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Optimization of preparative electrophoretic chiral separation of ritalin enantiomers

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Dedicated to Professor Gottfried Blaschke on the occasion of his 65th birthday

Abstract

Continuous free flow electrophoresis (CFFE) was applied to the preparative chiral separation of ritalin enantiomers. Sulfated β-cyclodextrin (sβ-CD) was used as the chiral additive. Liquid chromatography–mass spectrometry (LC–MS) experiments were applied to study the time averaged concentration of sβ-CD in the separation chamber. The distribution of sβ-CD in the separation chamber greatly influenced resolution and the angle of deflection. To optimize the separation, several parameters (methanol, concentration of sβ-CD in the cathodic wash and in the separation buffer, and the introduction of a low conductivity zone) were investigated. The dependence of the resolution and deflection angles of ritalin enantiomers on the concentration of sβ-CD in both the separation buffer and in the cathode wash solution appeared to be non-linear. Under close to optimal conditions, resolution of ritalin enantiomers was about 0.8 with an average processing rate of 0.5 mg/h. Overall, the enantiomeric purity of the individual isomers was $\sim 83\%$; however, of the 20 vials containing ritalin, the presence of both enantiomers was only detected in three vials.

Keywords: Ritalin; Methylphenidate; Chiral; Preparative; Separation; Continuous free flow electrophoresis; Capillary electrophoresis; Deflection angle

1. Introduction

It has been recognized that optical isomers might possess different biological properties. This is mainly due to the evolution of 'homochiral' biological structures, which ultimately impact preferential stereospecific drug–protein interactions. The stereospecificity of drug–protein interactions has resulted in the issuance of guidelines for drug development [1] advising that enan-

Abbreviations: CE, capillary electrophoresis; CFFE, continuous free flow electrophoresis; β-CD, beta cyclodextrin; sβ-CD, sulfated beta cyclodextrin; LC–MS, liquid chromatography mass spectrometer; μ, electrophoretic mobility; TIC, total ion current.

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tiomeric isomers should be treated as separate entities. Thus, it is increasingly desired that pure enantiomers of a pharmaceutical formulation be either synthesized or separated, so their properties can be evaluated early in the drug development process.

Ritalin is a commonly prescribed medication for children and adults with attention deficit hyperactivity disorder (ADHD). It is marketed as a racemic mixture of dl-threo-methylphenidate hydrochloride [2]. Its structure is shown in Fig. 1. Several independent experiments in animals [3], as well as in humans [4,5] suggested that the d-enantiomer is more active and binds specifically onto the dopamine receptor in the human brain, whereas binding of the l-enantiomer appears to be non-specific [6]. Small-scale chiral separations of ritalin enantiomers have been developed using chiral GC [7] and radio-analysis [8]; however, these methods were limited to analytical sample characterization and quality assurance purposes. To the best of our knowledge, only fractional recrystallization has been reported for the production of both enantiomerically pure isomers of d- and l-threo methylphenidate [9].

Chiral Free Flow Electrophoresis (FFE) has the potential to continuously separate enantiomers in mg/h yields. FFE is a free solution electrophoretic process, in which background electrolyte is continuously introduced through a series of inlet ports across one end of the separation chamber producing a buffer 'curtain'. Sample is continuously introduced at a single inlet into a separation chamber formed by two narrowly spaced (e.g. 0.1–0.4 mm) plates. In an alternate design, the plates of the separation chamber are about 1.3 cm apart and the inside of the chamber contains a heat exchanger

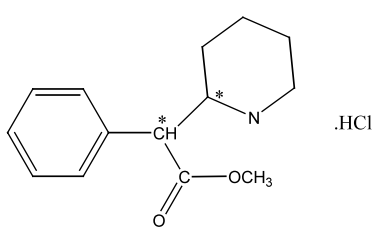

Fig. 1. Structure of methylphenidate hydrochloride. Methylphenidate hydrochloride has two chiral centers, thus possessing two pairs of enantiomers (four isomers). Ritalin is enantiomeric threo-methylphenidate.

consisting of closely spaced teflon capillary tubes through which chilled water is pumped, allowing heat transfer from the free buffer solution to the coolant. This mode of FFE is called Capillary Free Flow Electrophoresis (CFFE) [10,11].

Perpendicular to the sample and electrolyte flow, an electric field is applied while buffer is flowing; however, other modes, such as interval flow [12] have been also exploited. The ions are carried along the chamber and at the same time, are laterally displaced under the influence of the electric field towards the respective counter-electrode according to their electrophoretic mobilities (μ). At the outlet end of the electrophoretic chamber, separated sample components travel through an array of outlet tubes to a fraction collector. A schematic of the CFFE chamber is presented in Fig. 2a. Fig. 2b shows a photograph of the separation compartment of the CFFE instrument.

Although achiral FFE and CFFE has been known for more than 40 years [13–16], its wide use has been somewhat limited due to some challenges associated with flow instability (e.g. fluid convection, sedimentation, and boundary instabilities) [17,18]. Consequently, it has been studied as a separation technique in microgravity environments. However, despite some experimental drawbacks, successful separation of small ions [19], enzymes [20], proteins [21], DNA [22], and cells [23] has been reported.

