Focusing Review

Method Development of Enantiomer Separations by Affinity Capillary Electrophoresis, Cyclodextrin Electrokinetic Chromatography and Capillary Electrophoresis-Mass Spectrometry

Yoshihide Tanaka

Department of Analytical Chemistry, Nippon Boehringer Ingelheim Co. Ltd., 3-10-1, Yato, Kawanishi, Hyogo 666-0193, Japan

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Abstract

Capillary electrophoresis (CE) has become a powerful tool for enantiomer separations during the last decade. Since 1993, the author has investigated enantiomer separations by affinity capillary electrophoresis (affinity CE) with some proteins and by cyclodextrin electrokinetic chromatography (CDEKC) with some charged cyclodextrins (CDs). Many successful enantiomer separations are demonstrated from our study in this review article. In the enantiomer separations by affinity CE, the deterioration of detection sensitivity was observed under high concentration of the protein in running solutions. The partial filling technique was practically useful to solve the serious problem. It allowed operation at high protein concentrations, such as 500 μM, without the detection problem. Charged CDs had several advantages for the enantiomer separations over neutral ones. Strong electrostatic interactions between a charged CD and oppositely charged analytes should be effective for the formation of the complex. A large difference in electrophoretic mobility between the free analyte and the inclusion complex should also enhance the enantiomeric resolution. In CE-mass spectrometry (CE-MS), the partial filling technique was applied to avoid the introduction of nonvolatile chiral selectors into the CE-MS interface. By replacing the nonvolatile electrolytes in the running buffer by volatile ones, the separation conditions employed in CE with the UV detection method could be transferred to CE-MS.

Keywords: enantiomer separation, capillary electrophoresis, capillary electrophoresis-mass spectrometry, partial filling technique, protein, charged cyclodextrin

1. Introduction

Many of isomeric pharmaceutical drugs frequently exhibit some stereoselectivity for pharmacological activity and/or drug-protein binding. The separation of enantiomeric mixtures into individual isomers is one of the most important issues in pharmaceutical developments. Although high-performance liquid chromatography (HPLC) is the most widespread for this purpose, the method developments, such as the choice of chiral stationary phases (CSPs) and the optimization of separation conditions, are time-consuming. On the contrary, capillary electrophoresis (CE) has high resolving power and requires simple operating procedures. It is simply and rapidly performed to choose a chiral selector for the relevant enantiomer separation. Only small amounts of the chiral selectors are required for the method developments in comparison to HPLC. Thus, CE has become an attractive analytical tool for the enantiomer separation during the last decade. Successful examples of applications in our study, which have been obtained by CE and on-line CE-mass spectrometry (CE-MS) with several chiral selectors such as proteins and cyclodextrins (CDs), are reviewed. A partial filling technique was employed to avoid both the deterioration of detection sensitivity of UV photometry caused by the strong UV absorption of chiral selectors and the introduction of nonvolatile chiral selectors into the MS instrument.

Tel, +81-727-90-2322; fax, +81-727-93-8399;

2. Theory

Concerning the enantiomer separation by CE, Wren and Rowe [1] firstly proposed a simple theoretical model using CD as a chiral additive:

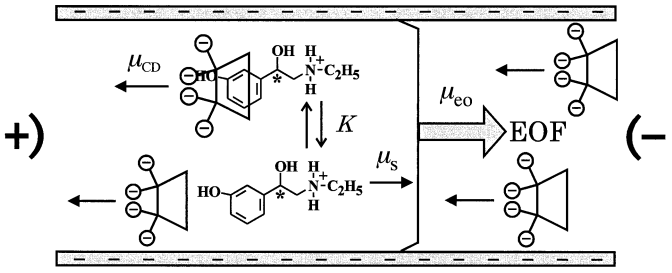


Figure 1. Schematic illustration of the migration behavior in the separation of a cationic analyte using an anionic CD.

$$\underset{\mu_s}{A} + CD \rightleftharpoons \underset{\mu_{CD}}{ACD} \qquad K_1 = \frac{[ACD]}{[A]\,[CD]} \tag{1}$$

$$\underset{\mu_s}{B} + CD \rightleftharpoons \underset{\mu_{CD}}{BCD} \qquad K_2 = \frac{[BCD]}{[B]\,[CD]} \tag{2}$$

where A and B are a pair of enantiomers having identical electrophoretic mobilities, μ_s; and ACD and BCD are diastereomeric inclusion complexes having approximately identical apparent electrophoretic mobilities, μ_{CD}. The formation constants for inclusion complexes, K_1 and K_2, are defined by the concentration of each species such as [A], [CD] and [ACD] as given in equations (1) and (2). A schematic diagram of the migration behavior of an anionic CD and a cationic analyte is shown in Figure 1. In order to obtain the enantiomer separation, it is required that both pairs of μ_s and μ_{CD}, and of K_1 and K_2 have different values. When the equilibrium between the free and bound forms is quickly established, the apparent electrophoretic mobility of A, $\mu_{app,A}$ is expressed:

$$\begin{aligned}\mu_{app,A} &= \mu_{eo} + \left(\frac{[A]}{[A] + [ACD]}\right)\mu_s + \left(\frac{[ACD]}{[A] + [ACD]}\right)\mu_{CD} \\ &= \mu_{eo} + \frac{\mu_s + \mu_{CD}K_1\,[CD]}{1 + K_1\,[CD]}\end{aligned} \tag{3}$$

where μ_{eo} is the electroosmotic mobility. The mobility difference between the enantiomers, $\Delta\mu$, is written as:

$$\Delta\mu = \frac{(\mu_s - \mu_{CD})\,(K_2 - K_1)\,[CD]}{1 + (K_1 + K_2)\,[CD] + K_1K_2\,[CD]^2} \tag{4}$$

From the above equation, the optimum concentration of CD that gives the maximum $\Delta\mu$, $[CD]_{opt}$, is mathematically obtained:

$$[CD]_{opt} = \frac{1}{(K_1K_2)^{1/2}} \tag{5}$$

Consequently, the enantiomeric resolution is enhanced by increasing the concentration of chiral selector below the $[CD]_{opt}$. The above theoretical model has been applied to the other chiral selectors, such as proteins and macrocyclic antibiotics.

