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Enantiomeric Separations of Terbutaline by CE with a Sulfated β-Cyclodextrin Chiral Selector: A Quantitative Binding Study

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Sulfated β-cyclodextrin, a negatively charged chiral selector, was used for the enantiomeric separation of racemic terbutaline by capillary electrophoresis. Chiral separation was found to increase with decreasing cyclodextrin concentration. Host−guest complex binding constants for this system were determined by UV difference spectroscopy ($K_{av} = 1490\ M^{-1}$) and by CE under conditions of minimal EOF and reversed polarity ($K_1 = 1730\ M^{-1}$, $K_2 = 1590\ M^{-1}$, $\alpha = 1.09$). The effect of organic modifiers, methanol, and acetonitrile was also studied over a wide range of modifier concentrations. Binding constants decreased while selectivity increased with increasing organic modifier concentration (10% MeOH: $K_1 = 1590\ M^{-1}$, $K_2 = 1130\ M^{-1}$, $\alpha = 1.41$. 10% ACN: $K_1 = 1320\ M^{-1}$, $K_2 = 870\ M^{-1}$, $\alpha = 1.52$). Experimental results are discussed in the context of existing separation models.

The separation of enantiomers has received a great deal of recent attention, specifically in the pharmaceutical industry. Because chiral drugs are commonly administered as racemic mixtures, and the two enantiomers often exhibit different pharmacological effects, it is of significant interest to develop analytical methods for chiral analysis. Still a relatively young technique, capillary electrophoresis (CE) has quickly established itself in the area of chiral separations.[1] The separation of enantiomers by CE is typically accomplished by exploiting stereospecific interactions between chiral analytes and a chiral selector that has been added to the background electrolyte. In brief, one enantiomer complexes more favorably with the chiral selector, establishing a dynamic equilibrium between the free analyte and the diastereomeric complex.

The separation of two enantiomers can take place if there is a difference in the binding or stability constants (K) between each enantiomer and the chiral selector as well as a difference between the mobility of the free analyte and the complex. Theoretical models based on binding equilibria have been shown to give an excellent account of the effects that changing the selector concentration has on enantioresolution.[2−4] Knowledge of binding constants can provide a better understanding of separation mechanisms and, consequently, can aid in predicting migration behavior and designing optimization strategies.[5] Spectroscopic methods have been used for the determination of host−guest complex binding constants.[6] More recently, however, CE has been adopted for binding constant determinations based on the migration equation that emerged from the work of Alberty and King in 1951.[7]

Cyclodextrins (CDs) are among the most prevalent selectors used in chiral CE. Currently, a wide range of native CDs (α, β, γ) as well as charged and neutral derivatives are commercially available and have been employed as chiral selectors.[8−12] Although neutral chiral selectors are the most frequently used, several reports have demonstrated the advantages of negatively charged selectors such as sulfobutylated CD,[13] sulfated CD,[14−16] dextran sulfate,[17] and heparin.[18] Tait et al. explained that the use of a negatively charged chiral selector effectively increases the "separation window", as the maximum opportunity for separation may exist when the analyte and chiral selector migrate in opposite directions.[13]

Several applications of anionic CDs to chiral separations have come with the applied voltage polarity reversed, under conditions of suppressed electroosmotic flow (EOF).[14,19] In this format, analytes are injected at the cathodic end of the capillary and detected at the anodic end, ensuring that the separated enantiomers will reach the detector only when complexed with the CD. A recent review by Janini and Issaq concerning the suppression of EOF pointed out that the use of coated capillaries can effectively diminish EOF and may be superior to the use of uncoated capillaries at low pH which suffer from instability and irreproducibility.[20]

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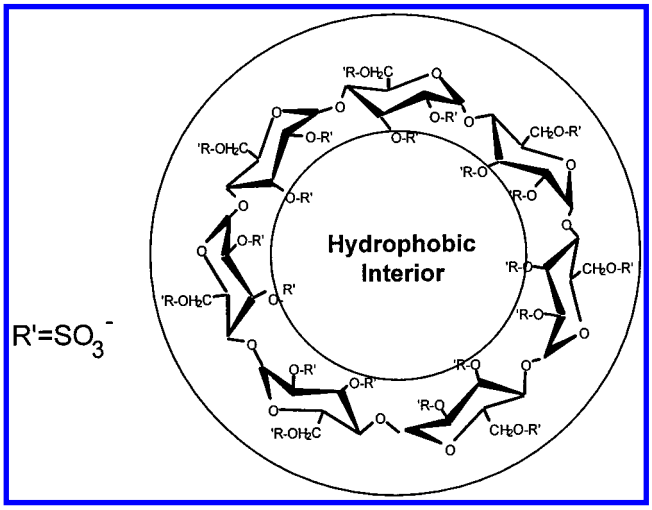

Figure 1. Sulfated β-cyclodextrin.