For chiral separations, thus far, only cyclodextrins and their derivatives have been used as the chiral additives in a CFFE separation buffer. The most commonly used cyclodextrin derivatives were uncharged. Glukhovski and Vigh used hydroxypropyl-β-cyclodextrin and isoelectric focusing for the preparative separation of dansyl phenylalanine enantiomers [24], and terbutaline enantiomers [25]; Thormann and coworkers used hydroxypropyl and carboxymethyl-β-cyclodextrin for the electrophoretic and isotachophoretic separation of methadone and chlorpheniramine enantiomers [26]. Despite potential solubility issues, the use of unchanged derivatives of cyclodextrins in CFFE separations may provide an advantage in that the chiral selector should remain essentially laterally stationary in the absence of electroosmotic flow in the separation chamber.

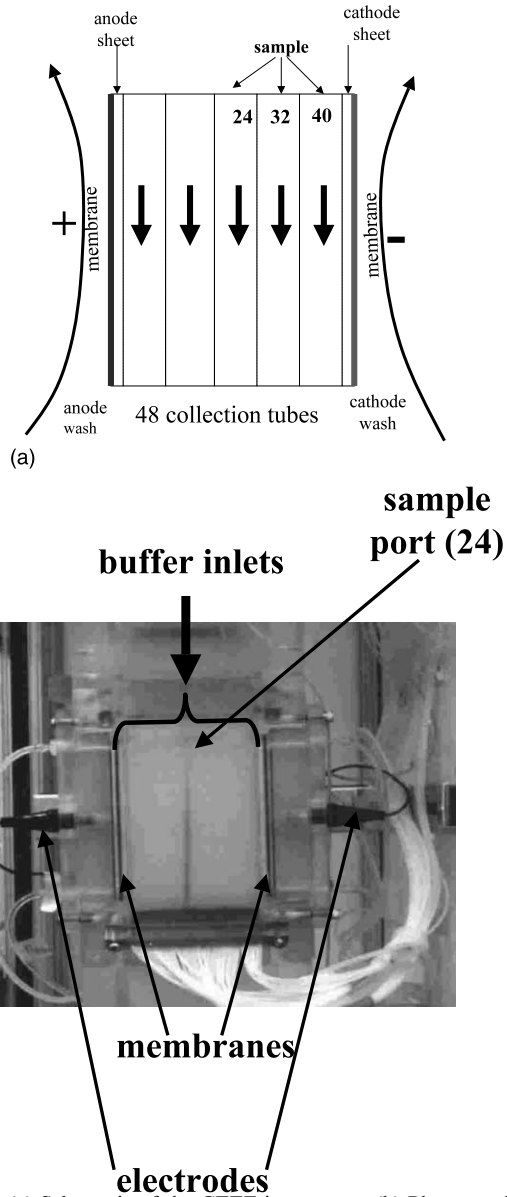

Fig. 2. (a) Schematic of the CFFE instrument. (b) Photograph of the CFFE separation chamber.

Polyanionic sulfated beta cyclodextrin (sβ-CD) has been very successful as a chiral selector in capillary electrophoresis (CE) [27,28]. Recently, Stalcup and co-workers reported the use of polyanionic sβ-CD as a chiral selector in CFFE for preparative chiral separations [29,30]. They reported the influence of several factors on chiral separation, such as voltage and concentration of sulfated cyclodextrin in the separation buffer. They found that sβ-CD forms accumulation and depletion zones in the electrophoretic chamber. It was also found that the interplay of the various experimental parameters in the CFFE is fairly complex [29,30].

In this work, the influence of methanol, concentration of sβ-CD in the separation buffer and in the cathode wash, and introduction of a low conductivity zone on the enantioseparation of ritalin were studied. CE was employed in the analysis of the fractions obtained from the CFFE, and it was also used as a developmental tool.

2. Experimental

2.1. Materials

The sβ-CD with nominal degree of substitution $\approx 13-15$, was obtained from Michigan Diagnostics, LLC (Troy, MI, USA). All experiments were conducted using sβ-CD from the same lot. Glacial acetic acid, ammonium acetate, ammonium hydroxide, and methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). Standard solutions of ritalin (dl-threo-methylphenidate hydrochloride 1 mg/ml methanol) were obtained from Radian International (Austin, TX, USA). Deionized water was used in all experiments.

2.2. Equipment

The preparative CFFE instrument used in this work is a prototype instrument on loan from Varian, Inc., (Wakefield, RI, USA) and was described previously [10,11,31]. The analytical CE separations were accomplished using a Bio-Rad (Richmond, CA, USA) Biofocus 3000 and/or a Beckman (Fullerton, CA, USA) MDQ CE instrument interfaced to a PC for instrument control and data handling. LC–MS experiments were performed using a Finnigan TSQ 700 triple quadrupole mass spectrometer (Thermofinnigan, San Jose, CA, USA) equipped with an API-1 source (Thermofinnigan) in electrospray mode and an HP1050 HPLC (Agilent Technologies, Palo Alto, CA, USA). The chromatographic column was a Colum-

bus 5 μm C8 reversed phase column (50 × 2.00 mm; Phenomenex, Torrence, CA, USA).