3. Affinity capillary electrophoresis using proteins

In CE, most chiral selectors are employed as a solution in a background solution unless they are immobilized in a gel or on capillary wall. CDs and their derivatives have been widely accepted as chiral selectors due to their characteristics of the high water solubility, the weak UV absorption and the excellent chiral discrimination toward various aromatic enantiomers [2]. However, all enantiomers are not always separated with native CDs or their derivatives. In HPLC, various kinds of CSPs have been developed to separate numerous different enantiomers. Among the CSPs, protein-based columns have been frequently employed because of their versatility and broad applicability. In the early 1990s, the usability of the proteins in CE had attracted the interest of several research groups [2]. We also started the study using some proteins such as egg white avidin [3, 4] and ovomucoid [4] in 1993. Because the chiral separations are based on the differential affinity of the proteins to individual enantiomers, this separation mode is particularly named “affinity capillary electrophoresis” (affinity CE). In this mode, a use of untreated fused-silica capillaries often brought about the deterioration of peak shapes of analytes due to the adsorption of the proteins and/or analytes on the inner wall of the capillary. Therefore, we used a linear polyacrylamide-coated capillary in affinity CE to solve the adsorption problem.

3.1. Conventional method

The most common procedure of chiral separations in CE is to fill a capillary with a separation solution containing a chiral selector. The separation conditions are commonly optimized by changing the concentration of the chiral selector and/or adding an organic modifier to the separation solution. Although the enantioselectivity should be mostly improved by increasing the protein concentration as shown in Figure 2, it was not preferable for high sensitive detection due to the background UV absorption of the protein solution. The effective concentration of the protein in HPLC columns was reported at mM level [5], whereas most applications in affinity CE should be performed at the protein concentrations of lower than 100 μM [3, 4]. Only a few successful separations were achieved under these low concentrations, suggesting the necessity of solving the detection problem to increase the number of successful chiral separations by affinity CE.

3.2. Partial filling technique

The partial filling technique was firstly introduced by Valtcheva et al. [6], and we modified the method to be able to perform the continuous and automatic operations using a conventional CE instrument [7]. The technique has been useful to solve the above-mentioned problem, i.e. the deterioration of detection sensitivity under high protein concentration solutions. The principle is schematically illustrated in Figure 3. After a capillary is washed with a running buffer without chiral selectors, a portion of the capillary is filled with a separation solution including a protein by

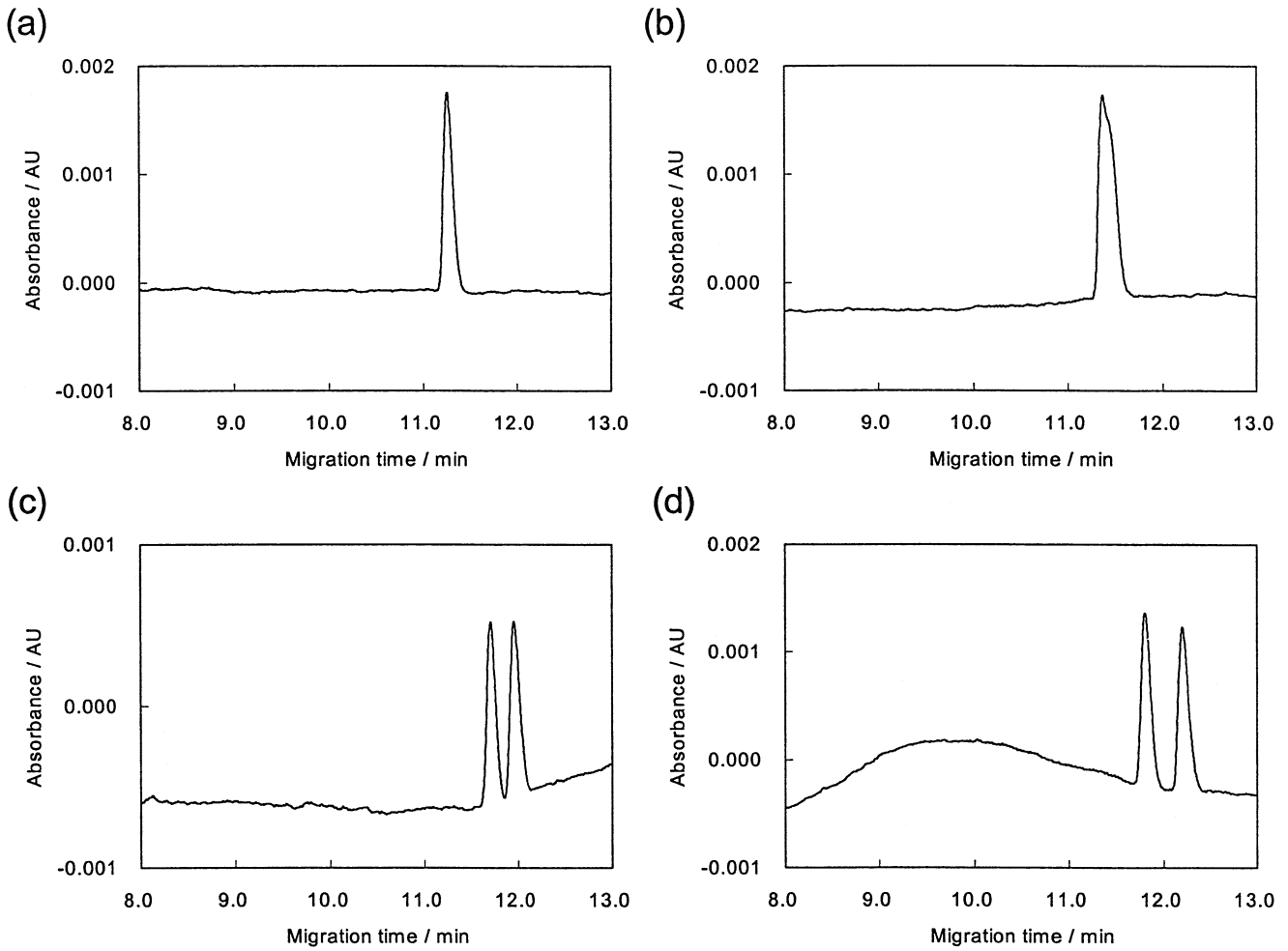


Figure 2. Effect of the concentration of AVI on the separation of racemic vanilmandelic acid [3]. Instrument, BioFocus 3000; separation solution, (a) 0 μM, (b) 10 μM, (c) 25 μM, (d) 50 μM AVI dissolved in 50 mM phosphate buffer (pH 4.0); applied voltage, −12 kV; sample injection, 6.9 kPa × 2s (sample concentration, 50 μg/mL); capillary temperature, 25 ℃; detection wavelength, 240 nm.