In the present study, chiral separations of the antiasthmatic drug terbutaline were performed using sulfated β-cyclodextrin (Figure 1) as the chiral selector in CE. Binding constants determined spectroscopically through the use of Benesi−Hildebrand plots[6] are presented and compared to those determined by capillary electrophoresis. The main objective of this work was to explore the effects that the organic modifiers, methanol (MeOH) and acetonitrile (ACN), have on the binding constants and enantioselectivity. Unlike the results of several related studies in which normal polarity was used,[4,21−22] the addition of an organic modifier to the run buffer was shown to enhance chiral selectivity for terbutaline.

EXPERIMENTAL SECTION

Materials. Sulfated cyclodextrin with ~13 sulfates/cyclodextrin (13 ds)[23] was obtained from Aldrich Chemical Co. (Milwaukee, WI). Racemic terbutaline was obtained from Sigma Chemical Co. (St. Louis, MO). The NaH_2PO_4, H_3PO_4, Na standards, and HPLC grade methanol and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA). CE buffers were prepared with distilled, deionized water, and reagent grade NaH_2PO_4 and adjusted to pH 3 with 85% H_3PO_4. For the experiments in which organic modifiers were used, the sulfated cyclodextrin was added to the hydroorganic mixture (w/w).

Apparatus and Methods. All CE experiments were performed using a Bio-Rad BioFocus 3000 automated capillary electrophoresis system (Bio-Rad Laboratories, Inc., Hercules,CA) interfaced with a Pentium personal computer (Gateway). Fused-silica capillaries with a polyacrylamide-coated inner surface (25-μm i.d., 24-cm total length, 19.6 cm to detector) were also obtained from Bio-Rad. Prior to each experiment, the run buffers were passed through a 0.2-μm filter and degassed. According to column care guidelines provided by the vendor, capillaries were flushed with water for 60 s and run buffer for 90 s before terbutaline was injected hydrodynamically (3 psi s). The applied voltage for all CE experiments was 10 kV. UV detection (λ = 211 nm) of 1 mM terbutaline was accomplished at the anodic end of the capillary. All CE experiments were conducted using 0−40 mM sulfated cyclodextrin and thermostated at 20 °C. Using the same cyclodextrin concentration range, buffer viscosity measurements were made using a Cannon-Ubbelohde No. 150 viscometer (Cannon Inst., State College, PA) also thermostated at 20 °C.

UV difference experiments were performed using a Hewlett-Packard 8452A diode array spectrophotometer and a 1-cm quartz cell. Solutions prepared for analysis were 25.0 μM terbutaline in pH 3 phosphate buffer. The range of sulfated-β-cyclodextrin concentrations was from 0.0 to 0.8 mM. Identical conditions were used in the presence of 10% (w/w) methanol.

Spectroscopic sodium measurements were made using a Plasma-Spec inductively coupled plasma atomic emission spectrometer with a photomultiplier detector (Leeman Labs Inc., Lowell, MA).

The sodium electrochemical measurements were made using an Orion model 86-11 Ross sodium ion-selective electrode (Beverly, MA). To each sodium standard and sample solution, equal volumes of 4 M NH_4Cl/NH_4OH were added to maintain constant ionic strength.

THEORY

The interaction between sulfated cyclodextrin (SCD) and the enantiomers of a chiral analyte is an equilibrium reaction which, assuming 1:1 complexation, may be represented as

$$\mathrm{A} + \mathrm{SCD} \overset{K}{\leftrightarrow} \mathrm{A{-}SCD} \qquad (1)$$

where A is the analyte, SCD is the sulfated CD, and A-SCD is the complex with a binding constant of K. In most techniques used to determine binding constants for enantiomers, the general strategy is to measure some system response (absorbance, mobility, retention time, etc.) to varying chiral selector concentration at constant analyte concentration. That response may then be related to the relative concentrations of free and bound analyte and subsequently to the selector−analyte binding constant.

