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Effect of the degree of substitution of cyclodextrin derivatives on chiral separations by high-performance liquid chromatography and capillary electrophoresis

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Abstract

Optical isomers of some basic racemic drugs (oxprenolol, AMEBD, ephedrine) were separated by high-performance liquid chromatography (HPLC) and/or capillary electrophoresis (CE) using carboxymethyl-β-cyclodextrin (CMBCD) with various degree of substitution (DS). The effects of the separation conditions (pH, concentration and DS of CMBCD) were studied and compared using CE and HPLC. The degree of substitution had a significant effect on the resolution of the optical isomers and the ionic strength of the separation media, hence the use of well characterized CD derivatives is crucial. Different optimum DS values for the same test samples were obtained when HPLC or CE was used.

Keywords: Mobile phase composition; Buffer composition; Enantiomer separation; Cyclodextrins

1. Introduction

Cyclodextrins and their derivatives are useful chiral mobile phase/buffer additives in the separation of racemic drugs using HPLC or capillary electrophoretic (CE) separations [1–3]. Derivatization, for example carboxymethylation of β-cyclodextrin (βCD), results in a substantially better soluble CD compound in aqueous solutions and is thus more suitable for CE and HPLC. Derivatization can lead to different degrees and patterns of substitution. Most of the CD derivatives are mixtures of differently substituted CD rings, therefore an average degree of substitution (DS) number can be given for the characterization of the CD derivatives.

Charged CDs have been successfully used for the separation of enantiomers of ionic and non-ionic molecules [4–7]. Enantioselective separations of ionic guest molecules with ionic CDs are determined at least by two phenomena: the complex-forming interaction between the hydrophobic cavity of the CD derivative and the analyte, and electrostatic interaction between the ionic substituents on the CD ring and the ionic guest compound [7]. The degree of substitution significantly influences the complex formation, and therefore differences in the enantiomer

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separation can also be observed [8]. In the case of ionic derivatives the ionic strength of the separation medium increases with increasing DS, which also affects the separation.

In our previous study [6], carboxymethyl- and carboxyethyl-βCD proved to be effective chiral selectors for the HPLC separation of basic drugs. As a continuation, in this study the effect of the DS of carboxymethyl-βCD (CMBCD) on the selectivity and the performance of the HPLC and CE separation was investigated. The effect of the buffer components and pH on the separation was also investigated.

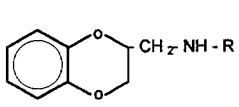
2. Experimental

2.1. Chemicals

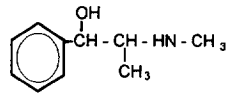
CMBCD (DS 3.5, CE purity) is a commercial product of Cyclolab (Budapest, Hungary). The other CMBCD samples (DS 2 and 6, analytical-reagent grade, and DS 8, crude) were also prepared at Cyclolab. The racemic solutes (AMEBD, ephedrine, oxprenolol) were of pharmaceutical grade. Their structures are shown in Fig. 1. Eluents were prepared from HPLC-grade solvents (Chemolab, Budapest, Hungary).

The buffers used for the CE separations were 100 m*M* phosphate (pH 2.45), 100 m*M* citrate (pH 3.50), 100 m*M* acetate (pH 5.45) and 100 m*M* Tris–borate (pH 8.20). All buffer reagents were of analytical-reagent grade and purchased from Fluka (Buchs, Switzerland) unless stated otherwise.

AMEBD
(Aminomethyl-benzodioxane derivative)



EPHEDRINE



OXPRENOLOL

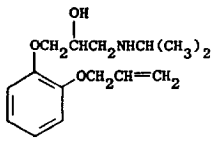


Fig. 1. Structures of the drugs used.

