Analytical- and Preparative-Scale Isoelectric Focusing Separation of Enantiomers

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Isoelectric focusing has been used to achieve the analytical- and preparative-scale separation of the enantiomers of amphoteric analytes. By considering the simultaneous multiple equilibria involved in the chiral recognition process, a model has been developed to describe the magnitude of the ΔpI value that develops between the enantiomers in the presence of a noncharged chiral resolving agent, such as a noncharged cyclodextrin. Theoretical analysis of the model indicates that three kinds of IEF enantiomer separations are possible: anionoselective and cationo-selective, when only the identically charged forms of the enantiomers bind selectively to the resolving agent, and duo-selective, when the differently charged forms of the enantiomers bind selectively to the resolving agent. The model predicts that the ΔpI vs cyclodextrin concentration curves approach limiting $\Delta p I$ values which can be as large as 0.1, even when the binding constants of the enantiomers differ only by 10%. The parameters of the model can be readily determined by free solution capillary electrophoretic or pressuremediated capillary electrophoretic experiments. The validity of the proposed model has been tested with hydroxypropyl β -cyclodextrin as resolving agent and dansyl phenylalanine as probe. Capillary IEF enantiomer separations have been achieved using both ampholytes and binary propionic acid-serine buffers (Bier's buffers). Preparative-scale IEF enantiomer separations with production rates as high as 1.3 mg/h have been achieved in an Octopus continuous free-flow electrophoretic system.

As indicated by numerous recent reviews and monographs,^{1–15} capillary electrophoretic (CE) enantiomer separations underwent spectacular development over the past few years. CE is a logical choice for the separation of water-soluble weak electrolyte enantiomers because, due to the high innate separation efficiency of CE, the enantiomers can be resolved even when the separation selectivity is as low as $\alpha = 1.005$. Successful enantiomer separations have been achieved early on in all major electrophoretic operation modes: in micellar electrokinetic chromatography,¹⁶ isotachophoresis,¹⁷ free solution capillary electrophoresis,¹⁸ and

- (5) Nguyen, N. T.; Siegler, R. W. J. Chromatogr., A 1996, 735, 123-150.
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capillary gel electrophoresis.¹⁹ Isoelectric focusing (IEF) techniques,²⁰ including, more recently, capillary isoelectric focusing (cIEF),²¹ have been used extensively to separate amphoteric compounds on the basis of their different isoelectric points (pI values). However, to the best of our knowledge, there is only one published report, by Righetti's group, on the successful use of IEF, in the slab gel format, for the separation of the enantiomers of dansyl amino acids.²² By adding 40 mM β -cyclodextrin (CD), 4 M urea, and 10% methanol to an immobilized pH-gradient gel, they were able to separate, using 6-h-long focusing times, the enantiomers of dansyl isoleucine (DNS-Ile), phenylalanine (DNS-Phe), and tryptophan (DNS-Trp). The pI values of the two CDcomplexed DNS amino acid enantiomers were found to differ by as much as 0.1. They argued that the enantiomer separation was feasible because CD altered the base strength (pK_b value) of the dimethylamino group of the DNS moiety more for L-DNS-Phe, than for D-DNS-Phe, but no equation was presented to describe the CD concentration dependence of the complexation-generated ΔpI value. Though they speculated that IEF enantiomer separations should be feasible in multicompartmental electrolyzers equipped with Immobiline membranes,24 no such follow-up report appeared in the literature.

The objectives of this paper are to (i) present an explicit relationship that predicts the magnitude of the ΔpI value that can be generated between the enantiomers upon the addition of a

- (6) St. Claire, R. L. Anal. Chem. 1996, 68, 569R-586R.
- (7) Camilleri, P. Chem. Commun. 1996, 16, 1851-1858.
- (8) Bressolle, F.; Audran, M.; Pham, T. N.; Vallon, J. J. J. Chromatogr., B 1996, 687, 303–336.
- (9) Vespalec, R.; Bocek, P. Electrophoresis 1997, 18, 843-852.
- (10) Fanali, S. J. Chromatogr., A 1997, 792, 227-267.
- (11) Vigh, Gy.; Sokolowski, A. D. Electrophoresis 1997, 18, 2305-2310.
- (12) Landers, J. P. Handbook of Capillary Electrophoresis, CRC Press: Boca Raton, 1997.
- (13) Chankvetadze, B. Capillary Electrophoresis in Chiral Analysis, J. Wiley: New York, 1997.
- (14) Beale, S. C. Anal. Chem. 1998, 70, 279R-300R.
- (15) Khaledi, M., Ed. High Performance Capillary Electrophoresis: Theory, Techniques and Applications, J. Wiley: New York, 1998.
- (16) Terabe, S.; Ozaki, H.; Ando, T. J. Chromatogr. 1985, 348, 39-51.
- (17) Jelinek, I.; Snopek, J.; Smolkova-Keulemansova, E. J. Chromatogr. 1987, 405, 379–384.
- (18) Fanali, S. J. Chromatogr. 1989, 474, 441-446.
- (19) Guttman, A.; Paulus, A.; Cohen, A. S.; Grinberg, N.; Karger, B. L. J. Chromatogr. 1988, 448, 41–53.
- (20) Righetti, P. G. Isoelectric Focusing: Theory, Methodology and Applications, Elsevier: Amsterdam, The Netherlands, 1983.
- (21) Hjerten, S.; Zhu, M. D. J. Chromatogr. 1985, 346, 265-270.
- (22) Righetti, P. G.; Ettori, C.; Chafey, P.; Wahrmann, J. P. *Electrophoresis* **1990**, *11*, 1–4.

