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Use of full-column imaging capillary isoelectric focusing for the rapid determination of the operating conditions in the preparative-scale continuous free-flow isoelectric focusing separation of enantiomers☆

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Abstract

A rapid, simple method is proposed here for the identification of the experimental conditions that lead to satisfactory preparative-scale isoelectric focusing enantiomer separations in continuous free-flow electrophoretic units. The method first calls for the use of a commercially available, full-column imaging capillary electrophoretic system to find the background electrolyte composition that generates the largest p*I* difference between the bands of the enantiomers. The method then calls for the finding of the minimum residence time that permits full development of the pH gradient across the separation chamber of the continuous free-flow electrophoretic unit by measuring the pH in the sample-free carrier electrolyte fractions collected during these runs. Finally, the quality of the predicted preparative-scale separation is verified by analyzing the enantiomer-containing collected fractions by capillary electrophoresis using a 14-sulfated, single-isomer cyclodextrin as resolving agent. The p*I* difference values and production rate values observed in this work agree well with the literature values that were obtained by much more time-consuming methods.

Keywords: Enantiomer separation; Full-column imaging capillary isoelectric focusing; Isoelectric focusing; Continuous free-flow isoelectric focusing; Dansyl tryptophan

1. Introduction

In the last decade, capillary electrophoresis (CE) became a very widely used method for the separation of enantiomers [1]. As the most recent review of the field indicates, its success as a powerful analytical tool also stimulated interest in the use of electrophoresis for the preparative-scale separation of enantiomers [2]. Preparative-scale electrophoretic separations have been achieved in the zone-electrophoretic mode with continuous free-flow electrophoretic devices using noncharged cyclodextrins [3], single-isomer sulfated cyclodextrins [4,5] and randomly sulfated cyclodextrins [6,7], and in the isoelectric focusing (IEF) mode using noncharged cyclodextrins, both in a continuous free-flow electrophoretic system [8], and in an isoelectric membrane-based multicompartmental electrolyzer [9]. The operating principles of the continuous free-flow electrophoretic system [10] and the multicompartmental analyzer [11] have been well described in the literature.

IEF is especially attractive for the preparative-scale separation of enantiomers because the focusing

☆Dedicated to the memory of Professor J.F.K. Huber.

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mechanism can mitigate, at least partially, the flow-related band broadening effects. The first analytical-scale IEF separation of enantiomers was achieved in a polyacrylamide slab gel (acting as an anticonvective medium) with β-cyclodextrin (CD) dissolved in a mixture of carrier ampholytes [12]. Later on, enantiomer separations have been obtained by capillary IEF using carrier ampholytes [8,13] and binary Bier's buffers [8] with noncharged cyclodextrins as chiral resolving agents. Based on a theoretical analysis of the pertaining multiple chemical equilibria, an analytical expression was obtained in Ref. [8] to describe the magnitude of the pI difference (ΔpI) that develops between the band centroids of the two enantiomers, RH and SH, in the presence of a noncharged chiral resolving agent, such as a noncharged CD:

$$\Delta \mathrm{p}I'_{\mathrm{R,S}} = \frac{1}{2}\log\left(\frac{1+K_{\mathrm{S}^-\mathrm{CD}}[\mathrm{CD}]}{1+K_{\mathrm{R}^-\mathrm{CD}}[\mathrm{CD}]}\,\frac{1+K_{\mathrm{H}^+\mathrm{RHCD}}[\mathrm{CD}]}{1+K_{\mathrm{H}^+\mathrm{SHCD}}[\mathrm{CD}]}\right) \quad (1)$$

where $K_{\mathrm{R}^-\mathrm{CD}}$, $K_{\mathrm{S}^-\mathrm{CD}}$, $K_{\mathrm{H}^+\mathrm{RHCD}}$, and $K_{\mathrm{H}^+\mathrm{SHCD}}$ are the complexation constants for the formation of the anionic complexes of the two enantiomers (R^-CD and S^-CD) and the cationic complexes of the two enantiomers (H^+RHCD and H^+SHCD), and [CD] is the species concentration of the free, noncharged cyclodextrin. Thus, if the respective K values were known, one could calculate the resulting ΔpI values and predict the optimum separation conditions. Rizzi et al. [14] carried out extensive, elegant capillary electrophoretic measurements to obtain such K values and confirm the correctness of Eq. (1). As an alternative, Glukhovskiy et al. developed a pressure-mediated capillary electrophoretic (PreMCE) method [15] to determine the pI values of ampholytic substances for use with the optimization of preparative-scale IEF enantiomer separations [16].