2.2. Instruments

HPLC

An HP 1050 HPLC system with a diode-array detector and an HPLC ChemStation were used. The detector wavelength was 220 nm, bandwidth (BW) 4, reference 350 nm, BW 80. Experiments were carried out on Nucleosil 300-5 C_4 and Nucleosil 120-7 CN cartridge columns (100 × 4 mm I.D.) (Macherey–Nagel, Düren, Germany). The mobile phase was 5 mg/ml aqueous CMBCD solution–ethanol (95:5) at a flow-rate of 1 ml/min in each run. The pH of the CMBCD solutions was adjusted with NaOH.

CE

The CE system consisted of a Spectra 100 variable-wavelength UV detector (Thermo Separation Products, Milford, MA, USA) and a reversible high-voltage power supply (Spellman, New York, USA). Fused-silica capillaries (I.D. 50 μm) were purchased from Polymicro Technologies (Phoenix, AZ, USA). The capillaries were air-cooled at room temperature (26°C) with a laboratory fan. Data analysis and acquisition were performed using the Caesar system (Analytical Devices, Alameda, CA, USA) and a PC.

2.3. Calculations

CE data analysis

Instead of the generally used resolution term, a modified resolution (R') was used to compare the resolution of poorly resolved peaks [8]. R' was calculated as follows:

$$R' = 100(H - H')/H \quad (1)$$

where H is the height of the first peak (mm) and H' is the height of the valley between the first and second peaks (mm). $R' = 100$ therefore means baseline resolution.

The separation selectivity was calculated according to well known equations [9].

3. Results and discussion

The CMBCD derivatives were characterized by their average degree of substitution and their titration curve. A representative titration curve of CMBCD (DS 3.5) is shown in Fig. 2. We found that a higher degree of substitution, where the number of acidic groups on the CD ring increased, resulted in higher pK_a values up to DS 6. At DS 8 the pK of CMBCD decreased. This phenomenon may be due to the esterification process; the carboxylic groups may esterify the alcoholic hydroxyls on the CD ring when CMBCD with a higher degree of substitution in prepared [10,11].

At pH below 4–4.5 all the CMBCD additives were in their protonated forms and at pH above 8 they were deprotonated.

3.1. HPLC

The ionic strength of CMBCD solutions increases with increasing degree of substitution. The use of neutral salts (NaCl or Na_2SO_4) for adjusting a constant ionic strength resulted in decreased resolution [5]. Therefore, in this work, the ionic strength of the mobile phase was not adjusted with neutral salts. Table 1 shows the conductivity values of the CMBCD solutions. Increasing conductivity was observed with increasing DS up to DS 6. The CMBCD sample of DS 8 showed a lower conductivity, owing to the esterification process [10,11].

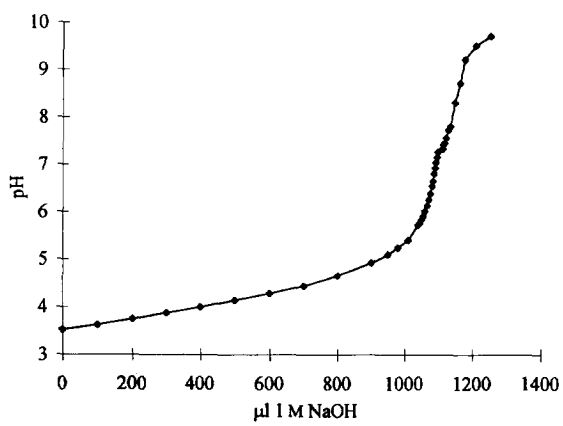


Fig. 2. Titration curve of 5 mg/ml CMBCD (DS 3.5) solution.

Table 1
Effect of degree of substitution of the CMBCD derivatives on the conductivity (κ) of the eluents used in the HPLC studies

DS[a]	pH[b]	κ (mS)
2	5.5	0.8
3.5	5.5	1.07
6	5.5	2.71
8	5.5	2.03

[a] CMBCD concentration 5 mg/ml.
[b] Adjusted with 0.1 *M* NaOH.

Effect of degree of substitution on capacity factor and resolution

The capacity factor of AMEBD (basic pK_a = 8.99, calculated with PCALC 3.1 software; Compudrug Chem., Hungary) has a minimum value at DS 3.5 over the entire pH range (4.5–6.0), as shown in Fig. 3A, suggesting the strongest interaction between AMEBD and CMBCD of DS 3.5 under the examined HPLC conditions. At a higher degree of substitution the capacity factor strongly increases.

