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Continuous free flow electrophoresis for preparative chiral separations of piperoxan using sulfated β-cyclodextrin

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Continuous free flow electrophoresis was investigated as a tool for the preparative chiral separation of piperoxan using a sulfated cyclodextrin chiral additive. In the absence of chiral additive, the sample stream was deflected cathodically. However, the presence of sulfated cyclodextrin in the run buffer caused anodic deflection and splitting of the sample stream into two streams, each enriched in one enantiomer. Although the sulfated cyclodextrin used was comprised of a mixture of homologues and isomers, this polydispersity did not seem to significantly impact band dispersion. Sample introduction rates ranged from approximately 0.9–7.2 mg h^{-1}. Maximum resolution was 0.53, using an applied voltage of 220 V, buffer composition of 0.075% sulfated cyclodextrin, 7.6 mM citrate (pH 3), 4.5 °C.

Introduction

Separation of enantiomers is an important topic to the pharmaceutical industry. Many of the drugs marketed in the US have at least one chiral center. The biological activities and bioavailabilities of the enantiomers often differ.[1] In some cases, one enantiomer may be inert; in other cases, both enantiomers may have therapeutic value but for different disease states. For example, the (*S*)-enantiomer of verapamil is effective as a calcium channel blocker whereas the (*R*)-enantiomer produces cardiac side effects but was reported to have potential value in reversing multiple drug resistance in cancer therapy.[2]

In the case of enantiomerically pure chiral drugs, the possibility of racemization or inversion either *in vivo* (*e.g.*, ibuprofen[3]) or during storage cannot be ruled out. Thus, despite the best efforts of our very creative synthetic colleagues, chiral separations will continue to be important.

The last two decades have seen the commercialization of a large number of different types of chiral liquid chromatographic stationary phases. In particular, the field has enjoyed tremendous success exploiting stereospecific interactions of enantiomers with biologically important molecules. For instance, many chiral separations have been achieved using proteins,[4–7] 'Pirkle-type' amino acid-derived ligands (*e.g.*, *N*-(3,5-dinitrobenzoyl)-phenyl-glycine),[8,9] macrocyclic antibiotics,[10–12] cellulosic and amylosic derivatives, as well as cyclodextrins.[15,16]

However, incomplete understanding of the chiral recognition mechanisms on many chiral phases hampers effective implementation. Multimodal retention leading to nonlinear mobile phase *vs.* retention factor plots and nonlinear thermodynamic behavior[17] complicate method development. Method development is further complicated by the lack of column ruggedness, compared to conventional columns, as well as the lot-to-lot and column-to-column variability. In addition, only a limited selection of chiral TLC plates are commercially available for screening conditions (with the exception of the ligand exchange TLC plates). As a consequence, chiral column selection is often reduced to identifying structurally similar analytes for which chiral resolution methods have been reported in the scientific literature or chromatographic supply catalogues, and adapting the reported method for the chiral pair to be resolved.

Preparative chromatography[18] (prep LC) is often the method of choice for preparative chiral separations. Prep chiral LC columns are costly (~ $5K) and are usually only commercially available on a 'special order' basis. Unfortunately, scaling up an analytical chiral separation to a preparative column magnifies some of the difficulties highlighted above for analytical chiral columns and introduces new challenges. For instance, most prep chiral LC employ an organic mobile phase but many underivatized chiral drugs have only limited solubility in these organic mobile phases. As a result, sample introduction of the native compound onto preparative chiral chromatographic columns often requires that packing material be removed from the head of the column, the sample be mechanically mixed with this packing material and this mixture then returned to the top of the column.

Chiral additives have been shown to be effective for chiral separations by capillary electrophoresis (CE)[19] and offer many advantages including rapid method development. Unlike HPLC, where flow is unidirectional, CE using chiral additives can exploit counter-current migration of oppositely charged analytes and additives to amplify chiral recognition.[20] However, CE is generally more suited to analytical separations than to preparative scale separations.

Although Karger and co-workers first demonstrated the utility of cyclodextrins immobilized in a polyacrylamide gel in capillary electrophoresis for enantiomeric separations,[21] Righetti *et al.*[22] were one of the first to demonstrate the utility of classical isoelectric focusing for the chiral separation of small molecules in a slab gel configuration. In their system, dansylated amino acids were enantiomerically resolved through complexation with β-cyclodextrin.