Though rigorous, the problem with both approaches described in Refs. [13,14,16] is that the methods are very time consuming and require great care if accurate K values are to be obtained. Therefore, a faster and simpler method would be desirable for the selection of the operating conditions in the preparative-scale IEF separation of enantiomers.

In most capillary IEF (CIEF) methods [17], the capillary is filled with a mixture of carrier ampholytes and the sample, the anode and cathode vials are filled with the respective acid and base solutions, the potential is applied to form the pH gradient and effect the separation, the content of the capillary (including the focused sample components which became stationary in different parts of the capillary) is mobilized by the detector (either by pressure or by altering the composition of the electrolyte in one of the electrode vials), and the detector trace is recorded [18]. In addition to requiring additional time, post-focusing mobilization causes band broadening and degrades peak resolution. As an alternative to post-separation mobilization, Pawliszyn et al. developed full-column imaging CIEF systems using both Schlieren-effect-based concentration gradient detectors [19,20] and absorbance detectors [21–23]. The latter became commercially available [24,25]; their applications have been reviewed recently [26,27].

The objective of this paper is to show that full-column imaging CIEF, combined with simple pH measurements in the collected sample-free carrier ampholyte fractions can be used for the rapid selection of the preparative-scale IEF separation conditions. The approach is demonstrated by separating the enantiomers of dansyl tryptophan in a continuous free-flow electrophoretic system, the Octopus.

2. Experimental

2.1. Chemicals

Phosphoric acid, lithium hydroxide, sodium hydroxide, triethanolamine and hydroxypropyl methyl cellulose (HPMC, average molecular mass 86 000) were obtained from Aldrich (Milwaukee, MI), carrier ampholytes pH 3–10 and pH 3–5 from Sigma (St Louis, MO). Hydroxypropyl-β-CD with a degree of substitution of 4.7 (HPβCD) was purchased from Cerestar (Hammond, IN). The sodium salt of heptakis(2-*O*-methyl-3,6-disulfo)-β-CD (HMediSuβ-CD) was synthesized and analytically characterized in our laboratory as described in Ref. [28]. The piperidinium salt of dansyl tryptophan (DNS-Trp) was obtained from NBS Biological (Huntingdon, Cambs, UK).

All solutions were freshly prepared using deion-

ized water from a Milli-Q unit (Millipore, Milford, MA). For the full-column imaging CIEF separations, the anolyte was 80 m*M* phosphoric acid, the catholyte was 100 m*M* sodium hydroxide. For the preparative-scale IEF separations, the anolyte was 50 m*M* phosphoric acid, the catholyte was 100 m*M* triethanolamine, and both solutions contained 0.2% HPMC. The background electrolyte used in the preparative-scale enantiomer separations contained 4% carrier ampholyte, 60 m*M* HPβCD and 0.2% (w/w) HPMC. The sample was 1.5 m*M* DNS-Trp, dissolved in the background electrolyte. The background electrolyte used for the CE analysis of the enantiomeric purity of the collected fractions contained 8 m*M* HMediSuβCD dissolved in 25 m*M* H_3PO_4 that was titrated to pH 2.13 with LiOH.

2.2. Equipment

All full-column imaging IEF separations were carried out on an iCE280 unit (Convergent Biosciences, Toronto, Canada) that was equipped with an ALCOTT 718AL autoinjector (ALCOTT, Norcross, GA), using a 5-cm long separation capillary (50 μm I.D.). The imaging detector was operated at 280 nm. The applied potential was 3000 V, the transfer time was 1.1 min, the focusing time 5 min. The acquired CIEF images were processed by the EZ Chrom software (Scientific Software, Inc., Pleasanton, CA).