The best resolution of AMEBD enantiomers was obtained with CMBCD of DS 3.5, at the capacity factor minimum, over the entire pH range (Fig. 3B), confirming that CMBCD with DS 3.5 has the strongest interaction with the molecule.

Effect of pH on capacity factor and resolution

The capacity factor of AMEBD has a minimum as a function of pH in the pH range 4–6.5 depending on the DS of the cyclodextrin derivative. At higher DS the minimum values are shifted towards higher pH (Fig. 4A). This observation is in accordance with the results of the titration curves, suggesting that the CMBCD derivatives have the strongest interaction with the guest molecule when the pH of the eluent is close to their pK_a values.

The resolution of enantiomers increases with increasing pH at lower DS value (Fig. 4B). At DS 6 the optimum of the separation is at pH 5.5,

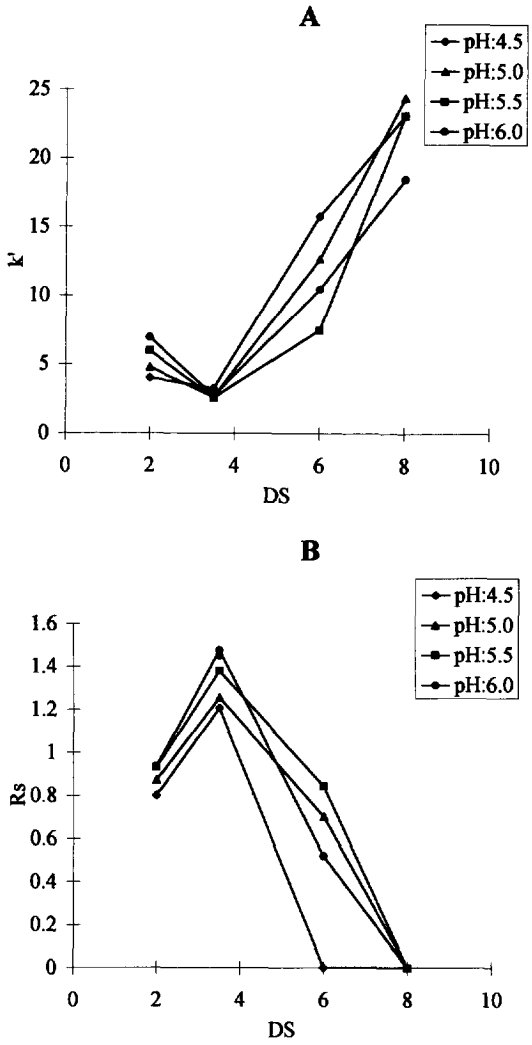


Fig. 3. Effect of degree of substitution on (A) capacity factor and (B) resolution of AMEBD enantiomers. Mobile phase, 5 mg/ml aqueous CMBCD solution–ethanol (95:5, v/v); column, Nucleosil 300-5 C_4.