2.3. Methods

For the CFFE buffers, pH adjustments were made with acetic acid and ammonium hydroxide, respectively, after the addition of sβ-CD. The separation buffer consisted of 7.5 and 10 mM ammonium acetate (pH 4.4). The cathode was washed with 10 mM acetic acid; the anode was washed with 10 mM acetic acid adjusted with ammonium hydroxide to pH 8.8. Composition of the sheath flows, which are the buffer streams adjacent to the membrane of the separation chamber was optimized to keep the pH constant across the chamber (as measured in the effluent). The anodic sheath was 20 mM ammonium acetate while the cathodic sheath was 20 mM ammonium acetate adjusted to pH 3.8 with acetic acid. Optimization of the composition of electrode washes and sheath flows respective to the separation buffer was needed to inhibit formation of a significant pH gradient (e.g. pH 2–9) across the separation compartment [10,32]. The concentration of sβ-CD in the cathodic wash matched that of the separation buffer. All buffers used in the CFEE were filtered through a 0.45 μm nylon filter prior to use.

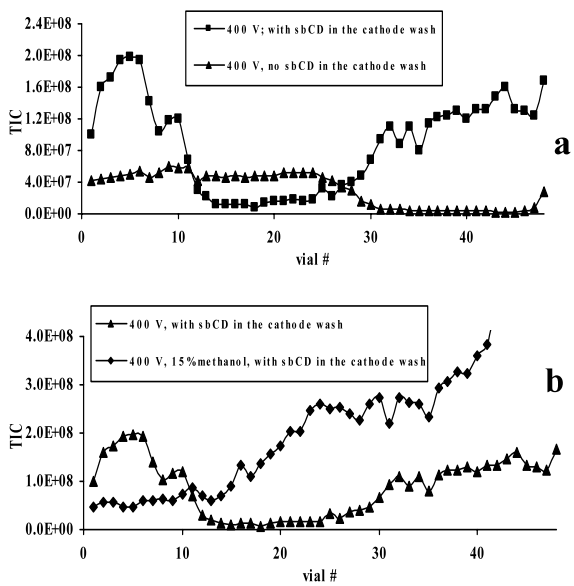

Fig. 3. Plot of total ion current (TIC), which is proportional to concentration of sβ-CD, vs. vial # at the outlets of CFFE chamber. (a) Illustrates the effect of the addition of sβ-CD into the cathode wash at 400 V, and (b) depict influence of the addition of 20% methanol in the separation buffer at 400 V with sβ-CD present in the cathode wash. Other conditions, 0.28 mM sβ-CD in the separation buffer, 1.88 mM sβ-CD in the cathode wash, buffer feed rate was 23 ml/min and sample feed rate was 0 ml/min.

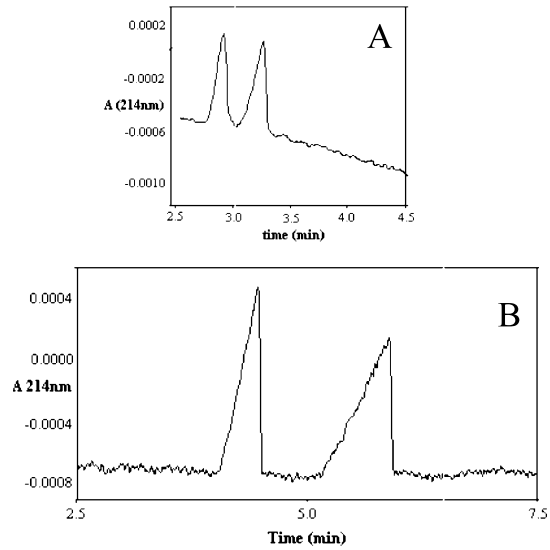

Fig. 4. Influence of methanol on chiral ritalin separation in 10 mM ammonium acetate pH 4.4 + 1% sβ-CD (A), and in 10 mM ammonium acetate pH 4.4 + 1% sβ-CD in 20% methanol (B). $U = 15$ kV, $L_{det} = 24$ cm.

For the preparation of the ritalin samples for CFFE, methanol from the standard solution (1 mg/ml) was evaporated using nitrogen. The sample was reconstituted in 8 ml of the separation buffer.

Chiral CE analysis of the CFFE fractions was accomplished using an untreated fused silica capillary (50 μm I.D., 24 cm total length, 19.6 cm to detector). The capillary was thermostated at 25 °C. The applied voltage was 17 kV, and UV detection was accomplished at the anodic end of the capillary at 214 nm. Typically, the CE run buffer contained 20% (vol) methanol, 1% sβ-CD in 7.5 mM ammonium acetate, adjusted to pH 4.2 with acetic acid. Run buffer was passed through a 0.45 μm filter prior to use in the CE. Aliquots of samples collected from the CFFE were injected hydrodynamically (20–40 psi s). Between electrophoretic runs, the capillary was rinsed with run buffer for 25 s.