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⁽¹⁾ Chankvetadze, B.; Endresz, G.; Blaschke, G. Chem. Soc. Rev. **1996**, 25, 141–152.

⁽²⁾ Nishi, H.; Terabe, S. J. Chromatogr., A 1996, 735, 3-27.

⁽³⁾ Nishi, H. J. Chromatogr., A 1996, 735, 57-76.

⁽⁴⁾ Fanali, S. J. Chromatogr., A 1996, 735, 77-121.

noncharged resolving agent, (ii) analyze this relationship to determine the advantageous regimes of IEF enantiomer separations, (iii) demonstrate the feasibility of analytical-scale cIEF enantiomer separations using both ampholyte and binary Bier buffers,²⁵ and (iv) demonstrate the feasibility of preparative-scale IEF enantiomer separations using the Octopus^{32–34} continuous free-flow electrophoretic unit. The history and recent advances of continuous free-flow electrophoresis were extensively reviewed recently.³³ The design and characteristics of the Octopus unit were also discussed in detail,³² and its long-term stability has been demonstrated.³⁴

THEORY

1. Complexation-Induced Shifts in the Effective pI Values of Enantiomers. To account for the effects of the noncharged cyclodextrin (D) concentration of the background electrolyte (BE) on the effective pI values of the individual enantiomers, both protonation equilibria and complexation equilibria must be considered simultaneously. Let the BE contain a buffer system to control the pH and a noncharged cyclodextrin, D, to create an anisotropic environment. Let the enantiomers of an amphoteric analyte be NRH and NSH. Cyclodextrin will interact with both the buffer components and the analytes. When the buffer concentration is much higher than that of D, and the concentration of D is much higher than that of the analytes, the concentration and original species distribution of D will remain practically the same whether analytes are present or absent. Therefore, in a first approximation, the equilibria between the buffer components and D can be omitted from consideration.

Once in solution, both solute enantiomers, NRH and NSH, participate in protic equilibria according to eqs 1-4. (Only the equations pertaining to the R enantiomer are shown, but identical expressions exist for the S enantiomer as well.)

$$NRH \rightleftharpoons H^+ NR^- \tag{1}$$

$$\mathrm{H}^{+}\mathrm{N}\mathrm{R}^{-} + \mathrm{H}_{2}\mathrm{O} \rightleftharpoons \mathrm{H}_{3}\mathrm{O}^{+} + \mathrm{N}\mathrm{R}^{-}$$
(2)

$$\mathrm{H}^{+}\mathrm{NR}^{-} + \mathrm{H}_{2}\mathrm{O} \rightleftharpoons \mathrm{OH}^{-} + \mathrm{H}^{+}\mathrm{NRH}$$
(3)

$$2H_2O \rightleftharpoons H_3O^+ + OH^- \tag{4}$$

Cyclodextrin will complex with all three forms of the enantiomers:

$$H^{+}NR^{-} + D \rightleftharpoons H^{+}NR^{-}D$$
 (5)

- (23) Righetti, P. G.; Wenisch, E.; Faupel, M. J. Chromaotgr. 1989, 475, 293– 309.
- (24) Rawjee, Y. Y. Thesis, Texas A&M University, College Station, 1994.
- (25) Rawjee, Y. Y.; Staerk, D. U.; Vigh, Gy. J. Chromatogr. 1993, 635, 291–306.
 (26) Rawjee, Y. Y.; Williams, R. L.; Vigh, Gy. J. Chromatogr. 1993, 652, 233–
- 245.
- (27) Rawjee, Y. Y.; Vigh, Gy. Anal. Chem. **1994**, 66, 619–627.
- (28) Bier, M.; Ostrem, J.; Marques, R. B. *Electrophoresis* 1993, *14*, 1011–1018.
 (29) Cai, H.; Vigh, Gy. *Anal. Chem.* 1998, *70*, 4640–4643.
- (30) Glukhovskiy, P.; Vigh, Gy. Electrophoresis 1998, 19, 3166-3170.
- (31) Righetti, P. G.; Gelfi, C.; Perego, M.; Stoyanov, A. V.; Bossi, A. *Electrophoresis* 1997, 18, 2145–2153.
- (32) Weber, G.; Bocek, P. Electrophoresis 1996, 17, 1896-1910.
- (33) Krivankova, L.; Bocek, P. Electrophoresis 1998, 19, 1064-1074.
- (34) Weber, G.; Bauer, J. *Electrophoresis* **1998**, *19*, 1104–1109.

$$NR^{-} + D \rightleftharpoons NR^{-}D \tag{6}$$

$$H^+NRH + D \rightleftharpoons H^+NRHD$$
 (7)