pressurization at 6.9 kPa for 190s. When a coated capillary of 50 μm I.D. and 36 cm in total length (31.5 cm to the detector) is employed, the zone length of the separation solution in the capillary is approximately 27 cm. Therefore, the rest portion of the capillary including the detection window is filled with the running buffer [Figure 3 (a)]. A sample solution is introduced at the end of the capillary filled with the separation solution [Figure 3 (b)]. Both ends of the capillary are dipped into the running buffer, and a constant voltage is applied for the separation. The partial filling technique has to be performed under the conditions where the protein does not migrate toward the detection end of the capillary. First, the enantiomer separation occurs when the enantiomers migrate through the separation solution zone [Figure 3 (c)], and thereafter both enantiomers migrate through the running buffer zone at an identical velocity. The analytes can be detected without interference with the high background UV absorption of the protein solu-

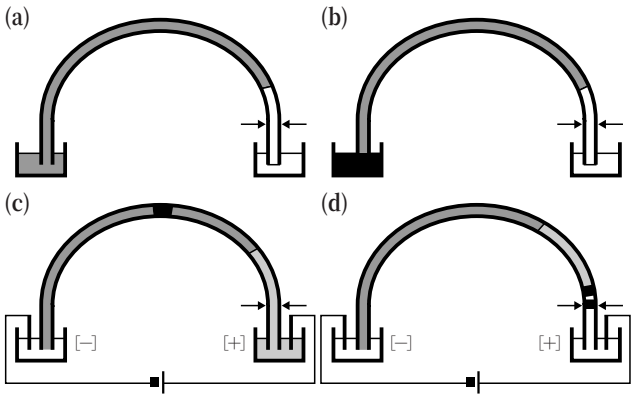


Figure 3. Schematic illustration of the partial filling technique.
= separation solution, = running buffer,
= sample solution, = detection window.

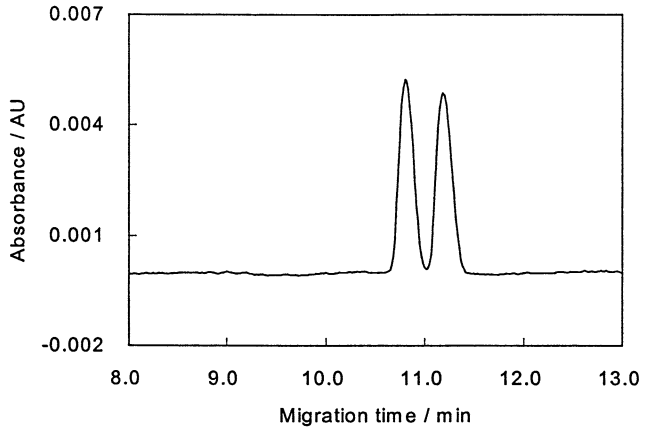


Figure 4. Enantiomer separation of racemic vanilmandelic acid by the partial filling technique with AVI [9]. Running buffer, 50 mM phosphate buffer (pH 4.0), separation solution, AVI dissolved in the running buffer (zone injection, 6.9 kPa × 190 s); detection wavelength, 210 nm. Other conditions are as in Figure 2.

tion [Figure 3 (d)]. As shown in Figure 4, the drift of baseline is significantly suppressed even if absorbance at 210 nm is employed. A number of enantiomer separations, which may be equivalent to those obtained using protein-based CSPs in HPLC, have been obtained by the partial filling technique with a high protein concentrations, such as 500 μM, as given in Table 1 [7-9].

3.3. Effects of biotin binding to avidin on enantioselectivity

Although egg white avidin (AVI) showed excellent enantioselectivity towards many acidic racemates, it was not known whether the enantioselectivity arose from the interaction between AVI and analytes on the biotin-binding sites. We investigated the effect of the formation of AVI-biotin complex on enantioselectivity [9]. Figure 5 shows the change in the enantiomer separation of racemic 4-fluoromandelic acid by the addition of D-biotin to the separation solution. Since the modification in the three-dimensional structure of AVI caused by the biotin binding was not well known, it was not concluded that the enantiomers are recognized at the biotin binding sites on AVI from these results. However, it was obvious that the enantioselectivity was drastically changed. When D-biotin was added to the separation solution at the molar ratio of 1:4/AVI: D-biotin, not only the enantioselectivity but also the interaction to the analytes was almost lost [9].

4. Capillary electrophoresis using charged cyclodextrins

4.1. Use of anionic CDs for the separation of basic and neutral enantiomers

Neutral CDs had been commonly used for the enantiomer separations by CE in the early 1990s [2]. Several charged CDs were commercially available in those days. A strong electrostatic interaction or a large binding constant between charged CDs and analytes having the opposite charge is expected. In addition, the large difference in the electrophoretic mobilities between free analytes and complexed analytes should be advantageous as given in equation (4). We attempted the separations of 50 basic racemates using anionic CDs as listed in Table 2 [10]. First, each anionic CD was dissolved in a 50 mM phosphate buffer (pH 5.0) as a separation solution. Owing to the short migration times caused by the strong EOF, insufficient separations were obtained using an untreated fused-silica capillary. In this study, therefore, a linear polyacrylamide-coated capillary was used to suppress the EOF. The coated capillary was also effective in preventing the cationic analytes from the adsorption on the capillary wall. As given in Table 3, 40 racemates of 50 basic racemates were successfully separated with $Rs > 1.2$ by choosing a suitable type and concentration of the anionic CD. The basic analyte is protonated under weakly acidic conditions, and hence migrates toward the cathode when it is free from the CD. On the other hand, the anionic CD and the analyte included by the anionic CD migrate toward the anode because the EOF is almost completely suppressed. The migration direction of the analyte depends on the values of μ_s, μ_{CD} and K as given by equation (3). Therefore, the cationic analyte will migrate toward the anode if it is strongly included into the anionic CD. Figure 6 shows two typical electropherograms, one is detection at the anodic side and another is at the cathode side. Most analytes were better resolved at a low pH and with a low CD concentration because the positive charge on the basic analyte decreases with an increase in pH. For the separation of azelastine racemates using β-CD-phosphate sodium salt (β-CD-Phos), however, no resolution was observed at pH 5.0 and hence the CD was dissolved in a phosphate buffer at pH 7.0 to obtain the successful separation. It was certain that only the non-dissociated forms of the enantiomers interacted selectively with β-CD-Phos.

The analytes were classified into three groups according to the concentration of the CD and the migration behavior as given in Table 3: (I) migrate toward the cathode with a low CD concentration; (II) migrate toward anode with a relatively high CD concentration; (III) migrate toward the cathode with a high CD concentration. The enantiomers of the group I have relatively large differences between K_1 and K_2, and thus easily resolve at low CD concentrations. However, the K-values of the analytes should be intermediate between those in the group II and group III. The enantiomers of the group II have large K-values and the analytes migrate as the complex toward the anode. Increasing the CD concentration is not effective to enhance the resolution, because the apparent mobilities of both enantiomers approach the maximum value or the mobility of the CD complexes, and the difference in the mobilities will not

Table 1. Enantiomer separation of racemates by the partial filling technique with proteins [7-9].

