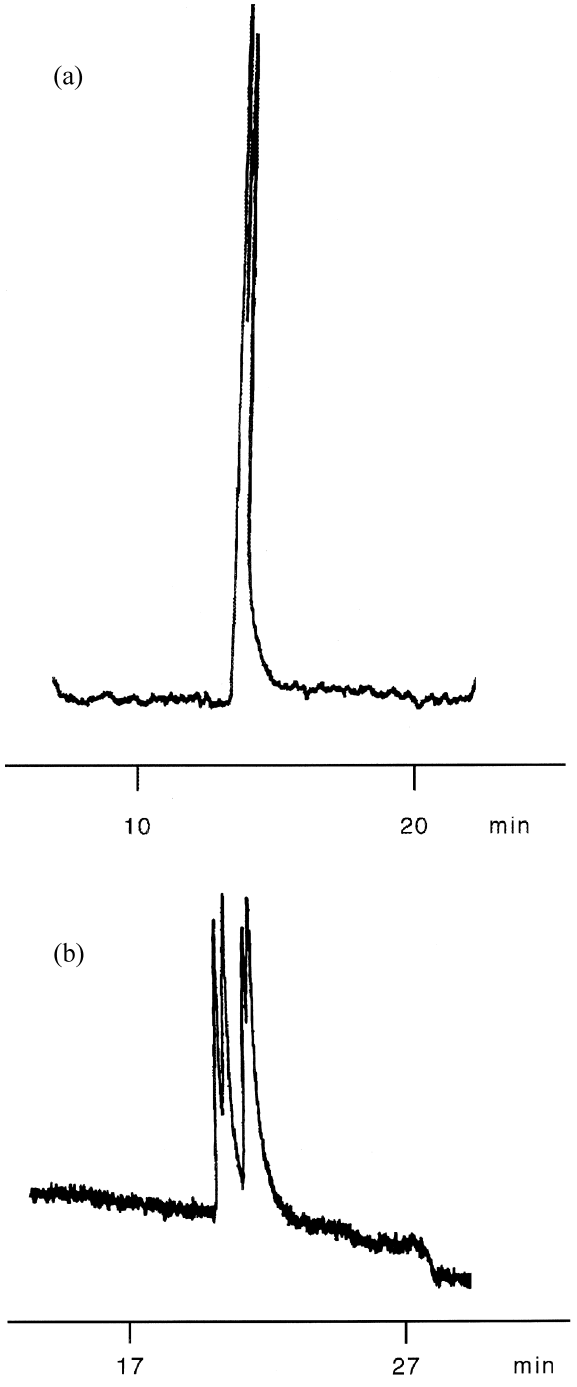

Fig. 6. Effect of S-β-CD (DS=4) concentration on enantioseparation of labetalol in formamide. Buffer: (A) 0.77 (~5 m*M*), and (B) 3.08% (w/v) (~20 m*M*) S-β-CD in 150 m*M* citric acid–100 m*M* Tris (pH* 5.1) in formamide. Field strength: 595 V/cm. From Ref. [13].