The equilibrium expressions for reactions 1-7 are

$$K_{a,R} = [NR^{-}][H_{3}O^{+}]/[H^{+}NR^{-}]$$
 (8)

$$K_{\rm b,R} = [{\rm H}^+ {\rm NRH}][{\rm OH}^-]/[{\rm H}^+ {\rm NR}^-]$$
 (9)

$$K_{\rm w} = [{\rm H}_3{\rm O}^+][{\rm OH}^-]$$
 (10)

$$K_{\rm H^+NR^-D} = [{\rm H^+NR^-D}]/[{\rm H^+NR^-}][{\rm D}]$$
 (11)

$$K_{\rm NR^-D} = [\rm NR^-D]/[\rm NR^-][D]$$
 (12)

$$K_{\rm H^+NRHD} = [\rm H^+NRHD]/[\rm H^+NRH][D]$$
(13)

The mass balance equations for the neutral, negatively charged, and positively charged R-related species are

$$c_{\rm H^+NR^-} = [{\rm H}^+ {\rm NR}^-] + [{\rm H}^+ {\rm NR}^- {\rm D}]$$
 (14)

$$c_{\rm NR^-} = [\rm NR^-] + [\rm NR^-D]$$
 (15)

$$c_{\mathrm{H}^{+}\mathrm{NRH}} = [\mathrm{H}^{+}\mathrm{NRH}] + [\mathrm{H}^{+}\mathrm{NRHD}]$$
(16)

The respective mole fractions of the neutral, negatively charged and positively charged noncomplexed species are

$$\alpha_{\rm H^+NR^-} = [{\rm H^+NR^-}]/c_{\rm H^+NR^-}$$
(17)

$$\alpha_{\rm NR^-} = [\rm NR^-] / c_{\rm NR^-} \tag{18}$$

$$\alpha_{\rm H^+NRH} = [{\rm H^+NRH}]/c_{\rm H^+NRH}$$
(19)

Analytical expressions can be obtained from eqs 8–19 for [H⁺NR⁻], [NR⁻], and [H⁺NRH] yielding

$$\alpha_{\rm H^+NR^-} = 1/(1 + K_{\rm H^+NR^-D}[D])$$
 (20)

$$\alpha_{\rm NR^-} = 1/(1 + K_{\rm NR^-D}[{\rm D}])$$
(21)

$$\alpha_{\rm H^+NRH} = 1/(1 + K_{\rm H^+NRHD}[\rm D])$$
 (22)

In the absence of a noncharged complexing agent, the ratio of the acid and base dissociation constants of the R enantiomer becomes

$$\frac{K_{\rm a,R}}{K_{\rm b,R}} = \frac{[\rm NR^{-}][\rm H_3O^{+}]^2}{[\rm H^{+}NRH]K_{\rm w}}$$
(23)

In the isoelectric point, $[H^+NRH] = [NR^-]$ and pH = pI, which leads to

$$pI_{R} = (1/2)(pK_{a,R} + (14 - pK_{b,R}))$$
(24)

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In the presence of a noncharged complexing agent, the ratio of the acid and base dissociation constants of the R enantiomer becomes

$$\frac{K_{a,R}}{K_{b,R}} = \frac{[NR^{-}][H_{3}O^{+}]^{2}}{[H^{+}NRH]K_{w}}$$
(25)

Equation 25 can be rewritten with eqs 17–22 as

$$\frac{K_{\mathrm{a,R}}}{K_{\mathrm{b,R}}} = \frac{\alpha_{\mathrm{NR}} - c_{\mathrm{NR}} - [\mathrm{H}_{3}\mathrm{O}^{+}]^{2}}{\alpha_{\mathrm{H}^{+}\mathrm{NRH}} c_{\mathrm{H}^{+}\mathrm{NRH}} K_{\mathrm{w}}}$$
(26)

If the pH at which $c_{NR^-} = c_{H^+NRH}$ is called p I'_R , the effective pI for enantiomer R in the presence of a noncharged complexing agent, then eq 26 becomes

$$pI_{R} = pI_{R} + \frac{1}{2}\log\frac{\alpha_{NR^{-}}}{\alpha_{H^{+}NRH}}$$
(27)

Similarly, pI'_{s} , the effective pI for enantiomer *S* in the presence of a noncharged complexing agent becomes

$$pI'_{\rm S} = pI_{\rm S} + \frac{1}{2}\log\frac{\alpha_{\rm NS^-}}{\alpha_{\rm H^+NSH}}$$
(28)

Thus, the complexation-induced effective p*I* difference between the enantiomers, $\Delta p I'_{R,S}$, becomes

$$\Delta pI_{R,S} = pI_{R} - pI_{S} = pI_{R} - pI_{S} + \frac{1}{2}\log\left(\frac{\alpha_{NR^{-}}}{\alpha_{NS^{-}}}\frac{\alpha_{H^{+}NSH}}{\alpha_{H^{+}NRH}}\right)$$
(29)

Since $pI_R = pI_S$, $\Delta pI'_{R,S}$ becomes

$$\Delta p I_{\rm R,S}^{\prime} = \frac{1}{2} \log \left(\frac{\alpha_{\rm NR^{-}}}{\alpha_{\rm NS^{-}}} \frac{\alpha_{\rm H^{+}NSH}}{\alpha_{\rm H^{+}NRH}} \right)$$
(30)

By substituting eqs 20-22 into eq 30, we obtain

$$pI_{R,S} = \frac{1}{2} \log \left(\frac{1 + K_{NS-D}[D]}{1 + K_{NR-D}[D]} \frac{1 + K_{H^+NRHD}[D]}{1 + K_{H^+NSHD}[D]} \right)$$
(31)

This means that, in chiral IEF separations, the resolving agentinduced $\Delta p I_{R,S}$ depends on both the material characteristics of the solutes (the complexation constants of the negatively and positively charged enantiomers) and the operating variable: the resolving agent concentration.

