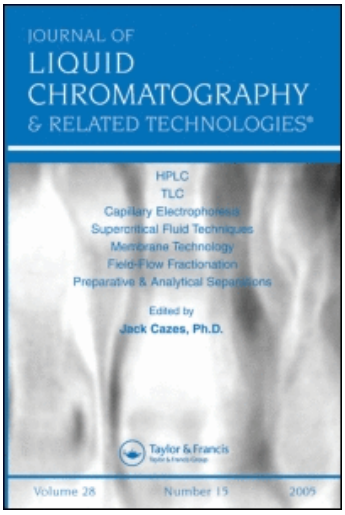

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ENANTIOMERIC SEPARATION OF A DRUG SUBSTANCE USING CAPILLARY ELECTROPHORESIS WITH SULFATED-β-CYCLODEXTRIN

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ABSTRACT

The enantioseparation of a weakly basic pharmaceutical drug substance is investigated using sulfated-β-CD as a chiral selector. The enantiomers are separated at acidic pHs in an anodic flow due to complexation with sulfated-β-CD, while under an electroosmotic flow (EOF) counter-current. At basic pHs, the EOF prevails and the analytes migrate toward the cathode, where improved separations can be obtained along with an apparent reversal of migration order. The optimal enantioseparation of the test compound is systematically explored by varying important factors, such as pH, concentration of sulfated-β-CD, temperature, and addition of organic modifier. The implications of the results on general enantioseparation of weakly basic pharmaceutical compounds are discussed.

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INTRODUCTION

Many pharmaceutical drug substances contain at least one chiral center. The fact that most enantiomers of chiral compounds exhibit significantly different pharmacological and toxicological properties has made the use of enantiomerically pure compounds desirable. With the introduction of Food and Drug Administration's (FDA) regulations[1] on the enantiomeric purity of drugs, separation and determination of enantiomers become pivotally important for the pharmaceutical industry. In the last decade, the development of capillary electrophoresis (CE) methods has rapidly grown and has been widely applied in enantioseparations due to numerous advantages including high efficiency, simplicity, short analysis time, small sample consumption, low cost, and high flexibility.[2–5] Among various CE methods, capillary zone electrophoresis (CZE) with cyclodextrins (CDs) and their derivatives has comprised most of the investigations and successful applications.[3–6] Cyclodextrins are 6-, 7-, and 8-membered (α, β, and γ, respectively) cyclic oligosaccharides consisting of α-[1,4]-linked glucopyranose units.[7] The truncated cone-shaped structure of CD with a hydrophobic cavity provides an ideal inclusion host for analytes through mainly hydrophobic interactions. The hydroxyl groups around the rims are amenable to chiral selectivity. Derivatizations at the hydroxyls with neutral, negative, or positive groups yield modified CDs with altered specificity and possibly higher affinity. Charged CDs exhibit higher solubility as well. Commonly available derivatized CDs include (2,6-di-O-methyl)-β-CD (DM-β-CD), (2,3,6-tri-O-methyl)-β-CD (TM-β-CD) and hydroxylpropyl-β-CD (HP-β-CD) in the neutral form, carboxymethyl-β-CD (CM-β-CD), sulfobutyl-β-CD (SBE-β-CD) and sulfated-β-CD in the anionic form, and methylamino-β-CD in the cationic form. They have been successfully employed in enantiomeric separations of a wide variety of analytes, such as pharmaceutical compounds,[2–5] PAHs,[8,9] amino acids,[10] peptides,[11,12] proteins,[12] etc. A combination of hydrophobic, electrostatic, van der Waals and dipole–dipole interactions may be involved in the host–guest complexation and enantioselection. Models concerning the complexation between enantiomeric molecules and chiral selectors have been proposed.[13,14] Estimation of association constants has also been seen.[15]