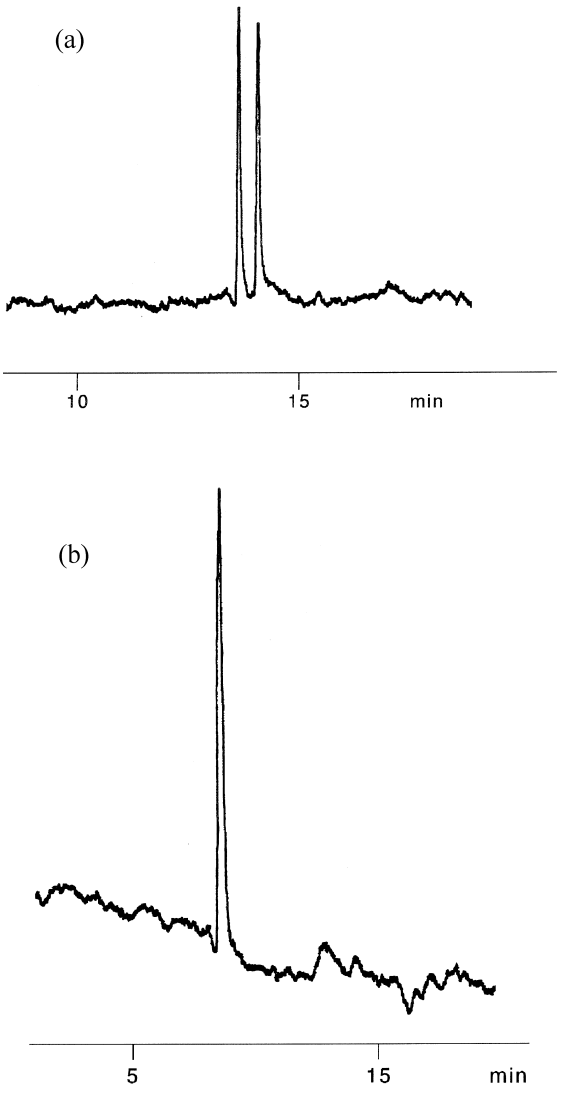

Fig. 7. Effect of degree of substitution (DS) on enantioseparation of trimeprazine. Buffer: (a) 3.08% (w/v) (~20 m*M*) S-β-CD (DS=4), (b) 3.08% (w/v) S-β-CD (DS=7–11) in 150 m*M* citric acid–100 m*M* Tris (pH* 5.1) in formamide. Field strength: 595 V/cm. From Ref. [13].

cationic, anionic and neutral racemates [36–38] in aqueous CE. One of the selectors, heptakis (2,3-diacetyl-6-sulfato)-β-CD (HDAS-β-CD), was also used in pure methanol [39]. They reported enantioseparation of a large number of small basic compounds. As expected, a large separation selectivity was obtained for positively charged analytes when the effective mobilities of the slower migrating

enantiomers were close to zero. No severe peak tailing was reported (Fig. 8). Enantioseparations of acidic or neutral compounds were not achieved in methanol due to the weak enantiomer–selector interaction in the pure organic solvent.

The separation of acidic enantiomers by a cationic CD, quaternary ammonium β-CD (QA-β-CD), in pure organic solvents has recently been reported [12]. No enantioseparation of the nonsteroidal anti-inflammatory drugs (profens) was achieved in NMF, methanol, dimethyl sulfoxide, and aqueous media, while separation of most amino acids was achieved in all of these solvents [12]. The application of NACE separation of ketoprofen racemate in a commercially available sample with only minimum sample preparation has been reported (Fig. 9). It was found that some of the profens and derivatized amino acids can be well separated by QA-β-CD in formamide [12]. Enantioseparation of dansyl amino acids by uncharged β-CD was also reported by Riekkola's group [40].

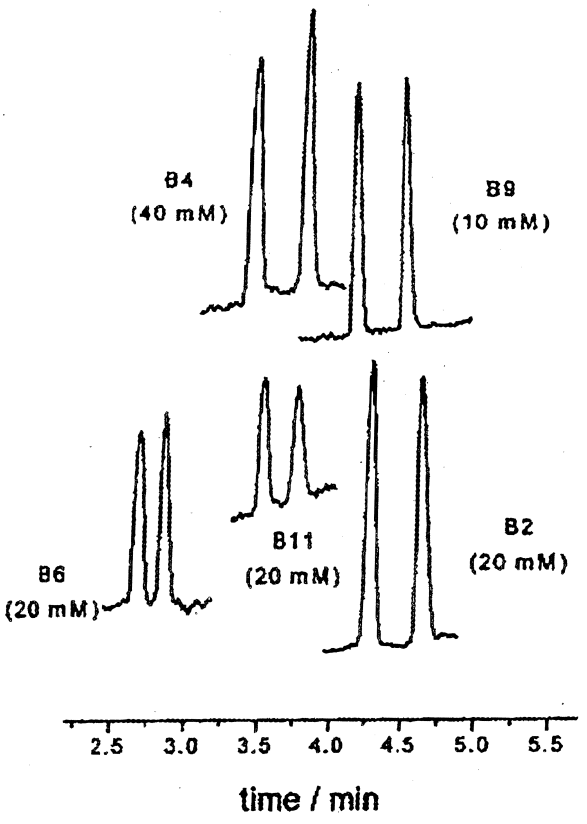

Fig. 8. Typical electropherograms of weak base analytes in acidic methanolic HDAS-β-CD BGEs. The numbers in parentheses indicate the CD concentration. From Ref. [39].

