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Reverse migration order of sibutramine enantiomers as a function of cyclodextrin concentration in capillary electrophoresis

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ABSTRACT

The current study demonstrates the reversal of enantiomer migration order (EMO) in capillary electrophoresis (CE) based separations of sibutramines (SIB) as a function of the concentration of two types of cyclodextrin (CD), native β-CD and acetyl-β-CD. At normal working concentrations (<10 mM) of either CD, (S)-SIB migrated first. However, at CD concentrations greater than 10 mM, (R)-SIB was the first to migrate. This study describes factors involved in determining EMO for sibutramine enantiomers at low and high concentrations of CDs. The reversal of EMO could be explained in terms of the opposing effects of the stability and the limiting complex mobility of the SIB-CD complexes. The enantioseparation of SIB with methyl- and 2-hydroxypropyl-β-CD was possible based on differences in the binding constants of complexes. However, reverse EMO was not observed because of equal mobilities of SIB enantiomers complexed with methyl- and 2-hydroxypropyl-β-CD.

1. Introduction

In chiral capillary electrophoretic analyses, the reverse enantiomer migration order (EMO) is an uncommon but important phenomenon. This is particularly true when analyzing enantiomers for which only small mobility differences are observed and becomes especially important when one enantiomer is regarded as an impurity or is present at very low concentrations. Changing the migration order can prevent fronting or tailing of major component peaks from overlapping with adjacent peaks from minor components, thereby decreasing detection and quantification limits [1–3]. Several approaches have been used to switch the EMO in CE [4]. The EMO can be reversed by using chiral selectors, such as CDs [5–11], with opposing chiral recognition mechanisms or by including chiral micelles with opposite configurations [2,12]. The experimental conditions also play an important role and tactics such as the elimination or reversal of electro-osmotic flow (EOF) [13], altering the polarity of the electrodes, changing the buffer type [14] or aqueous to non-aqueous buffer [15], changing the CE mode from CD-CZE to CD-MEKC [1], the addition of organic solvents [16,17], have been successful in reversing EMO. Varying the pH of the solution [13,18–27] is also effective when the overall charge of analytes, selectors, or complexes thereof, depend on solution pH. EMO inversion can also be achieved by varying the chiral selector concentration [14,27–35]. However, this last phenomenon is relatively rare. To our knowledge, EMO reversal as a function of CD concentration has been reported for 2-hydroxypropyl(HP)-β-CD, applied in enantiomeric separations of azole compounds [30,32] and dansylated amino acids [27–29], heptakis(2,3-dimethyl-6-sulfato)-β-CD, applied in CE separations of phenyl compounds [31], and γ-CD, applied in separations of phenothiazines, binaphthol, and their derivatives [14,32,33]. The most recent example of EMO reversal used dimethyl- and trimethyl-β-CD in the separation of 6,6′-dibromo-1,1′-binaphthyl-2,2′-diol [34,35].

As an anti-obesity drug, sibutramine (SIB), *N*-(1-(1-(4-chlorophenyl)-cyclobutyl)-3-methylbutyl)-*N*,*N*-dimethylamine (Fig. 1), decreases food intake and increases energy expenditure [36]. SIB is a weak basic compound containing a chiral carbon and two possible enantiomers exist. Both pharmacodynamic and pharmacokinetic data have revealed enantioselective behavior of SIB and its metabolites [37–40]. Although SIB has been marketed as a racemic mixture, an optically pure drug may be available in the near future. EMO reversal was observed during the devel-

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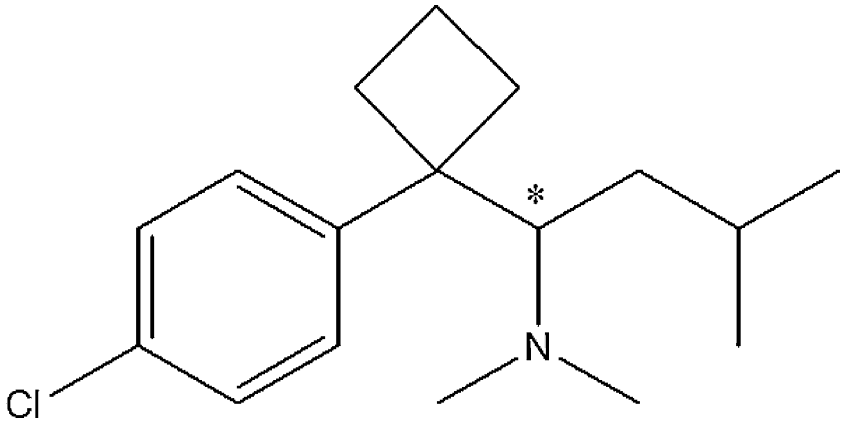

Fig. 1. Chemical structure of sibutramine. The chiral carbon is marked by the asterisk.

opment of an analytical method for the CE separation of SIB enantiomers.