2. Discussion of the $\Delta p I_{R,S}$ Model. It can be seen from the analysis of eq 31 that, depending on the relative magnitudes of the complexation constants, K_{NR-D} , K_{NS-D} , K_{H+NRHD} , and K_{H+NSHD} , there are three major types of chiral IEF separations: aniono-selective, cationo-selective, and duo-selective separations.

In an aniono-selective IEF enantiomer separation $K_{\text{H+NRHD}} = K_{\text{H+NSHD}}$; i.e., the positively charged enantiomers complex identically with the resolving agent. This simplifies eq 31 to

$$\Delta p I_{R,S}^{\prime} = \frac{1}{2} \log \left(\frac{1 + K_{NS^{-D}}[D]}{1 + K_{NR^{-D}}[D]} \right)$$
(32)

In a cationo-selective IEF enantiomer separation $K_{\text{NR-D}} = K_{\text{NS-D}}$; i.e., the negatively charged enantiomers complex identically with the resolving agent. This also simplifies eq 31 to

$$\Delta p I_{\rm R,S} = \frac{1}{2} \log \left(\frac{1 + K_{\rm H^+NRHD}[\rm D]}{1 + K_{\rm H^+NSHD}[\rm D]} \right)$$
(33)

The functions described by eqs 32 and 33 are similar: as the noncharged resolving agent concentration is increased toward infinity, the absolute value of $\Delta p I_{R,S}$ increases from zero to the limiting value, $\Delta p I_{R,S}^{max}$. When $K_{H+NRHD}[D] \gg 1$, $\Delta p I_{R,S} = \Delta p I_{R,S}^{max} = 1/2 \log(K_{H+NRHD}/K_{H+NSHD})$. Figure 1 shows the $\Delta p I_{R,S}$ vs [D] function calculated by eq 33; the top curve was obtained with $K_{H+NRHD} = 114$ and $K_{H+NSHD} = 150$ (estimated complexation constant values for a DNS-Phe type compound²⁴), yielding $\Delta p I_{R,S}^{max} = -0.06$.

The rate with which $\Delta p I'_{R,S}$ approaches $\Delta p I'_{R,S}^{max}$ depends on the magnitude of $K_{\text{H+NRHD}}$ and $K_{\text{H+NSHD}}$. This effect can be studied by introducing a new variable, ρ_{R,S^+} , the ratio of the complexation constants:

$$\rho_{\mathrm{R},\mathrm{S}^+} = K_{\mathrm{H}^+\mathrm{NRHD}} / K_{\mathrm{H}^+\mathrm{NSHD}} \tag{34}$$

With this definition, eq 33 can be rewritten as

$$\Delta p I_{\text{R,S}}^{\prime} = \frac{1}{2} \log \left(\frac{1 + \rho_{\text{R,S}+} K_{\text{H}+\text{NSHD}}[\text{D}]}{1 + K_{\text{H}+\text{NSHD}}[\text{D}]} \right)$$
(35)

In Figure 1, the $\Delta p I'_{R,S}$ vs [D] function is plotted at a constant $\rho_{R,S+}$ value ($\rho_{R,S+} = 0.76$) for increasing K_{H+NSHD} values (150 to 750 to 1500). $\Delta p I'_{R,S}$ ^{max} remains the same, but the rate with which this limiting value is approached increases as the complexation constant is increased 5-fold and then 10-fold. A cyclodextrin concentration as low as 15 mM can generate ~90% of the theoretically possible complexation-induced $\Delta p I'$ value, even for a moderately strong binding constant ($K_{\text{H+NSHD}} = 750$).

It is instructive to see how $\Delta p I'_{R,S}^{max}$ changes as ρ_{R,S^+} is varied, i.e., what kind of enantiomer binding strength difference leads to what kind of $\Delta p I'_{R,S}^{max}$ (Figure 2). Though the relationship is slightly nonlinear, the larger the ρ_{R,S^+} , the greater the $\Delta p I'_{R,S}^{max}$. In the 0.9 < ρ_{R,S^+} and 1.1 < ρ_{R,S^+} ranges, which cover the realistic, modest enantiomer binding strength differences of ~10% or greater, $\Delta p I'_{R,S}^{max}$ is 0.03 or larger.