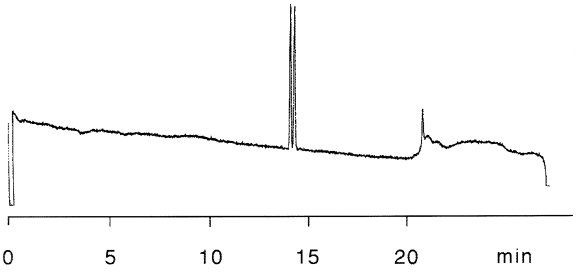

Fig. 9. Nonaqueous separation of ketoprofen enantiomers in Actron. Buffer electrolytes: 4.28% (w/v) (~20 m*M*) QA-β-CD in 100 m*M* Tris–100 m*M* acetic acid in formamide (pH* 7.5). Field strength: −476 V/cm. From Ref. [12].

Li et al. reported the enantioseparation of *N*-benzoyl phenylalamine methyl ester racemate (the compound has poor solubility in water) by β-CD in formamide [15].

4.2. Other chiral selectors

A chiral crown ether, (+)-18-crown-6 tetracarboxylic acid, has been successfully applied to separate amino acids [41,42], amines [43], and di- and tripeptides [44,45] in aqueous CE. This crown ether was used to separate compounds with primary amine groups adjacent to chiral centers in formamide (Fig. 10) [46].

Bjornsdottir et al. reported the enantioseparation of 15 basic drugs by (+)-*S*-camphorsulphonate in pure acetonitrile by an ion-pair mechanism [11]. No separation of these racemates by the same counterions was observed in pure aqueous media. It was believed that two point interactions between the analytes and selector via a charged sulphonate group and a hydrogen-accepting oxo-group contributed to the separation [11]. Tween 20 was used to enhance the resolution by reducing EOF. The migration order of enantiomers in (+)-*S*-camphorsulphonate buffer was opposite to that in (−)-*R*-camphorsulphonate (Fig. 11).

A cationic chiral selector, quinine, was also used for separation of some derivatized amino acids and acidic racemates in pure methanol [9]. It was found that selectivity decreased as the ammonium acetate

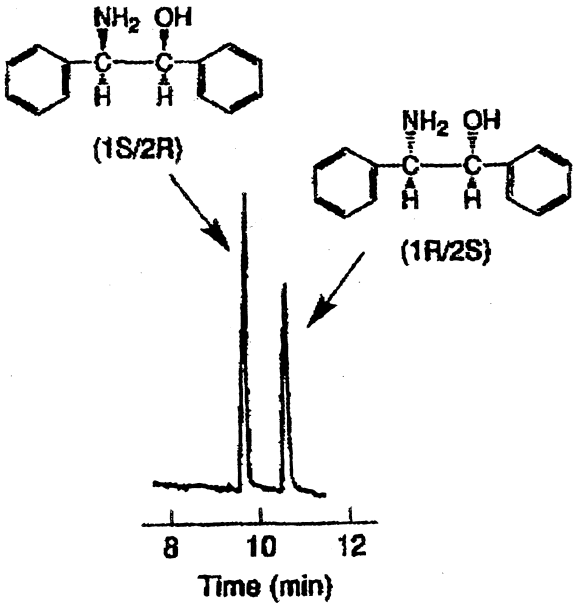

Fig. 10. Enantiomeric separation of (1*S*/2*R*), (1*R*/2*S*)-2-amino-1,2-diphenylethanol. Conditions: 10 m*M* 18-crown-6 tetracarboxylic acid in formamide, $\lambda = 300$ nm, +20 kV. From Ref. [46].

concentration increased. The separation selectivity increased with the addition of acetic acid. The separation was primarily based on ion-pair interactions. Recently, Piette et al. showed the enhancement of enantioseparation of N-derivatized amino acids with quinine and *tert.*-butyl carbamoylated quinine in an ethanol–methanol solvent mixture [14]. The optimized separation condition for DNB–Leu enantiomers was 10 m*M* *tert.*-butyl carbamoylated quinine in 12.5 m*M* ammonia–100 m*M* octanoic acid. The resolution of DNB–Leu was 64.3 with a separation efficiency of 127 000 plate counts (Fig. 12).

5. Effect of other parameters

Other experimental parameters, such as pH*, ionic strength, temperature and surface coating of the capillary, can also affect enantioseparation in NACE.