Negatively charged CDs have been shown to be particularly effective for the enantiomeric separation of basic compounds.[16–25] It is believed that the negative charges of the substituents provide electrostatic interactions to positively charged analytes. In addition, anionic CDs do not interact with the capillary wall (negatively charged silano groups) and counter-migrates against cationic analytes and the EOF of an untreated silica capillary. Among various negatively charged CDs, sulfated-β-CD has the highest degree of substitution and remains charged even at low pHs, making it a popular choice for chiral separation of basic analytes. Stalcup and Gahm[22] demonstrated that sulfated-β-CD efficiently separated enantiomers of close to 40 different types of basic pharmaceutical

compounds. Izumoto and Nishi[25,26] observed that, out of eight kinds of charged CDs, sulfated-β-CD gave relatively wide enantioselectivity for a variety of compounds. Chankvetadze et al.[21] reported the separation of basic racemic drugs using extremely low levels of SBE-β-CD. Other studies also indicated that sulfated CDs are more suitable for the separation of basic compounds than other types of CDs.[24,27] In most of the enantioseparations of basic compounds using sulfated CDs, an anodic migration of analytes with sulfated CDs at low pH proved to be effective and widely applicable.

In this study, CZE enantiomeric separation of a drug substance, made by Merck Process Chemists using sulfated-β-CD, was investigated. The compound, whose schematic structure is shown in Fig. 1, is a highly selective muscarinic M3 receptor antagonist (M3 antagonist) for the treatment of urinary tract disorders, irritable bowel syndrome, and respiratory disorders.[28,29] There are two neighboring chiral centers in the structure. The current study concerns the separation of the racemic mixture of the (*R*,*R*) and (*S*,*S*) enantiomers. The compound contains a protonable tertiary amine group and multiple aromatic ring systems. It is slightly basic and exhibits a pKa of 7.5. Enantioseparations of the compounds were investigated at both the acidic and basic pHs with sulfated-β-CD. Other experimental conditions, such as chiral selector type and concentration, pH, temperature, and addition of organic modifier, were systematically examined in an effort to obtain the best resolution. Separation results under different conditions were interpreted and their implications to optimization of enantiomeric separation are discussed.

EXPERIMENTAL

Instrumentation

All experiments were performed on a Hewlett Packard ^{3D}CE system equipped with a photodiode array detector (Hewlett Packard, Santa Clarita, CA). Uncoated fused silica capillaries (50 μm I.D. × 80.5 cm with effective length of

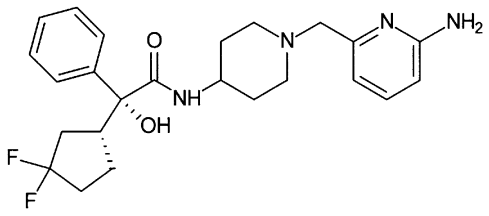

Figure 1. The structure of the (*R*,*R*) enantiomer of the drug substance used in the present study.

72 cm) were purchased from Hewlett Packard. Detection wavelength was set at 220 nm. Sample was introduced to the capillary by a 50 mbar pressure pulse for 5 seconds. Data were collected and analyzed with PE Nelson Access Chrom version 1.7 (PE Nelson, Cupertino, CA).

Reagents

The test compound (2R)-2-[(1R)-3,3-difluorocyclopentyl]-2-hydroxy-2-phenylacetamide and its enantiomer were provided by Process Research, Merck Research Laboratories (Rahway, NJ). Biological, pharmacological properties, and the synthesis of the compounds can be found in Refs. [28, 29] Citric acid anhydrous, methanol, and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Fairlawn, NJ). Triethylamine, α-, β-, γ-cyclodextrin, and sulfated β-cyclodextrin were obtained from Aldrich (Milwaukee, MI). Tris(hydroxylmethyl)aminomethane (electrophoresis grade) was supplied by Bio-Rad Laboratories (Richmond, CA). Hydroxylpropyl-, succinylated-, and carboxymethylcyclodextrin were obtained from Cerestar (Hammond, IN).

Buffer and Sample Preparations

All buffer solutions were filtered through membranes of 0.45 μm pore size before use. For buffers of pH 3 to pH 5, 50 mM citric acid solution was used and the pH was titrated with triethylamine to the desired values. For buffers of pH 7.7, 8.1, and 8.4, appropriate volumes of Tris buffer and citric acid solution were mixed. The buffer of pH 10.3 was made using TEA and citric acid. The diluent for all solution preparation of the test compounds was 80/20 (v/v) deionized water/acetonitrile. The (*R*,*R*) and (*S*,*S*) racemic mixture of the compound was prepared by mixing solutions of two optically pure enantiomers.