In UV difference spectroscopy, the absorbance spectrum of an unbound analyte is measured and subtracted from its spectra at several CD concentrations. K is then obtained from a Benesi-Hildebrand or double-reciprocal plot based on eq 2.[6]

$$(\Delta A_{\mathrm{obs}})^{-1} = (K\Delta A_{\mathrm{c}})^{-1}[\mathrm{B}]^{-1} + (\Delta A_{\mathrm{c}})^{-1} \qquad (2)$$

where ΔA_{obs} is the observed absorbance difference, at some particular λ, between the analyte−CD complex and the free analyte at different CD concentrations; ΔA_{c} is the absorbance difference at λ between the analyte−CD complex and the free analyte, [B] is the molar concentration of the cyclodextrin, and K is the binding constant.

In the case of CE, binding constants are determined using the relationship between the CD concentration and the electrophoretic mobility of the analyte. A variety of different equations and graphical methods have been employed to determine binding constants, depending on the format of the CE experiment (MEKC, EKC, reversed-flow EKC, enantiospecific complexation). How-

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ever, according to a recent review by Rundlett and Armstrong,[24] the migration equations for the various modes of CE are analogous and originate from the 1951 publication of Alberty and King.[7]

The relationship used in this work, eq 3, was originally derived by Kuhn et al.[25] to calculate binding constants for lectin−sugar systems and was later adapted by Tanaka et al.[26] for enantiose-

$$\frac{1}{\mu_{Cor} - \mu_f} = \frac{1}{(\mu_{CD} - \mu_f)K}\frac{1}{[CD]} + \frac{1}{\mu_{CD} - \mu_f} \qquad (3)$$

lective complexation using anionic cyclodextrins where μ_{Cor} is the corrected solute mobility at a given cyclodextrin concentration [CD], μ_{CD} is the electrophoretic mobility of the complexed solute, μ_f is the mobility of the free analyte in the opposite direction of the complexed analyte and is determined in the conventional polarity mode in the absence of the chiral selector, and K is determined by plotting $(\mu - \mu_f)^{-1}$ vs $[CD]^{-1}$ and is equal to (intercept/slope). This eliminates the need to measure μ_{CD}.

RESULTS AND DISCUSSION

Mobility Corrections. Many of the methods used to determine binding constants by CE require correction or normalization procedures to negate any changes in mobility caused by changes in bulk buffer properties (e.g., viscosity).[24] When neutral additives are used, mobilities are typically corrected by multiplying each value by the current ratio in the absence and presence of the additive.[2] However, in the case of polyvalent additives, measurements of current do not provide an accurate assessment of the additive's contribution to the viscosity because the additive also contributes to the conductivity of the background electrolyte (BGE). However, it is important to monitor current during electrophoresis because charged additives may generate large currents, possibly leading to Joule heating. This was not a source of concern in this work as currents ranged from 10 μA in the absence of SCD to 30 μA at the highest SCD concentration. Mobilities were corrected for viscosity changes by multiplying each mobility value by the ratio of the measured viscosity at zero SCD concentration to the viscosity at the concentration of interest. Measured viscosities ranged from 1.13 mm^2/s for 1 mM SCD to 1.37 mm^2/s for 40 mM SCD.

In this work, there was no measurable EOF in the absence of SCD with a pristine column. However, charged additives such as sulfated cyclodextrins may also adsorb onto the capillary's inner surface, thereby changing the electric double layer. Although the use of coated capillaries and low-pH buffers is thought to minimize this effect, it cannot be assumed that EOF is eliminated or even negligible. As illustrated in Figure 2, there is a small but nonzero EOF in the presence of SCD both in the presence and absence of organic modifiers. The EOF mobility was found to increase with increasing cyclodextrin concentration and is thought to arise from adsorption of the sulfated cyclodextrin on the capillary wall. This effect is illustrated in Figure 3, which shows two separate injections of terbutaline without cyclodextrin in the background electrolyte. Figure 3a, obtained using conventional polarity, shows