5.1. Apparent pH

The pH of the buffer determines the charges on

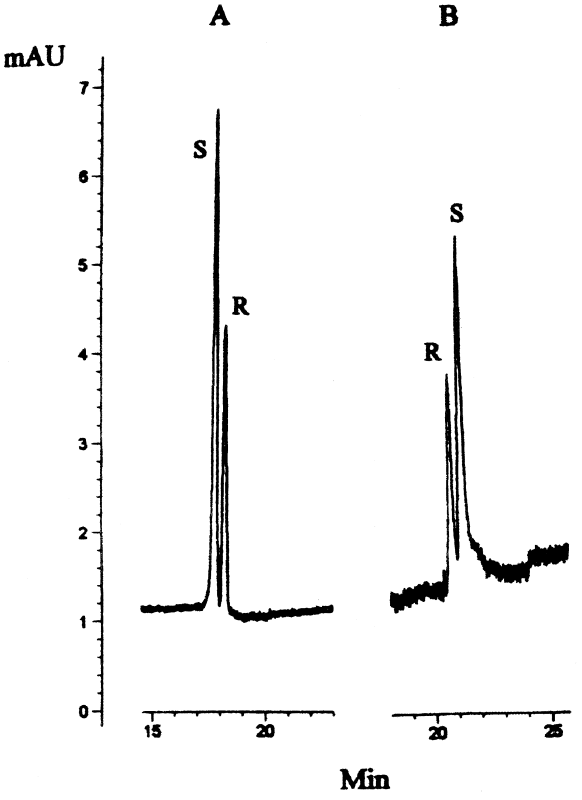

Fig. 11. Reversal of migration order of metoprolol in acetonitrile. Electrophoresis medium: 30 m*M* (+)-*S*-camphorsulphonate in 0.2 m*M* Tween 20+1 *M* acetic acid in acetonitrile. (A) *RS*-metoprolol, and (B) *RS*-metoprolol spiked with *S*-metoprolol. From Ref. [11].

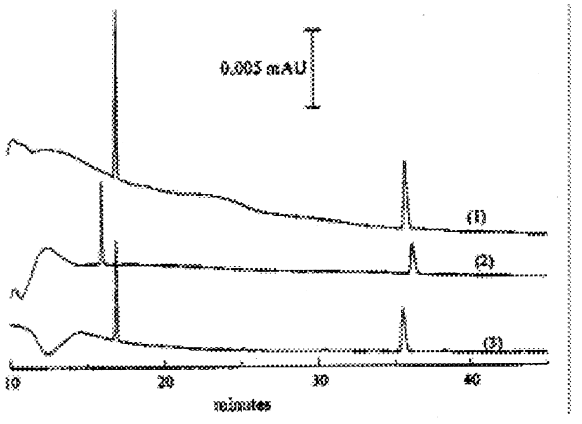

Fig. 12. Enantioseparation of N-derivatized amino acids by *tert.*-butyl carbamoylated quinine. Buffer: 10 m*M* *tert.*-butyl carbamoylated quinine in 100 m*M* octanoic acid–12.5 m*M* ammonia in methanol–ethanol (40:60). From Ref. [14].

analytes. Therefore, it has a pronounced effect on separation selectivity and resolution. Addition of organic solvents to an aqueous buffer can induce shifts in the pK_a values for both acidic and basic compounds. According to Sarmini and Kenndler, pK_a values for acidic solutes increase with increase in organic solvent percentage (for both alcohol and acetonitrile) [47–50]. They also found that separation selectivity was enhanced for acids with hydroxyl substituents, while the selectivity was reduced for acids without hydroxyl groups. A drawback of NACE is that knowledge about acid–base chemistry in organic media is very limited and it is difficult to relate the apparent pH (pH*) that is measured by a pH meter in organic media to that in aqueous solvent. However, pH* can be used to monitor the relative changes in pH in organic media to achieve reproducible results.

Fig. 13 shows the typical pH* effect on the electrophoretic mobilities for a group of amines using a sulfated CD. The mobility vs. pH* plots in formamide follow typical sigmoidal behavior, as shown in Fig. 13 for the first eluted enantiomers of the test solutes. At pH* <10, the mobilities of the solutes were positive, which indicates that the solutes bear a positive charge. Between pH* 6 and 8, there was no obvious change in the mobilities, which suggests that solutes are fully protonated. The pH* effect on the mobility difference was relatively small in the pH* range 6–9 since there was no drastic change in the mobilities of the enantiomers. With increase of pH*, the mobility of the basic solutes decreased. Finally, at pH* >10.3, negative mobilities were obtained for all enantiomers. This is the deprotonated amines that are associated with the negatively charged selector.