Stalcup *et al.*[23] also reported that chiral analytes could be enantiomerically resolved using classical gel electrophoresis while offering many of the same advantages as capillary electrophoresis. Indeed, CE was synergistically used in the

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method development of a classical gel electrophoretic chiral separation of piperoxan.[24] A continuous elution mini-prep electrophoretic apparatus was interfaced to an HPLC detector. In this work, baseline resolution was obtained for 0.5 mg of piperoxan.

While classical gel electrophoresis does allow the results obtained with CE to be scaled up, it is a batch process and therefore limited in sample throughput. Truly 'preparative' processes are ones in which a sample feed stream is continuously introduced while purified sample product streams are continuously produced.

Preparative continuous free flow electrophoresis (CFFE), first reported in 1958,[25] translates the tremendous resolving power of electrophoresis into a continuous feed process. In preparative continuous free flow electrophoresis, employing continuous buffer and sample feed, the buffer streams are introduced at several ports across one end of a thin, rectangular electrophoresis chamber to produce a buffer 'curtain' while the sample stream is introduced at a single port on the same end. At the same time, an electric field is imposed perpendicular to the flow (Fig. 1). Differential interaction between the various solutes and the applied electric field produce a lateral displacement of the individual analytes between the two electrodes. Individual sample components are collected at various locations positioned across the end of the chamber opposite to sample introduction. It is important to note that these separations are accomplished in free solution. For an extensive recent review of continuous free flow electrophoresis, the reader is directed to Křvávková and Boček[26]

Of course, the separations in these systems rely upon the flow stability in the electrophoretic chamber. Density and pH gradients, cooling and performance in microgravitational environments (*e.g.*, the space shuttle),[27] have all been used in an attempt to minimize the effects of heat generated in electrophoretic separations that destabilized the buffer and sample stream stabilities. Recent innovations in the design of a continuous free flow electrophoresis apparatus[28] have circumvented the heat dissipation and sample stream distortion inherent in most of the previous designs.

As in the case of classical gel electrophoresis, most of the separations obtained by CFFE have been in the purification of biopolymers. A CFFE system used for the separation of biopolymers (*e.g.*, ovalbumin and lysozyme) as well as smaller inorganic species[29] (*e.g.*, $[Co^{III}(sepulchrate)]^{3+}$ and $[Co^{III}(CN)_6]^{3-}$) reported sample processing rates of 15 mg h^{-1} for a mixture of Amaranth (M_r: 804) and Patent Blue VF (M_r: 1159).[30]

To effect the separation of enantiomers by preparative electrophoresis requires, as in CE, the addition of an auxiliary chiral selector to the buffer 'curtain'. Thormann and coworkers[31] used 2-hydroxypropyl-β-cyclodextrin as a neutral additive for the enantiomeric enrichment of methadone in two different recycling free-flow instruments using chiral recycling isotachophoresis in a batch process. Subsequent work[32] by the same group with an Octopus continuous free flow electrophoresis apparatus using both zone and isotachophoretic

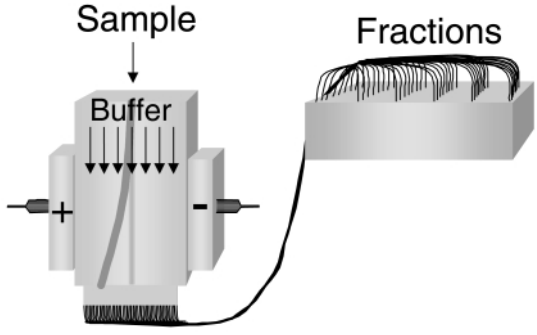

Fig. 1 Schematic of continuous free flow electrophoresis apparatus.

electrophoresis reported processing rates of the order of 10–20 mg h^{-1}. More recently, Glukhovskiy and Vigh reported the use of 2-hydroxypropyl-β-cyclodextrin as an additive in CFFE for the preparative chiral separation of dansyl phenylalanine enantiomers by isoelectric focusing.[33] In this work, they exploited the fact that the association between the cyclodextrin and the dansylamino acid shifted the pK_a of the acid.

In principle, any chiral selector and solute amenable to enantiomeric separations by capillary electrophoresis may be a candidate for CFFE. Because of experience gained through the preparative gel work reported previously, the general focus of this work was the use of sulfated cyclodextrins for the enantiomeric resolution of piperoxan. A more long-term goal of this project is to obtain pure enantiomers with subsequent recovery and reuse of the chiral additive. Thus, conditions for this initial study were chosen to minimize post-CFFE clean-up. System parameters investigated include the impact of the concentration of the chiral additive as well as the buffer concentration.

