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Enantiomer separation of acidic racemates by capillary electrophoresis using cationic and amphoteric β-cyclodextrins as chiral selectors

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Abstract

Enantiomer separations of various acidic racemates were performed by capillary electrophoresis (CE) using a commercial cationic β-cyclodextrin (β-CD), quaternary ammonium β-CD (QA-β-CD), and a commercial amphoteric β-CD (AM-β-CD) as chiral selectors. Eleven acidic racemates were successfully separated using QA-β-CD by changing the CD concentration and the buffer pH. These enantiomer separations were compared with the results using five neutral CD derivatives. Although most racemates were separated with some of the neutral CDs, relatively high CD concentrations were required to obtain baseline separations. In contrast, when QA-β-CD was employed, the enantiomer separations were successful at low concentrations below 5 m*M*. Enantiomers of five acidic racemates and ten dansylated amino acids (Dns-amino acids) were separated using AM-β-CD. Although the baseline separation of racemic 4-chloromandelic acid was not achieved with either QA-β-CD or five neutral CDs, AM-β-CD showed complete resolution. Furthermore, the simultaneous enantiomer separation of eight Dns-amino acids was also achieved with AM-β-CD. Both QA-β-CD and AM-β-CD were analyzed by CE and mass spectrometry (MS) in order to identify their compositions because they consisted of a mixture having different degrees of substitution. QA-β-CD consisted of six components having from one to six quaternary ammonium groups. The composition of AM-β-CD, however, was very complicated and could not be identified. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Buffer composition; Chiral selectors; Cyclodextrins; Acids

1. Introduction

Cyclodextrins (CDs) are chiral cyclic oligosaccharides composed of six to eight d-glucopyranose units, and can be successfully employed as chiral selectors in chromatographic techniques such as high-performance liquid chromatography (HPLC) [1] and gas chromatography [2]. In capillary electrophoresis (CE), CDs have been used for the separation of enantiomers by dissolving in an electrolyte solution [3,4]. Because the hydroxyl groups on the rim at positions 2, 3 and 6 of each glucopyranose unit of CD can be easily modified with various substitutes, various CD derivatives are synthesized and used to obtain both a high water solubility and different enantioselectivity. Up to now, a number of enantiomer separations have been reported using not only natural CDs but also derivatized CDs. In particular, methylated and hydroxypropylated CDs have shown high enantioselectivity to various racemates [3,4]. Several anionic CDs such as sulfobutyl ether β-CD and β-CD phosphate showed high enantioselectivity to numerous basic racemates as compared with neutral CD derivatives [3–8]. Not

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only the electrostatic interaction between anionic CDs and cationic analytes but also the large difference in the electrophoretic mobilities between free analytes and complexed analytes were effective for the enantiomer separations.

On the other hand, the use of cationic CDs has not been popular for the separations of anionic racemates. Mono-(6-β-aminoethylamino-6-deoxy)-β-CD, 6-methylamino-β-CD and 6A,6D-dimethylamino-β-CD have been reported as chiral selectors in CE [9–11]. Although several enantiomer separations were obtained using these CDs, the cationic CDs were not commercially available. Recently, some enantiomer separations were reported using a new cationic β-CD which had quaternary ammonium groups, quaternary ammonium β-CD (QA-β-CD), by HPLC [12] and CE [13]. Furthermore, because QA-β-CD is positively charged over a wide range of pH in comparison with the cationic CDs having amino groups, it is practically useful for the enantiomer separations by CE. In this paper, we employed a commercial QA-β-CD as a chiral selector for the separations of various acidic racemates. The enantiomer separations were compared with the results using neutral CD derivatives.

An amphoteric β-CD (AM-β-CD) which has both quaternary ammonium groups and carboxyl groups is also commercially available. The total charge of the CD can be varied by changing the pH of the running buffer. To our knowledge, AM-β-CD has not been used as a chiral selector in CE, and we tried the enantiomer separations of various acidic racemates with this CD.