5.2. Ionic strength

The effect of ionic strength was studied in NMF [8]. The increase of the ionic strength of the system upon increasing the concentration ratio of citric acid to Tris from 10/5 to 100/50 enhanced enantioseparation. No further improvement in separation of the racemate was observed at concentrations larger than 100/50 (data not shown). The mobilities of the test solutes and the electroosmotic flow decreased with increase of the ionic strength. The decrease in the electrophoretic mobilities of the enantiomers and EOF caused an increase in the resolution. The electrophoretic mobility of a solute in NACE is inversely proportional to the ionic strength of the

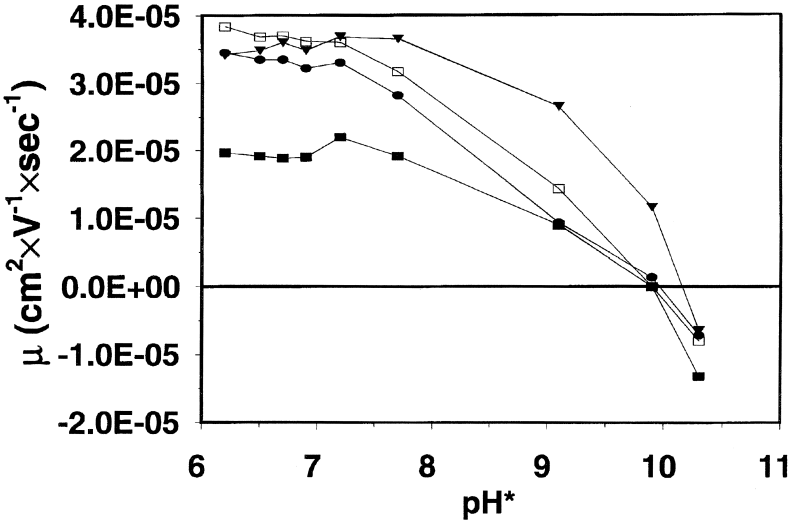

Fig. 13. Effect of pH* on the mobilities of compounds in formamide. Lines represent the first eluted peak for each enantiomeric pair. Buffer: 3.08% (w/v) (~20 m*M*) S-β-CD (DS=4) in formamide, the total concentration of acetic acid plus Tris was kept at 250 m*M*. Field strength: 595 V/cm. Compounds: trimipramine (filled square), ethopropazine (filled triangle), propiomazine (filled oval), promethazine (empty square). From Ref. [13].

medium, which is the same behavior observed in aqueous buffer.

5.3. Temperature

As the temperature decreased, better separation was achieved for trimipramine racemate [8]. This was because the decrease in temperature increased the viscosity of the medium, and therefore increased the migration time. A change in temperature can also change the binding constants and separation selectivity.

