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USE OF COMPLEXING AGENTS FOR SELECTIVE SEPARATION IN HIGH-PERFORMANCE CAPILLARY ELECTROPHORESIS

CHIRAL RESOLUTION VIA CYCLODEXTRINS INCORPORATED WITHIN POLYACRYLAMIDE GEL COLUMNS

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SUMMARY

The incorporation of a complexing agent within a polyacrylamide gel column provides a general means of manipulating the selectivity of a capillary electrophoresis separation. As an example of this approach, chiral resolution of dansylated amino acids by high-performance capillary electrophoresis has been achieved by adding β-cyclodextrin within the gel matrix. A retention model has been developed which can be used for selectivity optimization. Various parameters, such as concentration of cyclodextrin, addition of methanol to the buffer, and temperature, have been examined in terms of their influence on retention and selectivity. From this study high-performance separations have been developed. Efficiencies as high as 100 000 plates in 15 cm have been achieved.

INTRODUCTION

High-performance capillary electrophoresis (HPCE) is a method undergoing rapid development at the present time[1–7]. With on-column detection, the method has analogy to high-performance liquid chromatography (HPLC) in terms of its instrumental approach and potential for automation. HPCE is characterized as a rapid, highly efficient separation tool. This arises in part from the ability to operate the columns at high electric field for fast migration through the capillary and, when axial diffusion controls band broadening, for high efficiency. Significant Joule heat is produced at high power levels, and this heat must be removed to take full advantage of the high fields. Capillaries are advantageous in this regard since for a given power level the temperature gradient between the column center and the walls is proportional to the square of the tube radius[8].

As in HPLC, the versatility of HPCE can be extended via the incorporation of

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chemical selectivity into the migration process. The need for new approaches to manipulate retention in HPCE has been noted by others[9]. Micelles have been used in open tubes for separation of neutral species[10] and differential metal chelate complexation on the surface of the micelles for selective ligand exchange[11]. In addition, selective interaction with the buffering components or other additives has been employed in the open tube for hydrophobic[12] and chiral separations[13].

As a further example of selectivity manipulation, we have explored the use of cyclodextrins (CDs) as selective complexing agents in HPCE. CDs have been shown in HPLC to separate closely related species, including chiral species, via the formation of inclusion complexes[14]. Amino acids, barbiturates, prostaglandins and other drugs have been successfully resolved using CDs, either bound to the stationary phase or as an additive to the mobile phase[15]. Interestingly, positional isomers were resolved using a modified charged form of the CDs via micellar CE[16]. Also penicillins have recently been separated by capillary isotachophoresis with the addition of CD to the buffer[17].

CDs are non-ionic cyclic polysaccharides of glucose with a shape of a toroid or hollow truncated cone. The particular name of a CD depends on the number of glucose units; α-, β-, and γ-CD corresponds to six, seven and eight glucose units, respectively. The cavity is relatively hydrophobic while the external faces are hydrophilic, with the edge of the torus of the larger circumference containing chiral secondary hydroxyl groups[18].

Fig. 1 shows a possible complex of a dansylated amino acid (Dns-AA) with β-CD[19], as well as the dimensions of this CD. Note that the non-polar dansyl portion of the molecule is found inside the cavity and that the amino group forms hydrogen bonds with the hydroxyl groups at the rim of the toroid. Selectivity or differential complexation of individual Dns-AA results from the size of the hydrophobic group with respect to the ability of the solute to penetrate the cavity. In addition, chiral selectivity can arise from hydrogen bonding at the entrance of the cavity with the chiral glucose moiety.

We first tested CDs as additives to the buffer in open tube HPCE with

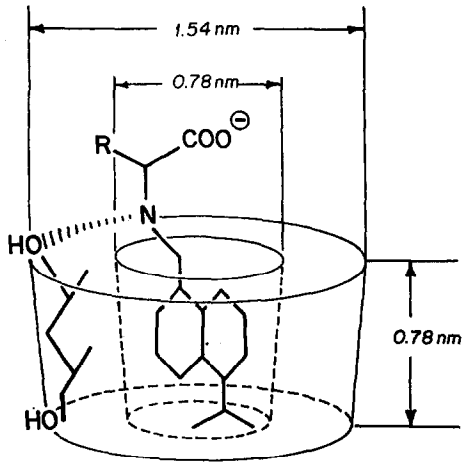

Fig. 1. Schematic diagram of the β-cyclodextrin–Dns-amino acid complex.

electroosmotic flow but found no influence on retention or separation of dansylated amino acids. Previously, we have reported on the successful use of HPCE polyacrylamide gel columns for the sodium dodecyl sulfate electrophoresis of peptide and proteins[20]. When we incorported CDs into polyacrylamide gel capillary columns, chiral separation was obtained. The addition of biorecognition agents into polyacrylamide gel slabs has also been used in affinity electrophoresis[21].