The overlapping of migration for SIB enantiomers was observed in the presence of methyl(M)-β-CD and HP-β-CD, while EMO reversal was observed using native β-CD and Acetyl(Ac)-β-CD at increased CD concentration. In the current study, binding constants and the complex mobilities of SIB-CD complexes were determined to explain why β-CD and Ac-β-CD resulted in reversed migration order as a function of CD concentration.

2. Experimental

2.1. Chemicals and reagents

Racemic SIB chloride and enriched (R)-SIB were provided by Taegu Catholic University. All reagents used in buffer preparations were of analytical grade. Citric acid, dibasic sodium phosphate, and phosphoric acid were purchased from Sigma–Aldrich (MO, USA). HP-β-CD (DS 0.58–0.73 per glucose unit), M-β-CD (DS 1.7–1.9), Ac-β-CD (DS 0.85–01.15) and β-CD were obtained from Wacker-Chemie GmbH (Burghausen, Germany). Water was purified through an ultra filtration system (Sinhan, Korea) and nylon membrane filters (0.2 μm) were obtained from Whatman (Maidstone, England).

2.2. Instrumentation

CE analyses were performed on an HP^{3D}CE system (Hewlett Packard, Germany) equipped with a diode array detector. Instrument control and data acquisition were performed using the HP^{3D}CE ChemStation software. Uncoated fused silica capillary (BGB Analytic, Goettingen, Germany) with a total length of 54 cm (effective length 45 cm) and an inner diameter of 50 μm was used as the separation capillary. The pH was adjusted using an ATI 370 pH meter (Orion, MA, USA).

2.3. CE conditions

The separation capillary temperature was maintained at 25 °C with an applied voltage of 25 kV. Compound migration was detected by monitoring absorbance at 223 nm. Samples were injected at the anodic end of the capillary using a pressure of 50 mbar for 5 s. New capillaries were rinsed sequentially with 1.0 M sodium hydroxide for 30 min, deionized water for 5 min, and with running buffer for 5 min prior to first use. Between runs, capillaries were treated with 0.1 M sodium hydroxide for 3 min, water for 3 min, and running buffer for 5 min. The background electrolyte (BGE) consisted of 10 mM citrate and 20 mM phosphate (pH 4.3, adjusted with 95% phosphoric acid). Stock solutions of racemic SIB (1.0 mg/mL in BGE) and (R)-SIB (1.0 mg/mL in BGE) were stored at −4 °C. Racemic SIB (0.1 mg/mL) spiked with (R)-SIB was injected and the migration order was confirmed by comparing peak sizes. All solutions were filtered through 0.2-μm membrane filters prior to injection into the CE instrument.

2.4. Stiochiometry of CD-SIB

Sibutramine and CD were dissolved in buffer at various molar ratios (1:9–9:1). The solution was equilibrated for 12 h at room temperature and UV absorbance was measured at 223 nm. The stiochiometry was determined by ΔA[SIB]/([CD] + [SIB]) plot against to [SIB]/([CD] + [SIB]).

2.5. Data treatment and calculations

Linear plots of experimental data were generated in Microsoft Excel XP; non-linear plot fits were generated in Microcal Origin (ver. 7.5). All data represent the average value of two or three repeated injections and all results were confirmed by two independent experiments. Effective mobilities were obtained from the experimental migration time of each analyte relative to that of an EOF marker (mesityl oxide), according to Eq. (1):

$$\mu_{\text{eff}} = \left(\frac{1}{t_1} - \frac{1}{t_0}\right)\frac{Ll}{V} \quad (1)$$

where t_1 and t_0 are the migration times of the enantiomer and the EOF, respectively. L and l are the total and effective capillary lengths, respectively. V is the run voltage.