5.4. Electroosmotic flow

Two general migration schemes are recognized in

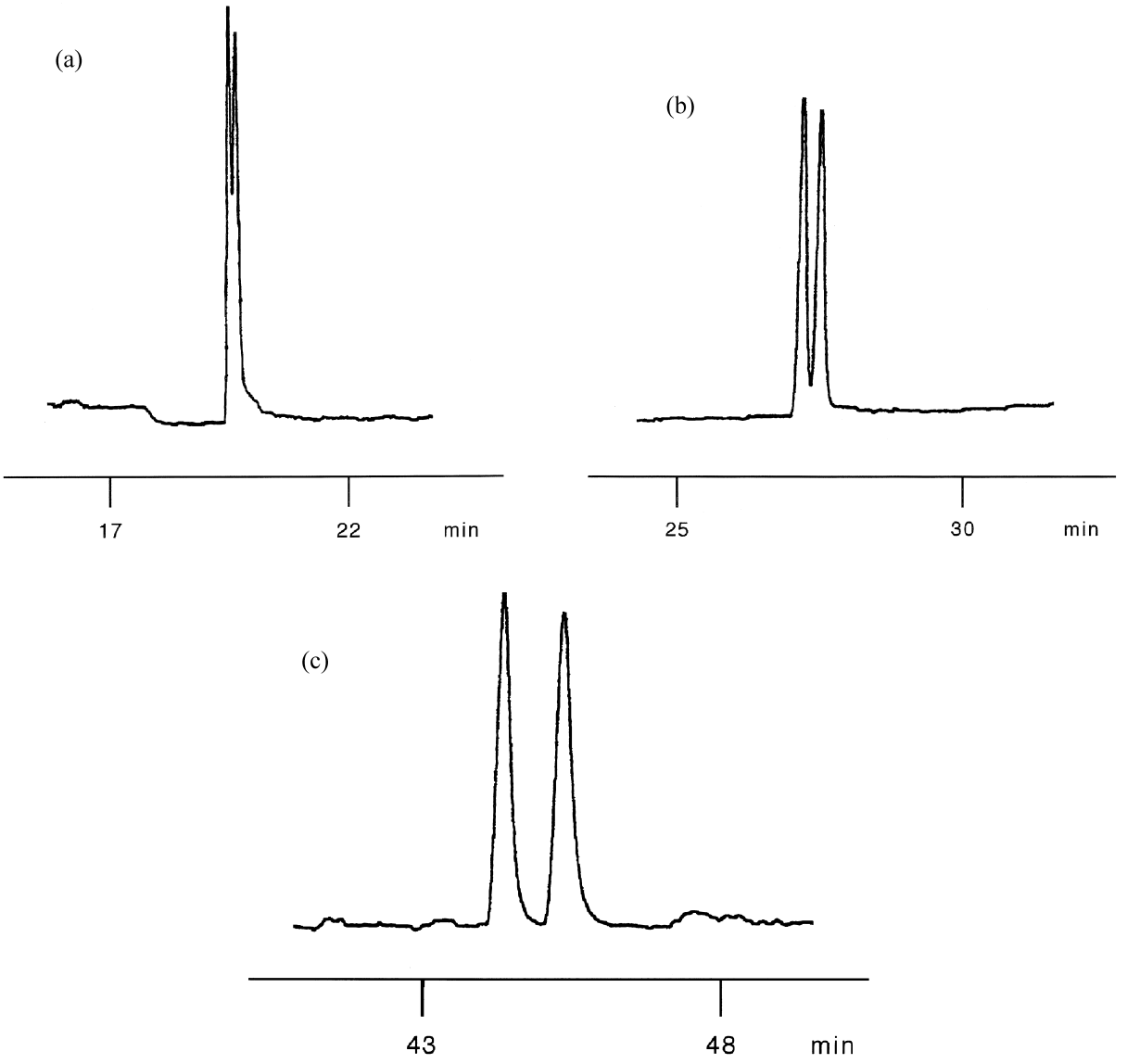

Fig. 14. Influence of tetraalkylammonium ion, TAA^+, on enantioseparation of trimipramine in formamide: (A) without TAA^+, (B) with 100 m*M* TBA^+, and (C) with 100 m*M* TMA^+. The running electrolyte was 150 m*M* citric acid, 100 m*M* Tris, 250 m*M* β-CD in formamide at pH* 5.1. From Ref. [8].

Table 1
Separation of enantiomers by NACE

Compound	Chiral selector	Buffer, solvent	Ref.
1,1′-Binaphthyl-2,2′diyl hydrogen phosphate	Quinine	13/26 m*M* Ammonium acetate in MeOH	[9]
	QA-β-CD (DS = 3.8)	21 m*M* Ammonium acetate–1% acetic acid in formamide–DMSO	[12]
1-Naphthylethylamine	Chira1 crown ether	2.5, 40, 100 m*M* Tetrabutylammonium perchlorate	[46]
1-Phenylethylamine	Chiral crown ether	2.5, 40, 100 m*M* Tetrabutylammonium perchlorate	[46]
2-Amino-1,2-diphenyl-ethnol	Chiral crown ether	2.5, 40, 100 m*M* Tetrabutylammonium perchlorate	[46]
Atenolol	HDA5-β-CD	50 m*M* DCAA–25 m*M* TEA in MeOH	[39]
	camphorsulphonate	1 *M* Acetic acid in ACN	[11]
Bifonazole	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Bisoprolol	Camphorsulphonate	1 *M* Acetic acid in ACN	[11]
Bunitrolol	Camphorsulphonate	1 *M* Acetic acid in ACN	[11]
Bupivacaine	HDAS-β-CD	50 m*M* DCAA–25 m*M* TEA in MeOH	[39]
Carprofen	QA-β-CD (DS = 3.8)	20 m*M* Ammonium acetate–1% acetic acid in formamide	[12]
Chlophedianol	HDAS-β-CD	50 m*M* DCAA–25 m*M* TEA in MeOH	[39]
	β-CD	150 m*M* Citric acid–100 m*M* Tris in formamide	[8]
Chlorcyclizine	γ-CD, M-β-CD	150 m*M* Citric acid–100 m*M* Tris in formamide	[8]
	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Chlorphedianol	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Chlorpheniramine	S-β-CD (D5 = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Cloperostine	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Cyclopentolate	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Dansyl-amino acids	β-CD	10 m*M* NaCl in NMF–formamide	[10,40]
	QA-β-CD (DS = 3.8)	21 m*M* Ammonium acetate–1% acetic acid in formamide–NMF–MeOH–DMSO	[12]
Dopa	Chiral crown ether	2.5. 40, 100 m*M* Tetrabutylammonium perchlorate	[46]
Doxylamine	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Drofenine	S-β-CD (DS = 4.0)	150 m*M* Citric acic–100 m*M* Tris in formamide	[13]
Econazole	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Ephedrine	Camphorsulphonate	1 *M* Acetic acid in ACN	[11]
Epinephrine	Camphorsulphonate	1 *M* Acetic acid in ACN	[11]
Ethopropazine	HP-β-CD	150 m*M* Citric acid–100 m*M* Tris in formamide	[8]