RESULTS AND DISCUSSION

Due to the presence of amine groups, the compound is positively charged at neutral or acidic conditions. At pH 8.4, CZE without any chiral selector added showed no separation of the enantiomers. A preliminary screening of chiral selectors revealed that most promising separations were obtained using sulfated-β-CD among a set of CDs, including also, α-CD, β-CD, γ-CD, HP-β-CD, succinylated-β-CD, CM-β-CD. This agrees with previously shown examples that opposite charges promote selector-selectand interactions.[16,18,21,30] A chiral selector with an opposite electrophoretic mobility to that of the analyte promises

a maximum potential for enantioseparation.[23,31] Thus, sulfated-β-CD was chosen as the chiral selector for further investigations.

In the present study using bare silica capillaries, the EOF migrates toward the cathode, whereas sulfated-β-CD migrates toward the anode. An initial study conducted at an alkaline pH, with the detector set at the cathode side, revealed that the compounds migrated after the EOF in the presence of sulfated-β-CD. Since the electrophoretic mobility of the compound is also cathodic, an apparent mobility slower than the EOF indicates a complexation with the anionic sulfated-β-CD. With a high degree of sulfate substitution, sulfated-β-CD apparently dominated the net charge of the drug/sulfated-β-CD complex, and a counter-EOF migration of the complex postponed the migration of the compounds relative to the EOF. Because the mobility of the EOF can be adjusted by changing the pH, and the counter-EOF mobility of the compounds can be tuned by varying the concentration of sulfated-β-CD, a control of the migration direction of the compounds toward either one of the electrodes is possible. Such design is particularly suitable for separations of basic analytes using sulfated-CDs, because a dominant anodic flow is made possible at low pHs, where sulfated-CDs are still highly negatively charged and EOF is minimized, while an apparent cathodic flow of the compound can be dominant at high pHs, where EOF is drastically increased and the electrostatic interactions between the compound and the chiral selector are weakened due to the loss of charge of the compounds. The use of highly charged sulfated-CDs helps to ensure the anodic flow of the compounds at the low pH region through electrostatic interactions. When experimental conditions are carefully tuned and the detector is properly placed, the compounds can be detected at either the cathode or the anode side of the capillary. Even though the relative binding affinities of the enantiomers for the chiral selector do not change, the migration orders of the enantiomers when detected at the cathode and the anode sides are reversed. Such apparent migration order reversal differs from "real" reversals caused by enantiomer-chiral selector affinity turnover, which can be obtained by, most commonly, altering the properties of the chiral selector but without changing the point of detection.[32,33] Migration order reversal may be necessary when the minor enantiomer is to be detected first for sensitive detection and measurement. In the present study, separations with the analytes migrating toward either the cathode or the anode were investigated. The effects of other important variables, such as pH, sulfated-CD concentration, temperature, etc. on the enantiomeric separation were examined and are discussed in the following sections.

Effect of pH

pH is of prime importance in chiral separations by CZE, since it directly influences the charge states of the analytes, chiral selectors, and the magnitude of

EOF. There is a consequent effect on analyte-chiral selector interactions and the chiral selectivity. Previously, enantioseparations using sulfated-CDs were normally performed at acidic pHs,[2,4,7,34] where the compound migrates towards the anode through electrostatic interactions with the chiral selector. In the present study, two types of separations, anodic migration and cathodic migration in the acidic and basic regions, respectively, were performed. For separations under acidic conditions, three pHs, 3.2, 4.0, and 5.0, were examined using 8% sulfated-β-CD (a relatively high level of sulfated-β-CD was used from the screening discussed later, the current was about -75 μA). For all the pHs evaluated, the analyte/sulfated-β-CD complex has a net negative charge and migrates toward the anode in the presence of relatively weak EOFs. Figure 2A shows the enantiomeric separations at different acidic pHs. At pH 3.2, the enantiomeric pair was well separated but not to baseline resolution. The (*S*,*S*) enantiomer was detected first, indicating a stronger interaction with sulfated-β-CD. Increasing pH to 4.0 prolonged the migration time and greatly improved the resolution between the enantiomers. The observation was primarily due to a slightly higher counter-current EOF. When pH was changed from 3 to 4, the relative migration time of the enantiomers, a measurement of selectivity, increased slightly from 1.02 to 1.03, but the resolution of the separation increased from 2.4 to 3.7. The higher resolution was attributable to a longer time that the enantiomers and sulfated-β-CD are able to interact. A longer interaction time has been shown to better magnify subtle differences in analyte-CD complexation.[24] At pH 5.0, the resolution and peak shapes deteriorated with the concomitant longer migration time. An increased counter-current EOF, which was competitive to the mobility of the sulfated-β-CD, was deemed responsible. As a conclusion, pH 4.0 was regarded as the best pH for the separation under acidic conditions.