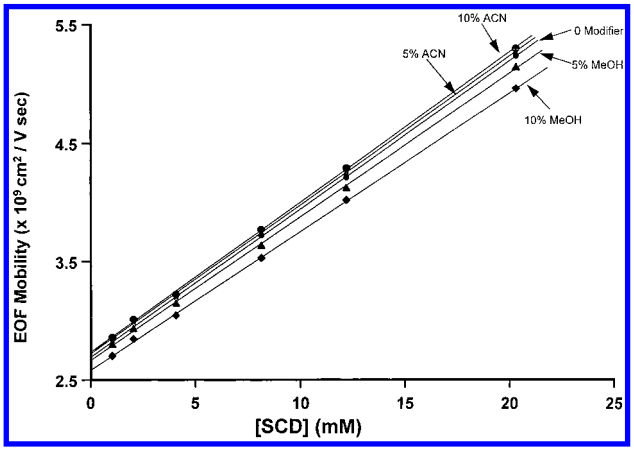

Figure 2. Effect of increasing SCD concentration on EOF mobility (nitromethane) in a 25-μm-i.d. coated capillary: applied voltage, 10 kV; buffer, 25 mM phosphate (pH 3); ●, no organic modifier; ▲, 5% methanol; ◆, 10% methanol; ★, 5% acetonitrile; half circle, 10% acetonitrile.

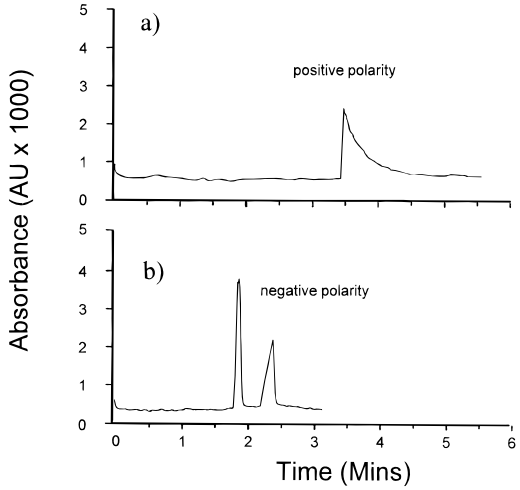

Figure 3. (a) Electropherogram for terbutaline (0.5 mM) injected into pristine capillary without sulfated cyclodextrin present in 25 mM phosphate buffer (pH 3). Applied voltage, 10 kV. (b) Electropherogram for terbutaline (0.5 mM) injected into that had previously been used with sulfated cyclodextrin-containing buffers. In this run there was no cyclodextrin in the 25 mM phosphate buffer (pH 3). Applied voltage, 10 kV.

terbutaline injected into a pristine, coated capillary. As expected, terbutaline migrates as a single peak with a migration time of 225 s. Figure 3b shows terbutaline injected into the same capillary after it was used with SCD-containing buffers. In this case, not only was terbutaline detected but the enantiomers were resolved, indicative of interactions between the enantiomers and adsorbed cyclodextrin. It should also be noted that terbutaline failed to elute from this column under conventional polarity; the electropherogram was obtained by reversing the polarity. Thus, the dynamic cyclodextrin layer is the only mechanism of analyte transport to the detector. Because this layer seems to be dynamic, no correction for adsorption was incorporated in the binding constant determination. The migration times for the separation in Figure 3b were comparable to those observed with buffer containing 5% SCD.

In this study, enantiomer mobilities were corrected for the measured EOF at each sulfated cyclodextrin concentration. After

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incorporating corrections for both viscosity and EOF, the μ_{cor} term in eq 3 is defined as

$$\mu_{cor} = [(\mu_{obs}) - (\mu_{eof})](\eta_0/\eta_x) \quad (4)$$

where μ_{obs} is the observed electrophoretic mobility for the analyte at a given set of experimental conditions, μ_{eof} is the mobility of a neutral marker under the same experimental conditions, η_0 is the buffer viscosity in the absence of SCD, and η_x is the buffer viscosity at a given SCD concentration. Note that because the migration of the neutral marker is opposite that of the analytes, μ_{eof} is effectively added to $\mu_{analyte}$.