In duo-selective IEF enantiomer separations, both the anionic and the cationic forms of the amphoteric enantiomers complex differently with cyclodextrin, i.e., $K_{\rm NR-D} \neq K_{\rm NS-D}$ and $K_{\rm H+NRHD} \neq$ $K_{\rm H+NSHD}$. Therefore, eq 31 cannot be simplified. As in the case of duo-selective CE enantiomer separations,^{25–27} the first and the second multiplier terms in the argument of the logarithm function in eq 31 have opposing effects. Therefore, depending on the numeric values of $K_{\rm NR-D}$, $K_{\rm NS-D}$, $K_{\rm H+NRHD}$, and $K_{\rm H+NSHD}$, the $\Delta p I'_{\rm R,S}$ vs [D] function will have two distinct shapes.



Figure 1. Effective p/ difference as a function of the cyclodextrin concentration in a cationo-selective IEF enantiomer separation. For all curves, $\rho_{R,S+} = 0.76$. Dash-dot line, $K_{H+NSD} = 150$. Dotted line, $K_{H+NSD} = 750$. Solid line, $K_{H+NSD} = 1500$.



Figure 2. Relationship between the $\Delta p I^{\text{max}}$ value and the complexation constant ratio ($\rho_{\text{R},\text{S}+}$) for a cationo-selective IEF enantiomer separation.

When $\rho_{R,S^+} > 1$ and $\rho_{R,S^-} > 1$, or $\rho_{R,S^+} < 1$ and $\rho_{R,S^-} < 1$, the first and second terms act antagonistically (they counteract each other's influence): there is a finite cyclodextrin concentration at which $\Delta p I_{R,S}$ is at its maximum. This $\Delta p I_{R,S}^{max}$ is smaller than the value one would observe in an aniono-selective or cationoselective case with the same ρ_{R,S^-} or ρ_{R,S^+} value. The shape of the $\Delta p I_{RS}$ vs [D] function is shown in Figure 3 for a realistic set of $K_{\rm NR-D}$, $K_{\rm NS-D}$ and $K_{\rm H+NRHD}$, $K_{\rm H+NSHD}$ values. (The complex formation constants for the anionic species are measured values²⁴ for DNS-Phe with hydroxypropyl β -cyclodextrin, $K_{\text{NR-D}} = 137$ and $K_{\rm NS-D} = 186$; the complex formation constants for the cationic species are estimated values, $K_{\text{H+NRHD}} = 114$ and $K_{\text{H+NSHD}} = 150$.) For the three curves, the numeric values of the formation constants increase in the 1:5:10 ratio, but the ρ_{RS} values are the same: $\rho_{R,S^+} = 0.736$ and $\rho_{R,S^-} = 0.76$. Obviously, $\Delta p I_{R,S}^{max}$ remains the same in all three cases, but the optimum CD concentration becomes lower as the complex formation constants increase, just as in a duo-selective free solution CE enantiomer separation.^{25–27}

The optimum concentration is at the point where the first derivative of the $\Delta p I_{R,S}$ (D) function is equal to zero. This occurs



Figure 3. Effective p/ difference for the enantiomers as a function of the cyclodextrin concentration in an antagonistic duo-selective IEF enantiomer separation. Dash-dot line: $K_{NS-D} = 186$, $\rho_{R,S-} = 0.76$, $K_{HNSH+D} = 150$, and $\rho_{R,S+} = 0.736$. Dotted line: $K_{NS-D} = 930$, $\rho_{R,S-} = 0.76$, $K_{HNSH+D} = 750$, and $\rho_{R,S+} = 0.736$. Solid line: $K_{NS-D} = 1860$, $\rho_{R,S-} = 0.76$, $K_{HNSH+D} = 1500$, and $\rho_{R,S+} = 0.736$.

where

$$\frac{K_{\rm NS^{-}D}}{1 + K_{\rm NS^{-}D}[D]} + \frac{K_{\rm H^{+}NRHD}}{1 + K_{\rm H^{+}NRHD}[D]} = \frac{K_{\rm NR^{-}D}}{1 + K_{\rm NR^{-}D}[D]} + \frac{K_{\rm H^{+}NSHD}}{1 + K_{\rm H^{+}NSHD}[D]}$$
(36)

i.e., where the positive root(s) of the quadratic equation obtained from the rearrangement of eq 36 are located:

$$[D]^{2}[K_{H^{+}NRHD}K_{NR^{-}D}(K_{NS^{-}D} - K_{H^{+}NSHD}) + K_{H^{+}NSHD}K_{NS^{-}D} \times (K_{H^{+}NRHD} - K_{NR^{-}D})] + 2[D](K_{H^{+}NRHD}K_{NS^{-}D} - K_{NR^{-}D}K_{H^{+}NSHD}) + (K_{H^{+}NRHD} - K_{NR^{-}D}) - (K_{H^{+}NSHD} - K_{NS^{-}D}) = 0$$
(37)

 $\Delta p I'_{R,S}^{max}$ depends only on the value of the $\rho_{R,S^+}/\rho_{R,S^-}$ ratio: the more this ratio differs from unity, the larger the $\Delta p I'_{R,S}^{max}$ value.