This paper reports the HPCE results with polyacrylamide gel columns on the chiral resolution of dansylated amino acids using CDs. In this work, plate counts of 50 000–100 000 in columns of 15 cm effective length have been obtained using fields up to 1000 V/cm. The principles of retention and selectivity optimization are developed. The general requirements for high-performance rapid separation in capillary electrophoresis using selective complexing agents within polyacrylamide gels are presented. This approach can readily be used with a wide variety of complexing agents for selective separations in HPCE, thus extending the scope of the methodology.

THEORY

Retention and selectivity

The retention model for separation with incorporated complexing agents follows that used in affinity electrophoresis[21,22]. In general, when a complexating agent C is present in a capillary electrophoretic system, a fraction of the solute A will occur as uncomplexed or free in solution (R) and the remainder will exist as a complex ($1 - R$). The net mobility of the solute μ will then be the weighted sum of the mobility of the free solute μ^f and the complexed solute μ^c

$$\mu = R\mu^f + (1 - R)\mu^c \tag{1}$$

The fraction R is equal to c^f/c^t where c^f = concentration of uncomplexed A and c^t = total concentration of A. R can be related to the formation constant for complexation K by

$$A + C \rightleftharpoons AC \tag{2}$$

$$K = \frac{[AC]}{[A]\,[C]} \tag{3}$$

and

$$R = \frac{c^f}{c^t} = \frac{1}{1 + K[C]} \tag{4}$$

$$1 - R = \frac{K[C]}{1 + K[C]} \tag{5}$$

The formation constant is an apparent value since concentrations are used instead of activities. Substitution of eqns. 4 and 5 into 1 yields the general mobility expression

$$\mu = \frac{\mu^f}{1 + K[C]} + \frac{K[C]\mu^c}{1 + K[C]} \tag{6}$$

The retention of a solute in electrophoresis t_R can be written as

$$t_R = \frac{l}{\mu E} = \frac{lL}{\mu V} \tag{7}$$

where l = effective length of the capillary tubing (from injection to detection point), E = electric field, V = applied voltage and L = overall length of the capillary between the two reservoirs. The relative retention α can be expressed as

$$\alpha = \frac{t_{R2}}{t_{R1}} = \frac{\mu_1}{\mu_2} \tag{8}$$

where subscripts 1 and 2 refer to the earlier and later migrating species, respectively (*e.g.*, L and D). Note that in comparison to chromatography, with gel capillary columns there is no bulk flow and therefore subtraction of the unretained solute time t_0 from t_R does not result. If open tubes with electroosmotic flow were employed, subtraction of t_0 would be required[2].

Combination of eqns. 6 and 8 yields the general expression for relative retention with complexing agents

$$\alpha = \frac{\mu_1^f}{\mu_2^f} \underbrace{\left\{\frac{1 + K_1[C]\,(\mu_1^c/\mu_1^f)}{1 + K_2[C]\,(\mu_2^c/\mu_2^f)}\right.}_{\text{I}} \cdot \underbrace{\left.\frac{1 + K_2[C]}{1 + K_1[C]}\right\}}_{\text{II}} \tag{9}$$

Three cases can be envisioned from eqn. 9. First, if the mobility of the uncomplexed solute is much greater than that of the complex AC, *i.e.*, $\mu^f \gg \mu^c$, then term I on the right hand side will be approximately unity and

$$\alpha = \frac{\mu_1^f}{\mu_2^f}\left(\frac{1 + K_2[C]}{1 + K_1[C]}\right) \tag{10}$$

As will be noted later, eqn. 10 is the relevant expression when CDs are incorporated within the gel matrix. When $K[C] \gg 1$ and for chiral pairs, eqn. 10 simplifies to

$$\alpha = \frac{K_2}{K_1} \tag{11}$$

where μ_2^f is assumed equal to μ_1^f (L and D isomers of uncomplexed species). Eqn. 11 represents the maximum value of α in the chiral system.