All preparative-scale IEF separations were completed in the Octopus continuous free-flow electrophoretic unit (Dr Weber GmbH, Kirchheim-Heimstetten, Germany), equipped with a pair of anolyte recirculating ports, a pair of catholyte recirculating ports, seven independently fed background electrolyte inlets, a central sample inlet, and a counter-flow inlet. All ports were fed by variable-rate, multichannel peristaltic pumps. The 96 sample collection ports at the exit end of the separation chamber offered a lateral resolution of about 1 mm/collection port. The depth of the separation chamber was set to 450 μm. Deionized water was used as counter-flow medium. The chamber coolant was thermostated at 10 °C. The residence time in the chamber was adjusted to 30 min, the sample feed rate was set at 0.9 ml/h. Once the focusing potential was turned on, the separation was continued for an hour to insure that the system was in steady state, then fractions were collected for 30 min. The collected samples were analyzed for enantiomeric purity by CE as described below. The pH of the collected fractions was measured with a solid state microelectrode, pH16-SS, and a Model IQ240 pH meter (IQ Inc., San Diego, CA).

The CE separations were completed on a P/ACE 5510 CE unit (Beckman-Coulter, Fullerton, CA) using an $L_d = 19$ cm, $L_t = 26$ cm, 25 μm I.D., 150 μm O.D. uncoated, fused-silica capillary (Polymicro Technologies, Phoenix, AZ), at an applied potential of 10 kV and cartridge coolant temperature of 25 °C. The UV detector of the system was operated at 214 nm. For quantitation, a five-point calibration curve was constructed using peak areas that were normalized for migration time.

3. Results and discussion

3.1. Determination of the complexation-induced ΔpI values

The HPβCD-induced Δp*I* values of the DNS-Trp enantiomers were determined with the iCE280 unit. Traditional p*I* markers cannot be used to characterize the pH gradient in the capillary when the carrier ampholyte contains a cyclodextrin because the p*I* markers form complexes with the CD. The complexes have p*I* values that are different from the p*I* values of the non-complexed markers and the extent of the CD-induced p*I* shift depends on the cyclodextrin concentration [8,13,14]. Thus, actual p*I* values cannot be assigned to the enantiomer bands. However, band position in the capillary with respect to the anode or cathode compartment can be determined accurately. In the iCE280 instrument, the capillary is imaged onto 2043 pixels of the detector. Therefore, the pH gradient in the capillary spans the 1–2043 pixel range and the position of the enantiomer bands in the pH gradient can be described in terms of pixels. Since the objective of the proposed method is to find the background electrolyte composition that leads to the largest separation of the enantiomer peaks, rather than to determine their actual p*I* values, lack of knowledge of the actual p*I* values does not pose a problem.

First, the separation cartridge of iCE280 was filled with a 1.5 m*M* solution of DNS-Trp dissolved in the stock solution that contained 0.2% hydroxypropyl cellulose and 4% of pH 3–10 carrier ampholytes. Then, the focusing potential was applied and the full-column images were displayed every 30 s by the data collection system. The images did not change after 5 min of focusing indicating that complete focusing has been achieved. The peak of DNS-Trp was located at 178 pixels which, assuming a linear pH gradient, corresponds to an approximate p*I* value of 3.6. Therefore, the separation was repeated with a narrower range carrier ampholyte, pH 3–5. The peak of DNS-Trp was located at 823 pixels, around the first third of the length of the separation cartridge.

Next, HPβCD was added in increasing concentrations to the DNS-Trp-containing pH 3–5 carrier ampholyte solution and the 5-min long focusing was repeated for each solution. Even with an HPβCD concentration of only 10 m*M*, two peaks appeared corresponding to the two DNS-Trp enantiomers. Their distance in pixels was obtained and plotted in Fig. 1. In agreement with the predictions of Eq. (1) [8], the peak separation distance (corresponding to the Δp*I* values) increases and approaches a limiting value (or a very shallow maximum) at an HPβCD concentration of about 60 m*M*. All of these experiments were completed in less than 5 h, a time much shorter than what would be required using conventional CIEF.