When the pH is increased, the higher EOF eventually overcomes the mobility of sulfated-β-CD and the analytes migrate in the same direction as the EOF. Compared to the electropherograms at low pHs, after changing the polarity of voltage and, therefore, switching the detector to the cathode side of the capillary, the enantiomers were observed in reversed order. Figure 2B shows the separation at high pHs using 3% sulfated-β-CD. At pH 7.7, the two enantiomers were well separated with a resolution of 3.2. Although the separation was good at this pH, the peak shapes and efficiencies were less satisfactory. When pH was increased to 8.4, the migration time and resolution changed drastically despite the small changes in the EOF (electrophoretic mobility of EOF was $\sim 3.6 \times 10^{-4}\,\mathrm{cm^2\,V^{-1}\,sec^{-1}}$ at pH 8.4 vs. $\sim 3.1 \times 10^{-4}\,\mathrm{cm^2\,V^{-1}\,sec^{-1}}$ at pH 7.7). The average migration time of the chiral pair at pH 8.4 was shortened to almost half of that at pH 7.7 and the resolution decreased to 1.7. The small change in EOF could not account for such a large drop in migration time. It is likely that the pH change from 7.7 to 8.4 also weakened the interactions between the compounds and the sulfated-β-CD, which consequently shortened the migration time and decreased the separation resolution. Considering the pKa of the compound is 7.5, a pH change from 7.7 to 8.4 would significantly

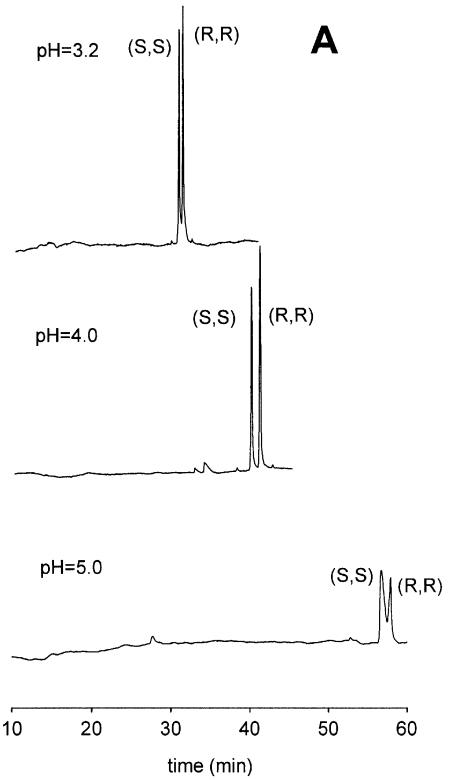

Figure 2. Comparison of the electropherograms of enantiomeric separations at different pHs. (A) Acidic buffers of pH 3.2, 4.0 and 5.0 (50 mM citric acid, pH adjusted with TEA). Voltage: −15 kV. Concentration of sulfated-β-CD: 8% (w/v). (B) Basic buffers of pH 7.7 (40 mM *Tris*/10 mM citric acid), pH 8.4 (45 mM *Tris*/5 mM citric acid) and pH 10.3 (40 mM TEA/10 mM citric acid). Voltage: 15 kV. Concentration of sulfated-β-CD: 3% (w/v). Other conditions used, experimental temperature: 20°C. Sample concentration: 0.4 mg/mL for each enantiomer. Size of the uncoated fused silica capillary: 50 μm I.D. × 80.5 cm (effective length 72 cm). Detection: 220 nm UV. Sample introduction: 50 mbar pressure pulse for 5 seconds.