These commercial CDs, QA-β-CD and AM-β-CD were expected to consist of a mixture having different degree of substitution. The substitution distribution must significantly influence the enantioselectivity and peak shapes. Therefore, we analyzed these commercial CDs by CE and mass spectrometry (MS) in order to identify the compositions of the CDs.

2. Experimental

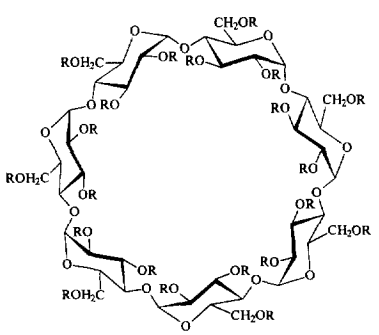
2.1. Apparatus

Unless otherwise stated, the experiments were performed with a BioFocus 3000 capillary electrophoresis system (Nippon Bio-Rad, Tokyo, Japan). Fused-silica capillary tubing of 50 μm I.D. (GL Science, Tokyo, Japan) was coated with linear polyacrylamide [14]. The capillary of 36 cm in total length (31.5 cm to the detector) was incorporated into a user assembled capillary cartridge. The instrument control and data collections were performed with an IBM PS/V computer. For some experiments such as analyses of CDs, a Hewlett-Packard 3D capillary electrophoresis system (Yokogawa Analytical Systems, Tokyo, Japan) was employed. The capillary of 33 cm in total length (24.5 cm to the detector) was used. The instrument control and data collections were performed with a Hewlett-Packard Vectra XM Series 3 (5/120) computer.

Mass spectrometric experiments were performed using a Perkin-Elmer Sciex API-300 (Perkin-Elmer Japan, Yokohama, Japan) equipped with an pneumatically-assisted electrospray (IonSpray) interface. A Macintosh computer (model 8500/120) was used for the instrument control and data collections.

2.2. Reagent

Quaternary ammonium β-CD (QA-β-CD) and amphoteric β-CD (AM-β-CD) were purchased from Supelco (Bellefonte, PA, USA). Chemical structures of the CDs are shown in Fig. 1. Each charged CD used was from a single batch. β-CD, heptakis(2,6-di-*O*-methyl)-β-CD (DM-β-CD) and heptakis(2,3,6-tri-*O*-methyl)-β-CD (TM-β-CD) were purchased from Nacalai Tesque (Kyoto, Japan). Hydroxypropyl-β-CD (HP-β-CD) whose average degree of substitution was 0.6 was purchased from Aldrich (Milwaukee, WI, USA). γ-CD was purchased from Wako (Osaka, Japan). (*cis,trans*)-Abscisic acid, adrenochrome semicarbazone sulfonate sodium salt, *p*-chlorowarfarin, chrysanthemum-monocarboxylic acid, menadione sodium bisulfite, suprofen and dansylated amino acids (Dns-amino acids) were purchased from Sigma (St. Louis, MO, USA); 3-phenylbutyric acid was from Aldrich; 2-phenoxypropionic acid, 2-phenylbutyric acid, 2-phenyllactic acid, 3-phenyllactic acid and vanilmandelic acid were from Tokyo Kasei (Tokyo, Japan); flurbiprofen, ibuprofen, ketoprofen and tropic acid were from Wako; 4-bromomandelic acid, 4-chloromandelic acid and 4-fluoromandelic acid were from Lankaster (Morecambe,



<Quaternary ammonium β-CD>

R = −CH$_2$CHCH$_2$N$^+$(CH$_3$)$_2$ Cl$^-$ (DS = 3.5)

OH

or

R = −H

<Amphoteric β-CD>

R = −CH$_2$CHCH$_2$N$^+$(CH$_3$)$_2$ Cl$^-$ (DS = 2)

OH

or

R = −CH$_2$COO$^-$Na$^+$ (DS = 2)

or

R = −H

Fig. 1. Molecular structures and characteristics of QA-β-CD and AM-β-CD used in this work. DS, average degree of substitution.

UK); mandelic acid was from Nacalai Tesque. Warfarin was donated by Tanabe Seiyaku (Osaka, Japan). All other reagents were of analytical grade or HPLC grade. Water was purified with a Milli-Q Labo system (Nihon Millipore, Yonezawa, Japan).