Protein	Conc. (μM)	Buffer pH	Additive	Enantiomers
AVI	50	4.0	none	4-Bromomandelic acid, 4-Fluoromandelic acid
	50	4.5	none	2-Phenylbutyric acid
	100	4.0	none	2-Phenyllactic acid, 3-Phenyllactic acid, 2-Phenoxypropionic acid, Vanilmandelic acid
	100	6.0	none	Menadione sodium bisulfite
	100	6.0	10% methanol	Abscisic acid
	100	6.0	10% ethanol	Warfarin
	100	6.0	10% 2-propanol	Flurbiprofen, Ibuprofen, Ketoprofen
	100	6.0	10% 1-propanol	Adrenochrome semicarbazone sulfonate Na
Suc-AVI	100	5.0	none	Trimipramine, Primaquine, Bupivacaine
STAV	100	5.5	none	DNS-Val, DNS-Nva, DNS-Asp
	100	6.0	none	Chlorpheniramine, Trimipramine, Primaquine, Warfarin
α_1-AGP	50	5.0	10% 1-propanol	Homatropine
	50	6.0	10% 1-propanol	Trihexyphenidyl
	100	6.0	10% ethanol	Pindolol
	100	6.0	10% 1-propanol	Epinastine, Trimebutine
	120	5.0	10% 1-propanol	Trimipramine
	200	5.0	10% 1-propanol	Nicardipine
	200	6.0	none	Ketamine
	200	6.0	10% 2-propanol	Oxyphencyclimine
	300	6.0	10% ethanol	Clorprenaline
	500	5.0	10% 1-propanol	Bupivacaine, Promethazine
	500	6.0	none	Etilefrin
	500	6.0	10% methanol	Denopamine, Fenoterol, Metoprolol, Trimetoquinol
	500	6.0	8% 2-propanol	Mexiletine
	500	6.0	10% 2-propanol	Arotinolol, Tolperisone
	500	6.0	10% 1-propanol	Eperisone, Primaquine, Verapamil
	750	6.0	10% 2-propanol	Atropine
	1000	6.0	none	Acebutolol, Metanephrine, Phenylephrine, Sulpiride, Terbutaline
BSA	500	6.0	none	Homochlorcyclizine, Propranolol, Oxyphencyclimine, Trimebutine
	750	6.0	none	Epinastine
OVM	500	5.0	10 mM CHAPS	Bunitrolol
	500	5.0	8% ethanol	Pindolol
	500	5.0	6% 2-propanol	Arotinolol
	500	5.0	8% 2-propanol	Oxyphencyclimine
	500	5.0	10% 2-propanol	Tolperisone, Verapamil
	500	5.0	8% 1-propanol	Chlorpheniramine, Primaquine, Trimebutine
CON	500	7.0	none	Trimetoquinol

Conditions : running buffer, 50 mM phosphate buffer containing an additive; separation solution, a protein dissolved in the running buffer; applied voltage, 12 kV for cationic racemates, −12 kV for anionic racemates. Other conditions are as in Figure 4. Abbreviations: AVI, avidin; Suc-AVI, succinylated avidin; STAV, streptavidin; α_1-AGP, bovine α_1-acid glycoprotein; BSA, bovine serum albumin; OVM, ovomucoid; CON, conalbumin; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DNS, dansyl; Val, valine; Nva, norvaline; Asp, aspartic acid.

increase. On the contrary, decreasing the CD concentration causes a decrease in the apparent mobilities, and will increase resolution. Some successful separations, e.g. aminoglutethimide and trihexyphenidyl using β-CD-SBE(IV), were obtained by decreasing the CD concentration. Although we tried to inverse the migration direction of the analytes to detect at the cathodic side by decreasing the CD concentration, the concentration had to be less than 0.05 mM and the enantiomer separations were nearly lost. The enantiomers of the group III have relatively small K-value and the difference between K_1 and K_2 is small. The anionic CDs are particularly useful for the enantiomeric separations of the group III. The electrostatic interaction will enhance the complex formation and a high concentration of the chiral selector can be easily obtained because of the high solubility of the charged CDs.

One limitation of neutral CDs as chiral selectors is that neutral racemates cannot be resolved unless a different discrimination mechanism such as ionic micellar solubilization is added [11]. The use of charged CDs offered new possibilities to separate the neutral racemates. These separations were performed using an untreated capillary and the EOF was used as a driving force for the neutral