Secondly, the complex may move much faster than the free solute, *i.e.*, $\mu^f \ll \mu^c$ and term I in eqn. 9 will now override term II. Note that in this case the elution order will reverse relative to that in eqn. 10. Finally, if AC moves at the same rate as A,

α approaches unity, and no separation is possible. It can be concluded that separation is best achieved when one of the extreme cases exists, *i.e.*, $\mu^c/\mu^f \gg 1$ or $\ll 1$.

Resolution

The resolution R_s of a chiral pair of solutes can be written as

$$R_s = \frac{\Delta t_R}{4\sigma_t} \tag{12}$$

where Δt_R = retention time difference of the D,L pair and σ_t = time based standard deviation of the band. Since

$$N = \left(\frac{t_R}{\sigma_t}\right)^2 \tag{13}$$

combination of eqns. 7 and 13 with 12 yields

$$R_s = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right) N^{1/2} \frac{\mu E}{l} \tag{14}$$

or

$$R_s = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right) \frac{\mu E}{H^{1/2} l^{1/2}} \tag{15}$$

The resolution is seen to be proportional to the field, if H (height equivalent to a theoretical plate) is independent of E. Also, as in chromatography, the value of $\alpha - 1$ can dramatically affect resolution when α is close to one[23].

EXPERIMENTAL

Apparatus

A regulated high voltage power supply, 0–30 kV and 0–1 mA, Model PS/EG 30P1-RV-II (Glassman High Voltage, Whitehouse Station, NJ, U.S.A.) was used. Electrophoresis was performed in 30 cm × 75 μm I.D. fused-silica capillary tubing (Scientific Glass Engineering, Austin, TX, U.S.A.). At 15 cm approximately 1 cm of polyimide coating was carefully burned off to expose the fused silica for on-column detection which was accomplished with a microbore UV system (ISCO, Lincoln, NE, U.S.A.). Care was exercised to position the fused silica in the light path of the source. The temperature of the fused-silica capillary was controlled by a specially designed water cooling system. Each end of the capillary was connected to a separate 3-ml vial (Wheaton Science, Mellville, NJ, U.S.A.) filled with buffer. Platinum wire electrodes were inserted into the two vials for connection to the electrical circuit. The electropherogram signals were acquired and stored on an IBM PC/XT computer via a Model 762 SB A/D interface (Nelson Analytical, Cupertino, CA, U.S.A.). Sample injection was accomplished by placing one of the capillaries in the sample vial and applying a field for a fixed length of time.

Materials

The dansylated D,L-amino acids (Dns-DL-AA) were purchased from Sigma (St. Louis, MO, U.S.A.) and used as received. The water was deionized and triply distilled. Before use, all buffer and sample solutions were filtered through a Nylon 66 filter unit of 0.2 μm pore size (Schleicher & Schuell, Keene, NH, U.S.A.) and then degassed. The polyacrylamide columns were made with appropriate amounts of a CD dissolved in the gel buffer solution prior to polymerization. The formation of the polyacrylamide gel columns thus incorporated the CD into the porous network.

RESULTS AND DISCUSSION

Retention and separation

Our initial efforts in achieving chiral selectivity involved the addition of β-CD, a complexing agent known to resolve Dns-DL-AAs[19], to the buffer of open tube capillaries. With a variety of conditions, no change in retention or separation was observed. This could mean no complexation took place. However, assuming complexation occurred, the change in mobility upon complexation may have been small. Eqn. 9 shows that no separation would take place, if $\mu_1^c = \mu_1^f$ and $\mu_2^c = \mu_2^f$. As clearly seen in this equation, the formed complex has to differ significantly in mobility from that of the free amino acid. One could consider manipulating the mass or charge of the cyclodextrin to create a difference in mobility of the complex and free species as employed in micelle CE[16] as well as more generally in affinophoresis[24]; however, we decided on an alternative approach.