3.2. Conversion of the imaging CIEF separations into preparative-scale IEF separations

The preparative-scale IEF separations were set up using the 60 m*M* HPβCD, 4% pH 3–5 carrier ampholyte, 0.2% HPMC solution as the background electrolyte in all seven feed ports of the Octopus unit. The required residence time for the separation in the Octopus unit was determined by filling the separation chamber with the sample-free carrier ampholyte mixture, setting the residence time to 10 min, applying the focusing potential, collecting the sample-free carrier ampholyte fractions, measuring their pH with a microelectrode and plotting the pH as a function of the fraction number. Then, the residence time was increased by 10 min and the experiment was repeated. As shown in Fig. 2, a quasi-linear pH gradient was obtained with a residence time of 30 min, indicating that at the available 3 kV separation potential, preparative-scale focusing was practically complete in 30 min. The slope of the pH gradient is a little steeper in the first 20 fractions than in the rest of the fractions indicating that the buffering capacity of the carrier ampholytes in the low p*I* range is slightly lower than above p*I* 3.5.

Next, the 1.5 m*M* DNS-Trp sample dissolved in

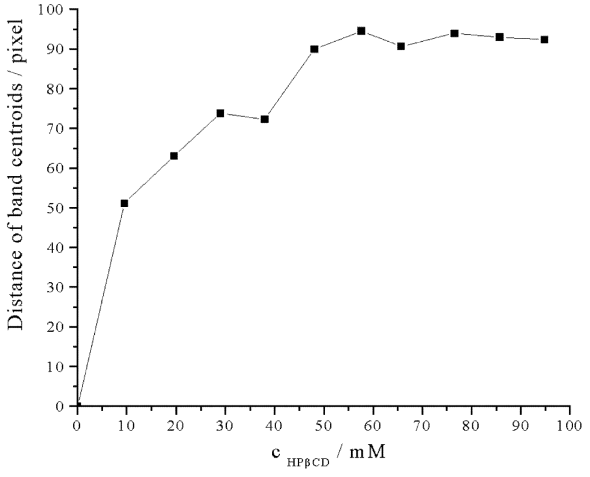

Fig. 1. Dynamically generated Δp*I* values (expressed as distance of the band centroids in pixels) as a function of the HPβCD concentration of the carrier ampholyte solution.

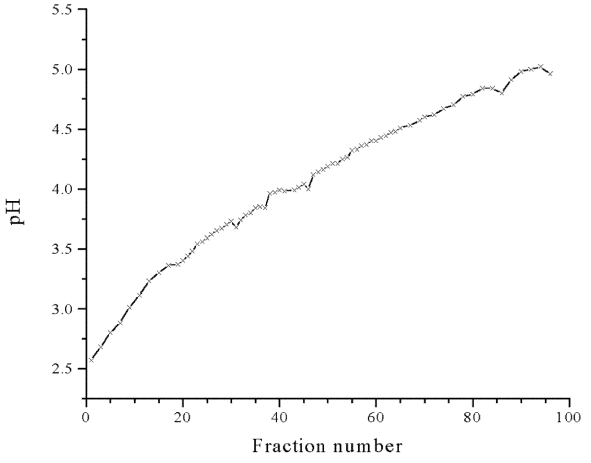

Fig. 2. Measured pH values in the sample-free carrier ampholyte fractions collected from the Octopus unit with a residence time of 30 min.

the 60 m*M* HPβCD, 4% pH 3–5 carrier ampholyte, 0.2% HPMC solution was fed into the system through the central sample injection port (2 cm above the background electrolyte feed ports) and the 60 m*M* HPβCD, 4% pH 3–5 carrier ampholyte, 0.2% HPMC solution was fed into all seven buffer feed ports. The focusing potential was applied and the system was allowed to reach steady state for 1 h before fraction collection began for 30 min. All these experiments were completed in the course of a working day.