When $\rho_{R,S^+} > 1$ and $\rho_{R,S^-} < 1$ or $\rho_{R,S^+} < 1$ and $\rho_{R,S^-} > 1$, the first and second terms in the argument of the logarithm in eq 31 act cooperatively: the $\Delta p I_{R,S}$ values will be much higher at any cyclodextrin concentration than in a corresponding anionoselective or cationo-selective case. Obviously, this is the most desirable scenario. The shape of the $\Delta p I_{R,S}$ vs [D] function is shown in Figure 4 for $\rho_{\rm R,S^-}$ = 0.76, $\rho_{\rm R,S^+}$ = 1.359 and $K_{\rm NS-D}$ = 186, 930, and 1860, K_{HNSH+D} = 150, 750, and 1500 (the same numeric values as in Figure 3, except that the R and S subscripts are interchanged). The shape of the curves is similar to that of an aniono-selective or cationo-selective $\Delta p I'_{R,S}$ vs [D] curve. As the K values increase, $\Delta p I_{RS}^{max}$ is approached more rapidly: $\Delta p I_{RS}^{max}$ depends only on the value of the multiple of ρ_{RS+} and $\rho_{R,S-}$. The more this multiple differs from unity, the larger the $\Delta p \textbf{I}_{R,S}^{max}$ value. In such a system, $\Delta p \textbf{I}_{R,S}$ values as large as 0.1 can be realized easily.

Obviously, there is yet another, theoretically possible case. This occurs when both the anionic and the cationic forms of the two



Figure 4. Effective p*l* differences for the enantiomers as a function of the cyclodextrin concentration in a cooperative duo-selective IEF enantiomer separation.. Dash-dot line: $K_{NS-D} = 186$, $\rho_{R,S-} = 0.76$, $K_{HNSH+D} = 150$, and $\rho_{R,S+} = 1.359$. Dotted line: $K_{NS-D} = 930$, $\rho_{R,S-} = 0.76$, $K_{HNSH+D} = 750$, and $\rho_{R,S+} = 1.359$. Solid line: $K_{NS-D} = 1860$, $\rho_{R,S-} = 0.76$, $K_{HNSH+D} = 1500$, and $\rho_{R,S+} = 1.359$.

enantiomers complex identically; i.e., $K_{\text{NR}-\text{D}} = K_{\text{NS}-\text{D}}$ and $K_{\text{H}+\text{NRHD}} = K_{\text{H}+\text{NSHD}}$. Clearly, $\Delta p I_{\text{R},\text{S}} = 0$ and there can be no IEF enantiomer separation.

Often, the pK_a and pK_b (or pI) values are known for the enantiomers, but the model parameters are not. However, reasonable parameter estimates can be obtained from free solution CE experiments (or PreMCE experiments) as discussed in refs 25-27. Once the complex formation constants are known, the type of the separation can be established (aniono-selective, cationoselective, or duo-selective), the best cyclodextrin concentration can be determined and the $\Delta p I_{R,S}^{max}$ value can be calculated. Naturally, the model developed here applies not only for uncharged cyclodextrins as resolving agents, but for any other noncharged resolving agent (such as maltodextrins, chiral crown ethers, etc.), as long as the interactions can be described by stoichiometric equilibrium reactions. The model covers the complexation-assisted IEF separation of nonenantiomeric analytes as well, with the added complexity that in such cases it is often true that the original p*I* values of the two analytes are not exactly identical: $pI_R \neq pI_S$.

Since protein cIEF separations have been achieved for ΔpI values smaller than 0.03–0.1, the $\Delta pI_{R,S}$ values predicted here,²¹ cIEF enantiomer separations—and even preparative IEF enantiomer separations—should be feasible.

EXPERIMENTAL SECTION

Chemicals. Both polydisperse ampholytes (ampholyte pH 3-5, Sigma, St. Louis, MO) and Bier's serine-propionic acid binary buffers²⁸ (OptiFocus, Protein Technologies, Tucson, AZ) were used as IEF background electrolyte components. Their pH range covered the p*I* value of the uncomplexed enantiomers. Dansylglycine (DNS-Gly), racemic and pure enantiomer dan-sylphenylalanine (DNS-Phe) were obtained from Sigma. Hydroxy-propyl β -cyclodextrin (HP β CD) was obtained from Cerastar (Hammond, IN). All solutions were freshly prepared using deionized water from a Millipore Q unit (Millipore, Milford, MA). The p*I* value of uncomplexed DNS-Phe in the serine-propionic



Figure 5. Simplified schematic of the Octopus free flow electrophoretic system.

acid binary Bier buffers²⁸ was determined using the modified fiveband PreMCE technique.^{29,30} The serine—propionic acid BEs used in the preparative-scale IEF separations contained 0.1% (w/w) hydroxypropylmethyl cellulose, HPMC (Aldrich, Milwaukee, WI), and 30 mM HP β CD. The enantiomeric purity of the fractions collected in the preparative IEF runs was determined in a 200 mM iminodiacetic acid buffer,³¹ which contained 200 mM HP β CD.

Equipment. All analytical-scale CE separations were completed on a P/ACE 5510 CE unit (Beckman Instruments, Fullerton, CA). Its UV detector was operated at 340 nm for the ampholyte pH 3–5 cIEF measurements and at 214 nm for all other BEs. All ampholyte-based separations and IDA buffer-based separations were carried out in $L_d = 19$ cm, $L_t = 26$ cm, 25 μ m i.d., 150 μ m o.d. uncoated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ). All cIEF separations with Bier's buffers were carried out in $L_d = 37$ cm, $L_t = 44$ cm, 25 μ m i.d., 150 μ m o.d. uncoated fused-silica capillaries were thermostated at 25 °C.