Experimental

Materials

The sulfated cyclodextrin (SCD; nominal degree of substitution ≈ 15) was obtained from Cerestar, Inc. (Hammond, IN, USA). All experiments were conducted using cyclodextrin from the same lot. Sodium phosphate monobasic, phosphoric acid and citric acid monohydrate was obtained from Fisher Scientific (Pittsburgh, PA, USA) and used as is. Piperoxan was obtained from Sigma Chemical Company (St. Louis, MO, USA). Distilled, deionized water (Barnstead Dubuque, IA, USA) was used in all experiments. Patent Blue VF was used as an electroosmotic flow (EOF) indicator.

Equipment

The preparative CFFE used in this study is a prototype instrument on loan from R & S Technology (Wakefield, RI, USA). In the CFFE apparatus, the electrophoresis chamber is approximately 8 cm in width, 14 cm in length and 3 cm in depth. The buffer occupies the interstitial volume between closely aligned capillary Teflon tubing, oriented parallel to the buffer flow. Cooling water is pumped through these Teflon tubes while the separation is performed in the spaces between the capillaries. The cooling allows fairly high voltages (*e.g.*, 260 V with currents of ≈ 250 mA) to be well tolerated.

In the apparatus used in this study, with the electrophoretic chamber oriented in a vertical position, there are seven ports for the continuous introduction of buffer and three sample ports (only one used at a time) for the introduction of the sample stream into the top of the chamber. At the bottom of the chamber, there are forty-eight ports which are connected through Teflon tubing to an array of forty-eight sample receptacles. Vial 1 corresponded to the anodic edge of the chamber. In the present study, the sample was always introduced through the sample port midway between the electrodes. This position corresponds to sample vial number 24, for a sample with no intrinsic electrophoretic mobility.

The electrodes, which are isolated from the electrophoresis chamber by a membrane and extend the entire length of either side of the electrophoresis chamber, are continually washed with fresh buffer. The constant exchange of the electrode buffers allows longer run times using buffers with lower ionic strength (*e.g.*, 5–10 mM) than are typically encountered in CE (*e.g.*, 50–100 mM). The various buffer and sample streams are pumped using peristaltic pumps. Typical flow rates for the

buffer were ≈26 mL min^{-1} while the sample flow rate was on the order of 0.1 mL min^{-1}.

Bio-Rad (Richmond, CA, USA) Biofocus 3000 and Biofocus 2000 capillary electrophoresis systems with computer control were used for chiral analysis of an aliquot of each fraction collected from the CFFE instrument.

Methods

It should be noted that different lots of sulfated cyclodextrins with differing degrees of substitution obtained from different vendors can affect the pH of aqueous solutions to different extents. Thus, pH adjustments must be made after the addition of the cyclodextrin. The citric acid concentration varied from 6.7 to 8.6 mM. All buffers used in the CFFE were filtered through a 0.45 μm nylon filter prior to use. The sequence used in running the instrument is given in Table 1. Details of the various buffer compositions used in the CFFE experiments are presented along with the results.

Chiral CE analysis of the individual fractions collected from the CFFE was accomplished using an untreated fused silica capillary (50 μm id, 24 cm total length, 19.6 cm to detector). The capillary was thermostatted at 20 °C. The applied voltage was 8 kV and UV detection (λ = 214 nm) was accomplished at the anodic end of the capillary. Aliquots of samples collected from the CFFE were injected hydrodynamically (2 psi s^{-1}). After each run, the capillary was rinsed with run buffer for 60 s.

Typically, the CE run buffer contained 2% sulfated cyclodextrin and 10 mM sodium phosphate buffer, adjusted to pH 3.0 with phosphoric acid. Run buffer was passed through a 0.2 μm filter and degassed prior to use in the CE. The data was used to generate a histogram of the enantiomeric distribution in the sample vials.