For LC–MS analysis, samples were collected from the CFFE and a 10 μl aliquot injected onto a C8 reversed phase column and eluted with 75% methanol in water (v/v). The flow rate was 250 μl/min. Mass spectra were obtained in the negative ion mode scanning from 200 to 900 (m/z) in 0.5 s. The electrospray voltage was 4.5 kV and the capillary temperature was maintained at 200 °C. Nitrogen was used for both sheath and auxiliary gases. For relative quantitation of the sβ-CD in the samples, the total ion current (TIC) of the HPLC peak representing sβ-CD was used. The calibration curve was linear in the range 50–500 ppm.

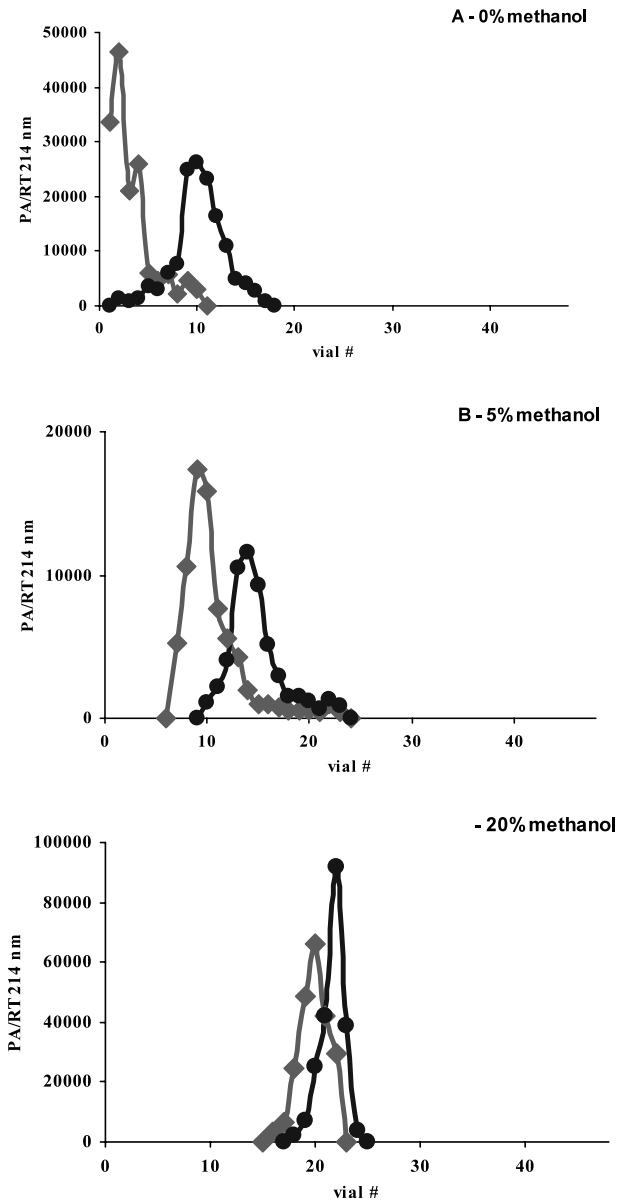

Fig. 5. Histograms of CFFE chiral ritalin separation with methanol as a buffer additive. Other conditions, buffer feed rate = 23 ml/min; sample feed rate = 0.11 ml/min; $U = 400$ V; sampling position 32; anode wash 10 mM acetic acid; cathode wash 10 mM acetic acid + NH_4OH pH 8.8 + 1.88 mM sβ-CD; separation buffer 7.5 mM ammonium acetate + 0.38 mM sβ-CD pH 4.2.

3. Results and discussion

3.1. Background

The angle of lateral deflection, Θ, of the sample stream for charged species depends on the apparent electrophoretic mobility of the solute, μ_i, the linear velocity of the electrolyte, v, and the field strength across the electrophoretic chamber (V/I), according to [11]:

$$\tan \Theta = \frac{\mu_i V}{v \times I} \quad (1)$$

For the separation of enantiomers in a buffer containing a chiral selector (e.g. cyclodextrin), to a first approximation, the difference of the angles of deflection can be written as [25].

$$\Theta_1 - \Theta_2 \approx a\left[\frac{(\mu_f - \mu_c)(K_1 - K_2)[CD]}{(1 + K_1[CD])(1 + K_2[CD])}\right] \quad (2)$$

where $a = V/vl$, μ_f is the mobility of the free solute, μ_c is the mobility of the complex, and K_1 and K_2 are the enantiomer-cyclodextrin binding constants [33].