2.3. Procedure

For the enantiomer separations of acidic racemates, a running solution was prepared by dissolving a CD as a chiral selector in a 50 m*M* phosphate buffer. Every running solution was filtered through a 0.45 μm syringe type membrane filter prior to use. Stock solutions of racemic samples (ca. 0.5 mg/ml) were prepared in water or methanol. Sample solutions for CE analysis were prepared by tenfold dilution of the stock solution with water. The capillary was rinsed with water for 30 s and the running solution for 45 s at 690 kPa (100 p.s.i.) prior to each run. The beginning and end of each day, the capillary was washed with the capillary wash solution (Bio-Rad, Cat No. 148-5022) and water at 690 kPa (100 p.s.i.) for more than 5 min each. After sample solution was injected at 6.9 kPa (1 p.s.i.) for 2 s, a constant voltage of either 12 kV or −12 kV was applied for the separation. The capillary was thermostated at 20°C. Migrating analytes were detected at 210 nm.

For the CE analyses of CDs by an indirect detection method, the Hewlett-Packard 3D system was used. All running solutions were prepared in a 50 m*M* phosphate buffer by dissolving additives such as pyridinium chloride. Sample solutions were prepared by dissolving a CD in water (ca. 20 mg/ml). The coated capillary was rinsed with water and the running solution for 3 min at 94 kPa (940 mbar) prior to each run. The beginning and end of each day, the capillary was washed with the capillary wash solution (Bio-Rad, Cat No. 148-5022) and water at 94 kPa (940 mbar) for more than 5 min each. Sample solution was injected at 5 kPa (50 mbar) for 4 s, and 8 kV was applied. The capillary temperature was 20°C, and detection wavelength was 254 nm. For the analysis of AM-β-CD by a direct detection method, the Hewlett-Packard 3D system and a fused-silica capillary were used. A running solution was a 50 m*M* borate buffer (pH 9.3). AM-β-CD was dissolved in water (ca. 40 mg/ml). The capillary was rinsed with 1 mol/l NaOH, water and the running solution for 3 min at 94 kPa (940 mbar) respectively, prior to each run. The beginning and end of each day, the capillary was washed with 1 mol/l NaOH and water at 94 kPa (940 mbar) for

more than 5 min each. Sample solution was injected at 5 kPa (50 mbar) for 4 s, and voltage was 10 kV. The capillary temperature was 20°C, and detection wavelength was 195 nm.

For the analyses by IonSpray-MS, sample solutions were prepared by dissolving a CD in 20 m*M* ammonium acetate solution including 50% acetonitrile (ca. 0.1 mg/ml). The sample solutions were infused into the IonSpray interface directly at 2 μl/min with a Harvard Apparatus model 11 syringe pump (South Natick, MA, USA). The IonSpray voltage and orifice potential were maintained at 5 kV and 10 V, respectively. Spectra were acquired by scanning Q1 from *m/z* 300 to *m/z* 1500.

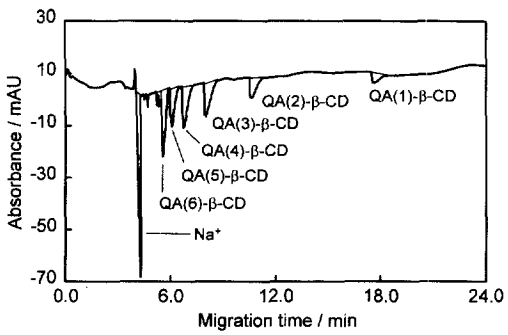


Fig. 2. Analysis of QA-β-CD by CE with indirect UV detection. Conditions: instrument, Hewlett Packard 3D System; sample solution, QA-β-CD (Lot No. LA57337) in water (20 mg/ml); capillary, 33 cm×50 μm I.D. polyacrylamide coated capillary; separation solution, 20 m*M* pyridinium chloride in 50 m*M* phosphate buffer (pH 6.0); voltage, 8 kV; detection, 254 nm.