The use of charged additives such as SCD changes the ionic strength of the background electrolyte, which can affect analyte mobility in two different ways. First, increased ionic strength decreases the effective field strength experienced by the analyte thereby decreasing its electrophoretic mobility. Second, in uncoated fused-silica capillaries, increased ionic strength causes decreased EOF as a result of a decreased ζ potential. However, in this study, as seen in Figure 2, wall adsorption of the sulfated cyclodextrin with the attendant EOF confounds this relationship.

It should be noted that polyvalent species with high charge densities such as these sulfated cyclodextrins may not contribute as much to ionic strength as their nominal degree of substitution suggests. Counterion condensation theory predicts that if the linear charge density of a polyionic covalent structure exceeds a critical value, there will be a layer of condensed counterions along the length of the polyion, effectively reducing its linear charge density.[27] In the case of micelles, it has been demonstrated that only a fraction of the total charge is carried because counterions are bound to the micelle to reduce electrostatic repulsion between head groups. Specifically, SDS micelles bind approximately three-quarters of their counterions in the absence of alcohol or electrolyte.[22] Ion-selective electrode (ISE) measurements of sodium activity in SDS solutions show a significant decrease in [Na^+] at the critical micelle concentration, indicative of counterion binding upon micelle formation.[28−29]

The possibility of counterion condensation was explored by measuring the total atomic sodium in an SCD solution using inductively coupled plasma atomic emission spectroscopy (ICP-AES) and comparing the value to the sodium concentration at an ion-selective electrode. Solutions of sulfated β-cyclodextrin were prepared to be 50 ppm in total Na^+, assuming the degree of substitution provided by the manufacturer was correct. The results of these measurements are shown in Table 1 and are reasonably close to the expected values.

The results of the ISE analysis of the aqueous SCD solutions containing 50 ppm total Na^+ are also displayed in Table 1. As can be seen from the table, the more highly substituted cyclodextrin exhibits a greater discrepancy between the two experimentally determined values than the more lightly substituted cyclodextrin, which is consistent with the counterion condensation theory.

Table 1. Na^+ Measurement (ppm) in SCCD Samples[a]

sample	ICP-AES	ISE
4 ds SCD (50 ppm Na^+)	48.6	45.7
15 ds SCD (50 ppm Na^+)	51.5	26.9

[a] See Experimental Section of text for conditions.

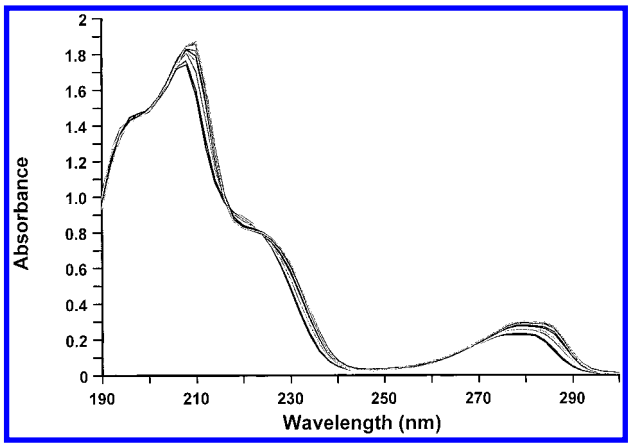

Figure 4. UV spectra illustrating SCD−terbutaline complexation and the effect of increasing the SCD concentration while maintaining constant terbutaline concentration at 1.25×10^{-4} M. SCD concentration range, 1.25×10^{-5}−2.5×10^{-4} M. The spectrum with the highest absorbance corresponds to the largest SCD concentration. The solvent for all spectra was 25 mM phosphate buffer (pH 3), which was subtracted as the blank.

The current CE models for binding constant determinations contain no explicit correction for ionic strength although, to some extent, compensation for the ionic strength is incorporated in the EOF corrections. One measure of the validity of neglecting ionic strength effects may be a comparison of binding constants determined by an alternate technique.

UV Difference Spectroscopy. Figure 4 shows the absorbance spectra of 1.25×10^{-4} M racemic terbutaline at SCD concentrations ranging from 0.0 to 2.5×10^{-4} M. The spectrum of racemic terbutaline in the absence of SCD exhibits a maximum absorbance of 1.7 with λ_{max} at 208 nm. As cyclodextrin is added, the maximum absorbance value reaches 1.9 and λ_{max} is shifted to 212 nm.