All preparative-scale IEF separations were completed with an Octopus continuous free-flow electrophoretic unit³²⁻³⁴ (Dr. Weber GmbH, Kirchheim-Heimstetten, Germany). The schematic of the unit is shown in Figure 5. The unit is equipped with a pair of anolyte recirculating ports, a pair of catholyte recirculating ports, seven independently fed BE inlets, a central sample inlet, and a counterflow inlet. All these ports are fed by a variable rate, multichannel peristaltic pump. There are 96 sample collection ports at the exit end of the separation chamber that offer a lateral resolution of 1.04 mm/collection port. The 400-µm-thick IEF separation chamber spacer was used in each experiment. The chamber coolant was thermostated at 10 °C. The residence time in the chamber was adjusted to 12.5 min; the sample feed rate was set at 3 mL/h. Fraction collection was continued until 2-mL aliquots were obtained at each port; these aliquots were analyzed for enantiomeric purity as described in Procedures.

Procedures. The p*I* value of the uncomplexed DNS-Phe in the serine—propionic acid Bier buffers²⁸ was determined using the five-band PreMCE method³⁰ and was found to be p*I* = 3.65. The cIEF separations with 2.0% ampholyte pH 3–5, and 15, 30, 60, or 120 mM HP β CD, were obtained at 18 kV in electroosmotic flow mobilization mode, with UV detection at 340 nm.

The cIEF separations with the 0.1% HPMC, 30 mM HP β CD, serine—propionic acid binary Bier buffers were obtained by first filling the capillary with a 0.1% HPMC, 30 mM HP β CD, 90 mM

serine-10 mM propionic acid mixture, pH 3.9, using the injection pressure of the P/ACE. Next, a 1-s-wide band of nitromethane marker, dissolved in the pH 3.9 BE was injected. Then, this band was moved into the capillary by pushing it with a 0.1% HPMC, 30 mM HP β CD, 40 mM serine-60 mM propionic acid mixture, pH 3.4, using the injection function of the P/ACE. The band was stopped 9.25 cm away from the detector window. Next, a 1-s-wide band of the DNS-Phe sample, dissolved in the pH 3.4 BE was injected. Then, it was pushed into the capillary with a 0.1% HPMC, 30 mM HP β CD, 35 mM serine-65 mM propionic acid mixture, pH 3.3, using the injection function of the P/ACE and was stopped 27.75 cm away from the detector window. The detector-side vial (cathode vial) was filled with the pH 3.9 BE; the injector side vial (anode vial) was filled with the pH 3.3 BE. The separation potential (25 kV) was turned on for 48 min, followed by the injection of a 1-s-wide band of nitromethane, dissolved in the pH 3.3 BE. Finally, the content of the entire capillary was pushed out, using the pH 3.3 BE and the injection pressure, and the UV detector trace was recorded. Since the push velocity is constant during the last step, the time variable can be directly translated into position in the capillary.

The preparative, continuous free-flow IEF enantiomer separations were completed by first filling the Octopus separation chamber with deionized water and removing all air. Then, the anode compartment was filled with 40 mM serine-360 mM propionic acid and the cathode compartment was filled with 360 mM serine-40 mM propionic acid. The inlet ports were pumped at 13.5 mL/h with 0.1% HPMC, 30 mM HP β CD, x mM serine/y mM propionic acid/pH *z* BEs as follows: first port, 50/150/3.37; second port, 60/140/3.42; third port, 30/130/3.49; fourth port, 80/ 120/3.55; fifth port, 90/110/3.59; sixth port, 100/100/3.63; seventh port, 110/90/3.70. The sample, 2 mM DNS-Phe, dissolved in the pH 3.55 BE, was injected through the central sample port, at a rate of 3 mL/h. The counterflow, 0.1% HPMC in deionized water, was pumped at 55.0 mL/h. Once the flows were established, 1700-V potential was applied across the 10-cm-wide separation channel and the current was monitored; it became steady in ${\sim}0.5$ h. Fractions of 2 mL were then collected in 96-well deep titer plates, and the pH of each fraction was determined using a combined glass electrode and model 05669-20 precision pH meter (Cole-Parmer, Vernon Hills, IL).

Then, 200 μ L of 8.4 mM DNS-Gly was added to each 2-mL aliquot taken from the collected fractions and passed through methanol-activated, C18 solid-phase extraction cartridges (Adsorbex, E. Merck, Darmstadt, Germany). The cartridges were washed with 1.5 mL of deionized water and then eluted with 1 mL of methanol. The methanol solutions were evaporated to dryness in an AES1010 SpeedVac drier (Savant Industries, Holbrook, NY), reconstituted in 300 μ L of 200 mM IDA, 200 mM HP β CD BE, and analyzed for enantiomeric purity on the P/ACE.