Results and discussion

Theory

In preparative continuous free flow electrophoresis, the differential interaction between the various solutes and the electric field produce a lateral displacement of the individual analytes between the two electrodes. The angle of the deflection, Θ, of the solute in the electric field depends on the apparent or intrinsic electrophoretic mobility of the solute, μ_i, the linear velocity of the buffer, v, and the current through the chamber, i, and can be described as

$$\tan\Theta = \frac{\mu_i i}{q\kappa v} \quad (1)$$

where q is the cross section of the separation chamber and κ is the specific conductance of the buffer.[26] Substituting the conductance, $G = \kappa l/q = 1/R = i/V$ for κ yields

$$\tan\Theta = \frac{\mu_i V}{lv} \quad (2)$$

where V/l is the field strength.

Table 1 CFFE operation sequence

Time/min	Operation
0–10	Begin pumping buffer; no applied voltage
10–20	Begin pumping sample; no applied voltage
20–23	Apply initial voltage (typically ≈60 V)
23–30	Increase voltage stepwise to final operating voltage
50	Begin fraction collection

For the separation of enantiomers, we are interested in $\Theta_1 - \Theta_2$. Using the expression relating the apparent mobility of an analyte to its binding constant with an additive and the concentration of the additive, substituting $a = i/q\kappa v$ or V/lv and using a series expansion of tan Θ, it can be shown that, to a first approximation, the difference in the angle of deflection for the two enantiomers can be expressed as[34]

$$\Theta_1 - \Theta_2 \approx a(\mu_1 - \mu_2) \quad (3)$$

$$\Theta_1 - \Theta_2 \approx a\left[\frac{(\mu_{f1} + \mu_{c1})K_1[\mathrm{CD}]}{1 + K_1[\mathrm{CD}]} - \frac{(\mu_{f2} + \mu_{c2})K_2[\mathrm{CD}]}{1 + K_2[\mathrm{CD}]}\right] \quad (4)$$

where the subscripts f and c refer to the free and complexed analyte, respectively, and the numbered subscripts refer to the two enantiomers, 1 and 2. Because the mobilities of the free enantiomers are the same and assuming, to a first approximation, that the mobilities of the complexes formed by each of the enantiomers with the cyclodextrin are the same, eqn. (4) can be rearranged to:

$$\Theta_1 - \Theta_2 \approx a\left[\frac{(\mu_f - \mu_c)(K_1 - K_2)[\mathrm{CD}]}{(1 + K_1[\mathrm{CD}])(1 + K_2[\mathrm{CD}])}\right] \quad (5)$$

which is virtually the same expression as derived by Wren and Rowe[35] for CE. Eqn. (5) predicts that, as in CE, separation depends upon differences in the mobilities of the free and complexed analyte as well as differences in the binding constants, mediated by the dimensions of the chamber and the specific conductance and linear velocity of the buffer. Eqn. 4 also implies that there is an optimum cyclodextrin concentration, as predicted for CE using chiral additives by Wren and Rowe.

As can be seen from this equation, several factors need to be considered when optimizing the electrophoretic conditions. For instance, closer inspection of the constant reveals that a high linear buffer velocity is deleterious to the separation. In essence, the linear velocity of the buffer in CFFE is analogous to detector-directed electroosmotic flow in capillary electrophoresis but projected orthogonal to the electrophoretic separation. Eqn. (5) also predicts better separations at higher field strengths.

The impact of various experimental parameters on the CFFE separation of piperoxan is detailed in the following sections.

Effect of presence of chiral additive on distribution of piperoxan

As the analyte moves through the electrophoresis chamber, the sample stream is subject to dispersion. In addition to instrumental sources of dispersion and dispersion arising from the diffusion of the solute away from the sample stream, an additional source of sample stream dispersion may arise from the polydispersity of the chiral additive. Most commercially available derivatized cyclodextrins, including the material used in this study, are complex mixtures of homologues and isomers. Each of the isomers and homologues may have different affinities for the piperoxan enantiomers. In addition, anionic cyclodextrins with different degrees of substitution should have different electrophoretic mobilities, thus contributing to sample band dispersion.

Fig. 2a illustrates the histogram obtained for piperoxan in the absence of sulfated cyclodextrin in the buffer stream; Fig. 2b illustrates the sample distribution for the two enantiomers of piperoxan in the presence of sulfated cyclodextrin. As can be seen from the histograms, the presence of the sulfated cyclodextrin does not seem to significantly aggravate the sample stream dispersion.

Cyclodextrin concentration

As can be seen from Fig. 2, in the absence of cyclodextrin, the piperoxan peak maxima are shifted toward the cathodic end of the chamber (vial no. > 24) whereas, in the presence of the cyclodextrin, the piperoxan enantiomers are shifted toward the anode (vial no. < 24). Repeat experiments yielded the same results.