We reasoned that incorporating the CD into a polyacrylamide gel matrix would substantially slow down or immobilize the Dns-AA–CD complex. First, by virtue of the neutrality of CDs, these agents by themselves would not move in a gel matrix under the influence of an electric field. Secondly, as seen in Fig. 1, the distance across the wide edge of the toroid for β-CD is *ca.* 1.6 nm which is relatively wide for a non-polymeric species. Both from charge and size considerations, the Dns-AA–CD complex would be expected to migrate slowly, if at all, through the porous network. In this manner, there is direct analogy to chromatography when a CD is attached to a stationary chromatographic phase[14].

While it is possible to attach the CD covalently to polyacrylamide gels[21], we further reasoned that the simplest and most reproducible approach would be to incorporate the complexing agent directly into the porous matrix without bonding during the polymerization step. This was accomplished by simply adding the CD to the solution prior to free radical polymerization.

Fig. 2A shows the separation of Dns-Glu, Dns-Ser and Dns-Leu in a gel without any CD present. The retention order is in the expected direction of decreasing electrophoretic mobility with the doubly charged Dns-Glu migrating fastest and the singly charged bulky Dns-Leu migrating slowest. As expected, no chiral resolution is observed since a chiral complexing agent is not present. Fig. 2B shows the same sample injected on a gel column now polymerized from a solution containing 75 m*M* α-CD. Separation is similar to Fig. 2A, and no chiral resolution is found. It is known that the cavity size of α-CD is too small for effective penetration of dansylated amino acids, and no chiral resolution is expected[14].

Fig. 2C shows the baseline separation of the D,L pairs of each of the three

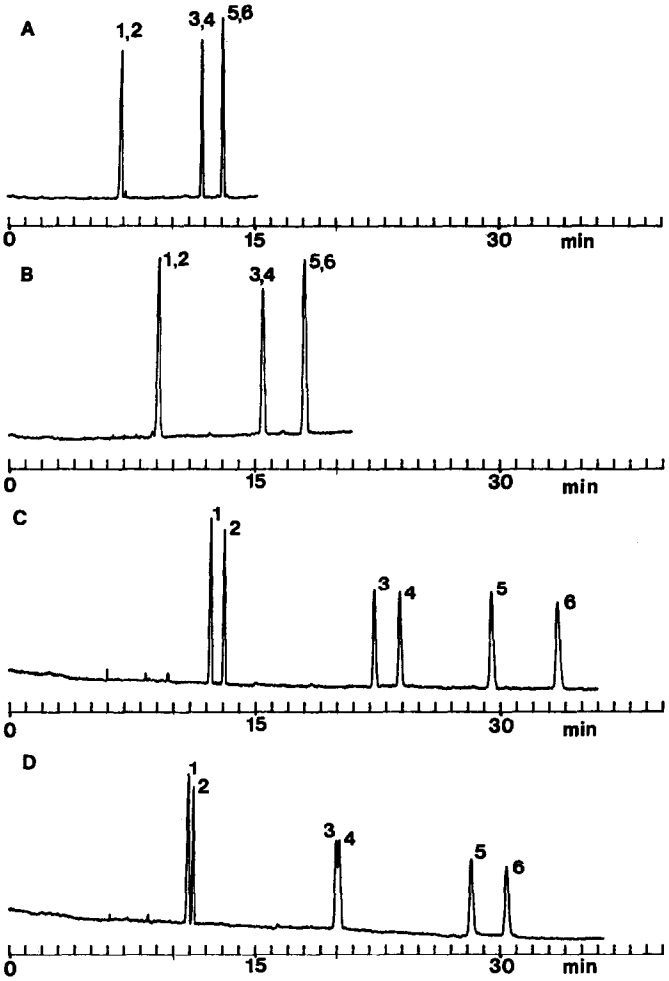

Fig. 2. Separation of Dns-DL-AAs. 1 = Dns-L-Glu; 2 = Dns-D-Glu; 3 = Dns-L-Ser; 4 = Dns-D-Ser; 5 = Dns-L-Leu; 6 = Dns-D-Leu. (A) Buffer: 0.1 *M* Tris–0.25 *M* boric acid (pH 8.3), 7 *M* urea, Gel: T = 5%, C = 3.3%, 0.1 *M* Tris–0.2 *M* boric acid (pH 8.3), 7 *M* urea. Capillary: 150 mm × 0.075 mm I.D., 400 V/cm, 8 μA. Electroinjection: 250 V/cm, 5 μA, 30 s, detection wavelength, 254 nm. (B) Addition of 75 m*M* α-CD to the buffer and the gel mixture. (C) Addition of 75 m*M* β-CD to the buffer and the gel mixture. (D) Addition of 75 m*M* γ-CD to the buffer and the gel mixture.