3. Results and discussion

3.1. Analysis of CD derivatives

Recently it was noted that the substitution pattern of CD derivatives significantly influences the enantioselectivity because most commercial CD derivatives consist of mixtures having different degrees of substitution [15,16]. Moreover, it is discussed that the peak tailing for some enantiomer separations using charged CDs is due to electrodispersion resulting from a mobility mismatch between buffer ions and ionic analyte-CD complexes. These will also hold for QA-β-CD and AM-β-CD. Analyses of CD derivatives such as DM-β-CD [17,18], sulfobutyl ether β-CD [19–21] and carboxymethylated β-CD [21,22] were reported using CE, MS and nuclear magnetic resonance (NMR) spectroscopy. We performed the analyses of QA-β-CD and AM-β-CD by CE first.

QA-β-CD cannot be detected directly by UV absorbance due to weak absorbance as well as the other CDs. An indirect UV detection was employed for this analysis. From the results of a preliminary examination using several cationic UV absorbing co-ions, the pyridinium ion was the most suitable for this analysis. As shown in Fig. 2, QA-β-CD consisted of more than six components having different degree of substitution. However, the charge number of each CD could not be elucidated by the electropherogram only because we had no single CD derivative having known degree of substitution. Therefore, the substitution distribution of QA-β-CD was characterized by IonSpray-MS. As shown in Fig. 3, not only molecular ions of the CDs but also various adduct ions were observed. However, it was clarified that QA-β-CD consisted of a mixtures having from 1 to 5 substituents at least. Accordingly, the last peak at 17.7 min in Fig. 2 was assigned to QA(1)-β-CD, which has one quaternary ammonium group, and then the preceding peak at 10.7 min was QA(2)-β-CD. The other peaks were assigned to QA(3)-β-CD, QA(4)-β-CD, QA(5)-β-CD and QA(6)-β-CD in the same way. The composition of QA-β-CD was calculated from the corrected peak areas (peak area divided by the migration), and listed in Table 1. The average degree of substitution calculated was 3.7, which agreed with the degree of substitution given by the supplier (degree of substitution was 3.5). Moreover, the electropherogram showed that sodium ion was detected at 4.3 min besides the QA-β-CDs. The amount of sodium ion was calculated from the peak area of the standard solution of sodium chloride, being ca. 4% in the QA-β-CD. In fact, the current rose beyond 100 μA in our experiments when more than 15 m*M* of the CD was used. The high current should be caused not only by the charged CD but also by the impurity of sodium chloride.

AM-β-CD has both cationic and anionic substituents. When the degree of substitution is equal between cationic and anionic substituents, the CDs

