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Systematic approach to treatment of enantiomeric separations in capillary electrophoresis and liquid chromatography. I. Initial evaluation using propranolol and dansylated amino acids

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Abstract

A systematic approach is outlined for treatment of enantiomeric separations in capillary electrophoresis (CE) and liquid chromatography (LC) using chiral mobile phase additives. General equations and data analysis methods are presented to relate mobilities or capacity factors to equilibrium constants in binding equilibria, and to maximise mobility or retention time differences as a function of selector concentration. The use of cyclohexanol as a competitor is shown to be beneficial in optimising chiral separations of species which bind strongly to β-cyclodextrins. This general treatment has been applied with the test systems 1: propranolol and β-cyclodextrin and 2: dansylated amino acids and β-cyclodextrin. Chiral separations and binding constants, determined using LC with β-cyclodextrin as a mobile phase additive or a chiral stationary phase, are compared with results using the same selector in CE for system 2. Mobile phase equilibria defined by CE reveal more complex stationary phase binding equilibria in LC. Our studies make a link between LC and CE which may allow rational separation strategies to be transferred between the two fields.

1. Introduction

Chiral analysis in the separation sciences has become increasingly important in recent years due to differences in biological activity of the enantiomers of pharmacologically active compounds [1]. In liquid chromatography (LC) analysis of the different enantiomers is brought about by use of either a chiral stationary phase, or by the addition of chiral additives in the mobile phase. Sybilska and co-workers [2,3] have developed a systematic treatment for use with cyclodextrins (CDs) acting either via complexation in the mobile phase or as a dynamically generated stationary phase. Equations giving the dependence of capacity factors on the CD concentration have been used to determine enantioselective binding constants for a series of chiral barbiturates with β-CD. Similar equations have been used for considering variation of separation factor with β-CD concentration for some positional isomers [4]. In the emerging field of chiral capillary electrophoresis (CE) separation is carried out by adding to the run-

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ning buffer additives such as bile salts [5], chiral surfactants [6], and cyclodextrins [7]. Some recent papers have looked for a strategy to optimise chiral CE with the use of cyclodextrin additives. Wren and Rowe [8] have developed a theoretical model relating mobility to the concentration of a cyclodextrin selector. Their analysis suggested that an optimum CD concentration exists for a particular chiral separation, and this was observed using propranolol and methyl-β-cyclodextrin (Me-β-CD) as a model system. In our previous paper we have extended this treatment [9], showing how binding constants could be derived and giving an application to the system tioconazole and hydroxypropyl-β-cyclodextrin (HP-β-CD). Maximum mobility difference was shown to occur when the selector concentration equalled the reciprocal of the average binding constant. Rawjee and co-corkers [10–12] have developed a multiple-equilibria-based model to account for separation of chiral weak acids and bases as a function of both pH and β-CD. In this paper we aim to find a master approach linking the methods of processing results from chiral CE, and chiral LC methods through the use of both mobile phase additives and chiral stationary phases. In short, can CE methods of optimisation be applied to LC?

2. Theory

2.1. Determination of binding constants, and prediction of resolution in CE

Chiral separations can take place in CE by the addition of a chiral selector, such as cyclodextrin, to the mobile phase. Binding constants can be determined from the dependence of mobility of the analyte on the selector concentration,

$$KC = \frac{\mu_0 - \mu}{\mu - \mu_\infty} \tag{1}$$

where K is the binding constant, and μ, μ_0, μ_∞ are the mobilities of the analyte at concentration of free selector C, mobility of free analyte, and mobility of the analyte:selector complex, respectively. Whilst μ and μ_0 are measured experimentally, μ_∞ and K are obtained by a non-linear least squares fit of the data to Eq. 1 [13]. The experimental set of data for a pair of enantiomers gives the values of binding constants K_1 and K_2 for the two enantiomers. When viscosity varies with selector concentration, observed mobilities, μ_{eff}, should be converted to corrected mobilities before fitting to Eq. 1 [8].

$$\mu = \mu_{\text{eff}} \frac{\eta}{\eta_0} \tag{2}$$

where η_0 and η are viscosities at selector concentration zero and C, respectively.