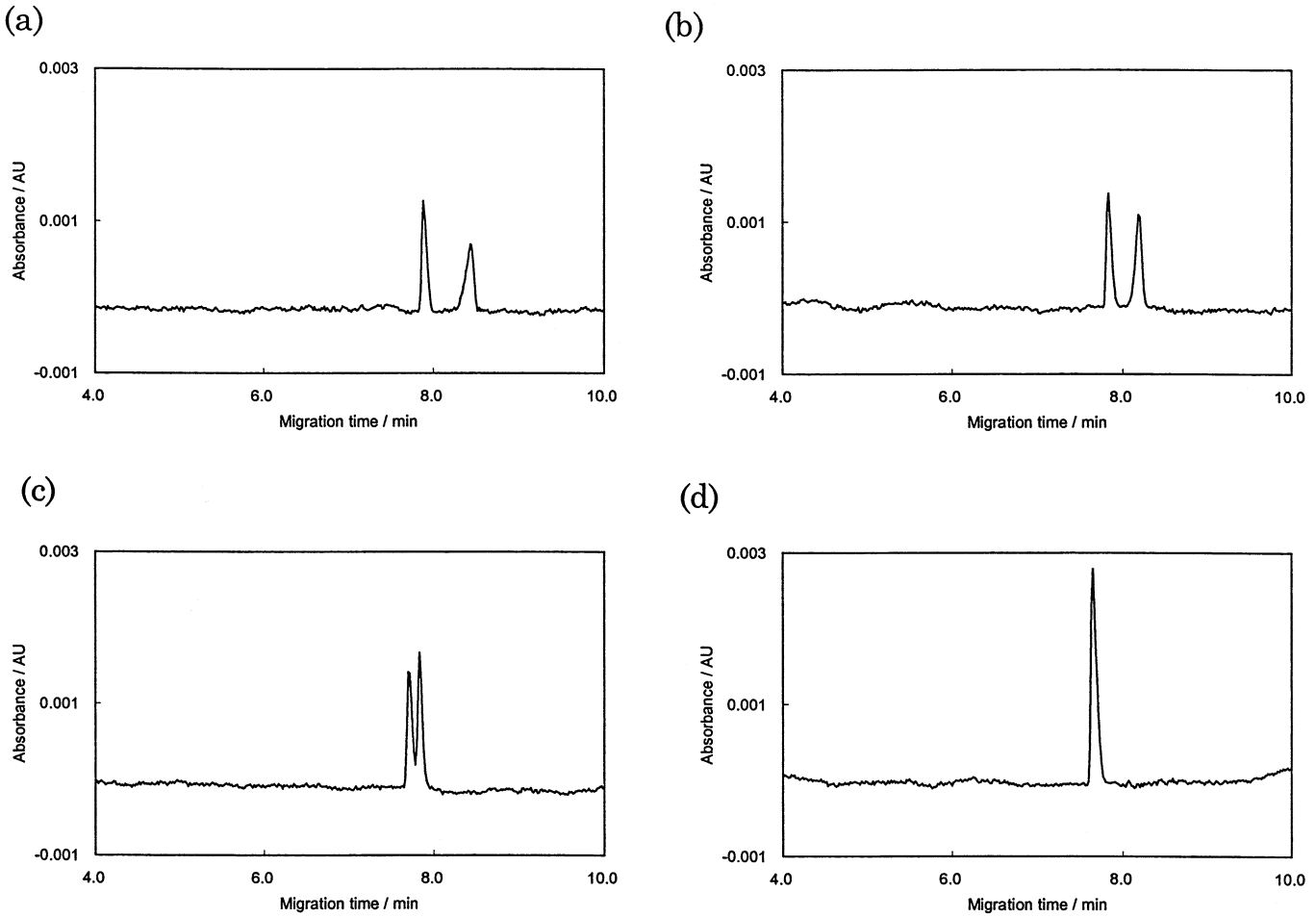


Figure 5. Effect of biotin binding to AVI on the separation of racemic 4-fluoromandelic acid [9]. Running buffer, 50 mM phosphate buffer (pH 4.0); separation solution, 100 μM AVI in the running buffer containing (a) 0 μM, (b) 100 μM, (c) 200 μM, (d) 400 μM D-biotin. Other conditions are as in Figure 4.

Table 2. Description of charged CD derivatives used.

CD type	CD derivative	Abbreviation	DS[a)]	MW[b)]	Average formula
Anionic	Carboxymethylated β-CD	CM-β-CD	3.6	1338	$C_{49}H_{77}O_{42}$
	β-CD phosphate sodium salt	β-CD-Phos	6	1879	$C_{42}H_{64}O_{53}P_6Na_{12}$
	Sulfobutylether(IV) β-CD sodium salt	β-CD-SBE(IV)	3.5	1683	$C_{56}H_{94.5}O_{45.5}S_{3.5}Na_{3.5}$
	Carboxymethylated γ-CD	CM-γ-CD	3.2	1472	$C_{54}H_{86}O_{46}$
	γ-CD phosphate sodium salt	γ-CD-Phos	6	1997	$C_{48}H_{76}O_{58}P_6Na_{10}$
Cationic	Quaternary ammonium β-CD	QA-β-CD	3.5	1664	$C_{63}H_{119}O_{.38.5}N_{3.5}Cl_{3.5}$
Amphoteric	Amphoteric β-CD	AM-β-CD	QA[c)]: 2 CM[d)]: 2	1593	$C_{58}H_{96}O_{41}N_2Cl_2Na_2$

Abbreviations: a) degree of substitutions, b) average molecular mass, c) quaternary ammonium group, d) carboxymethyl group.

Table 3. Successful separation of basic enantiomers using anionic CDs [10].

CD [a]	Enantiomers	[CD] mM	Migration direction[b]	Group[c]
β-CD-Phos	Clorprenaline, Imazalil	0.2	+	I
	Salbutamol, Trimebutine, Verapamil	0.4		
	Metanephrine	0.5		
	Ketamine	0.7		
	Azelastine	1[d]		
	Aminoglutethimide	3	–	II
	5 basic racemates Phenylephrine, Primaquine etc.	5		
	Bunitrolol, Nicardipine	5	+	III
	Acebutolol, Bupivacaine, Sulpiride	10		
β-CD-SBE(IV)	5 basic racemates Chlorpheniramine, Epinastine etc.	0.2	+	I
	Denopamine, Nicardipine	0.5		
	Etilefrin, Phenylephrine, Sulpiride	1		
	Atenolol, Mexiletine, Trimebutine	2		
	Trihexyphenidyl	2	–	II
	Aminoglutethimide, Verapamil	3		
	10 basic racemates Chlormezanone, Oxyphencyclimine, Meclizine, Sulconazole etc.	5		
	Acebutolol, Bunitrolol, Bupivacaine, Salbutamol	3	+	III
CM-γ-CD	Epinastine, Primaquine, Promethazine	0.1	+	I
	Imazalil	0.5		
	7 basic racemates Azelastine, Nicardipine etc.	1		
	Clorprenaline	2		
	Meclizine	3	–	II
	Thioridazine, Trimipramine	5		
	Pindolol, Trimebutine	5	+	III
	Bupivacaine, Sulconazole,Trimetoquinol	10		
γ-CD-Phos	Epinastine	0.1	+	I
	Primaquine	0.2		
	Clorprenaline	0.5		
	Chlorpheniramine, Denopamine, Verapamil	1		
	Mexiletine, Trimetoquinol	2		
	Fenoterol, Promethazine, Thioridazine, Trimipramine	5	–	II
	Tolperisone	3	+	III
	Nebracetam	4		
	Bisoprolol, Bupivacaine, Eperisone, Etilefrin, Metanephrine	5		
	Bunitrolol, Metoprolol	10		

CD was dissolved in 50 mM phosphate buffer (pH 5.0). Other conditions are as in Figure 6.
a) see Table 2. b) +, toward the cathode (applied voltage, 12 kV); –, toward the anode (applied voltage, –12 kV). c) see text. d) The CD was dissolved in the buffer at pH 7.0.

analytes. The migration times and the resolution were affected by the change in the EOF velocity. Successful enantiomer separations are listed in Table 4.