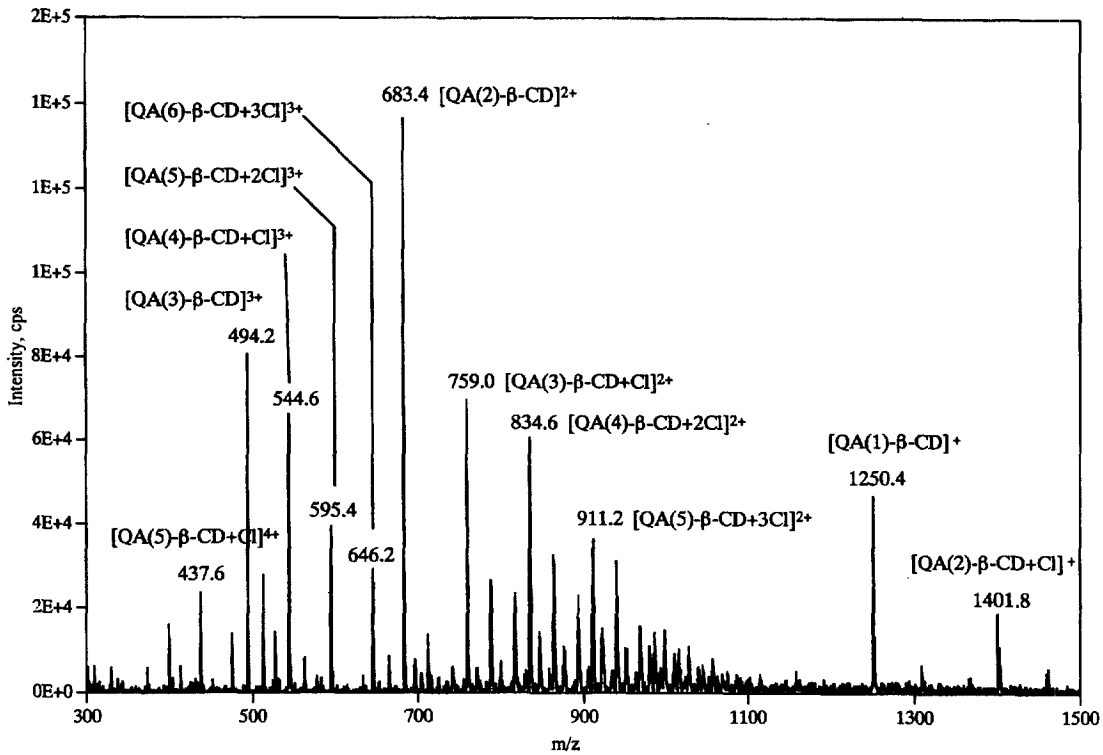


Fig. 3. Mass spectrum of QA-β-CD with an pneumatically-assisted electrospray (IonSpray) interface. Conditions and abbreviations are given in the text.

have no net charge in a buffer solution unless the pH is extremely low or high. It was not known whether the CDs were charged or not in a buffer solution. When a polyacrylamide coated capillary is used, the uncharged CDs cannot be detected because the mobility of the uncharged CDs is zero and the electroosmotic flow is almost completely suppressed. AM-β-CD was analyzed by capillary zone electrophoresis using an uncoated fused-silica capillary, although the uncharged CDs could not be separated in this method. A 50 m*M* borate buffer at pH 9.3 was used as the running solution in order to obtain the simultaneous detection of both anionic and cationic CDs. The direct detection at 195 nm was employed because AM-β-CD has several carboxyl groups. As a result, many peaks were detected as shown in Fig. 4, where the migration time of neutral analytes were 4.2 min as determined using mesityl oxide. It was shown that many anionic CDs and cationic CDs were included in AM-β-CD. Although we also tried the mass spectrometric analysis of AM-β-CD by IonSpray-MS as described for QA-β-CD, we could not identify the composition of the CD because the mass spectra were very complicated, suggesting that AM-β-CD contained many components.

From the above results, it was concluded that the commercial QA-β-CD and AM-β-CD consisted of mixtures having various degrees of substitution.

Table 1
Composition of QA-β-CD calculated by the CE analysis

CD[a]	Composition (%)[b]
QA(1)-β-CD	10.2
QA(2)-β-CD	15.8
QA(3)-β-CD	21.5
QA(4)-β-CD	20.4
QA(5)-β-CD	14.1
QA(6)-β-CD	18.0

[a] Abbreviations are given in the text.
[b] Average of three determinations.

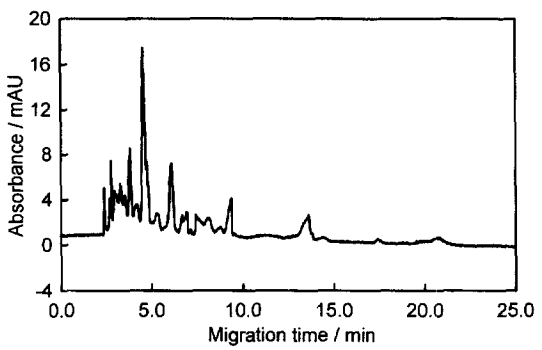


Fig. 4. Analysis of AM-β-CD by CE with direct detection. Conditions: instrument, Hewlett-Packard 3D System; sample solution, AM-β-CD (Lot No. LA46828) in water (40 mg/ml); capillary, 33 cm×50 μm I.D. fused-silica capillary; separation solution, 50 m*M* borate buffer (pH 9.3); voltage, 10 kV; detection, 195 nm.