Figures 5 and 6 are the difference spectra and the resulting Benesi−Hildebrand plot from which the binding constant was obtained. The experimentally determined value for K was 1490 ($\pm$60) M^{-1}. It is important to point out that this K is an average binding constant because UV lacks the ability to discriminate between the two different terbutaline enantiomer−cyclodextrin complexes.

Mobility and Binding Constant. Figure 7 illustrates the affect that varying SCD concentration has on the mobility and chiral separation of terbutaline. As the selector concentration increases, solute mobility increases continuously and levels off as the mobility of the analyte−SCD complex is approached. The mobility difference between enantiomers increases with decreas-

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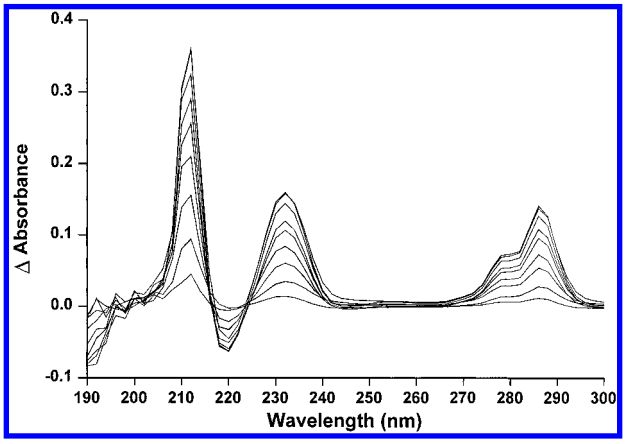

Figure 5. UV difference spectra for SCD−terbutaline complexation. Conditions shown in Figure 4. Each spectrum is the result of subtracting the free terbutaline spectrum from that at each sulfated cyclodextrin concentration.

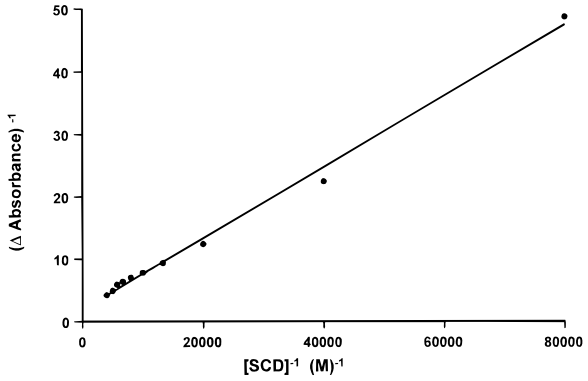

Figure 6. Benesi−Hildebrand plot for SCD−terbutaline complexation resulting from the difference spectra in Figure 5. The binding constant was calculated using eq 2 and found to be $K = 1450 \pm 60$ M^{-1}.

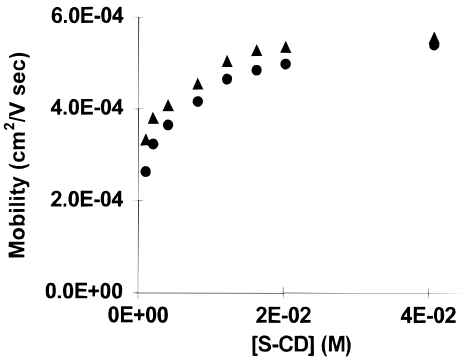

Figure 7. Effect of increasing SCD concentration on the mobility of terbutaline enantiomers. Buffer was 25 mM phosphate (pH 3) containing 0−40 mM SCD: coated capillary: −10 kV; 20 °C; ▲, first enantiomer; ●, second enantiomer.

ing SCD concentration but is limited by broad peaks and long migration times at the lowest extreme.

Mobility data, corrected for viscosity and EOF, were plotted against SCD concentration using a double-reciprocal format (Figure 8) and related to K according to eq 3. Experimental values for K were 1730 ± 50 and 1590 ± 38 M^{-1} for the two enantiomers. These values were in fairly good agreement with the average value determined by the spectroscopic method and are indicative of relatively strong binding between terbutaline and SCD.