The selectivity α can be calculated from,

$$\alpha = \frac{K_2}{K_1} \tag{3}$$

The mobility difference $\Delta\mu$ for the enantiomers is dependent on ΔK, the difference of binding constants via the relationship [9,13],

$$\frac{\Delta\mu}{\mu_0 - \mu_\infty} = \frac{-\Delta K}{\bar{K}} \cdot \frac{\bar{K}C}{(1 + \bar{K}C)^2} \tag{4}$$

where $\bar{K}$ is the average binding constant $(K_1K_2)^{1/2}$. Fitting data to this equation allows the difference in binding constants of the two enantiomers to be determined with greatest precision. By differentiation of Eq. 4 with respect to C, it can be shown that the maximum value of the mobility difference occurs when the concentration of cyclodextrin is the reciprocal of the average binding constant, allowing prediction of the optimum concentration to use. An equation for predicting the concentration for maximum resolution has also been developed [13]. Whilst previous treatments of resolution have assumed μ to be a function of concentration, whilst D, the diffusion coefficient is constant, we adopt a self-consistent approach in which Eq. 1 is used to give the variation of both μ and D with concentration of free selector C.

Resolution R_s, is given by,

$$R_s = \frac{F\,\Delta K(\mu_0 - \mu_\infty)}{4\sqrt{2}\bar{K}} \left[\frac{Vlze}{LkT\mu_\infty(\mu_\infty + \mu_{eo})}\right]^{1/2} \tag{5}$$

where V is the applied voltage, L the length of the capillary, l the length to the detector, z the charge on the analyte, e the electronic charge, k the Boltzmann constant, T the absolute temperature, μ_{eo} the electroosmotic mobility and,

$$F = \frac{\bar{K}C}{(1+\bar{K}C)(\beta+\bar{K}C)^{1/2}(\gamma+\bar{K}C)^{1/2}}$$

$$\gamma = \frac{\mu_0 + \mu_{eo}}{\mu_\infty + \mu_{eo}}$$

$$\beta = \frac{\mu_0}{\mu_\infty}$$

Differentiation of Eq. 5 with respect to $\bar{K}C$ gives the condition for maximising R_s. Thus with a knowledge of the electroosmotic flow and electrophoretic mobility of the analyte and analyte–selector complex, a prediction of resolution can be made at any selector concentration.

2.2. LC, with and without chiral mobile phase additives

Various cases for chiral discrimination in LC can be considered. The following treatment builds on that given by Sybilska et al. [2]. They gave equations relating capacity factors and selector concentration, for our cases 1 and 4, and we extend this to discuss optimisation of selector concentration and links with CE.

Case 1. Chiral mobile phase additives and achiral stationary phase. All discrimination in the mobile phase. *Case 2.* Chiral mobile phase additives partially bound to achiral stationary phase. Discrimination in both mobile and stationary phase. *Case 3.* Dynamically coated chiral stationary phase. All discrimination in the stationary phase. *Case 4.* Covalently bonded chiral stationary phase. All discrimination in the stationary phase.

Case 1

This is analogous to CE with mobile phase additives as discussed in section 2.1, and therefore an identical rational separation strategy applies for optimising the selector concentration in the two techniques. In this case, k', the capacity factor (or retention factor [14]), versus C, the concentration of the free selector, is a binding curve analogous to the CE binding curve of μ vs. C, with equations for the curve

$$k'_1 = \frac{k'_A}{1+K_1C}$$

$$k'_2 = \frac{k'_A}{1+K_2C} \quad (6)$$

where k'_1 and k'_2 are capacity factors for the two enantiomers and k'_A the capacity factor for the free analyte. Data fitting of k' as a function of C allows binding constants to be determined.

Upon rearrangement we obtain,

$$\frac{\Delta t}{t_A - t_0} = \frac{\Delta K}{\bar{K}} \frac{\bar{K}C}{(1+\bar{K}C)^2} \quad (7)$$

where t_0 is the time for unretained species to elute, t_A the elution time for the free analyte A, and Δt the difference in enantiomer elution times. It should be noted that the right hand side of this equation is identical in magnitude to Eq. 4 for CE, and predicts that Δt will go through a maximum at a concentration of free selector equal to the reciprocal of the average binding constant.