(continued)

deprotonate the chargeable amine group. Since the ion-pairing interactions are critical to the complex formation, a decrease in positive charge of the compound understandably decreases the degree of complexation and consequently the enantioselectivity. On the other hand, faster migration at pH 8.4 resulted in higher efficiencies of the peaks. At pH 10.3, almost no separation could be seen between

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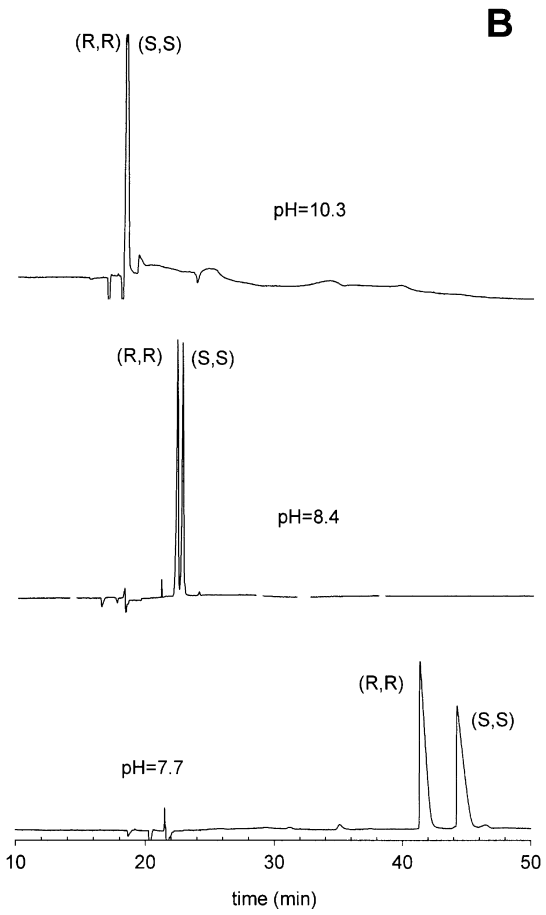

Figure 2. Continued.

the two enantiomers. The enantiomers peaks were seen to co-migrate with EOF, indicating a significant decrease of complexation likely due to an elimination of electrostatic interactions.

Effect of Sulfated-Cyclodextrins Concentration

The concentration of CD has been shown, theoretically and experimentally, to have important effects on enantioseparations.[7,13] An existence of maximum mobility difference of the enantiomeric pair as a function of the concentration of the

chiral selector is predicted by theoretical works.[13] However, whether the optimal mobility difference is achievable depends on other factors, including solubility of the chiral selector and the affinity of the analyte for the chiral selector.

The effects of sulfated-β-CD concentration on the separation of the present enantiomers were examined at four pH values, 4.1, 7.7, 8.1, and 8.4, with sulfated-β-CD concentrations from 1% to 8% (w/v). The electropherograms for the study at pH 8.1 are shown in Fig. 3, while a plot of enantioseparation resolution vs. sulfated-β-CD concentration is shown in Fig. 4 for all the tested pHs. At pH 8.1, addition of 1% sulfated-β-CD barely separated the two enantiomers. Increasing sulfated-β-CD concentration to 3%, 5%, and 8% gradually enhanced the separation of the chiral pair, indicating longer time of the analytes complexed with sulfated-β-CD. It was also observed that the migration time of the EOF and the compounds also increased. This is due to increased viscosity and ionic strength of the background electrolytes (BGE) with the addition of sulfated-β-CD, as well as increased complexation between the compound and sulfated-β-CD. Similar trends were observed at all other pHs tested.

The dependence of enantioseparation resolution on sulfated-β-CD concentration is shown, in detail, for four pH values in Fig. 4. All the curves show the same trend of higher sulfated-β-CD concentration giving higher resolution in enantioseparation. The relationship holds to the highest concentration tested without displaying any sign of a plateau or maximum. The buffer pH plays a dominant role in regulating separation time (through changing EOF), peak efficiency, and separation resolution. pH 8.1 appears to be a compromise among these factors giving the best resolution at high sulfated-β-CD concentrations (Fig. 4).