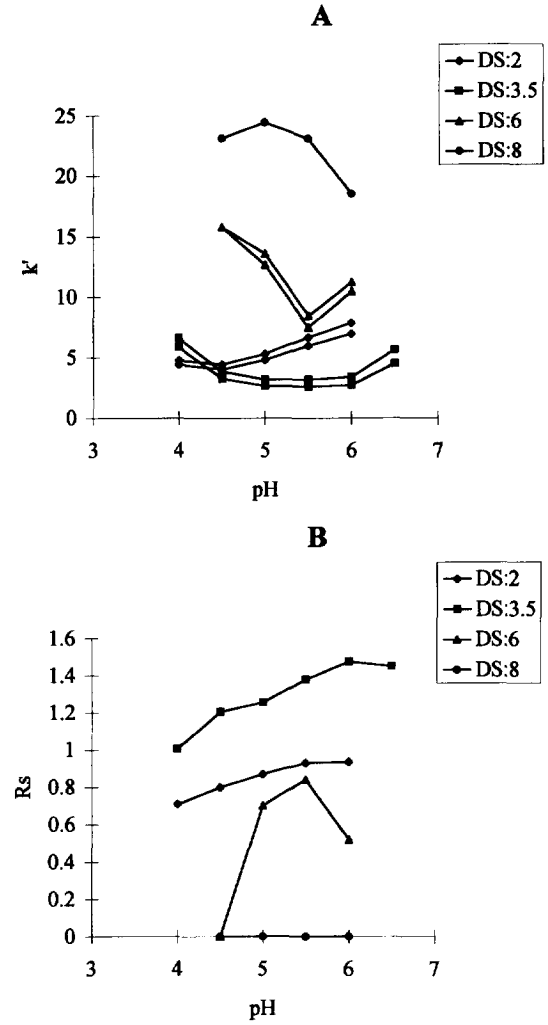


Fig. 4. Effect of pH on (A) capacity factor and (B) resolution of AMEBD enantiomers. Mobile phase, 5 mg/ml aqueous CMBCD solution–ethanol (95:5, v/v); column, Nucleosil 300-5 C_4.

whereas AMEBD (±) could not be resolved using CMBCD of DS 8 at any pH value in the observed pH range.

Effect of stationary phase on resolution

Most of the experiments were carried out on a Nucleosil 300 C_4 column, which proved to be good for this purpose in previous work [6], but the separation of AMEBD enantiomers was also examined on a Nucleosil 120 CN stationary phase. As can be seen in Fig. 5, the resolution is different using different types of HPLC stationary phase, giving another dimension for optimization of the separation. Better resolution was found on the Nucleosil 300 C_4 column at DS 2 and 3.5, whereas at higher DS, where the resolution is decreased, the CN column was more effective. Fig. 6 shows the separation of AMEBD enantiomers on a Nucleosil 300 C_4 column using CMBCD of DS 3.5 as chiral selector at pH 6.

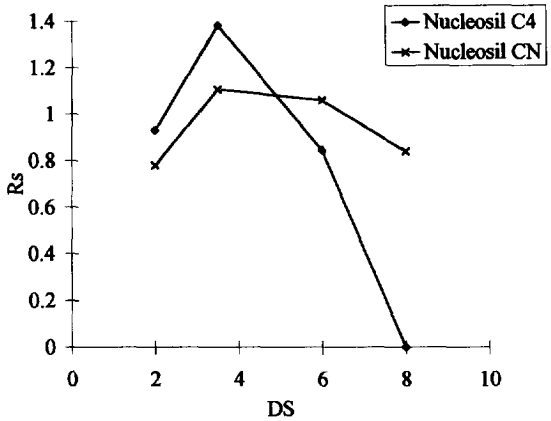


Fig. 5. Resolution of AMEBD enantiomers on different HPLC columns as a function of degree of substitution. Mobile phase, 5 mg/ml aqueous CMBCD (DS 3.5) solution–ethanol (95:5, v/v); pH = 5.5.

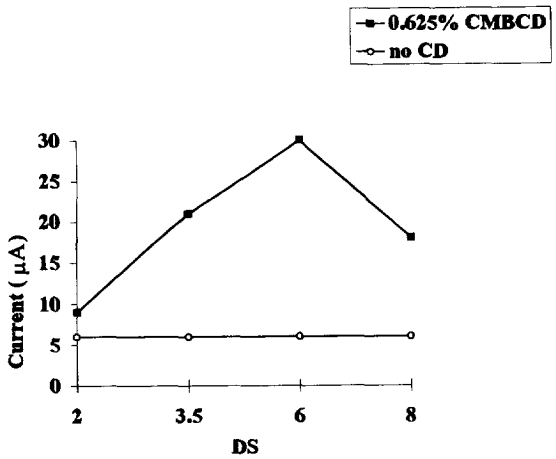


Fig. 7. Effect of degree of substitution on the current. Conditions: fused-silica capillary, $L = 35$ cm, $l = 22$ cm × 50 μm I.D.; eluent, 100 m*M* borate (pH 8.2); $E = 150$ V/cm.