Moreover, QA-β-CD contained a considerable amount of sodium chloride. However, we used the CDs as received in this work because the primary purpose of the research was to explore the capability of the cationic and amphoteric CDs as chiral selectors on comparison with the neutral CDs. The further purification of the CDs will be preferable for obtaining the high plate number and good reproducibility. The supply of a pure single CD derivative is strongly recommended in order to provide good reproducibility for enantiomer separations.

3.2. Separation of acidic racemates using QA-β-CD

So far, various separations of acidic racemates have been reported with neutral CD derivatives by CE [23–27]. However, relatively high CD concentrations were necessary for the baseline separations. The use of charged CDs has an advantage that enantiomer separations are possible with low CD concentrations because of strong electrostatic interaction between the charged CD and oppositely charged analytes and also the large difference in the electrophoretic mobilities between free analytes and complexed analytes. Several racemates were completely separated with QA-β-CD at the concentration below 5 m*M* as given in Table 2, and representative electropherograms are shown in Fig. 5. The optimum separation conditions were simply found by changing the CD concentration and the buffer pH. In order to enhance the resolution, the CD concentration was increased [23]. Buffer solutions of pH 4–7 were employed because pK_a values of most acidic racemates were between 3 and 6, and the analytes should be negatively charged in this method using polyacrylamide coated capillary. It should be noted that these results were obtained using the commercial multi-component CD derivative without further purification. In fact, the broad peak shapes were obtained for some enantiomers such as racemic 4-bromomandelic acid and racemic 3-phenylbutyric acid.

Table 2
Baseline separations of acidic racemates by CE using QA-β-CD

Compounds	[CD]/m*M*	Buffer pH	Migration[a] direction
(*cis,trans*)-Abscisic acid	1	6.0	–
Adrenochrome semicarbazone sulfonate sodium salt	3	4.0	–
4-Bromomandelic acid	3	4.0	–
p-Chlorowarfarin	0.3	7.0	–
Chrysanthemum monocarboxylic acid	2	4.0	+
Menadione sodium bisulfite	1	4.0	–
2-phenylbutyric acid	5	5.0	–
3-Phenylbutyric acid	0.4	5.0	–
2-Phenyllactic acid	4	5.0	–
2-Phenoxypropionic acid	4	4.0	–
Tropic acid	5	5.0	–

Conditions: capillary, 36 cm×50 μm I.D. polyacrylamide coated capillary; separation solution, a CD was dissolved in a 50 m*M* phosphate buffer; voltage, −12 kV or 12 kV.
[a] –, toward the anode (−12 kV); +, toward the cathode (12 kV).

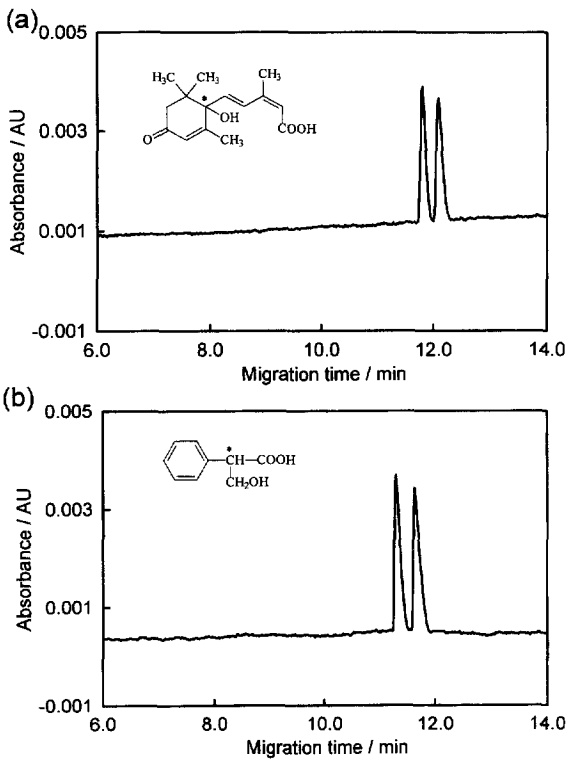


Fig. 5. Representative electropherograms for the enantiomer separations by CE using QA-β-CD. Sample: (a) (*cis,trans*)-abscisic acid, (b) tropic acid. Conditions: capillary, 36 cm×50 μm I.D. polyacrylamide coated capillary; separation solution, (a) 1 m*M* QA-β-CD in 50 m*M* phosphate buffer (pH 6.0), (b) 5 m*M* QA-β-CD in 50 m*M* phosphate buffer (pH 5.0); voltage, −12 kV; detection, 210 nm.