To improve on CFEE separations, several authors have developed computer simulation and optimization programs. The simulations can predict concentration inhomogeneities of various species within the electrophoretic chamber. However, it should be noted that these models may only provide a qualitative guide for describing the electrophoretic behavior distribution of highly charged ions such as sβ-CD in CFFE [30]. For instance, many of these models do not consider specific interactions between various buffer components and the analyte.

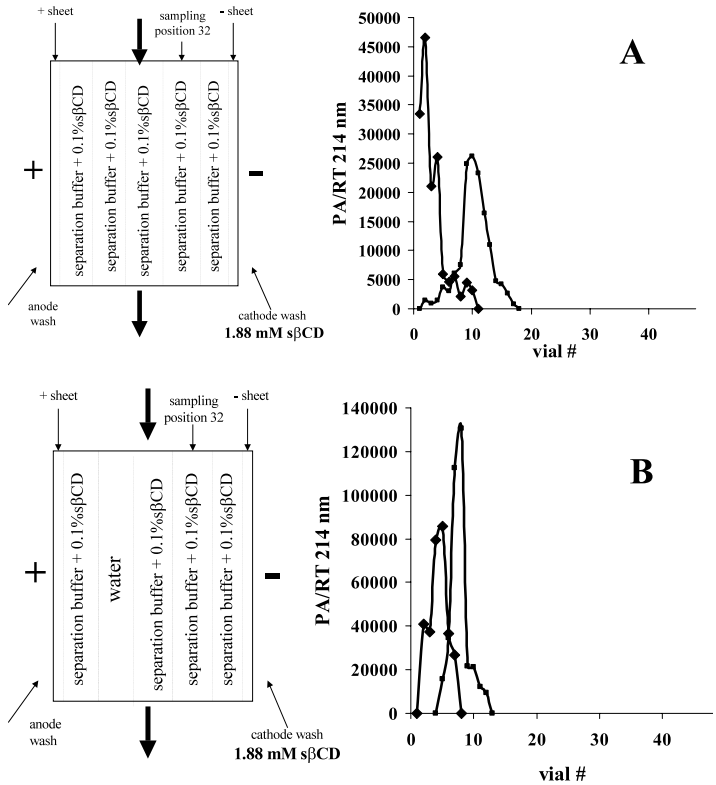

Fig. 6. Histograms of separation of ritalin enantiomers representing influence of the introduction of water into CFFE chamber. (a) No artificial depletion zones; (b) water introduced into the system. Other conditions, buffer feed rate, 23 ml/min; sample feed rate, 0.0.086 ml/min; $U = 400$ V; sampling position 32; anode wash: 10 mM acetic acid; cathode wash, 10 mM acetic acid pH 8.8 + 1.88 mM sβ-CD; separation buffer 7.5 mM ammonium acetate + 0.38 mM sβ-CD pH 4.2.

The extent (e.g. width, depth) of concentration inhomogeneities have been predicted to be dependent on several experimental conditions (e.g. electroosmosis, μs, voltage thickness of the chamber, etc.). Indeed, Stalcup and coworkers [30] noted formation of depletion and accumulation zones when using sβ-CD as a chiral additive in CFFE. Of course, these concentration inhomogeneities, regardless of the origin, would be expected to impact local field strength and conductivities, density and Joule heat gradients and likely be accompanied by a loss of separation [34–38].

3.2. Characterization of the concentration inhomogeneities of sβ-CD in the CFFE separation chamber

Although sβ-CD concentration variation across the CFFE outlets has been reported previously [30], the impact of other experimental parameters (e.g. methanol and presence of sβ-CD in the cathode wash) on the sβ-CD distribution was further investigated in this study. The concentration of sβ-CD in the individual CFFE outlets was determined with LC–MS. TIC was plotted versus vial # at the chamber outlets (Fig. 3). As reported previously, application of the electric field resulted in the formation of an accumulation zone of the sβ-CD on the anodic side of the separation chamber with a corresponding depletion zone on the cathodic side. In addition, the width of the sβ-CD depletion zone increased while the magnitude and the width of the accumulation zone decreased with increasing applied voltage, suggesting that higher voltages may overcome the membrane resistance to sβ-CD transport [30].

Fig. 3a shows the distribution of sβ-CD across the chamber at 400 V with and without sβ-CD present in the cathode wash. Although the addition of sβ-CD into the cathode wash appeared to minimize the depth of the depletion zone at the cathodic end, it did seem to induce formation of another narrower depletion zone in the center of the chamber.

In an effort to eliminate the depletion zone in the separation chamber, 15% methanol was added in to the separation buffer and the sheath flows. Concentration of methanol was chosen based on CE experiments in which the addition of 15% methanol into the CE buffer gave reasonable migration times with good resolution. The corresponding LC–MS histogram of sβ-CD concentration is shown in Fig. 3b. As can be seen from the figure, the depletion zone of sβ-CD was eliminated, likely arising from the increased viscosity of the buffer and decreased μ of sβ-CD in 15% methanol when compared with pure aqueous solutions. Nevertheless, a gradient of sβ-CD concentration across the chamber was formed.