4.2. Relationship between the separation conditions and binding constants

In order to support the three classifications based on the difference in binding constants as given in Table 3, the binding constants were measured for three racemates, clorprenaline, primaquine, and sulpiride, chosen from each group. The binding constant, K, is obtained from the following equations when it is assumed that μ_{eo} is negligible.

$$\frac{\mu_s - \mu_{app}}{\mu_{app} - \mu_{CD}} = K\,[CD] \tag{6}$$

$$\frac{1}{\mu_{app} - \mu_s} = \frac{1}{(\mu_{CD} - \mu_s)\,K} \times \frac{1}{[CD]} + \frac{1}{\mu_{CD} - \mu_s} \tag{7}$$

As shown in Figure 7, they were calculated from the slopes of each straight line according to the least-square method. However, four points should be noted in this experiment: (i) although a single lot of β-CD-Phos was employed, it consisted of a mixture of the CD

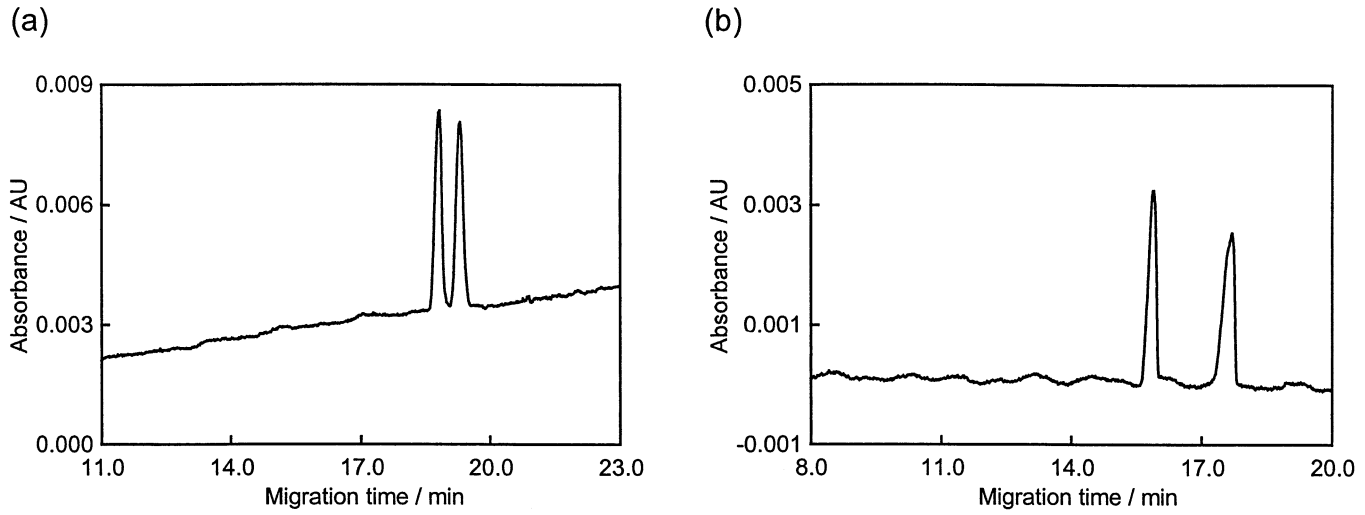


Figure 6. Representative electropherograms of the chiral separation of basic enantiomers by CE with an anionic CD derivative [10]. Samples, (a) racemic trimebutine (50 μg/mL), (b) racemic trimipramine (50 μg/mL); sample injection, 6.9 kPa × 2s; instrument, BioFocus 3000; capillary, 36 cm (31.5 cm to the detector) × 50 μm polyacrylamide-coated capillary; separation solution, 5 mM CM-γ-CD in 50 mM phosphate buffer (pH 5.0); migrate direction, (a) toward the cathode, (b) toward the anode; applied voltage, (a) 12 kV, (b) −12 kV; capillary temperature, 20 ℃; detection wavelength, 210 nm.

Table 4. Successful separation of neutral enantiomers with anionic CDs [10].

CD [a)]	[CD] mM	Buffer pH	Enantiomers
CM-β-CD	10	6.5	Methyl mandelate
	10	7.0	Benzoin
β-CD-Phos	10	6.5	Benzyl mandelate
	2.5	7.0	Benzoin
β-CD-SBE(IV)	10	6.0	Methyl mandelate, Ethyl mandelate, 1-Phenylethylalcohol
	10	6.5	12 PTH-amino acids[b)]
	10	7.0	1-Acenaphthenol

Conditions : capillary, 36 cm (31.5 cm to the detector) × 50 μm I.D. untreated fused-silica capillary; separation solution, CD dissolved in 50 mM phosphate buffer; applied voltage, 12 kV. Other conditions are as in Figure 6.
a) see Table 2. b) PTH, phenylthiohydantoin; 12 amino acids, alanine, α-aminobutylic acid, isoleucine, leucine, methionine, norleucine, norvaline, phenylalanine, proline, tryptophan, tyrosine, valine.

derivatives having different degrees of substitution; (ii) although [CD] was not equal to the added CD concentration, but it was not corrected; (iii) the EOF was not completely suppressed and hence the absolute mobility could not be obtained. (iv) viscosity changes with increasing [CD] were not taken into account. The rough binding constants were given as follow:

Clorprenaline (Group I) $K_1 = 2.4 \times 10^2\ M^{-1}$
$K_2 = 4.4 \times 10^2\ M^{-1}$ $\alpha = 1.8$
Primaquine (Group II) $K_1 = 1.7 \times 10^3\ M^{-1}$
$K_2 = 2.1 \times 10^3\ M^{-1}$ $\alpha = 1.2$
Sulpiride (Group III) $K_1 = 2.36 \times 10^2\ M^{-1}$
$K_2 = 2.37 \times 10^2\ M^{-1}$ $\alpha = 1.0$

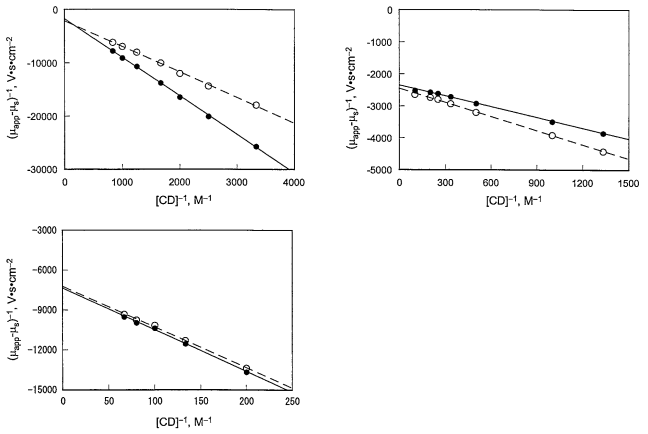
where α is K_2/K_1. The (α-value for the enantiomers of sulpiride was calculated to be 1.004 with the binding constants.