3. Results and discussion

3.1. Binding constants of SIB with CD

The formation of transient diastereoisomeric complexes between enantiomers and the chiral selector results in differential mobilities for different enantiomers and allows separation by CE. The ability of a given analyte to form a complex with CD can be quantified by calculating binding constants (K). Several mathematical approaches have been used to determine binding constants, including linear model in double reciprocal (Eq. (2)), y-reciprocal (Eq. (3)), and x-reciprocal methods (Eq. (4)) [41]:

$$\frac{1}{\mu_{\text{eff}} - \mu_{\text{f}}} = \frac{1}{\mu_{\text{c}} - \mu_{\text{f}}} \cdot \frac{1}{K[C]} + \frac{1}{\mu_{\text{c}} - \mu_{\text{f}}} \quad (2)$$

$$\frac{[C]}{\mu_{\text{eff}} - \mu_{\text{f}}} = \frac{[C]}{\mu_{\text{c}} - \mu_{\text{f}}} + \frac{1}{\mu_{\text{c}} - \mu_{\text{f}}} \cdot \frac{1}{K} \quad (3)$$

$$\frac{\mu_{\text{eff}} - \mu_{\text{f}}}{[C]} = -K(\mu_{\text{eff}} - \mu_{\text{f}}) + K(\mu_{\text{c}} - \mu_{\text{f}}) \quad (4)$$

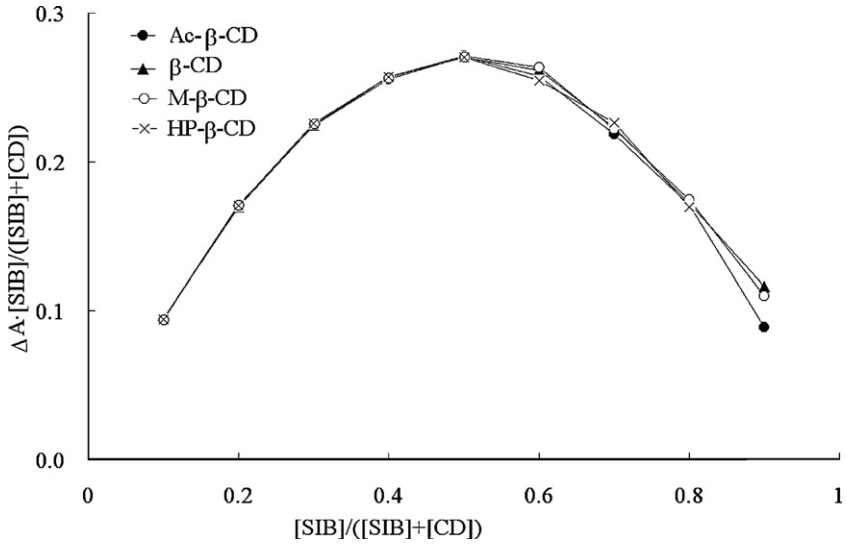

Fig. 2. Continuous variation plot for sibutramine and various types of CD. Sibutramine and CD were dissolved in buffer and UV absorbance was measured at 223 nm.

μ_{eff} is the effective mobility of the (S)- or (R)-enantiomer at a given CD concentration [*C*], μ_f is the effective mobility of free SIB, μ_c is the effective mobility of the SIB enantiomer complexed with CD, and *K* is the binding constant of the complex.

The continuous variation plot of racemic SIB and used CD showed the maximal absorbance difference at 0.5 of [SIB]/([SIB] + [CD]), indicating that the complexes have 1:1 stoichiometry in the case of native, Ac-, M- and HP-β-CD (Fig. 2). Binding constants calculated according to linear methods are given in Table 1. High binding constants are indicative of stable SIB-CD complexes. Binding constants calculated by different methods were very similar except Ac-β-CD as chiral selector. However, for all SIB-CD complexes, the binding constants of those formed with (R)-SIB were higher than those formed with (S)-SIB. The observed difference in mobility between the two enantiomers is caused not only by the different binding constants, but also by the differences in complex mobility for the (R)- or (S)-diastereoisomeric complexes [42,43]. In most cases, however, these differences in complex mobility for the (R)- or (S)-diastereoisomeric complexes were negligible [42]. The complex mobility for each SIB-CD complex was calculated by a nonlinear fit of the data [28,35] and the results are listed in Table 2. (R)- and (S)- diastereoisomeric complexes, when HP-β-CD and M-β-CD were used as chiral selector, exhibited similar complex mobilities, indicating that enantioseparation was based primarily on differences in complex stability. Conversely, enantioseparation of SIB complexes with Ac-β-CD and β-CD was due to differences in both complex mobility and stability.