3.3. Influence of methanol on the chiral separation

Evidence from the LC–MS experiments indicated that addition of methanol into the separation buffer, as well as into the sheath produced a fairly homogeneous concentration of sβ-CD in the chamber. It was expected that homogeneous distribution of sβ-CD would result in the most efficient chiral separation of ritalin enantiomers. Thus, the influence of methanol on the resolution of ritalin enantiomers was investigated by both CE and CFFE.

Fig. 4 shows the CE separation of ritalin enantiomers in the separation buffer with and without methanol. As can be seen in the figure, the addition of methanol into the CE buffer appears to significantly increase the resolution of ritalin

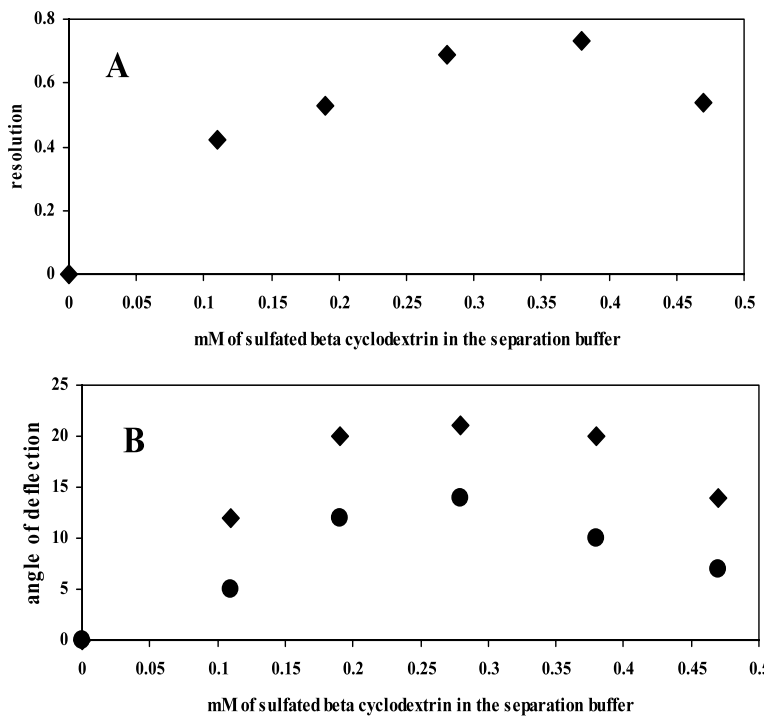

Fig. 7. Dependence of the resolution (A) and angles of deflections (B) of ritalin enantiomers on concentration of sβ-CD in the separation buffer. Other conditions, buffer feed rate 23 ml/min, sample feed rate 0.11 ml/min, $U = 400$ V, sampling position 32 (1.88 mM sβ-CD was added into the cathode wash).

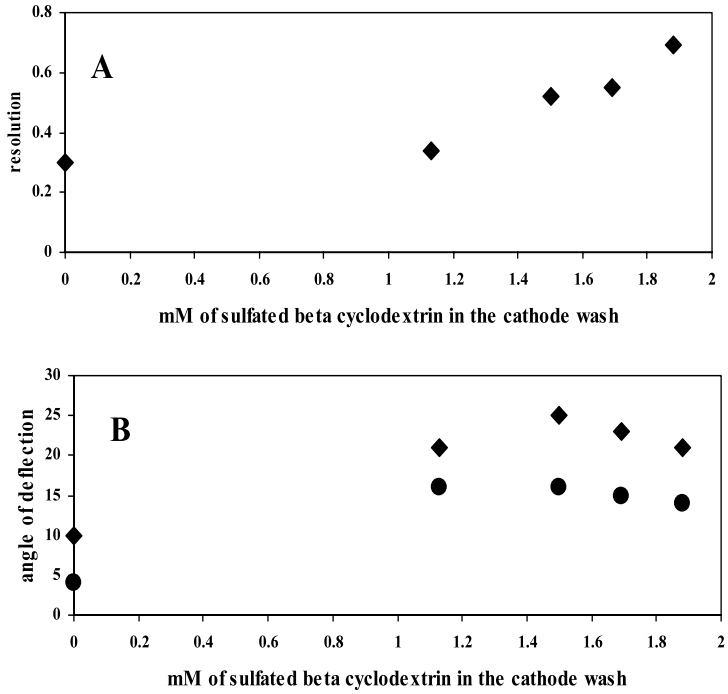

Fig. 8. Dependence of the resolution (A) and angles of deflections (B) if ritalin enantiomers on concentration of sβ-CD in the cathode wash. Other conditions, buffer feed rate 23 ml/min, sample feed rate 0.11 ml/min, $U = 400$ V, sampling position 32 (0.28 mM sβ-CD in the separation buffer).

enantiomers. This is consistent with results obtained by Gratz and Stalcup for terbutaline [39].