3.2. Capillary electrophoresis

Addition of CMBCD derivatives to CE buffers resulted in increased buffer conductivity. Fig. 7 shows the effect of DS on the measured current when 100 m*M* borate (pH 8.2) was used with 0.625% CMBCD in the buffer. With increasing DS, a strong increase in the current is observed up to DS 6. At DS 8 the current decreases. It is worth mentioning that although the high solubility of CMBCD would permit the use of higher concentrations of the chiral selector, the buffer capacity of the applied buffer might be a limiting factor when charged CDs are used. This is

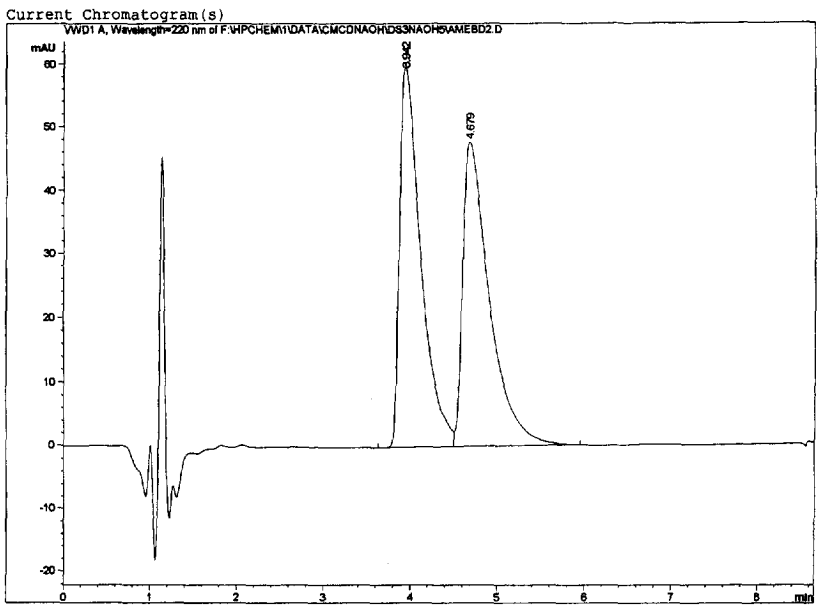


Fig. 6. Separation of AMEBD enantiomers. Mobile phase, 5 mg/ml aqueous CMBCD (DS 3.5) solution–ethanol (95:5, v/v); pH = 6; column, Nucleosil 300-5 C_4.

Table 2
Effect of CMBCD concentration on the pH of the buffer systems used in CE

CMBCD (%, w/v)	DS	pH			
		Phosphate (100 m*M*)	Citrate (100 m*M*)	Acetate (100 m*M*)	Borate (100 m*M*)
2.5	2	2.45		4.95[a]	5.07[a]
1.25		2.45		5.10[a]	5.70[a]
0.625		2.45	3.50[a]	5.43	8.02
0.313		2.45	3.50[a]	5.45	8.20
0.0		2.45	3.50[a]	5.45	8.20
2.5	3.5	2.45		4.85[a]	4.50[a]
1.25		2.45		5.05[a]	5.05[a]
0.625		2.45	3.50[a]	5.30	7.80
0.313		2.45	3.50[a]	5.45	8.20
0.0		2.45	3.50[a]	5.45	8.20
2.5	6	2.45		4.80[a]	3.50[a]
1.25		2.45		5.00[a]	4.72[a]
0.625		2.45	3.50[a]	5.12	7.50
0.313		2.45	3.50[a]	5.40	7.90
0.0		2.45	3.50[a]	5.45	8.20
2.5	8	2.45		4.85[a]	4.10[a]
1.25		2.45		5.00[a]	5.05[a]
0.625		2.45	3.50[a]	5.25	7.86
0.0		2.45	3.50[a]	5.45	8.20

[a] These buffers were not used in further experiments.

demonstrated in Table 2, where the pH values of CMBCD solutions dissolved in different buffers are shown. As can be seen, the use of borate buffer is limited to CMBCD concentrations of 0.625 mG/ml or less, since the pH of the buffer solution decreases dramatically when higher concentrations of the CMBCD are used.