Table 1
Stability constants of CD-SIB complexes obtained by different calculation methods.

CD type	Complexed enantiomer	Binding constants (M^{-1}) calculated by		
		Double-re[a]	*y*-re[b]	*x*-re[c]
HP-β-CD	S	119 ± 27	130 ± 17	128 ± 16
	R	150 ± 25	153 ± 18	156 ± 12
M-β-CD	S	207 ± 51	193 ± 56	202 ± 45
	R	247 ± 57	231 ± 66	240 ± 55
β-CD	S	204 ± 1	201 ± 4	199 ± 4
	R	232 ± 25	231 ± 30	228 ± 4
Ac-β-CD	S	731 ± 40	591 ± 19	765 ± 78
	R	1531 ± 60	712 ± 22	1005 ± 125

[a] Binding constants calculated by double reciprocal method with intercept/slope.
[b] Binding constants calculated by *y*-reciprocal method with slope/intercept.
[c] Binding constants calculated by *x*-reciprocal method with slope.

The magnitude of the binding constant reflects the ability of CD to bind SIB and differences observed between enantiomers indicate differences in binding selectivity [44].

3.2. Reverse migration order as a function of CD concentration

The migration order in CE is determined by the relative apparent mobility of the analytes. The binding constant (*K*) and the limiting complex mobility (μ_c) of each enantiomer were calculated based on the effective mobility. Migration order was reversed when these two factors (*K* and μ_c) exhibited opposite effects on the two enantiomers [35,44]. Tables 1 and 2 show that in the presence of β-CD or Ac-β-CD, the binding constants of complexes formed with (R)-SIB were higher than those of (S)-SIB, and the complex mobility of the (R)-SIB-CD was higher than that of the (S)-SIB-CD. In the experimental conditions used here, SIB carries a positive charge while β-CD and Ac-β-CD are neutral. The effective mobility of the enantiomer therefore decreases with complex formation. At low CD concentrations (<10 mM), the migration order is determined primarily by the relative stability of the complexes because most of the enantiomers in solution are unbound. Thus, (S)-SIB migrates faster than (R)-SIB. At higher β-CD and Ac-β-CD concentrations, most of the enantiomers exist as CD complexes and the migration order is determined by the relative mobility of the complexes. Thus, (R)-SIB-CD migrates faster than (S)-SIB-CD. As a result, a reversal of migration order was observed (Fig. 3). With 10 mM CD, a single peak corresponding to both enantiomers was observed in electropherograms of both the β-CD and Ac-β-CD complexes, indicating that the effects of binding constant and complex mobility on the separation of SIB enantiomers cancelled each other out at this concentration. At higher concentrations of M-β-CD or HP-β-CD, most of SIB enantiomers exist as CD complexes and complex mobilities play more important role than binding constant. In this case,

Table 2
The complex mobility of SIB-CD.

CD type	Mobility[a] of CD complexed with	
	(S)-SIB	(R)-SIB
HP-β-CD	4.02 ± 0.87	4.05 ± 0.92
M-β-CD	3.47 ± 0.20	3.51 ± 0.24
β-CD	3.11 ± 0.18	3.90 ± 0.28
Ac-β-CD	3.00 ± 0.02	3.45 ± 0.07

[a] In 10^{-3} cm^2 V^{-1} s^{-1}, $n = 3$.