Dns-AAs in a gel column containing 75 m*M* β-CD. This complexing agent is known to be ideal for these substances[15]. In addition, the retention order is found to be L migrating faster than D. This order agrees with the literature where it is known that the Dns-L-AA forms the weaker complex with β-CD[14]. It can also be seen that for any Dns-AA, retention is longer in the case of β-CD than α-CD, no doubt primarily due to the retardation caused by complexation with β-CD. It is to be noted that 7 *M* urea has been included in the buffer in all cases of Fig. 2. It was found that the urea increased relative retention, thus providing improved separation. It is known that additives to the mobile phase in LC affect selective binding to CDs[14,15].

TABLE I

CHIRAL SELECTIVITY (α) FOR Dns-DL-AMINO ACIDS WITH β-CYCLODEXTRIN IN A POLYACRYLAMIDE GEL COLUMN

Column, 100 m*M* β-CD in a 5%T, 3%C gel, 7 *M* urea; buffer, 0.1 *M* Tris–0.25 *M* boric acid (pH 8.3), T = 25°C.

Dns-DL-AA	α	
	Buffer (400 V/cm, 8 μA)	*Buffer + 10% methanol (700 V/cm, 10 μA)*
Dns-DL-Leu	1.18	1.12
Dns-DL-Ser	1.12	1.09
Dns-DL-Val	1.09	1.12
Dns-DL-Glu	1.09	1.06
Dns-DL-Asp	1.08	1.09
Dns-DL-Met	1.09	1.11
Dns-DL-Thr	1.09	1.12
Dns-DL-Norleu	1.11	1.13
Dns-DL-NorVal	1.07	1.09
Dns-DL-α-amino-*n*-butyric acid	1.06	1.08
Dns-DL-Phe	1.04	1.08
Dns-DL-Trp	1.025	1.05

Finally, Fig. 2D shows a similar separation with 75 m*M* γ-CD. Chiral resolution is in general poorer with this CD, relative to β-CD. This result is again expected, since it is known that if the cavity is too wide, poor selectivity results[14]. As noted above, the optimum fit for the inclusion complex with Dns-AAs occurs with β-CD.

Further data with β-CD can be found in Table I which presents α values for twelve Dns-AAs both in the aqueous buffer and a buffer containing 10% (v/v) methanol. It can be seen that, as expected[14,15], relative retention varies with addition of methanol. In some cases, *e.g.*, Dns-DL-Leu, α is lower with added methanol; however, for the aromatic amino acids there is a substantial increase in α. As already noted, eqns. 14 and 15 show that improvement in α, when the relative retention is close to unity, can greatly increase resolution. This can be clearly seen in Fig. 3 where Dns-DL-Phe and Dns-DL-Trp are separated with and without the addition of methanol. The improvement in separation in the case of added methanol is self-evident. As in chromatography, organic modifier in the buffer can be used to manipulate selectivity when CDs are in the system. Finally, it can be noted in Table I and Fig. 3 that a much higher field with added methanol produces approximately the same current as a lower field with the aqueous buffer. This result is a consequence of the decreased dielectric constant in the solution of 10% (v/v) methanol.

Factors influencing chiral resolution

Eqn. 10 is an expression similar to that found in HPLC when secondary chemical equilibria are present[25]. As already noted, α will be greatest when $K[C]\mu^f \gg 1$. Below this limit, the concentration of β-CD in the gel matrix should influence α. Fig. 4 shows plots of α *versus* concentration of β-CD in the polymerizing solution, with the α value tending to a plateau at a high concentration of β-CD. The greatest curvature, as