(*Continued on next page*)

Table 1 (*continued*)

Compound	Chiral selector	Buffer, solvent	Ref.
Fenoprofen	QA-β-CD (DS = 3.8)	20 m*M* Ammonium acetate–1% acetic acid in formamide	[12]
Flurbiprofen	QA-β-CD (DS = 3.8)	20 m*M* Ammonium acetate–1% acetic acid in formamide	[12]
Homochlorcyclizine	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Indoprofen	QA-β-CD (DS = 3.8)	20 m*M* Ammonium acetate–1% acetic acid in formamide	[12]
Isoproterenol	HDAS-β-CD	50 m*M* DCAA–25 m*M* TEA in MeOH	[39]
Ketoprofen	QA-β-CD (DS = 3.8)	20 m*M* Ammonium acetate–1% acetic acid in formamide	[12]
Labetalol	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Meclizine	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Metaproterenol	HDAS-β-CD	50 m*M* DCAA–25 m*M* TEA/25 m*M* DCAA–50 m*M* TEA in MeOH	[39]
Metoprolol	Camphorsulphonate	1 *M* acetic acid in ACN	[11]
Mianserin	β-CD, γ-CD, M-β-CD, HP-β-CD	150 m*M* Citric acid–100 m*M* Tris in formamide	[8]
	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
N-[1-(1-Naphthyl)ethyl]-phthalamic acid	Quinine	13/26 m*M* Ammonium acetate in MeOA	[9]
N-3,5-Dinitrobenzoylated amino acids	Quinine	13/26 m*M* Ammonium acetate in MeOA	[9]
Nefopam	β-CD, γ-CD, M-β-CD, HP-β-CD	150 m*M* Citric acid–100 m*M* Tris in formamide	[8]
	S-β-CD (DS = 4.0)	150 m*M* Citric acic–100 m*M* Tris in formamide	[13]
Noradrenaline	Chiral crown ether	2.5, 40, 100 m*M* Tetrabutylammonium perchlorate	[46]
Norphedrine	Chiral crown ether	2.5, 40, 100 m*M* Tetrabutylammonium perchlorate	[46]
Oxyphencyclimine	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Oxyphenonium	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Phenylalanine	Chiral crown ether	2.5, 40, 100 m*M* Tetrabutylammonium perchlorate	[46]
Pindolol	HDAS-β-CD	50 m*M* DCAM–25 m*M* TEA/25 m*M* DCAA–50 m*M* TEA in MeOH	[39]
	Camphorsulphonate	1 *M* Acetic acid in ACN	[11]
Piperoxan	HDAS-β-CD	50 m*M* DCAA–25 m*M* TEA in MeOH	[39]
Primaquine	M-β-CD	150 m*M* Citric acid–100 m*M* Tris in formamide	[8]
	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Propiomazine	β-CD	150 m*M* Citric acid–100 m*M* Tris in formamide	[8]
	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]

Table 1 (*continued*)