The ability of methanol to dampen the formation of sβ-CD accumulation and depletion zones in the CFFE and improve the chiral resolution in CE suggested that its addition into the separation buffer for CFFE might benefit the chiral separation. Fig. 5 shows the effect of methanol concentration in the CFFE separation buffer on the chiral separation of ritalin enantiomers. As the concentration of methanol in the separation buffer increased, the overall deflection and resolution of both ritalin enantiomers decreased. The reduced deflection may be partially explained by the decreased association with the anionic sβ-CD and also by the increased viscosity of the buffers containing methanol. The decreased residency time of ritalin enantiomers within the separation chamber, as a result of the decreased deflection, leads to loss of resolution as can also be seen in Fig. 5.

In carrier mode, CE with sβ-CD and little or no EOF directed toward the column inlet, inhibition of the binding through the addition of methanol leads to higher resolution. Under these conditions, ritalin enantiomers essentially see a longer column, the column residency time increases and the separation window is effectively widened leading to higher resolution [40]. However, CFFE seems more analogous to CE in non-carrier mode. Addition of methanol into the CFFE separation buffer decreases the μ of sβ-CD and ritalin enantiomers, decreases association with sβ-CD, causing reduced deflection and decreased analyte residency time in the separation chamber.

Despite the relative homogeneity of the sβ-CD distribution in the separation chamber (LC–MS), it was concluded that effects other than just homogeneity of sβ-CD influence the chiral separation.

3.4. Introduction of a low conductivity zone

To conserve sβ-CD and to decrease the overall system current, the separation buffer was replaced by water in some of the buffer inlets into the CFFE chamber and the effect on the chiral separation of ritalin was studied. Fig. 6 shows an example of the impact of the water introduction on the resolution of ritalin enantiomers. It is important to note that the schematics of the experimental set-up shown in Fig. 6 represents only experimental conditions prior to voltage application. It does not represent a true buffer distribution after the voltage has been applied, because of the electromigration of the ions (acetate, ammonium, and sβ-CD) in the system. As can be seen in Fig. 6, although the enantioresolution of ritalin decreased with the introduction of a depletion zone, the enantiomerically enriched zones were sharpened. Interestingly, with the deliberate introduction of a depletion zone, while the band having the highest affinity for sβ-CD was not deflected to the same extent as in the case where a uniform buffer system was used throughout, the weaker binding enantiomer was deflected more. Several arguments could be made explaining this observation. It is possible that water introduction lowers the current in the system and may decrease band dispersion due to the heat dissipation. In addition, the presence of a low conductivity zone could also locally amplify the field strength resulting in band sharpening in a mechanism similar to that of field amplification used in CE for sample refocusing [41].

As water introduction into the separation chamber showed some interesting and promising results, more detailed study is the subject of ongoing research.

3.5. Effects of sβ-CD concentration on chiral separation

Previously, it has been reported that an increase of sβ-CD in the separation buffer led to lower

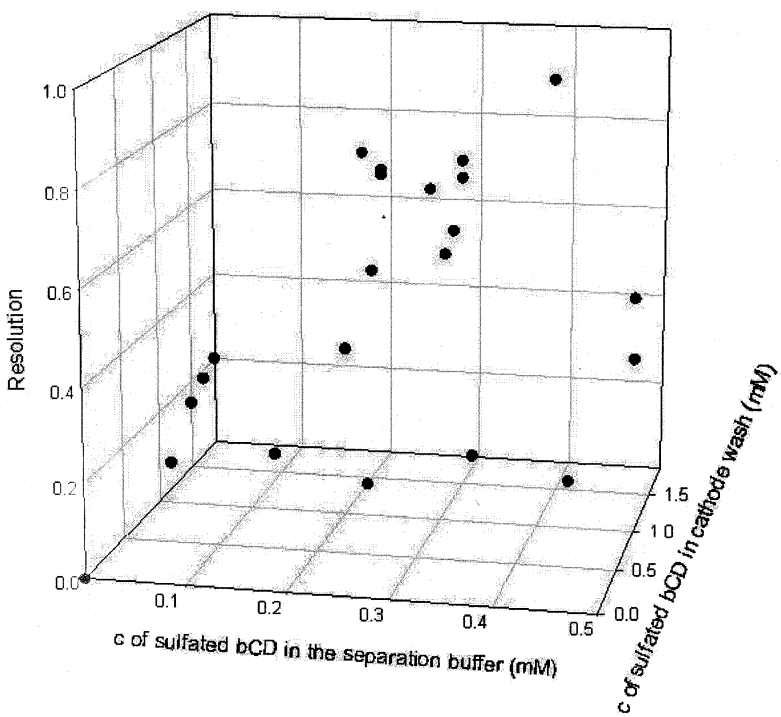

Fig. 9. Three-dimensional scatter plot of the resolution versus concentration of sβ-CD in the separation buffer and in the cathode wash. Other conditions, buffer feed rate 23 ml/min, sample feed rate, 0.086 ml/min, $U = 400$ V, sampling position 32.