The volume of each collected fraction (which varied slightly due to an interplay of the resistance of the collecting lines and the local flow-rate of the counter-flow) was measured with a gas-tight microsyringe and used for the calculation of the material balance. Next, the pH of each fraction was measured. Finally, the collected fractions were analyzed by CE to determine the enantiomeric purity of DNS-Trp. The electropherogram of the racemic feed mixture is shown in Fig. 3. Initially, every fourth fraction was analyzed to locate the approximate band boundaries, followed by the analysis of every fraction between the boundaries. The results are shown in Fig. 4, along with the measured pH of the fractions. Fig. 4 indicates that complete preparative-scale separation

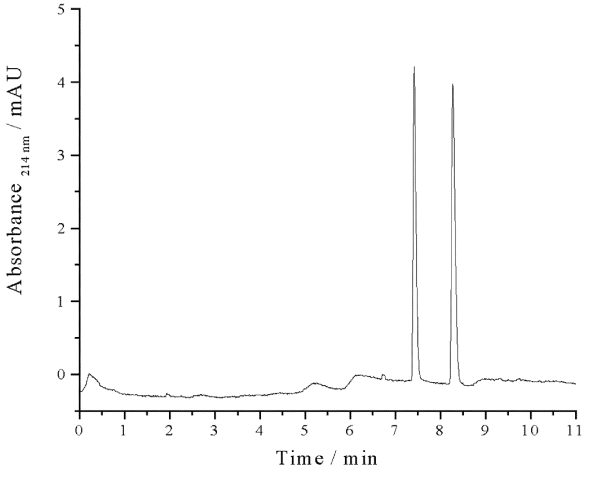

Fig. 3. Electropherogram of the racemic DNS-Trp feed mixture obtained on the P/ACE 5510 CE unit. Background electrolyte: 8 m*M* HMediSuβCD dissolved in 25 m*M* H_3PO_4 that was titrated to pH 2.13 with LiOH. Capillary: $L_d = 19$ cm, $L_t = 26$ cm, I.D. = 25 μm, O.D. = 150 μm, uncoated fused-silica, applied potential: 10 kV, temperature: 25 °C.

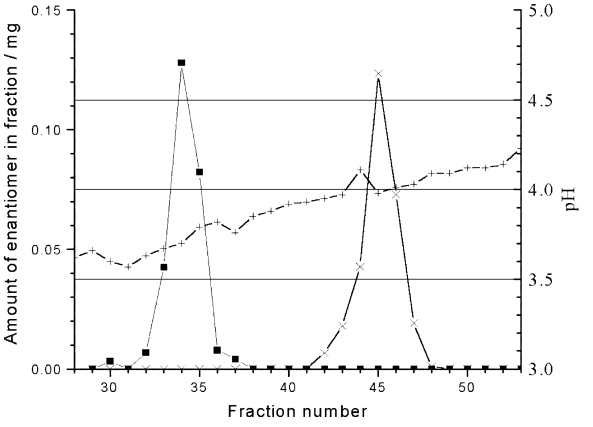

Fig. 4. Amount of the respective DNS-Trp enantiomers and the pH in the fractions collected during the preparative-scale IEF enantiomer separation using a background electrolyte of 0.2% HPMC, 60 m*M* HPβCD and 4% pH 3–5 carrier ampholytes in the Octopus unit. Residence time: 30 min, applied potential: 3000 V.

of the enantiomers has been achieved with a production rate of 1.6 mg enantiomer/h, and the concentration of the undesired enantiomer was below the detection limit in each fraction. These production rates and purities are comparable to what has been achieved in the past (1.3 mg/h) for other enantiomers using an optimization scheme that was based on a detailed CE study of the enantiomers [8].

The centroid of the first enantiomer band is in fraction 34 with a p*I* of 3.7, the centroid of the second enantiomer band is in fraction 45 with a p*I* of 4.0. This $\Delta pI = 0.3$ value agrees very well with the $\Delta pI = 0.28$ value found by Rizzi et al. [14] using capillary CE. All these experiments were completed in another day.