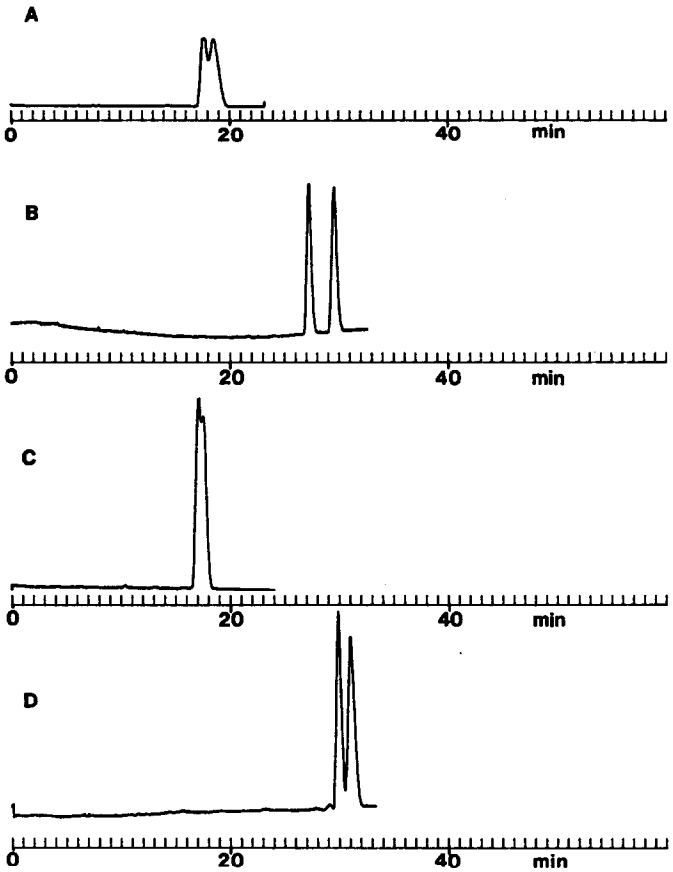

Fig. 3. Effect of methanol on chiral selectivity, conditions as in Fig. 2C, except as noted. (A) Dns-DL-Phe, E = 400 V/cm. (B) Dns-DL-Phe, E = 700 V/cm, buffer contains 10% (v/v) methanol. (C) Dns-DL-Trp, E = 400 V/cm. (D) Dns-DL-Trp, E = 700 V/cm, buffer contains 10% (v/v) methanol.

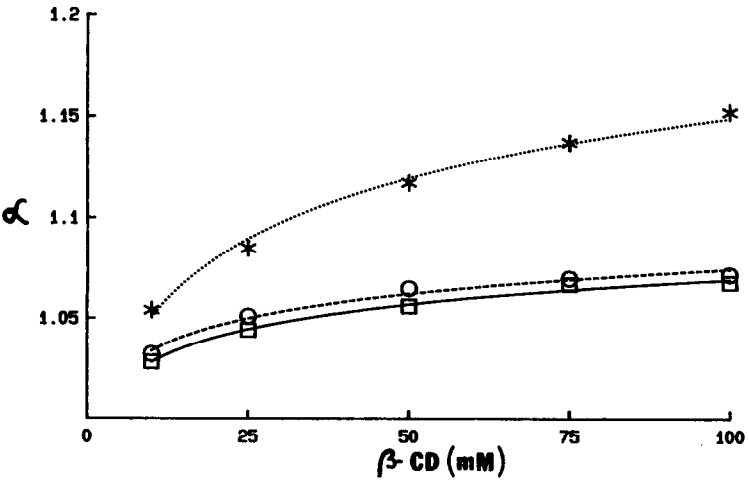

Fig. 4. Dependence of chiral selectivity, α, on β-CD concentration in the gel. Test mixture: * = Dns-DL-Leu; ○ = Dns-DL-Ser; □ = Dns-DL-Glu. All other conditions are given in Fig. 2C.

TABLE II

Dns AMINO ACID–β-CYCLODEXTRIN ASSOCIATION CONSTANTS (K) IN A POLYACRYLAMIDE GEL COLUMN

Temperature, 25°C; buffer, 0.1 *M* Tris–0.25 *M* boric acid (pH 8.3); no methanol added.

Dns-DL-AA	$K\ (M^{-1})$
Dns-L-Glu	6.4
Dns-D-Glu	7.6
Dns-L-Ser	7.3
Dns-D-Ser	8.6
Dns-L-Leu	13.3
Dns-D-Leu	17.6

expected, occurs with Dns-DL-Leu which has the highest binding constant of the amino acids in this figure (see Table II). The concentration of β-CD has not yet reached a sufficient level to yield the maximum value of α, *i.e.*, the plateau has not yet been reached.