The sulfated-β-CD concentrations required for the enantioseparation in this study were relatively high compared to other studies using the same or similar chiral selector, where common concentration range of 1 to 5% for sulfated-CD or SBE-β-CD was sufficient for most of the applications.[3] For most of the pHs examined here, high sulfated-β-CD concentrations of up to 8% were used (with $<85\,\mu$A current), but no instability in the baseline was observed. No higher concentration was tried due to concerns with respect to Joule heating. According to the mobility difference model of enantioseparation proposed by Wren et al.,[13] the concentration of the chiral selector at which the maximum mobility difference between the enantiomeric pair occurs is inversely proportional to the binding constants of the enantiomers to the chiral selector. The high concentration of chiral selector used in the present system is indicative of weak analyte/sulfated-β-CD interactions.

Effect of Temperature

Temperature is considered a key parameter in enantioseparations, since it affects the kinetics and thermodynamics of the complexation process. The

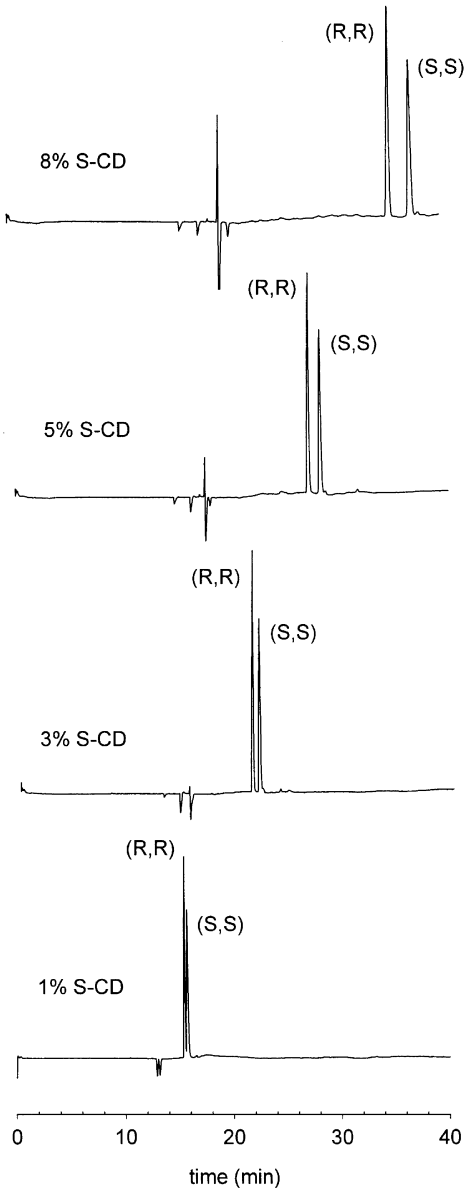

Figure 3. Comparison of electropherograms of enantiomeric separations with different sulfated-β-CD concentrations (labeled on the graphs). Buffer: 80 mM *Tris*/15 mM citric acid, pH 8.1. Voltage: 20 kV. Sample concentration: 0.8 mg/mL for each enantiomer. Refer to Fig. 2 legend for other conditions when not specified.