Case 3 and 4

We assume that all binding to the stationary phase occurs at the chiral selector sites. Whilst individual binding constants cannot be obtained directly from LC without knowledge of phase ratios, the ratio of capacity factors is equal to the ratio of binding constants,

$$\frac{k'_2}{k'_1} = \frac{K_2}{K_1} = \alpha \quad (8)$$

This equation will hold using a dynamically coated or covalently bonded chiral selector, either in LC or electrochromatography.

Case 2

This is intermediate between case 1 and cases 3 and 4. In general, competition between chiral discrimination in the mobile and stationary phase

is expected to lead to overall discrimination less than cases 3 and 4.

3. Experimental

Capillary electrophoresis experiments were carried out on a P/ACE 2100 system (Beckman, High Wycombe, UK), thermostatted at 25°C. Each experiment was run in triplicate, with mesityl oxide as a neutral marker. Relative viscosity was determined by taking the ratio of the current I, at [CD] = 0 and at [CD] = C ($I_0/I = \eta/\eta_0$) [8].

Methyl-β-cyclodextrin was a gift from Wacker Chemicals (Halifax, UK). All other materials were from Aldrich (Gillingham, UK). The fused-silica separation capillary for the propranolol work had an internal diameter of 50 μm, a total length of 57 cm and a length of 50 cm from inlet to detector. A voltage of 20 kV was used for the separation, and detection was at 200 nm. The samples were loaded by a 1-s pressure injection (corresponding to 1 nl) from a 0.6 mM solution in run buffer. The pH 7.4 buffer was prepared by titrating 200 mM Na_2HPO_4 with 5 M phosphoric acid, and diluting 5-fold. The pH 3.0 buffer was prepared by titrating 40 mM LiOH with 5 M phosphoric acid.

The fused-silica separation capillary for the dansyl-amino acid work had an internal diameter of 20 μm, a total length of 27 cm and a length of 20 cm from inlet to detector. Separation voltage was 30 kV, and detection at 254 nm. The samples were loaded by a 3-s pressure injection (corresponding to 0.2 nl) from a 0.5 mM solution of dansyl-amino acid in run buffer that had been diluted by a factor of 10 with water, to induce stacking. The pH 6.8 buffer (total final ionic strength = 200 mM) was prepared by mixing 50 mM Na_2HPO_4 and 50 mM NaH_2PO_4; methanol was added in the ratio methanol:buffer (20:80), and then cyclodextrin was added in varying amounts.

The HPLC system consisted of a ternary gradient pump (ACS, Model 352), an injection valve (Rheodyne 7152) with a 20-μl loop, and a variable-wavelength UV detector (ACS, 750/12) operating at 254 nm. The UV data were collected and analysed on an integrator (Trivector Trio). The column was thermostatted at 25°C. In direct chiral analysis a β-CD bonded chiral stationary phase (244 × 4 mm I.D., ChiraDex, E. Merck, Darmstadt, Germany) was used. A mixture of methanol–phosphate buffer, 200 mM, pH 6.8 (20:80) was used as the mobile phase at a flow-rate of 0.8 ml min^{-1}. The concentration of 0.9 mM of Dns-Glu in mobile phase was injected on column. For the mobile phase additive method, a C_{18} column (250 × 4.6 mm I.D.; HPLC Technology, Macclesfield, UK) was used. The mobile phase additive was a mixture of methanol–phosphate buffer, 200 mm, pH 6.8 (20:80) containing a specified concentration of β-CD.