RESULTS AND DISCUSSION

The cIEF separations of the enantiomers of DNS-Phe were obtained in 15, 30, 60, and 120 mM HP β CD, 2.0% ampholyte pH 3–5 BEs. As predicted by eq 31 for an antagonistic duo-selective case, the two DNS-Phe enantiomer bands were well separated from each other and there was not much change in the $\Delta pI'$ values as the HP β CD concentration was varied from 15 to 120 mM. The enantiomer bands were separated from each other by 3.6, 3.4,



Figure 6. EOF-mobilized cIEF separation of the enantiomers of DNS-Phe in 2.0% ampholyte pH 3–5, 30 mM HP β CD background electrolyte. For other conditions, see text.



Figure 7. cIEF separation of the enantiomers of DNS-Phe in Bier's serine–propionic acid, 0.1% HPMC, 30 mM HP β CD background electrolyte. Separation time: 48 min, 25 kV, pressure mobilization. For other conditions, see text.

3.6, and 2.9 cm, respectively, in the 10, 30, 60, and 120 mM HP β CD BEs. The electropherogram obtained in the 30 mM HP β CD, 2.0% pH 3–5 ampholyte BE with electroosmotic mobilization is shown in Figure 6. Though the pH 3–5 ampholyte BE permits the analytical-scale separation, the separated enantiomer bands also contain the ampholytic components. This is undesirable in a preparative application.

Therefore, the cIEF separation was repeated using 30 mM HP β CD serine—propionic acid binary Bier buffers, as shown in Figure 7. To follow more easily what happened during the separation, the time axis is replaced by the distance (from the detector window) axis. The neutral marker, which was placed 9.25 cm away from the detector window and marked the boundary between the pH 3.9 and pH 3.4 buffers, is now 4.8 cm away from the window. This 4.4 cm movement was caused by the residual electroosmotic flow (EOF) during the 48-min-long separation. The D- and L-DNS-Phe enantiomers were originally 18.5 cm away from the nitromethane neutral marker. For the D-enantiomer, this distance decreased to ~6 cm; the high-pH side of the peak is sharper, the low-pH side is broader, reflecting focusing. For the



Figure 8. Preparative continuous free-flow IEF separation of the enantiomers of DNS-Phe using Bier's serine-propionic acid, 0.1% HPMC, 30 mM HP β CD background electrolyte in the Octopus unit. Residence time: 12.5 min, 1700 V. For other conditions, see text.

L-enantiomer, the distance decreased to 19 cm; the low-pH side of the peak is sharper, once again reflecting focusing. In 48 min, the D- and L-enantiomers became separated by 13 cm. When the focusing time was almost doubled to 90 min, the neutral marker was swept past the detector by the residual EOF, but the distance between the D- and L-enantiomers increased only by \sim 1 cm, to a total of 14 cm, indicating that the final migration position in the pH gradient was almost completely achieved. When the focusing time was further increased to 120 min, even the D-enantiomer was swept past the detector by the EOF.

Figure 8 shows the enantiomeric analysis results of the fraction streams collected during the continuous free-flow preparative-scale IEF separation in the Octopus unit. The pH profile is superimposed on the graph (right axis). It shows that the L-DNS-Phe band is located at around pH 3.54 where pH varies evenly from fraction to fraction. The D-DNS-Phe band is located in an eight-fraction-wide constant pH region, at pH 3.59. The band shape of L-DNS-Phe indicates that there is good focusing on the low-pH side. With the 12.5-min-long residence time in the separation chamber, there is not enough time for D-DNS-Phe to migrate out of the constant pH zone and approach its final focusing position in channels somewhere above 60. Nevertheless, the D-DNS-Phe enantiomer is completely resolved from the L-DNS-Phe enantiomer. When there is no electric field applied, the racemic D- and L-DNS-Phe

mixture can be collected in channels 48–52 (over a distance of \sim 0.4 cm). With the electric field on, both bands are only five channels wide (covering a distance of \sim 0.5 cm), indicating that the detrimental electrohydrodynamic band-broadening processes³⁵ are well controlled in the system. The accuracy of the analytical system is good: the collected fractions were found to contain 1.988 mg of L-DNS-Phe and 2.012 mg of D-DNS-Phe, vis-à-vis the added 2.0-mg quantities. This represents a production rate of 1.33 mg/ h, with an enantiomeric excess of better than 99.99% in the combined fractions.

CONCLUSIONS

A rigorous theoretical model has been developed to describe chiral IEF separations that rely on complexation-generated pI'differences between the enantiomers. Though all the model parameters can be determined by straightforward PreMCE or conventional CE experiments, only the pI of the uncomplexed analyte need to be known to set up successful IEF separations. Since the predicted $\Delta pI'$ values are about 0.05–0.1, narrow, nonnatural pH gradients generated by simple binary buffers, such as Bier's buffers, can be used to resolve the cyclodextrincomplexed enantiomers. This simplifies the downstream processing of the collected fractions prior to their intended end use. With the Octopus continuous free-flow electrophoretic unit, production rates of 1.33 mg/h yielding an enantiomeric excess of better than 99.99% have been demonstrated.

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⁽³⁵⁾ Rhodes, P. H.; Snyder, R. S.; Roberts, G. O.; Baygents, J. C. Appl. Theor. Electrophor. 1991, 2, 87–98.