Presumably, as the concentration of sulfated cyclodextrin increases, both the peak maxima for both piperoxan enantiomers should be increasingly shifted toward the anode (smaller vial number) as the apparent electrophoretic mobility of the cationic piperoxan becomes more dominated by association with the polyanionic cyclodextrin. However, as can be seen in Table 2, this does not seem to be the case. One possible explanation for the shifts of the peak maxima back toward the center vial at higher concentrations of sulfated cyclodextrin may be the result of inadequate heat dissipation from the higher currents obtained at higher concentrations of sulfated cyclodextrin. In CE, insufficient cooling is often revealed by nonlinearity in current *vs.* applied voltage plots. Fig. 3 shows the relationship between current and applied voltage in the CFFE for a buffer containing 0.075% sulfated cyclodextrin. The currents obtained were consistent with the currents found in the CFFE using the various concentrations of sulfated cyclodextrin. The linearity obtained suggests that insufficient cooling does not seem to be a problem in this system under these conditions.

To determine if the buffer adequately compensated for possible pH changes during the electrophoretic run, the pH was measured in each of the collection vials (data not shown); however, no significant differences in pH were observed between the vials at the buffer flow rates used in these experiments.

It should be noted that the shift of the peak maxima back towards the center suggests that either the association between the cyclodextrin and the analyte is reduced at higher cyclodextrin concentrations or that the complex is experiencing a diminished effective electric field strength. Accumulation of the highly anionic sulfated cyclodextrin on the membrane separating the anode and anodic electrode buffer stream from the bulk electrophoresis chamber could produce a potential opposing the applied electric field. The extent of accumulation should be cyclodextrin concentration-dependent as well as dependent on membrane thickness. Indeed, preliminary results obtained using thicker membranes showed an even more dramatic reduction in both apparent field strength (*e.g.*, position of peak maxima) and reduced separation. Additional preliminary results obtained using just the citrate buffer in the buffer stream nearest the anode but inside the electrophoresis chamber showed some improvement in peak shape.[34] Further studies are on-going.

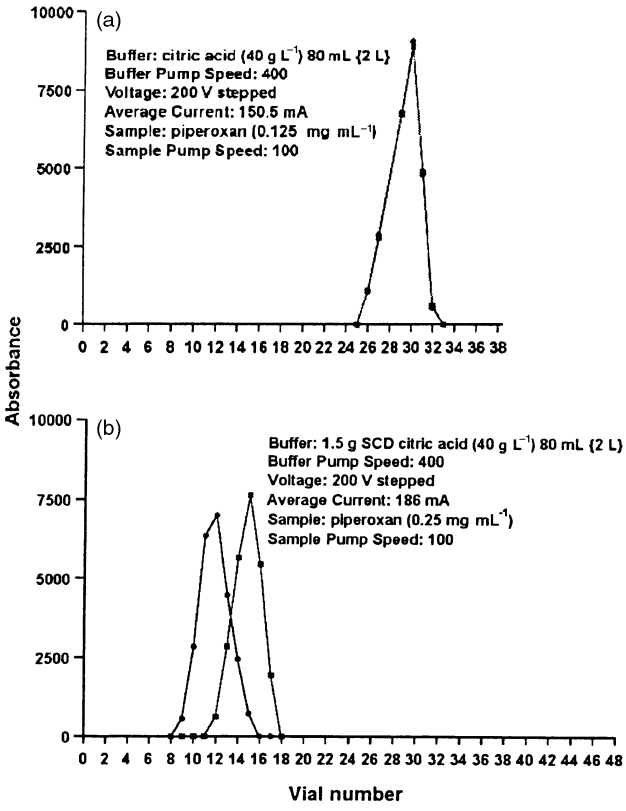

Fig. 2 Histograms showing the distribution of piperoxan enantiomers in the absence (a) and presence (b) of sulfated cyclodextrin in fractions collected from CFFE.