As further evidence of the model, Fig. 5 shows plots of retention time of dansylated D-amino acids *versus* β-CD concentration. The linear behavior can be seen by combination of eqns. 6 and 7 and noting that $\mu^c \ll \mu^f$.

$$t_R = \frac{l(1 + K[C])}{\mu^f E} \tag{16}$$

Eqn. 16 suggests that the greatest slope of the plot of t_R *versus* E would be expected for Dns-D-Leu which has the strongest binding (see Fig. 5). In addition, μ^f for Dns-D-Leu

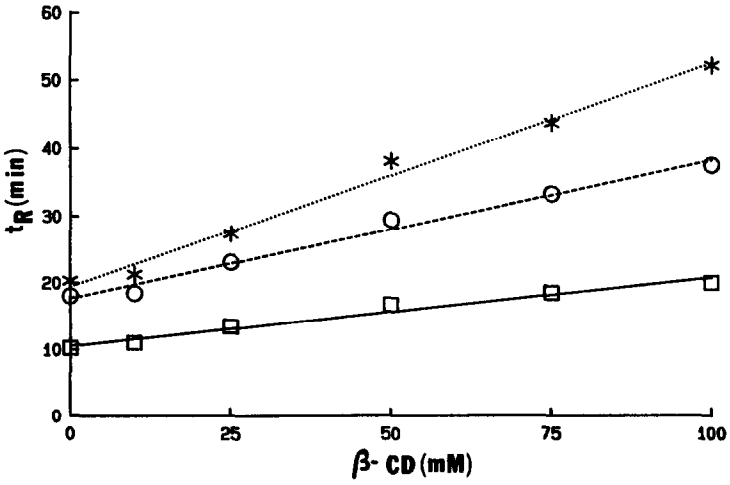

Fig. 5. Dependence of solute retention time, t_R, on β-CD concentration in the gel. Test mixture: * = Dns-D-Leu; ○ = Dns-D-Ser; □ = Dns-D-Glu. All other conditions are given in Fig. 2C.

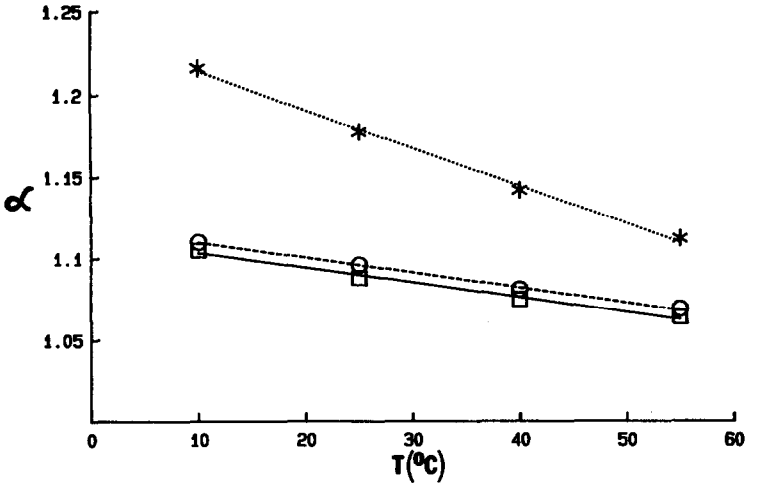

Fig. 6. Dependence of chiral selectivity, α, on column temperature, *T*. Test mixture is same as in Fig. 4. The buffer and the gel contained 10% (v/v) methanol. All other conditions are given in Fig. 2C.

is the lowest of the three Dns-AAs and therefore this substance would be expected to have the largest intercept value, as also observed in Fig. 5. Eqn. 16 and Fig. 5 can be used to calculate the apparent formation constants from the slope of the plots. Results for these constants in buffer are shown in Table II.

We next explored the influence of column temperature on separation. In this study, the column temperature was varied between 10°C and 55°C. Retention decreased with increasing temperature, as K is known to become smaller at elevated temperatures[26] and μ^f is larger at higher temperatures (see eqn. 16). The α value was also found to decrease with temperature, as shown in Fig. 6. Interestingly, the rate of change of α with temperature was greatest for the strongest binding amino acid, Dns-Leu. With the largest K values for the Dns-DL-Leu pair, the $\Delta(\Delta H)$ (difference in heats of complexation for the enantiomeric pair) is found to be the greatest. Fig. 6 shows that, as in chromatography, temperature can be used for manipulation of selectivity.