Effect of degree of substitution on separation

The selectivity of the separation is influenced by the DS of the chiral selector (Fig. 8A). For AMEBD, CMBCD with DS 6 proved to give the best selectivity, but for the other two basic drugs (ephedrine and oxprenolol), the selectivity of the separation increased slightly with increasing DS values.

The resolutions of the enantiomers were calculated according to Eq. 1 in order to be able to compare separations where the peaks were not completely resolved [8]. As shown in Fig. 8B, baseline resolution was obtained for AMEBD when CMBCD of DS 6 or above was used. For the other two basic drugs, none of the chiral selectors resulted in baseline (or near baseline) resolution under the given electrophoretic conditions. The degree of substitution, however, did slightly affect the resolution.

As demonstrated in Fig. 8A and B, the optimum DS value has to be determined for each individual drug separately.

Effect of pH on separation

Table 3 summarizes the effect of pH on the electrophoretic mobility of the chiral drug and the electroosmotic flow when similar conditions [0.625% or 0% CMBCD (DS = 2), $l = 25$ cm, $E = 150$ V/cm] were used.

At pH 2.5, most of the silanol groups on the capillary wall are protonated, resulting in low electroosmotic flow (EOF) values. Also, the CMBCD derivatives are protonated, hence they will move with the EOF. On addition of the chiral selector to the buffer, no significant change

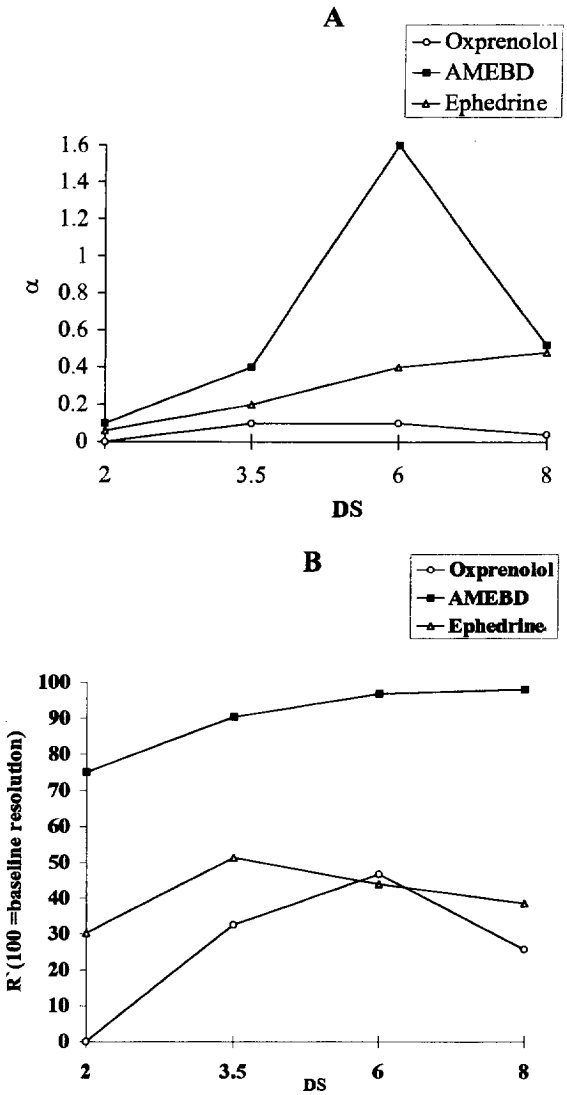


Fig. 8. Effect of degree of substitution on (A) selectivity and (B) resolution of the separation using 0.625% CMBCD derivative in the buffer. Conditions: fused-silica capillary, $L = 35$ cm, $l = 13$ cm × 50 μm I.D.; eluent, 100 mM borate (pH 8.2); $E = 200$ V/cm.

in the EOF is observed. Using CMBCD of DS 2 in the electrolyte, a small difference in the electrophoretic mobility of the (+)- and (−)-forms of the AMEBD can be observed. Although the difference in the electrophoretic mobility of the enantiomers is small, baseline resolution can be obtained owing to the long separation time.