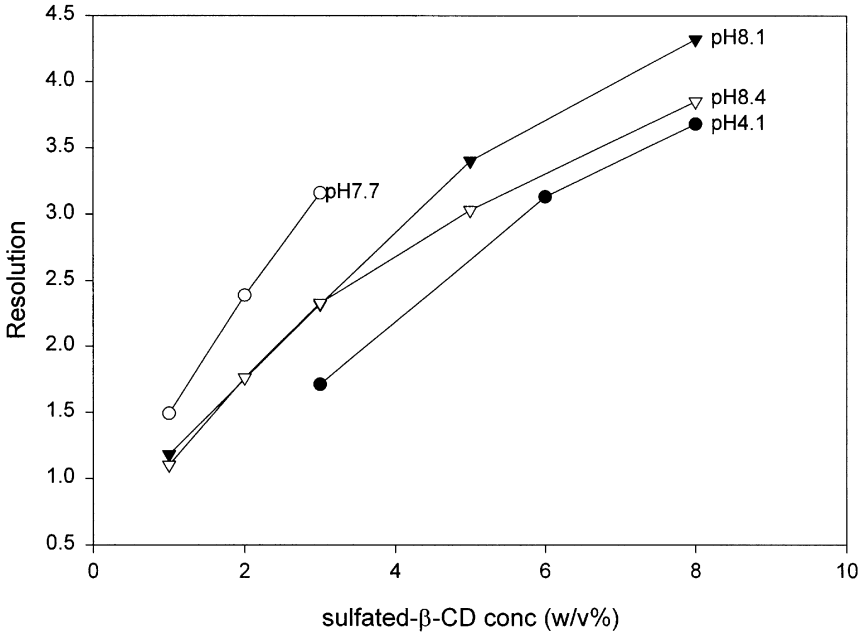

Figure 4. Effect of sulfated-β-CD concentration on enantiomeric resolution at different pHs. Symbol coding: ○ pH7.7 (40 mM *Tris*/10 mM citric acid), ▼ pH8.1 (80 mM *Tris*/15 mM citric acid), ▽ pH8.4 (80 mM *Tris*/10 mM citric acid), ● pH4.1 (50 mM citric acid/TEA). Voltage: 20 kV for runs with buffers of pH 7.7 to 8.4, −15 kV for the run with the buffer of pH 4.1. Refer to Fig. 2 legend for other conditions when not specified. Compared to the (*S*,*S*) enantiomer, the (*R*,*R*) enantiomer migrated faster in buffers of pH 7.7 to 8.4, but slower in the buffer of pH 4.1.

examination of the temperature influence on the separation using 8% sulfated-β-CD at pH 4.0 is shown in Fig. 5. Investigations at other pHs showed a very similar trend. Compared to 20°C, separation at 35°C resulted in shortened analysis time and improved peak efficiency, but deteriorated resolution. Increasing temperature lowers the complexation constant between the analytes and the chiral selector.[35,36] As discussed before, the present compound interacts weakly with sulfated-β-CD; at a chiral selector concentration below optimum, a higher temperature leads to weaker analytes-chiral selector complex and worsened separation. On the other hand, lowering temperature to 15°C gave slightly improved resolution due to enhanced complex formation, while a longer analysis time was evident. For a compromise among resolution, efficiency, and analysis time, 20°C is a reasonable choice.

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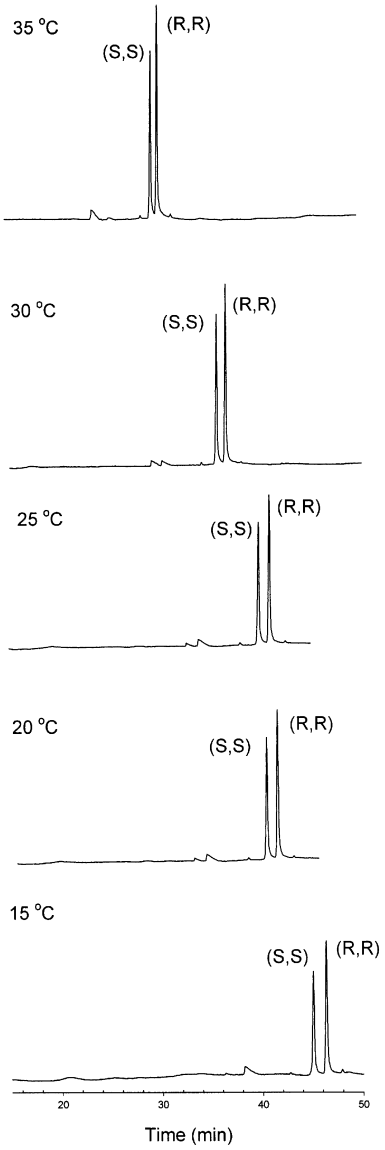

Figure 5. Comparison of electropherograms of enantiomeric separations with different temperatures at pH 4.1. Temperatures are labeled on the graphs. Background electrolyte: 50 mM citric acid/TEA, pH 4.1. Voltage: −15 kV. Concentration of sulfated-β-CD: 8% (w/v). Sample concentration: 0.4 mg/mL for each enantiomer. Refer to Fig. 2 legend for other conditions when not specified.