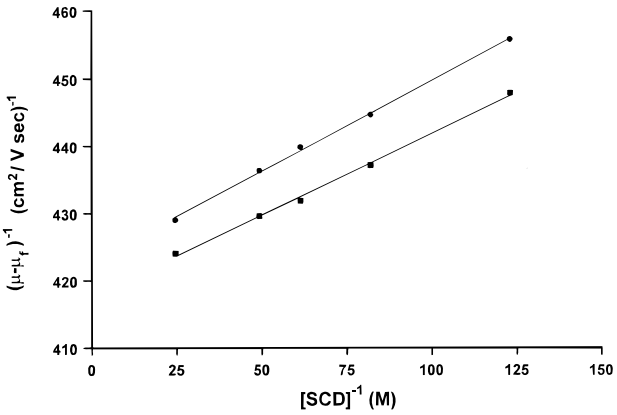

Figure 8. Terbutaline−SCD binding constants calculated using eq 3 and the data shown in Figure 7: coated capillary −10 kV; 20 °C; ▲, first enantiomer; ■, second enantiomer.

Organic Modifiers. Addition of an organic modifier to the buffer in CE has become fairly routine. Consequently, several groups have studied the effect that organic modifiers have on the differential binding of enantiomers to cyclodextrins.[4,20−22] One conclusion shared by most authors of such work is that the addition of organic modifiers such as methanol or acetonitrile decreases the magnitude of the CD−analyte binding constants. The most common explanation for this is that the organic modifier reduces the affinity of the analyte for the CD cavity and increases it for the bulk buffer. Alternatively, the modifier is thought to compete with the solute for the CD cavity. Goodall et al.[4] reported that selectivity remained constant from 0 to 15% MeOH or ACN in the chiral separation of tioconazole using native β-CD. The authors concluded that the constant selectivity observed supported the "solvent effect" explanation and that a simple 1:1 competitive binding scenario seemed unlikely for weakly binding species such as methanol.[21]

In contrast, Janini and Issaq[20] proposed that the effect of the organic modifier is different in the reversed-polarity MEKC format where the effect of EOF is suppressed. In this case, the addition of an organic modifier favors the partitioning of hydrophobic solutes into the immobile aqueous phase, which increases solute migration times as well as the resolution.

Figure 9 illustrates the effect that methanol has on the chiral separation of terbutaline. As the methanol concentration increases from 5 to 25%, with the SCD concentration remaining constant, separation between enantiomers increases from 2.6 to 21 min. The mobility difference between terbutaline enantiomers is plotted as a function of organic concentration in Figure 10. As can be seen in Figure 10, the mobility difference, $\Delta\mu$, increases with increasing organic content and the effect of acetonitrile is greater than that of methanol.

Binding constants in the presence of several concentrations of MeOH and ACN were determined as described above. As in the aqueous buffer systems, mobilities used to calculate K values were corrected for changes in both viscosity and EOF (see Figure 2) caused by the presence of the organic modifier. Table 2 reports values of K_1, K_2, and α, at each modifier concentration. As expected, the binding constants decrease with increasing organic concentration and again acetonitrile has a greater influence than methanol. It was also observed that the binding constant of the

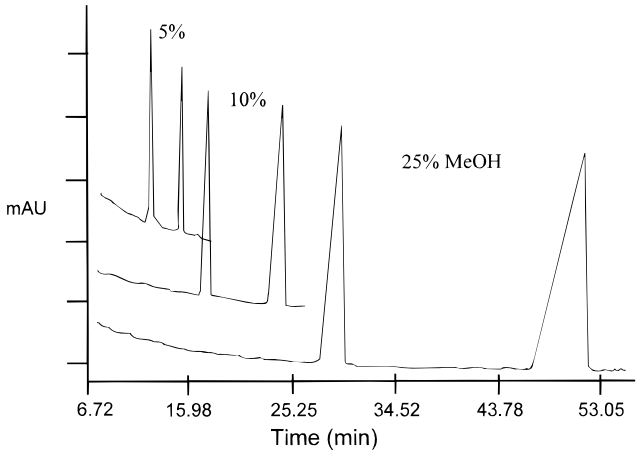

Figure 9. Overlaid electropherograms of terbutaline chiral separation. SCD concentration was held constant at 2% in 25 mM phosphate buffer (pH 3): Methanol concentrations 5, 10, and 15% (w/w); coated capillary; −10 kV; 20 °C.