4.3 Comparison between anionic CDs and neutral CDs

In order to compare the anionic CDs with neutral CDs, we tried chiral separations of basic racemates using five neutral CDs: 10 mM β-CD and 7 M urea, 10-40 mM heptakis(2,6-di-*O*-methyl)-β-CD (DM-β-CD), 10-20 mM heptakis(2,3,6-tri-*O*-methyl)-β-CD (TM-β-CD), 10-40 mM hydroxypropyl-β-CD and 10-40 mM γ-CD [10]. Most racemates that were successfully separated using an an-

ionic CD were also successfully separated using a 50 mM phosphate buffer (pH 5.0) containing one of the neutral CDs. However, eleven racemates, acebutolol, bisoprolol, bunitrolol, chlormezanone, meclizine, metoprolol, nebracetam, oxyphencyclimine, sulconazole, sulpiride and trimipramine, could not be resolved at all in our experiments with the neutral CDs. Furthermore, the separation of eight racemates, atenolol, azelastine, ketamine, metanephrine, nicardipine, pindolol, salbutamol and thioridazine were insufficient even with 40 mM DM-β-CD. The complete separation of salbutamol racemate was reported by Rogan et al. [12] using 112 mM DM-β-CD. Although relatively high concentrations as high as 5 mM were necessary for the separation of most above mentioned racemates, they were all successfully resolved using charged CDs. However, these concentrations were much lower than those employed for neutral CD derivatives. The use of anionic CDs was considerably effective for the enantiomer separation of basic racemates.

Figure 7. Plot of $(\mu_{app}-\mu_s)^{-1}$ versus $[CD]^{-1}$ for the calculation of the binding constants with β-CD-Phos [10]. Samples, (a) clorprenaline, (b) primaquine, (c) sulpiride. Conditions, see Table 3.



4.4. Use of cationic and amphoteric CDs for the separation of acidic enantiomers

Because it was expected that cationic CDs showed high enantioselectivity to anionic enantiomers as the anionic CDs did to basic racemates, we performed several enantiomer separations of acidic racemates using a commercial cationic β-CD derivative having quaternary ammonium groups (QA-β-CD). QA-β-CD is positively charged over the wide range of pH in comparison with the cationic CDs having amino groups, and is practically useful for the enantiomer separations by CE. Successful enantiomer separations are listed in Table 5. The enantioselectivity of QA-β-CD was compared with several neutral CDs. As the results, three racemates which were not resolved with neutral CDs, i.e., racemic (*cis, trans*) -abscisic acid, racemic 4-bromomandelic acid and racemic chrysanthemum-monocarboxylic acid, were separated completely with QA-β-CD [13].

An amphoteric β-CD derivative (AM-β-CD) having both quaternary ammonium groups and carboxyl groups was also commercially available. Because the degrees of the substitutions are equal between cationic and anionic substituents, the amphoteric CD has

Table 5. Successful separation of acidic enantiomers using cationic and amphoteric CDs [13].

CD [a]	Enantiomers	[CD] mM	Buffer pH	Migration direction [b]
QA-β-CD	*p*-Chlorowarfarin	0.3	7.0	–
	3-Phenylbutylic acid	0.4	5.0	–
	Menadione sodium bisulfite	1	4.0	–
	(*cis*, *trans*)-Abscisic acid	1	6.0	–
	Adrecochrome semicarbazone sulfonate Na	3	4.0	–
	4-Bromomandelic acid	3	4.0	–
	2-Phenoxypropionic acid	4	4.0	–
	2-Phenyllactic acid	4	5.0	–
	2-Phenylbutyric acid, Tropic acid	5	5.0	–
	Chrysanthemum monocarboxylic acid	2	4.0	+
AM-β-CD	4-Bromomandelic acid, 2-Phenoxypropionic acid Chrysanthemum monocarboxylic acid	10	4.0	–
	10 DNS-amino acids[c]	10	6.0	–
	4-Chloromandelic acid, 3-Phenylbutyric acid	20	4.0	–

CD was dissolved in 50 mM phosphate buffer. Other conditions are as in Figure 6.
a) see Table 2. b) +, toward the cathode (applied voltage 12 kV); −, toward the anode (applied voltage −12 kV). c) DNS, dansyl; 10 amino acids, α-aminobutylic acid, asparaginic acid, glutaminic acid, leucine, methionine, norleucine, phenylalanine, serine, threonine, valine.

no net charge in a buffer solution unless the pH is extremely low or high. AM-β-CD showed the different enantioselectivity as compare with the other CDs, e.g. racemic 4-chloromandelic acid was separated completely only with AM-β-CD [13].

5. Enantiomer separation by capillary electrophoresis-mass spectrometry

We have performed several application studies by CE-MS, that is, composition analysis of charged CDs [14] and ionic surfactants [15] by capillary zone electrophoresis-MS (CZE-MS), a simultaneous determination of charged and neutral analytes by CZE-MS, separations and detections by EKC-MS using sodium dodecyl sulfate, β-CD-SBE(IV) and 4-sulfonated calix[6]arene as pseudo-stationary phases [16], and enantiomer separations by CE-MS [17, 18]. The electrospray ionization (ESI) interface is employed in the above mentioned experiments. This review article is focused on the enantiomer separations by CE-MS. Coupling of CE with MS detection offers several advantages over the UV detection method. The MS detector has relatively high sensitivity as compared to the conventional UV detector, and structural information of the analytes is also obtained simultaneously on the separation. Even if some interfering compounds are co-eluted, target analytes are separately detected with high sensitivity according to the specific molecular mass. In addition, some analytes having no strong UV absorption can be detected by MS detection. Concerning the enantiomer separations by CE-MS, nonvolatile components, such as chiral additives and nonvolatile electrolytes, contaminated the ESI nozzle of the CE-MS interface and/or the orifice plate during the run. They may also cause a decrease in ionization efficiency of the analytes at the ESI interface. Furthermore, the nonvolatile components are blown into the orifice plate when a capillary is washed with a separation solution prior to the run. As an electrolyte, either ammonium formate or ammonium acetate buffer is usually employed. In order to avoid the introduction of the chiral selector into the interface, we employed the partial filling technique described as Section 3.2. The separation conditions as listed in Table 1 were employed for CE-MS by changing the buffer electrolytes from sodium phosphate to ammonium acetate, and enantiomer separations were successfully obtained as shown in Figure 8. The optical purity test is one of the important items in chiral separation. The determination of optical purity of commercial camphorsulfonic acid enantiomers was performed. The enantiomeric separation was achieved using DM-β-CD as a chiral selector. From 1% to 2% of the enantiomeric impurity was easily detected as shown in Figure 9.