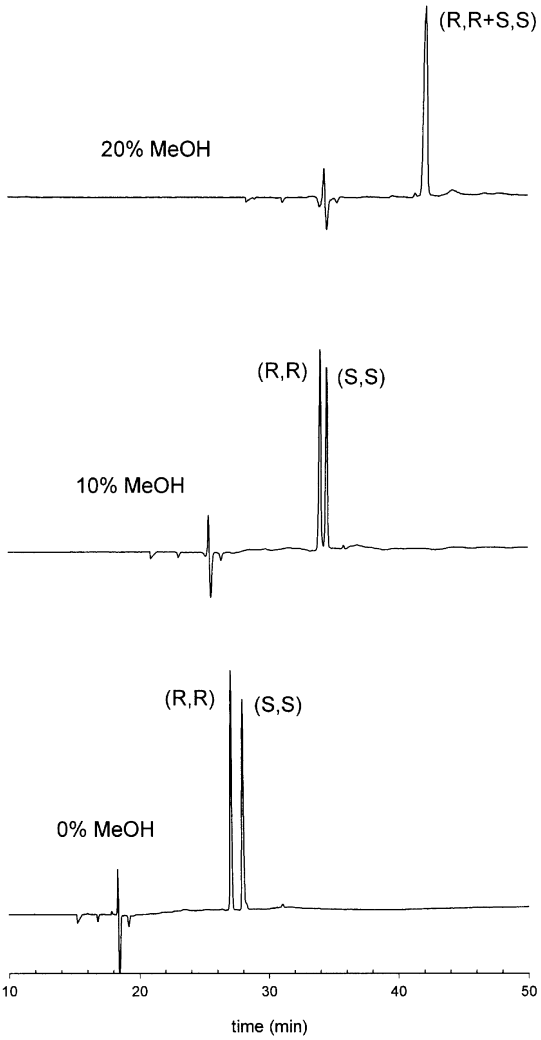

Figure 6. Comparison of electropherograms of enantiomeric separations with different methanol additions. The volume percentages of added methanol are labeled on the graphs. Background electrolyte: 80 mM *Tris*/10 mM citric acid, pH 8.4. Voltage: 20 kV. Concentration of sulfated-β-CD: 8% (w/v). Sample concentration: 1.0 mg/mL for each enantiomer. Refer to Fig. 2 legend for other conditions when not specified.

Effect of Organic Additive

Organic additives are usually used to modify the interaction equilibria of the analyte-CD complexation.[3] Improvement in enantioseparation occurs when the chiral selector is used above its optimal concentration. It is worthwhile to mention, that addition of organic additive also modifies the solubility of the chiral selector, the conductivity of the background electrolyte, and the viscosity of the media. In the present study, methanol was added to the background electrolyte to test any potential effects on the separation. Electropherograms of the separations at pH 8.4 with methanol addition up to 20% are shown in Fig. 6. Addition of methanol drastically lowered the resolution of the enantioseparation. As an extreme, at 20% methanol the chiral separation was lost. At the same time, methanol addition increased the viscosity and decreased the conductivity, which led to longer migration times of the EOF and the analytes/sulfated-β-CD complex. Similar effects of methanol addition were observed on the separation at other pHs. The reason underlying the negative effect of methanol addition is similar to that of raising temperature discussed above. When sulfated-β-CD was used below its optimal concentration, adding organic modifier decreases the association constant and, thus, the separation resolution.

CONCLUSIONS

Enantioseparation of a weakly basic pharmaceutical compound was accomplished using sulfated-β-CD as chiral selector under acidic and basic conditions. The test compounds were shown to exhibit a low affinity for sulfated-β-CD within a wide range of pHs and the primary interactions are electrostatic. Baseline separations were observed at an acidic pH employing a common design for the enantioseparation of basic compounds. However, basic background electrolyte was shown to provide a better interplay between the migration forces from EOF and the sulfated-β-CD complex, where a better combination of analysis time, resolution, and sulfated-β-CD concentrations was obtained. An apparent migration order reversal of the enantiomers was also recorded when changing from acidic to basic pHs. Combining the separation schemes at acid and basic pH regions results in a highly powerful and versatile method for enatioseparation of basic analytes.

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