Columns of high efficiency are possible with complexing agents incorporated into the gel matrix. Fig. 7A shows the separation of the three enantiomeric pairs in which the bands of the Dns-Ser pair each achieve roughly 100 000 theoretical plates in the column of 15 cm effective length and a buffer of 10% (v/v) methanol. For Dns-DL-Ser, the resolution is 6.4, in spite of the fact that α is only 1.12. More typically, *ca.* 50 000–80 000 plates are obtained, as found for the Dns-DL-Leu pair. The high efficiency arises in part from the high field of 700 V/cm. Attention was paid to minimize the extra column effects of injection (*ca.* 5 nl) and the detector (*ca.* 2nl). With further reduction in extra column band broadening, increases in efficiency will probably be possible. As a further example, Fig. 6B shows an identical separation to Fig. 6A except that the field was increased to 1000 V/cm with the 10% methanol buffer. The separation is now achieved in roughly half the time, or 10 min.

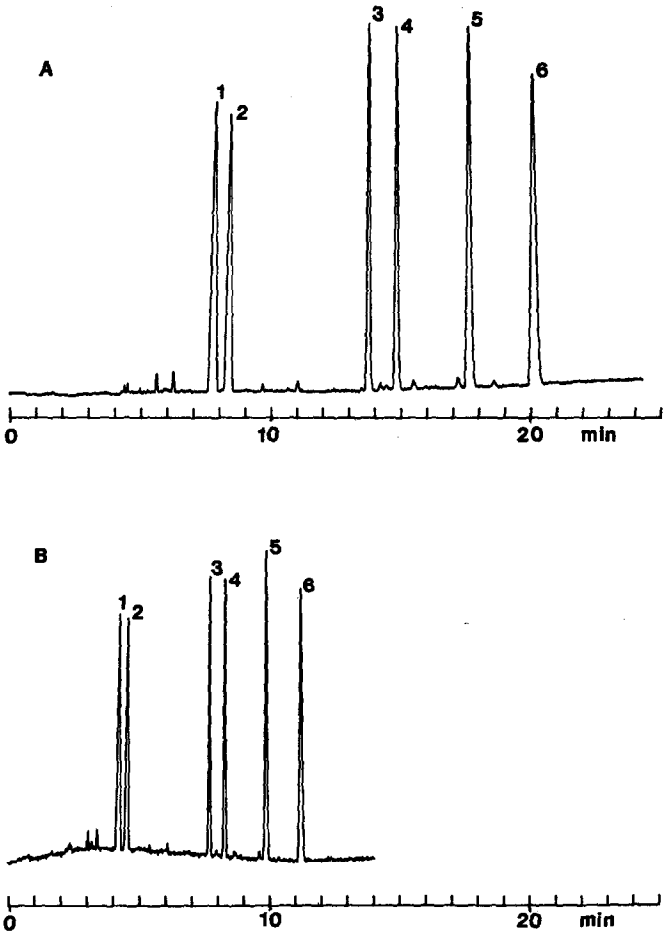

Fig. 7. High efficiency separation of Dns-DL-AAs. The test mixture is the same as in Fig. 2. All the other conditions are given in Fig. 6. (A) E = 700 V/cm; (B) E = 1000 V/cm.

CONCLUSIONS

High-performance capillary gel electrophoresis in which a complexing agent is incorporated into the gel matrix expands the potential of HPCE. We have found that high-performance, rapid separation and controlled selectivity are possible with these columns. High-performance chiral separation of Dns-DL-AAs has been achieved using β-CD. A model of the separation equilibria has been developed, from which it is emphasized that the mobility of the free species and the complex must differ significantly from one another in order to utilize the complexation equilibria to the fullest. Undoubtedly, the neutral character of the CDs themselves as well as the width of the toroid play a role in creating a low mobility of the solute–CD complex. In addition, the rates of association and dissociation must be rapid to take advantage of the high efficiency possible with the electrophoresis system. It is straightforward to

visualize tailor-made columns for high-performance separation of microamounts of specific samples. Separated fractions can be conveniently collected for further characterization, as recently shown by us[27] and others[28].

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