Their peak shapes would be improved using a single CD derivative, but we did not perform the purification of the CD in this study. The batch-to-batch reproducibility of the CD compositions is also important for the enantiomer separations when the mixed CD is used as a chiral selector. We should assess the difference in enantiomer separations using different batches of the CD. However, we have not performed yet because only one batch product has been supplied until now. These investigations will be necessary in the future to obtain good reproducibility for the enantiomer separations.

Enantioselectivity was compared among QA-β-CD and five neutral CDs. Although most racemates could be separated using one of the neutral CDs as given in Table 3, relatively high CD concentrations were necessary for the baseline separations. Among these neutral CDs, TM-β-CD was the most widely applicable for the separation of acidic racemates. However, these acidic racemates were successfully separated with below 5 m*M* QA-β-CD except for a few racemates. Three racemates, i.e., racemic (*cis, trans*)-abscisic acid, racemic 4-bromomandelic acid and racemic chrysanthemum-monocarboxylic acid, were only separated completely with QA-β-CD. On the contrary, racemic mandelic acid, racemic 3-phenyllactic acid and racemic vanilmandelic acid were not successfully separated with QA-β-CD. Furthermore, QA-β-CD was not useful for the enantiomer separations of hydrophobic compounds such as arylpropionic acids and warfarin probably because of too strong interactions with the CD.

3.3. Separation of acidic racemates using AM-β-CD

Another CD derivative having quaternary ammonium groups, AM-β-CD is also commercially available as well as QA-β-CD. Therefore, AM-β-CD was also employed for the enantiomer separations of various acidic racemates. AM-β-CD has both carboxymethyl and quaternary ammonium groups and consists of a mixture having different numbers of two substituents. As shown in Fig. 4, various cationic, neutral and anionic species were included in AM-β-CD. When a phosphate buffer of the pH from 4 to 6, where most acidic analytes were dissociated, were employed, baseline separations of five acidic racemates and ten Dns-amino acids were attained as shown in Table 4. Furthermore, simultaneous enantiomer separation of eight Dns-amino acids excluding Dns-methionine and Dns-threonine were also achieved with AM-β-CD. Typical electropherograms are shown in Fig. 6. In addition, 3-phenyllactic acid and tropic acid were partly separated (data not shown). The successful separation of racemic 4-chloromandelic acid should be noted because this racemate was not separated completely using either the five neutral CDs or QA-β-CD. It was confirmed that AM-β-CDs have different enantioselectivity compared with the other CDs, although the composition of AM-β-CD was very complicated.

Table 3
Baseline separations of acidic racemates by CE using neutral CDs

Compounds	CD[a]	[CD]/m*M*	Buffer pH
Adrenochrome semicarbazone sulfonate sodium salt	TM-β-CD	80	4.0
p-Chlorowarfarin	TM-β-CD	40	6.0
	HP-β-CD	5	6.0
Flurbiprofen	TM-β-CD	40	6.0
Ibuprofen	TM-β-CD	20	6.0
Ketoprofen	TM-β-CD	80	6.0
Mandelic acid	DM-β-CD	40	4.0
	TM-β-CD	120	4.0
	γ-CD	120	4.0
Menadione sodium bisulfite	β-CD	15	4.0
	γ-CD	20	4.0
2-Phenylbutyric acid	TM-β-CD	10	5.0
3-Phenylbutyric acid	TM-β-CD	40	5.0
	HP-β-CD	1	5.0
2-Phenyllactic acid	TM-β-CD	40	4.0
	HP-β-CD	40	4.0
	γ-CD	40	4.0
3-Phenyllactic acid	β-CD	10	4.0
2-Phenoxypropionic acid	β-CD	10	4.0
	TM-β-CD	20	4.0
Suprofen	TM-β-CD	80	6.0
Tropic acid	TM-β-CD	20	5.0
	HP-β-CD	10	5.0
Vanilmandelic acid	DM-β-CD	40	4.0
	HP-β-CD	120	4.0
Warfarin	DM-β-CD	5	6.0
	TM-β-CD	5	6.0
	HP-β-CD	10	6.0
	γ-CD	120	6.0