Table 2 CFFE data collected at various SCD concentrations[a]

SCD concentration (%)	$Vial_{max}$ (1)	$Vial_{max}$ (2)	Peak width (1) (no. of vial)	Peak width (2) (no. of vial)	Current/mA	R_s
0.050	11	15	12	12	173.0	0.36
0.075	12	15	9	8	186.0	0.40
0.10	13	15	11	11	203.4	0.20
0.15	16	18	9	8	226.6	0.27
0.20	18	20	11	9	250.6	0.22

[a] Experimental conditions: temperature, 4.5 °C; buffer composition, 7.6 mM citric acid, varied SCD; voltage applied, 200 V; sample, 0.25 mg mL^{-1} piperoxan; buffer pump speed, 26 mL min^{-1}; sample pump speed, 0.12 mL min^{-1}.

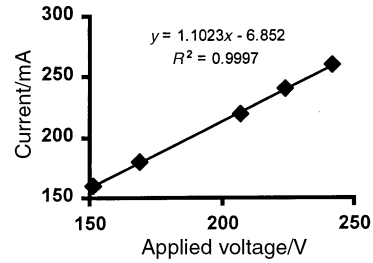

Fig. 3 Plot of current *vs.* voltage obtained in CFFE using 0.075% SCD, 7.6 mM citric acid (pH 3).

Table 3 CFFE data collected for variable piperoxan concentration experiments[a]

Piperoxan/mg mL^{-1}	Sample feed rate/mg h^{-1}	$Vial_{max}$ (1)	$Vial_{max}$ (2)	Peak width (1) (no. of vial)	Peak width (2) (no. of vial)	Current/mA	R_s
0.125	0.9	11	12, 13	9	8	186.0	0.27
0.250	1.8	12	15	9	8	186.0	0.40
0.500	3.6	13	17	11	11	186.3	0.40
1.000	7.2	19	24	19	16	184.2	0.29

[a] Experimental conditions: temperature, 4.5 °C; buffer composition, 0.075% SCD, 7.6 mM citric acid; voltage applied, 200 V; buffer pump speed, 26 mL min^{-1}; sample pump speed, 0.12 mL min^{-1}.

Piperoxan concentration

The impact of solute concentration was also assessed (Table 3). At 0.5 mg mL^{-1} and below, the distribution of the enantiomers was fairly constant but at 1 mg mL^{-1}, there was a significant increase in the number of vials across which the sample was distributed, thus suggesting a possible overload of the system using these conditions.

EOF

As in the previous gel and capillary work, low pH was used to minimize EOF. Indeed, no EOF was observed (*e.g.*, visual inspection of sample stream obtained using Patent Blue VF, a zwitterionic dye molecule) in this system. It should be noted, however, that EOF should be less in this system at any pH than is observed in uncoated fused silica capillaries employed in capillary electrophoresis because most of the internal surface area of the electrophoretic chamber is comprised of Teflon cooling tubes which typically engenders much less EOF than silica surfaces. In addition, the Teflon tubes are aligned parallel to the buffer flow and perpendicular to the applied electric field; therefore, most of the surface area of the Teflon tubing is discontinuous in the direction of the applied electric field.

Buffer and sample flow velocities

Data in Tables 4 and 5 was obtained using different flow rates for the buffer (Table 4) and sample stream (Table 5). As predicted in eqn. (1), higher buffer flow velocities produced slightly lower deflections (*e.g.*, larger $vial_{max}$ number) for both sample streams. However, the best resolution was obtained at the intermediate flow rate. It may well be that solute diffusion becomes an issue at low buffer flow rates but that higher flow rates diminish the opportunity for chirally selective interactions with the chiral selector. Given the relative flow rates of the sample and buffer streams, the sample flow rate should not significantly impact the separation. Indeed, as can be seen in Table 5, peak widths and positions appear to be very insensitive to sample stream flow rate.

Method robustness and reproducibility

Table 6 lists data obtained using three slightly different concentrations of citric acid. As can be seen from the data, the concentration of the citric acid appeared to have little effect on either the position of the sample peak maxima or on the enantioresolution which is desirable for method robustness.

Results are presented in Table 7 from data that was collected on three separate days to aid in assessing the method robustness. Between Day 2 and Day 22, many other experiments were run using a variety of conditions (*e.g.*, various buffer and chiral additive concentrations) and yet, as can be seen from the data, the results are entirely consistent not only with regard to current but also to peak maxima position, peak widths and resolution. In principle, changing system variables (*e.g.*, pH, ionic strength, additive concentration) should be very easy in the CFFE instrument because all the interactions take place in free solution. In addition, there is no 'stationary phase' to be prequilibrated as in chromatography. Indeed, CFFE did not seem to be plagued with the 'wall effects' typically observed in capillary electrophoresis.