Compound	Chiral selector	Buffer, solvent	Ref.
Propranolol	HDAS-β-CD	50 m*M* DCAA–25 m*M* TEA in MeOH	[39]
	Camphorsulphonate	1 *M* Acetic acid in ACN	[11]
	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Salbutamol	Camphorsulphonate	1 *M* Acetic acid in ACN	[11]
Suprofen	QA-β-CD (DS = 3.8)	20 m*M* Ammonium acetate–1% acetic acid in formamide	[12]
Terbutaline	HDAS-β-CD	50 m*M* DCAA–25 m*M* TEA/25 m*M* DCAA–50 m*M* TEA in MeOH	[39]
Tetramisole	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Thioridazine	β-CD, γ-CD, M-β-CD	150 m*M* Citric acid–100 m*M* Tris in formamide	[8]
	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Trihexyphenidyl	HDAS-β-CD	50 m*M* DCAA–25 m*M* TEA in MeOH	[39]
	M-β-CD	150 m*M* Citric acid–100 m*M* Tris in formamide	[8]
	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Trimepazine	β-CD, HP-β-CD	150 m*M* Citric acid–100 m*M* Tris in formamide	[8]
	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Trimipramine	β-CD, γ-CD, HP-β-CD	150 m*M* Citric acid–100 m*M* Tris in formamide	[8]
	S-β-CD (DS = 4.0)	150 m*M* Citric acid–100 m*M* Tris in formamide	[13]
Tryptophan	Chiral crown ether	2.5, 40, 100 m*M* Tetrabutylammonium perchlorate	[46]

CE; one is a co-electroosmotic flow (co-EOF setup), where the ions and EOF migrate in the same direction, and the other is a counter-electroosmotic flow (counter-EOF setup), where the ions migrate in the opposite direction of EOF. In the counter-EOF setup case, however, higher resolution can be achieved as the term $(\mu_{avg} + \mu_{eo})$ in the denominator of the resolution equation becomes smaller. According to Eq. (5), higher resolution results if the analyte migrates in the opposite direction to EOF. This is achieved at the expense of longer analysis times. In certain situations, separation might be achieved through controlling EOF even when other parameters such as selector concentration or pH are not under optimum conditions.

In enantioseparations by aqueous CE, both long-chain cationic surfactants and short-chain surfactants have been used to enhance separation [51,52]. It has been shown that short-chain surfactants have certain advantages, such as better capillary wall coverage at low pH, no micelle formation, and less inclusion complexation with CDs [52]. Tetramethylammonium (TMA^+) and tetrabutylammonium (TBA^+) cations were used in formamide to test the possibility of controlling EOF. Since tetraalkylammonium (TAA^+) cation competes with enantiomers to complex with β-CD, little separation was observed when 100 m*M* β-CD and 100 m*M* TMA^+ coexisted in formamide. Higher concentrations of β-CD were used with TAA^+ to monitor the influence on enantioseparation. Fig. 14 shows the effect of addition of TAA^+ on the separation of trimipramine racemate in 250 m*M* β-CD. Partial separation was achieved in the absence of short-chain cationic surfactants due to the higher than optimum concentration of chiral selector used (Fig. 14A). The addition of 100 m*M* TBA^+ and TMA^+ (Fig. 14B and C) caused the migration of the test solute to increase from 23 to 50 min. Baseline

separations were achieved in TMA^+ solution. TMA^+ was more effective than TBA^+ for control of EOF. The EOF could not be reversed in formamide at any TBA^+ or TMA^+ concentration. The addition of short-chain surfactants to NMF had very little influence on both migration time and enantioseparation, and almost no influence in DMF.

6. Nonaqueous capillary electrochromatography

Krause et al. reported the application of a wide-pore aminopropyl silica gel coated with helically chiral poly diphenyl-2-pyridylmethyl methacrylate as chiral stationary phase for enantioseparation of neutral racemates in methanol media [16]. They compared the enantioseparations using four different separation techniques, i.e. conventional HPLC, capillary HPLC, pressure-assisted CEC, and CEC. Capillary LC provided better separations.

7. Conclusions

The use of nonaqueous media in enantioseparation by CE has attracted attention for separations that have solubility and/or stability problems in aqueous media. Table 1 shows a list of reported applications of NACE in separations of racemates. In addition, the use of organic solvents leads to different separations due to differences in ion solvation, acid–base chemistry, and even recognition of enantiomers. Although the research reviewed in this paper focused on enantioseparation in pure organic solvents, method development is not limited to the use of single-solvent systems. Mixed solvents can be of great value in enhancing separation of many racemic mixtures as well as broadening the range of CE applications in separations. There is a need, however, for a better understanding of the solvent effects on migration behavior and separation mechanisms.

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