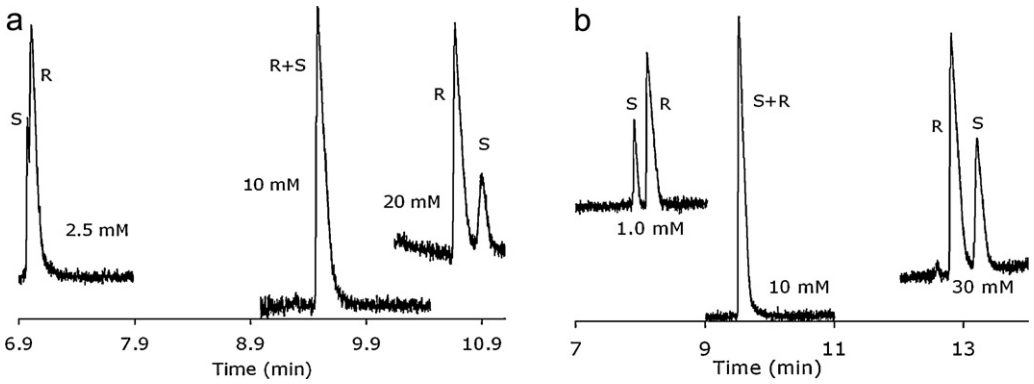

Fig. 3. An electropherogram demonstrates the reversal of sibutramine enantiomer migration order using (a) native β-CD and (b) Ac-β-CD as chiral selectors. CE conditions: fused-silica capillary (uncoated, 50 μm i.d. × 54 cm, effective length 45 cm); injection, 50 mbar, 5 s; applied voltage, 25 kV; temperature, 25 °C; buffer, 20 mM phosphate/10 mM citrate containing chiral selector, pH 4.3; detection, 223 nm. The highest concentration of β-CD and Ac-β-CD used in this experiment is 20 and 100 mM, respectively.

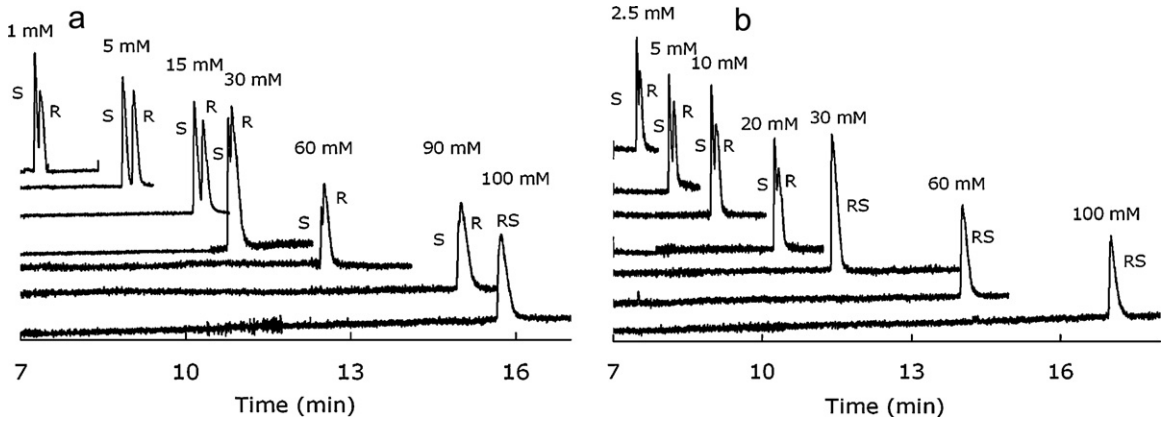

Fig. 4. An electropherogram shows overlapping peaks of sibutramine enantiomers using (a) M-β-CD and (b) HP-β-CD as chiral selectors. CE conditions same as Fig. 3.

the complex mobilities of two enantiomers were similar (Table 2), hence, the two enantiomer peaks overlapped (Fig. 4). Enantioselectivity caused by differences in stability at lower concentration disappeared when most of the enantiomers were complexed with M-β-CD or HP-β-CD at higher concentrations, resulting in overlapping peaks.

Enantioseparation in CE can be explained in terms of enantioselectivity and differences in the mobility of enantiomers, *i.e.*, $\Delta\mu \neq 0$ in Eq. (5) [4,42].

$$\Delta\mu = \frac{K_R K_S [C]^2(\mu_{SC} - \mu_{RC}) + \mu_f[C](K_R - K_S) + [C](\mu_{SC}K_S - \mu_{RC}K_R)}{(1 + K_R[C])(1 + K_S[C])} \quad (5)$$

This equation indicates that complex mobility and stability constant play a very important role in the separation of an analyte by CD-medicated CE. Moreover, this equation implies that complex mobilities might be more important for the enantioseparation at higher CD concentration, but binding constant at lower CD concentration. The enantioseparation of SIB with Ac-β-CD, β-CD, M-β-CD, and HP-β-CD as chiral selectors could be well explained with this equation.