The EOF values are significantly affected when buffers close to the pK_a value of the individual CMBCD are applied. With increasing pH the EOF increases, but on addition of CMBCD to the electrolyte one would expect a decrease in the EOF (at pH 5.5) owing to the increasing ionic strength of the buffer. In the experiments, however, a significant increase in the EOF was observed, indicating a possible interaction between the chiral selector and the capillary wall in the given pH range. At higher pH, the increasing difference in the electrophoretic mobility of the (+)- and (−)-forms of AMEBD suggests stronger complexation with the chiral selector than was observed at low pH values. The increased EOF resulted in a faster separation.

As the pH increases further, the EOF also increases, but the difference in the EOF with and without the chiral selector decreases, indicating that at higher pH the interaction between the wall and the βCD derivative is negligible (the CMBCD is also negatively charged). As a consequence of the increasing EOF, a shorter separation time is obtained.

The pH of the electrolyte strongly influences the host–guest complex formation, as shown in Table 3. In the case of AMEDB, at pH values of 5.5 or higher good resolution of the enantiomers was obtained, indicating that the ionic substituents on the CMBCD molecule enhance the complex formation and thus the separation.

The CMBCD molecules with higher degrees of substitution showed similar pH "sensitivity" with respect to both the EOF values and the resolution; however, an increase in DS resulted in better resolution even in the lower pH ranges (Fig. 9).

4. Conclusion

CMBCD added to the mobile phase/electrolyte proved to be a good selective agent for the resolution of racemic test compounds. Both the HPLC and the CE results showed that the separation efficiency is strongly influenced by the DS of CMBCD. The optimum DS value depends

Table 3
Effect of pH on the electrophoretic mobility of the (+)- and (−)-AMEBD test compounds

pH	Electrophoretic mobility ($\times 10^{-5}$ cm^2/V·s) in 100 m*M* buffer					
	0.625% CMBCD (DS 2)			No. CMBCD		
	(+)-AMEBD	(−)-AMEBD	EOF	(+)-AMEBD	(−)-AMEBD	EOF
2.45	6.5	5.9	6.6	16	16	6.7
5.45	35	33	29	34	34	14
8.2	48	47	58	49	49	57