4. Results and discussion

4.1. Competitive binding of cyclohexanol for β-CD

Fig. 1 shows the dramatic difference in separation of tioconazole enantiomers brought about by addition of 0.1% v/v cyclohexanol to a running buffer containing 5 mM β-CD. Whereas little resolution is evident without the cyclohexanol, near baseline resolution is seen in its presence. Cyclohexanol has a high association constant with β-cyclodextrin ($K = 501\ M^{-1}$) [15] and is thus an effective competitor for analyte binding to cyclodextrin. For the separation of enantiomers of tioconazole with β-CD by CE, the binding constants for (−)- and (+)-tioconazole were measured to be $1.32 \cdot 10^3$ and $1.60 \cdot 10^3\ M^{-1}$, respectively. Addition of just 0.1% cyclohexanol to the background electrolyte with all other conditions being identical resulted in an apparent binding constant for (−)- and (+)-tioconazole from data fitting to Eq. 1 of 223 and 259 M^{-1}, respectively. Using a quantitative treatment of competitive binding [13], this six-fold decrease was shown to be consistent with competition between cyclohexanol and tioconazole for β-CD. From Eq. 5 we can calculate that in the absence of cyclohexanol we would require 0.9 mM β-CD concentration in

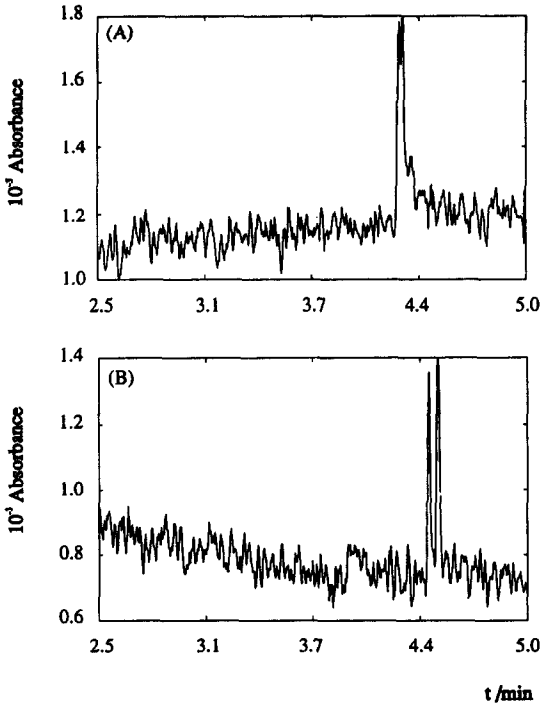

Fig. 1. Effect of competitive inhibition on the separation of tioconazole enantiomers. (A) No cyclohexanol, (B) 0.1% (v/v) cyclohexanol in the running buffer. Buffer: 20 m*M* phosphate/citrate pH 4.3 with 5 m*M* β-cyclodextrin; temperature: 25°C; injection: 1 s pressure injection (1 nl); sample: 0.1 m*M* tioconazole in run buffer; capillary: 50 μm internal diameter, 57 cm length; detection: 230 nm.

the background electrolyte for maximum resolution, but that with 0.1% (v/v) cyclohexanol we would require 5.3 m*M* β-CD (the concentration in Fig. 1) for maximum resolution. Eq. 1 shows that mobility is dependent upon the concentration of free selector, and an excess of selector over analyte is required. Due to the short pathlength of the on-column UV detection in CE, relatively high concentrations of analyte are required when working with weakly absorbing species such as tioconazole. Thus, with the use of cyclohexanol it is possible to bring very strongly binding species that require micromolar [CD] for optimum separation to a more convenient millimolar CD concentration.

4.2. Propranolol: β-cyclodextrin

Nicole et al. [16] determined the binding constant for propranolol binding to β-cyclodextrin to be $K = 220 \pm 20\ M^{-1}$, using an LC method with pH 7.4 phosphate buffer as the mobile phase. Using the same buffer conditions as in LC, we have determined the binding constant by CE from the variation of electrophoretic mobility with β-CD concentration (Fig. 2). Electrophoretic mobilities observed were corrected for buffer viscosity changes as discussed in sections 2.1 (Eq. 2) and 3. The value from CE obtained