Conclusions

Clearly, chiral separations, particularly preparative, present such a challenging problem that no single technology can completely satisfy. Although still in its infancy, preparative chiral free flow electrophoresis may represent an important technological advance in chiral separations and has the potential to complement preparative chiral chromatographic methods as well as chiral CE complements chiral analytical chromatography. In principle, any chiral solute amenable to enantiomeric separations by capillary electrophoresis may be a candidate for CFFE.

Although the sulfated cyclodextrin used was comprised of a mixture of homologues and isomers, this polydispersity did not seem to significantly impact band dispersion for the piperoxan enantiomers. Results obtained on separate days were entirely consistent with regard to peak width, peak position and resolution. Thus, continuous free flow electrophoresis may offer a viable alternative to preparative chiral LC for the chiral resolution of enantiomers.

Table 4 CFFE data collected for varying buffer pump speeds[a]

Buffer pump speed/mL min^{-1}	$Vial_{max}$ (1)	$Vial_{max}$ (2)	Peak width (1) (no. of vial)	Peak width (2) (no. of vial)	Current/mA	R_s
20	10	14	11	13	181.0	0.36
26	12	15	9	8	186.0	0.40
33	13	16	11	11	184.0	0.30

[a] Experimental conditions: temperature, 4.5 °C; buffer composition, 0.075% SCD, 7.6 mM citric acid; voltage applied, 200 V; sample pump speed, 0.12 mL min^{-1}; sample, 0.25 mg mL^{-1} piperoxan.

Table 6 CFFE data from variable citric acid concentration experiments[a]

Citrate concentration/mM	$Vial_{max}$ (1)	$Vial_{max}$ (2)	Peak width (1) (no. of vial)	Peak width (2) (no. of vial)	Current/mA	R_s
6.7	12	15	11	9	187.1	0.33
7.6	12	15	9	8	186.0	0.40
8.6	12	15	10	11	195.0	0.32

[a] Experimental conditions: temperature, 4.5 °C; buffer composition, 0.075% SCD, varied citric acid; voltage applied, 200 V; sample, 0.25 mg mL^{-1} piperoxan; buffer pump speed, 26 mL min^{-1}; sample pump speed, 0.12 mL min^{-1}.

Table 5 CFFE data collected at varying sample pump speeds[a]

Sample pump speed/mL min^{-1}	Sample fee rate/mg h^{-1}	$Vial_{max}$ (1)	$Vial_{max}$ (2)	Width (1) (no. of vial)	Width (2) (no. of vial)	Current/mA	R_s
0.073	1.1	11	13, 14, 15	9	9	188.6	0.25
0.097	1.5	11	14	6	9	188.7	0.46
0.121	1.8	12	15	9	8	186.0	0.40
0.145	2.2	12	15	11	9	185.4	0.33
0.170	2.6	12	16	11	9	185.6	0.44

[a] Experimental conditions: temperature, 4.5 °C; buffer composition, 0.075% SCD, 7.6 mM citric acid; voltage applied, 200 V; buffer pump speed, 26 mL min^{-1}; sample, 0.25 mg mL^{-1} piperoxan.

Table 7 CFFE data collected for method robustness and variable voltage experiments[a]

Run ID	Voltage/V	$Vial_{max}$ (1)	$Vial_{max}$ (2)	Peak width (1) (no. of vial)	Peak width (2) (no of vial)	Current/mA	R_s
Day 1	200	12	15	9	8	186.0	0.40
Day 2	200	12	15	10	9	184.7	0.35
Day 22	200	12	15	9	10	186.0	0.35
Day 22	200	12	15	10	8	189.2	0.38
Day 22	200	12	15	10	8	189.2	0.38
Day 2	160	13	16	10	9	151.4	0.35
Day 2	180	12	15	11	9	169.0	0.33

[a] Experimental conditions: temperature of electrophoretic chamber, 4.5 °C; buffer composition, 0.075% SCD, 7.6 mM citric acid; sample, 0.25 mg mL^{-1} piperoxan at 0.12 mL min^{-1}; buffer pump speed, 26 mL min^{-1}; sample pump speed, 0.12 mL min^{-1}.