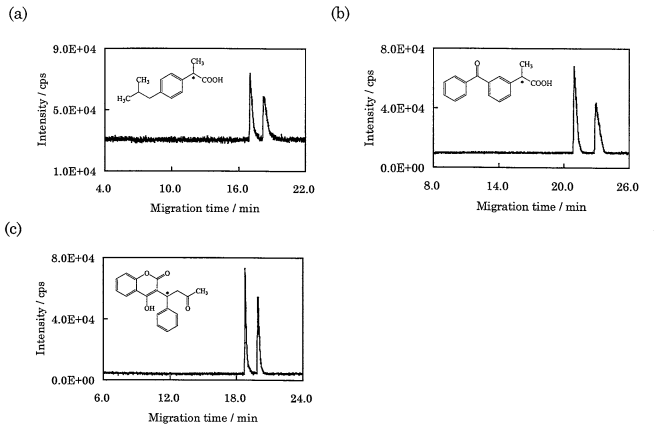


Figure 8. Enantiomer separation of (a) racemic ibuprofen, (b) racemic ketoprofen and (c) racemic warfarin by affinity CE-MS with AVI [17]. Capillary, 50 μm i.d. × 80 cm polyacrylamide-coated capillary; running buffer, (a), (b) 40 mM ammonium acetate buffer (pH 6.0) containing 10% (v/v) 2-propanol, (c) 40 mM ammonium acetate buffer (pH 6.0) containing 10% (v/v) ethanol; separation solution, 100 μM AVI in the running buffer; sheath liquid, a mixture of the running buffer and methanol (1:1), 5 μL/min; voltage, −30−(−4.5) kV; detection, (a) *m/z* 205.0, (b) *m/z* 253.0 and (c) *m/z* 306.6 (negative ion mode).

6. Conclusion

During the last decade, CE has become a useful analytical tool for chiral separations through the numerous efforts by many researchers. We believe that our investigations have contributed to the progress of chiral separations by CE. In this review article, many successful enantiomer separations are demonstrated. The excellent results suggest the wide application range for chiral separa-

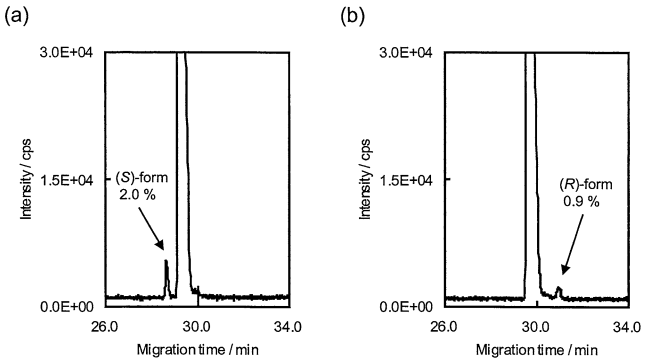


Figure 9. Optical purity test of camphorsulfonic acid enantiomers [17]. Conditions: sample solution, (a) *(R)*-(+)-camphorsulfonic acid enantiomer and (b) *(S)*-(−)-camphorsulfonic acid enantiomer in water (100 μg/mL); running buffer, 40 mM ammonium formate buffer (pH 4.0); separation solution, 50 mM DM-β-CD in the running buffer; detection, *m/z* 230.8 (negative ion mode). Other conditions are as in Figure 8.

tions by CE as well as conventional HPLC method. The method development for separation conditions can be also performed easily in a short-time.

Coupling of CE with MS detection offers several advantages over the UV detection method. However, nonvolatile chiral additives such as proteins and CDs are incompatible with either ESI interface or MS instrument in CE-MS. The partial filling technique has been practically useful to solve the problem. Although several applications are introduced in this paper, the type of chiral selectors does not restrict the MS detection by the partial filling technique. A number of applications of enantiomer separations have been already reported using UV detection, and the separation conditions should be transferred to CE-MS by changing the buffer electrolyte from the nonvolatile species to volatile one. CE-MS combined with the partial filling technique can be applied to a wide range of the separations.

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References

[1] Wren, S. A. C.; Rowe, R. C. *J. Chromatogr.* **1992**, *603*, 235.

[2] Terabe, S.; Otsuka, K.; Nishi, H. *J. Chromatogr. A* **1994**, *666*, 295.

[3] Tanaka, Y.; Matsubara, N.; Terabe, S. *Electrophoresis* **1994**, *15*, 848.

[4] Terabe, S.; Ozaki, H.; Tanaka, Y. *J. Chin. Chem. Soc.* **1994**, *41*, 251.

[5] Ahmed, A.; Ibrahim, H.; Pastoré, F.; Lloyd, D. K. *Anal. Chem.* **1996**, *68*, 3270.

[6] Valtcheva, L.; Mohammad, J.; Pettersson, G.; Hjertén, S. *J. Chromatogr.* **1993**, *638*, 263.

[7] Tanaka, Y.; Terabe, S. *J. Chromatogr.* A **1995**, *694*, 277.

[8] Tanaka, Y.; Terabe, S. *Chromatographia* **1997**, *44*, 119.

[9] Tanaka, Y.; Terabe, S. *Chromatographia* **1999**, *49*, 489.

[10] Tanaka, Y.; Yanagawa, M.; Terabe, S. *J. High Resoult. Chromatogr.* **1996**, *19*, 421.

[11] Terabe, S.; Ozaki, H.; Otsuka, K.; Ando, T. *J. Chromatogr.* **1985**, *332*, 211.

[12] Rogan, M. M.; Altria, K. D.; Goodall, D. M. *Electrophoresis* **1994**, *15*, 808.

[13] Tanaka, Y; Terabe, S. *J. Chromatogr. A* **1997**, *781*, 151.

[14] Tanaka, Y.; Kishimoto, Y.; Terabe, S. *Anal. Sci.* **1998**, *14*, 383.

[15] Tanaka, Y.; Kishimoto, Y.; Otsuka, K.; Terabe, S. *Bunsekikagaku* **1998**, *47*, 563.

[16] Tanaka, Y.; Kishimoto, Y.; Otsuka, K.; Terabe, S. *J. Chromatogr. A* **1998**, *817*, 49.

[17] Tanaka, Y.; Kishimoto, Y.; Terabe, S. *J. Chromatogr. A* **1998**, *802*, 83.

[18] Tanaka, Y.; Otsuka, K.; Terabe, S. *J. Chromatogr. A* **2000**, *875*, 323.