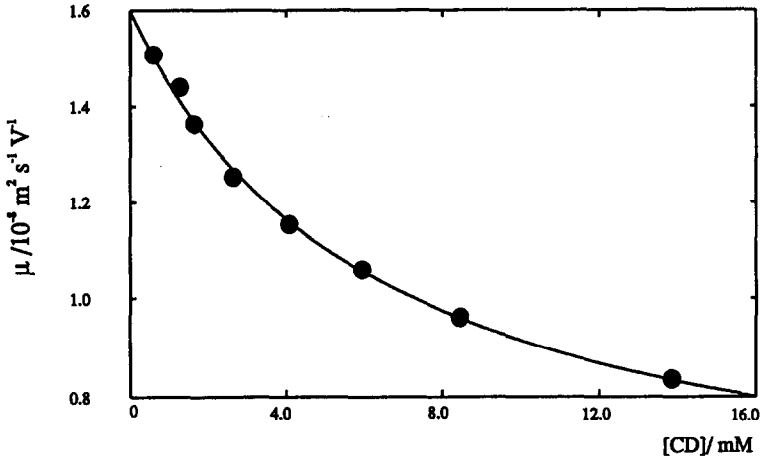

Fig. 2. Electrophoretic mobility of propranolol, corrected for buffer viscosity variation, as a function of [β-CD]. Data fitted to binding equilibrium curve giving $K = 160.4 \pm 3.1\ M^{-1}$. Buffer: 20 m*M* aqueous phosphate buffer pH 7.4; temperature: 25°C; voltage: 20 kV; capillary: 50 μm internal diameter, 57 cm length; detection: 200 nm.

for K, $160 \pm 3\ M^{-1}$, is comparable to that from LC.

Under the HPLC buffer conditions no chiral resolution was achieved in CE, due to the electroosmotic flow being too high at pH 7.4. Chiral resolution, although not baseline, was achieved using the conditions developed by Wren and Rowe [8] with Me-β-CD, at pH 3.0 in a lithium phosphate buffer. Binding constants under these conditions were found to be similar, although with larger errors than at pH 7.4, this being due to the inability to measure the low electroosmotic flow accurately. Both sets of experiments at pH 3.0 and pH 7.4 were under conditions well below the pK_a of propranolol ($pK_a = 9.5$), and no change in binding constant or selectivity was expected on changing the pH for a fully charged species.

4.3. Dansylated amino acids: β-CD

Fujimara et al. [17] used CD-bonded stationary phases in LC to chirally resolve dansylated amino acids. With the use of a 20-μm I.D. capillary it was possible to directly transfer the LC buffer conditions to CE (Fig. 3a). Using β-CD the binding constants between (D,L)-dansylated-glutamate (Dns-Glu) and β-CD were determined to be $K_2 = 220 \pm 4\ M^{-1}$ and $K_1 = 187 \pm 4\ M^{-1}$, and between (D,L)-dansylated-leucine (Dns-Leu) and β-CD $K_2 = 170 \pm 4\ M^{-1}$ and $K_1 = 141 \pm 4\ M^{-1}$. By spiking mixtures with pure L-Dns-amino acids assignments were made as L = 1, D = 2.

Comparison of mobile phase additives in LC (Fig. 3b), bonded phase LC (Fig. 3c) and CE in Table 1 reveal a number of interesting features. Firstly in the *mobile phase additive* work k'_A for Dns-Leu cannot be measured ($k'_A \geq 50$) but it can for Dns-Glu ($k'_A = 8$). This may be due to a partition into the non-polar stationary phase favouring a singly charged analyte (Dns-Leu) in comparison with a doubly charged analyte (Dns-Glu). As previously mentioned, the right hand sides of Eqs. 4 and 7 have the same magnitude, predicting that the left hand sides should be identical. This is indeed the case when looking at the data for Dns-Glu with 7 mM β-CD in LC and CE; $\Delta t/(t_a - t_0) = 0.042$, and under the same conditions in CE $\Delta\mu/(\mu_0 - \mu_\infty) = 0.041$. The data points of k' for Dns-Glu with β-CD as a mobile phase additive do not fit smoothly over the full range of β-CD concentration to the expected theoretical curve predicted by Eq. 6, revealing a more complex binding behaviour. This suggests that it may be a case 2 situation.

When comparing resolution (Fig. 3, Table 1), CE is revealed to be the technique to give the highest resolution for Dns-Glu. The high resolution attainable in CE is due to the electroosmotic flow and the electrophoretic mobility (which feature in the denominator of the resolution Eq. 5) being in opposing directions but of similar magnitude [13]. Table 1 also shows that there is good agreement between experimentally observed resolution at optimum cyclodextrin concentration and values calculated from Eq. 5.