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References

1 W. D. Hooper and M. S. Qing, *Clin. Pharmacol. Ther.*, 1990, **48**, 633.
2 S. C. Stinson, *Chem. Eng. News*, 1993, September 27, 38.
3 W. J. Wechter, D. G. Loughhead, R. J. Reischer, G. J. Van Giessen and D. G. Kaiser, *Biochem. Biophys. Res. Commun.*, 1974, **61**, 833.
4 J. Hermansson and M. Eriksson, *J. Liq. Chromatogr.*, 1986, **9**, 621.
5 G. Schill, I. W. Wainer and S. A. Barkin, *J. Liq. Chromatogr.*, 1986, **9**, 641.
6 S. Allenmark, *J. Liq. Chromatogr.*, 1986, **9**, 425.
7 M. Okamoto and H. Nakazawa, *J. Chromatogr.*, 1990, **508**, 217.
8 W. H. Pirkle and T. C. Pochapsky, *Adv. Chromatogr.*, 1987, **27**, 73.
9 W. H. Pirkle and T. C. Pochapsky, *J. Am. Chem. Soc.*, 1986, **108**, 352.
10 D. W. Armstrong, Y. Tang, S. Chen, Y. Zhou, C. Bagwill and J. R. Chen, *Anal. Chem.*, 1994, **66**, 1473.
11 A. Berthold, Y. Liu, C. Bagwill and D. W. Armstrong, *J. Chromatogr.*, 1996, **731**, 123.
12 D. W. Armstrong, Y. Liu and K. H. Ekborgott, *Chirality*, 1995, **7**, 474.
13 R. Isaksson, P. Erlandsson, L. Hansson, A. Holmberg and S. Berner, *J. Chromatogr.*, 1990, **498**, 257
14 Y. Okamoto, R. Aburatani, K. Hatano and K. Hatada, *J. Liq. Chromatogr.*, 1988, **11**, 2147.
15 S. M. Han and D. W. Armstrong, in *Chiral Separations by HPLC*, ed. A. M. Krstulovic, John Wiley & Sons, New York, 1989, pp. 208–287.
16 D. W. Armstrong, T. J. Ward, R. D. Armstrong and T. E. Beesley, *Science*, 1986, **232**, 1132.
17 S. Jönsson, A. Schön, R. Isaksson, C. Pettersson and G. Pettersson, *Chirality*, 1992, **5**, 505.
18 E. Küsters, *Chim. Oggi*, 1996, **14**, 39.
19 T. J. Ward, *Anal. Chem.*, 1994, **66**, 632A.
20 S. R. Gratz and A. M. Stalcup, *Anal. Chem.*, 1998, **70**, 5166.
21 A. Guttman, A. Paulus, A. S. Cohen, N. Grinberg and B. L. Karger, *J. Chromatogr.*, 1988, **448**, 41.
22 P. G. Righetti, C. Ettori, P. Chafey and J. P. Wahrmann, *Electrophoresis*, 1990, **11**, 1.
23 A. M. Stalcup, K. H. Gahm, S. R. Gratz and R. M. C. Sutton, *Anal. Chem.*, 1997, **70**, 144.
24 R. M. C. Sutton, S. R. Gratz and A. M. Stalcup, *Analyst*, 1998, **123**, 1477.
25 M. C. Roman and P. R. Brown, *Anal. Chem.*, 1994, **68**, 86A.
26 L. Křivávková and P. Boček, *Electrophoresis*, 1998, **19**, 1064.
27 R. E. Allen, P. H. Rhodes and R. Snyder, *Sep. Purif. Methods*, 1997, **6**, 1.
28 Y. Tarnopolsky, US Patent, 5 104 505, 1992.
29 M. E. Ketterer, G. E. Kozerski, R. Ritacco and P. Painuly, *Sep. Sci. Technol.*, 1997, **32**, 641.
30 Y. Tarnopolsky, M. C. Roman and P. R. Brown, *Sep. Sci. Technol.*, 1993, **28**, 719.
31 M. Lanz, J. Caslavska and W. Thormann, *Electrophoresis*, 1998, **19**, 1081.
32 P. Hoffmann, H. Wagner, G. Weber, M. Lanz, J. Caslavska and W. Thormann, *Anal. Chem.*, 1999, **71**, 1840.
33 P. Glukhovskiy and G. Vigh, *Anal. Chem.*, 1999, **71**, 3814.
34 G. Weber and J. Bauer, *Electrophoresis*, 1998, **19**, 1104.
35 S. A. C. Wren and R. C. Rowe, *J. Chromatogr.*, 1993, **635**, 113.