Conditions: voltage, −12 kV (detection, toward the anode); other conditions are as in Table 2.
[a] Abbreviations are given in the text.

Table 4
Baseline separations of acidic racemates by CE using AM-β-CD

Compounds	[CD]/m*M*	Buffer pH
4-Bromomandelic acid	10	4.0
4-Chloromandelic acid	20	4.0
Chrysanthemum monocarboxylic acid	10	4.0
3-Phenylbutyric acid	20	4.0
2-Phenoxypropionic acid	10	4.0
10 Dns-amino acids	10	6.0

Conditions: voltage, −12 kV (detection, toward the anode); other conditions are as in Table 2.

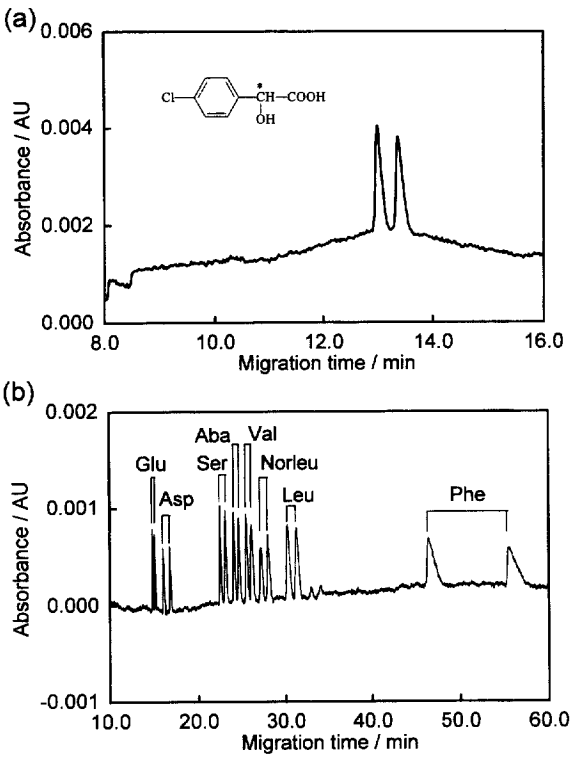


Fig. 6. Representative electropherograms for the enantiomer separations by CE using AM-β-CD. Sample: (a) 4-chloromandelic acid, (b) 8 Dns-amino acids. Conditions: capillary, 36 cm×50 μm I.D. polyacrylamide coated capillary; separation solution, (a) 20 m*M* AM-β-CD in 50 m*M* phosphate buffer (pH 4.0), (b) 10 m*M* AM-β-CD in 50 m*M* phosphate buffer (pH 6.0); voltage, −12 kV; detection, (a) 210 nm, (b) 254 nm. Abbreviations: Glu, glutaminic acid; Asp, asparaginic acid; Ser, serine; Aba, α-aminobutyric acid; Val, valine; Norleu, norleucine; Leu, leucine; Phe, phenylalanine.

4. Conclusion

The commercial cationic and amphoteric CDs, QA-β-CD and AM-β-CD, were successfully used as chiral selectors to separate the various acidic racemates by CE. As compared to neutral CD derivatives, QA-β-CD was effective for the enantiomer separations at low CD concentrations due to the strong electrostatic interaction between cationic CD and anionic analytes. On the other hand, 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. From the results of the analyses by CZE and MS, both QA-β-CD and AM-β-CD consisted of multi-components having different degrees of substitution, and QA-β-CD included 4% sodium chloride as an impurity. Single CD derivative will be required as a chiral selectors for good reproducibility in enantiomer separations.

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