3.3. Optimum CD concentrations and enantioselectivity

Optimal concentrations and the related information shown in Table 3 were obtained by analyzing several CE runs with solutions containing various CD concentrations or estimated from the binding constants of both enantiomer complexes. The optimal experimental concentration for both M-β-CD and HP-β-CD was 5.0 mM, which is in agreement with the optimal concentrations estimated from binding constants, 4.6 and 5.6 mM, respectively. These CDs exhibited the same mobility when complexed with either (R)- or (S)-SIB; enantioseparation was therefore a function of the different binding constants. However, β-CD complexes exhibited large discrepancies between the observed and calculated optimal CD concentrations. This indicates that enantioseparation was not determined solely by differences in the binding constants of the two complexes. The discrepancy, observed in peak resolution and binding selectivities (α_b) at low concentrations of β-CD, were likely caused by antagonistic effects in the complex mobility of the two enantiomers. The discrepancies between the observed and calculated optimal concentrations, and between the resolution and binding selectivity for Ac-β-CD, were very small. The ratios of

Table 3
Optimal CD concentrations, enantioselectivity, and peak resolution for sibutramine enantiomers analyzed with various CDs.

CD type	Conc. range (mM)	$[C_{op}]_{exp}$ [a] (mM)	$[C_{op}]_{cal}$ [b] (mM)	Rs[c]	α[d]	Config.[e]
β-CD	0–10	2.5	4.6	0.32	1.3	S
	10–20	20*	–	1.1	1.2[+]	R
Ac-β-CD	0–10	1.0	0.9	1.58	1.68	S
	10–100	100*	–	2.86	1.13[+]	R
M-β-CD	0–100	5.0	4.6	1.3	1.2	S
HP-β-CD	0–100	5.0	5.6	0.9	1.1	S

[a] Optimal CD concentration observed, except *, where the maximum CD concentration was used.
[b] Optimal CD concentration calculated according to $[C_{op}]_{cal} = (K_R \cdot K_S)^{-1/2}$ [45].
[c] Resolution at $[C_{op}]_{exp}$.
[d] Selectivity at $\alpha_b = K_R/K_S$, except [+], where $\alpha_c = \mu_R/\mu_S$ [43,46].
[e] Configuration of the first migrated enantiomer.

complex mobility selectivity (α_c) to binding selectivity (α_b) were 0.9 and 0.7 for β-CD and Ac-β-CD, respectively, indicating that the magnitude of the antagonistic effect with β-CD was greater than that of Ac-β-CD.

Theoretically, the complex mobility of β-CD or Ac-β-CD becomes important for the enantioseparation at concentrations higher than 10 mM. Thus, the peak resolution should increase with CD concentration until all of the available enantiomers exist as CD complexes. The maximum experimental resolution of Ac-β-CD, observed at 100 mM, was 2.86, which is significantly higher than the selectivity of 1.13 predicted based solely on differences in complex mobility. However, the low solubility of β-CD did not permit the maximum resolution (1.1) to exceed the complex mobility selectivity (1.2). Additionally, increasing CD concentration affects not only complexation dynamics, but also the viscosity of buffer. This indicates that one would observe a continual increase in resolution with increases in CD concentration, limited only by the solubility of those CDs that exhibit the required antagonistic effects on complex stability and mobility, such as β-CD and Ac-β-CD.

4. Conclusions

This paper reports a new example for EMO reversal in CE separations of SIB enantiomers using certain CDs as chiral selectors. Both native β-CD and acetyl-β-CD induced a reversal of EMO. However, at higher concentrations, M-β-CD and HP-β-CD resulted in significant peak overlap. The influences of CDs on enantioselectivity and migration order were evaluated in terms of binding constants and complex mobility. These two factors, and the relative magnitude of their effects, decide the migration order and the degree of enantioselectivity. Binding constants were more important with M-β-CD and HP-β-CD. EMO reversal with native β-CD and acetyl-β-CD was caused by opposing effects on the different enantiomers; the degree of this effect was greater with β-CD than with Ac-β-CD. These results provide valuable information for the development of an enantioselective CE method for SIB separations.

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