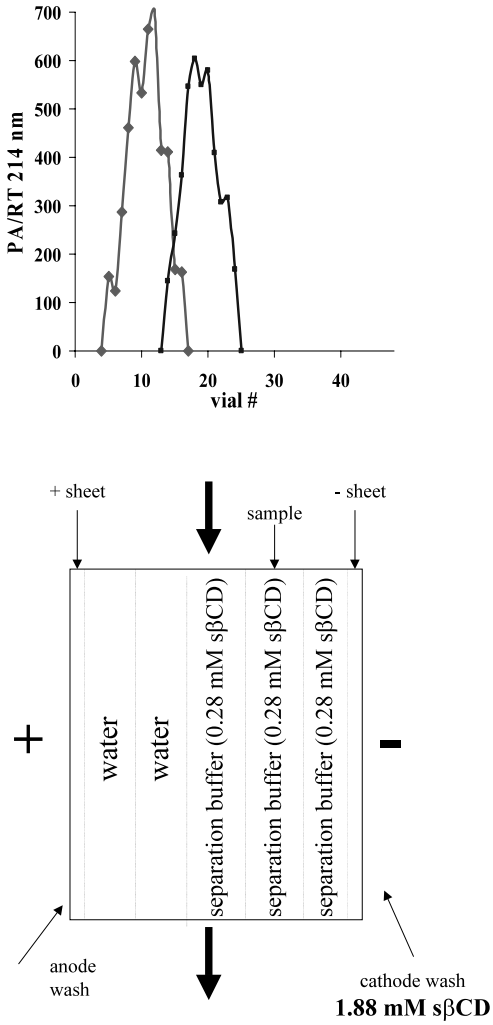

Fig. 10. CFFE chiral separation of ritalin in optimized separation conditions. Buffer feed rate = 23 ml/min; sample feed rate = 0.086 ml/min; $U = 400$ V; sampling position 32; anode wash 10 mM acetic acid; cathode wash 10 mM acetic acid + NH_4OH pH 8.8 + 1.9 sβ-CD; separation buffer 10 mM ammonium acetate + 0.28 mM sβ-CD pH 4.2.

angles of deflection and smaller resolution [29,30]. This behavior could be attributed to the formation of the depletion and accumulation zones, thereby, affecting the magnitude of the μ of free and cyclodextrin complexed molecules and the effective field strength within the separation chamber. Fig. 7a shows the dependence of the enantioresolution of ritalin on the concentration of sβ-CD in the separation buffer all other parameters held constant. Enantioresolution had a maximum value at 0.38 mM sβ-CD. A similar effect was observed for the angle of deflection, Θ; however, the angle of deflection for both enantiomers was largest at 0.28 mM sβ-CD. This plot can be found in Fig. 7b.

Fig. 8a and b show analogous representation of the influence of the concentration of sβ-CD in the cathode wash on the resolution and the angles of deflection with all other parameters held constant. It was found that enantioresolution of ritalin was not greatly affected at concentrations of sβ-CD in the cathode wash lower than 1.2 mM. However, while the resolution increased as concentration of sβ-CD increased in the cathode wash, the angles of deflection exhibited a maximum value at 1.5 mM sβ-CD. These experiments suggested that an optimum combination of a composition of the separation buffer and the cathode wash could lead to better separations.

Sixteen experiments, combining four concentrations of sβ-CD in the separation buffer with four concentrations of sβ-CD in the electrode wash, when water was introduced at the inlet ports # 5 and # 6 were performed. Fig. 9 shows a three-dimensional scatter plot of the resolution versus the concentration of sβ-CD in the separation buffer and in the cathode wash. The best resolution of ritalin enantiomers was recorded at 0.28 mM sβ-CD in the separation buffer, and 1.88 mM sβ-CD in the cathode wash, when water was introduced into buffer inlets # 5 and # 6. The histogram generated in this set of conditions is shown in Fig. 10. Under these conditions, the enantiomer with the highest affinity for the sβ-CD had an enantiomeric purity of ~84%, while the other enantiomers had an enantiomeric purity of ~82%; however, it should be noted that of the 20 vials containing ritalin, the presence of both enantiomers was only detected in three vials.

It was found that a three-dimensional plot of the dependence of ritalin enantioresolution on concentration of sβ-CD in the separation buffer and the cathode wash, respectively, had some curvature thereby confirming the interrelatedness of the various experimental parameters and complexity of this system. These additional effects will be the subject of future studies.

4. Conclusions

Chiral separation of ritalin enantiomers was optimized with implementation of several experimental approaches. Methanol appeared to decrease selectivity of sβ-CD for ritalin enantiomers and deteriorate resolution in CFFE conditions, despite buffer and sheath flows produces the most homogeneous distribution of sβ-CD. Introduction of water improved the peak widths and the peak shapes, however, resolution was partially lost. Analogous to CE experiments, the concentration of sβ-CD in the separation buffer exhibited an optimum value for the best separation. However, resolution appeared to increase with increasing concentration of sβ-CD in the cathode wash. The best resolution obtained in this study for ritalin enantiomers was about 0.8 at processing rate 0.5 mg/h.

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