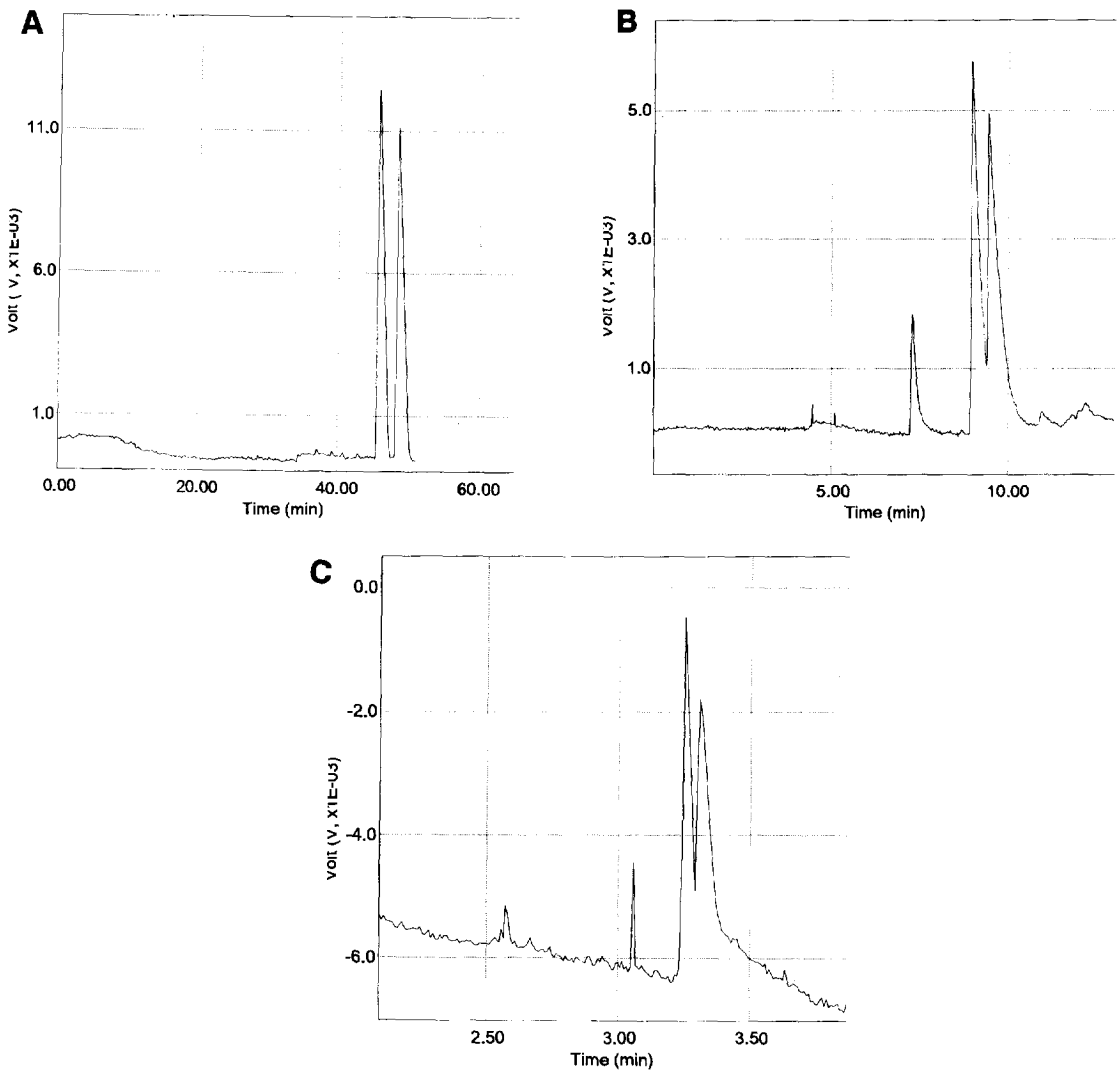


Fig. 9. Effect of pH on the separation of AMEBD enantiomers using 0.625% CMBCD (DS 2) in the buffer. Conditions: fused-silica capillary, 50 μm I.D.; (A) $L = 72$ cm, $l = 47$ cm; eluent, 100 m*M* phosphate (pH 2.45); $E = 17$ kV; (B) $L = 72$ cm, $l = 47$ cm; eluent, 100 m*M* acetate (pH 4.45); $E = 17$ kV; (C) $L = 72$ cm, $l = 25$ cm; eluent, 100 m*M* borate (pH 8.20); $E = 17$ kV.

on the molecular characteristics of the test compounds, and therefore the separation had to be optimized individually for each case.

Using a chiral selector in the eluent/buffer has several advantages: economic HPLC columns/uncoated fused-silica capillaries can be used; and two additional variables (CMBCD type and concentration) offer greater flexibility in the method development.

The ionic βCD derivatives changed the ionic strength/conductivity of the separation media. The change in the ionic strength was DS dependent, and therefore the use of well characterized βCD derivatives is strongly recommended. The buffer capacity of the applied buffers has to be taken into consideration when the operating CMBCD concentration is determined.

Comparing the two analytical techniques, different optimum DS values for the same test samples were obtained when HPLC or CE was used. Using CE, a wider range of pH can be used than in HPLC and thus sample stability issues can be addressed.

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