In the case of β-CD bonded *chiral stationary phases* in LC using Dns-Glu and Dns-Leu, the α values obtained by Fujimara et al. [17], and in the present work under the same buffer conditions on the ChiraDex column, differ. For Dns-Glu, α from CE is greater than α from both chiral stationary phases. The situation is reversed when comparing results for Dns-Leu, where a particularly high value of α is seen on the ChiraDex stationary phase. All these results imply that the binding at the critical points for selectivity in a chiral stationary phase may be affected by the tether to the support.

CE reveals that the ratio of the average binding constant for Dns-Glu and Dns-Leu is approximately equal to unity. However, the ratio of k' for Dns-Leu and Dns-Glu is ca. 6. It has been noted that Dns-Glu is strongly bound to a C_{18} stationary phase, and we may postulate a possible contribution to k' for Dns-Glu from bonding interactions to the hydrophobic spacer material of the CD tether. It should also be significant that Dns-Glu is doubly negatively charged, whereas Dns-Leu is singly charged. In the paper by Fujimara et al. use of a high ionic strength buffer was observed to be particularly

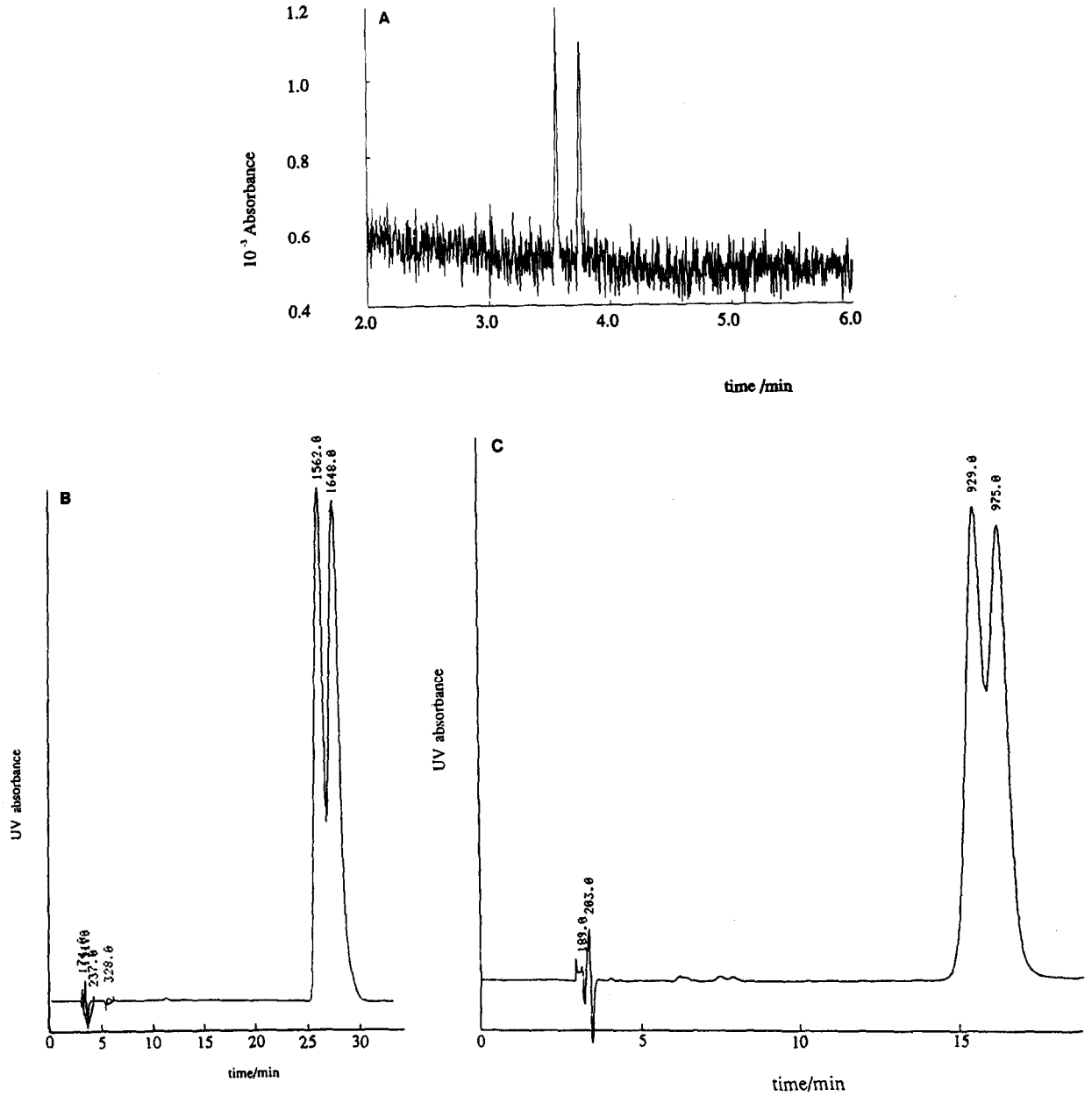

Fig. 3. Comparison of the chiral separation of dansyl-glutamate by: (A) capillary electrophoresis with mobile phase additive, (B) liquid chromatography with mobile phase additive and (C) liquid chromatography using a chiral stationary phase. Conditions: (A) 200 m*M* phosphate buffer pH 6.8, 20% methanol, with 10 m*M* β-CD in mobile phase, (B) 200 m*M* phosphate buffer pH 6.8, 20% methanol, with 7 m*M* β-CD in mobile phase and (C) 200 m*M* phosphate buffer pH 6.8, 20% methanol, no mobile phase additives, ChiraDex β-CD chiral stationary phase. Other conditions as in text.

beneficial in binding and chiral resolution of Dns-Glu, implying the need for charge screening for optimum bonding of Dns-Glu. All of these results have allowed CE to define the binding conditions and selectivity appropriate for free cyclodextrin, showing that the situation is made