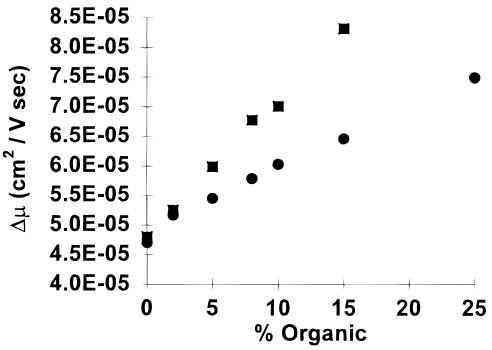

Figure 10. Terbutaline enantiomer mobility difference plotted against organic composition: 2% SCD; coated capillary; −10 kV, 20 °C; ■, acetonitrile, ●, methanol.

Table 2. Binding Constants of Terbutaline with Sulfated *β*-Cyclodextrin[a]

conditions	K_1	K_2	α
0% modifier	$1.73 \times 10^3 \pm 50$	$1.59 \times 10^3 \pm 38$	1.09 ± 0.04
5% MeOH	$1.70 \times 10^3 \pm 90$	$1.37 \times 10^3 \pm 21$	1.24 ± 0.07
10% MeOH	$1.59 \times 10^3 \pm 60$	$1.13 \times 10^3 \pm 52$	1.41 ± 0.08
5% ACN	$1.61 \times 10^3 \pm 90$	$1.26 \times 10^3 \pm 20$	1.28 ± 0.07
10% ACN	$1.32 \times 10^3 \pm 80$	$8.70 \times 10^2 \pm 90$	1.52 ± 0.18

[a] See Experimental Section of text for conditions.

second-eluting enantiomer was affected to a greater extent than the first enantiomer. The effect of methanol on the binding constant was also evaluated by UV difference spectroscopy. In the presence of 10% methanol, K was 1320 ± 50 M^{-1}, supporting the CE results of a decreased binding constant in the presence of organic modifiers. Unfortunately, a more exhaustive UV study was not possible because of insufficient sulfated cyclodextrin.

The selectivity, defined as $\alpha = K_2/K_1$,[28] was substantially affected by both modifiers. As can be seen from the table, α increases from 1.09 ± 0.04 with 0% organic to 1.41 ± 0.08 and 1.52 ± 0.18 at 10% methanol and acetonitrile, respectively.

It is important to recognize that the binding constant is related to the free energy associated with the transfer of the analyte from the bulk buffer to the cyclodextrin. While the energy associated with the inclusion of the analyte in the cavity may or may not change with bulk buffer composition, the energy of the analyte in free solution certainly does. Thus, it seems reasonable that the second-eluting enantiomer, which has lower affinity for the cyclodextrin, should exhibit greater differences in binding constant with bulk buffer composition, and thus greater selectivity, even though both enantiomers have the same energy in the bulk buffer. This increase in selectivity with increasing organic content may seem counterintuitive when compared to HPLC. However, CE can exploit potential countercurrent migration of analyte and additive while HPLC flow is unidirectional.

CONCLUSIONS

This study has shown that, for the chiral separation of racemic terbutaline with sulfated *β*-CD, separation increases with decreasing selector concentration but is limited by broad peaks and long migration times at the lowest extreme. The addition of methanol or acetonitrile to the CE run buffer increased enantioselectivity for terbutaline under conditions of reversed polarity and minimal EOF. It was also found that the presence of sulfated cyclodextrin did induce a concentration-dependent EOF despite the use of a coated capillary. Electrophoretic mobilities required adjustment due to the changes in viscosity caused by the presence of the chiral selector as well as changes in the electroosmotic flow caused by adsorption of SCD in the capillary. AES and ISE results indicate that counterion condensation occurs with these highly charged additives. A binding study was performed to approximate binding constants for this system and the effect that organic modifiers have on binding constants. Values for K determined spectroscopically compared well with those determined by CE. Furthermore, CE and UV difference spectroscopy revealed a substantial decrease in the values for K upon addition of organic modifier to the buffer.

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