4. Conclusions

Using the enantiomers of DNS-Trp as model substances, this paper has shown that experimental conditions that lead to a satisfactory preparative-scale IEF separation in a continuous free-flow electrophoretic unit (such as the Octopus) can be determined in less than 3 days, using the iCE280 full-column imaging system for the identification of the desired composition of the background electrolyte, and pH measurements of the collected sam-

ple-free carrier ampholyte fractions for the determination of the required minimum residence time in the preparative system. CE analysis of the enantiomeric purity of DNS-Trp in the collected fractions proved that the predicted preparative-scale separation of the enantiomers has indeed been achieved at a production rate of 1.6 mg/h for each enantiomer with essentially 100% purity. This method development approach is at least an order of magnitude faster than what was available in the past, and is currently used in our laboratory to study pharmaceutically relevant systems.

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References

[1] A. Amini, Electrophoresis 22 (2001) 3107.
[2] A. Rizzi, Electrophoresis 22 (2001) 3079.
[3] P. Hoffman, H. Wagner, G. Weber, M. Lanz, J. Caslavska, W. Thormann, Anal. Chem. 71 (1999) 1840.
[4] P. Glukhovskiy, Gy. Vigh, Electrophoresis 21 (2000) 2010.
[5] P. Glukhovskiy, Gy. Vigh, Electrophoresis 22 (2001) 2639.
[6] A.M. Stalcup, R.M.C. Sutton, J.V. Rodrigo, S.R. Gratz, E.G. Yanes, P. Painuly, Analyst 125 (2000) 1719.
[7] S.R. Gratz, E. Schneiderman, T.R. Mertens, A.M. Stalcup, Anal. Chem. 73 (2001) 3999.
[8] P. Glukhovskiy, Gy. Vigh, Anal. Chem. 71 (1999) 3814.
[9] P. Glukhovskiy, T.A. Landers, Gy. Vigh, Electrophoresis 21 (2000) 762.
[10] G. Weber, J. Bauer, Electrophoresis 19 (1998) 1104.
[11] P.G. Righetti, E. Wenisch, M. Faupel, J. Chromatogr. 475 (1989) 293.
[12] P.G. Righetti, C. Ettori, P. Chafey, J.P. Wahrmann, Electrophoresis 11 (1990) 1.
[13] A.M. Rizzi, L. Kremser, Electrophoresis 20 (1999) 2715.
[14] A.M. Rizzi, L. Kremser, Electrophoresis 20 (1999) 3410.
[15] W.A. Williams, Gy. Vigh, Anal. Chem. 68 (1996) 1174.
[16] P. Glukhovskiy, Gy. Vigh, Electrophoresis 19 (1998) 3125.
[17] S. Hjerten, M.D. Zhu, J. Chromatogr. 346 (1985) 265.
[18] P.G. Righetti, C. Gelfi, M. Chiari (Ed.), Capillary Electrophoresis in Analytical Biotechnology, CRC Series in Analytical Biotechnology, CRC Press, Boca Raton, FL, 1996.
[19] J.Q. Wu, J. Pawliszyn, J. Chromatogr. 608 (1992) 121.
[20] J.Q. Wu, J. Pawliszyn, Anal. Chem. 64 (1992) 224.
[21] J.Q. Wu, J. Pawliszyn, J. Liq. Chromatogr. 16 (1993) 1891.
[22] J.Q. Wu, J. Pawliszyn, Electrophoresis 14 (1993) 469.
[23] J.Q. Wu, J. Pawliszyn, Anal. Chem. 66 (1992) 867.
[24] J.Q. Wu, J. Pawliszyn, J. Chromatogr. B 657 (1994) 327.
[25] J.Q. Wu, J. Pawliszyn, Electrophoresis 16 (1995) 670.
[26] X.H. Fang, C. Tragas, J.Q. Wu, Q.L. Mao, J. Pawliszyn, Electrophoresis 19 (1998) 2290.
[27] X.Z. Wu, J.Q. Wu, J. Pawliszyn, LC GC North America 19 (2001) 526.
[28] D.K. Maynard, Gy. Vigh, Carbohydr. Res. 328 (2000) 277.