Table 1
Comparison of resolution and selectivity data for Dns-Glu and Dns-Leu

Technique	Parameter	Dns-Glu	Dns-Leu
CSP[a]	k'_L	2.79	17.1
	k'_D	3.12	19.7
	α	1.12	1.15
	R_s^b	1.19	0.88
CSP: ChiraDex	k'_L	3.84	13.17
	k'_D	4.11	22.14
	α	1.07	1.68
	R_s^b	0.79	4.34
HPLC-mpa[d]	k'_D	8.34	$\geqslant 50$
	k'_L	8.84	$\geqslant 50$
	α^c	1.06	–
	R_s^b	0.83	–
CE	K_D	$220 \pm 4\ M^{-1}$	$170 \pm 4\ M^{-1}$
	K_L	$187 \pm 4\ M^{-1}$	$141 \pm 4\ M^{-1}$
	α	1.18	1.21
	R_s^b	6.7	5.4
	R_s theoretical	6.6	8.0

[a] Data from Fujimura et al. [17].
[b] R_s calculated using: $(x_2 - x_1)/\frac{1}{2}(w_1 + w_2)$.
[c] k'_D/k'_L at 7 mM β-CD.
[d] mpa = Mobile phase additive.

more complex by cyclodextrin bound to a chiral stationary phase.

5. Conclusions

By measuring mobility as a function of selector concentration in CE, analyte–selector binding constants can be obtained. In enantioselective binding, a general treatment allows mobility difference and resolution at any selector concentration to be calculated. Similar equations apply to determining binding constants and retention time differences in LC using mobile phase additives, provided that all discrimination is in the mobile phase. This is the first of four general cases identified for chiral separations in LC. The other cases which allow simple treatment in terms of single equilibria are when all binding occurs at an immobilised selector, held either by dynamic coating or covalent bonding to a stationary phase support. Here the ratio of capacity factors of the enantiomers is the ratio of the binding constants.

Application of these ideas using β-cyclodextrin as selector shows satisfactory agreement between binding constants measured for propranolol as analyte in CE and LC. Using Dns-D/L-Glu as analytes, chiral resolution in CE is shown to be in excellent agreement with theoretical prediction, and resolution is considerably better in CE than in LC. When comparing a β-CD bonded stationary phase with β-CD as mobile phase additive in LC and CE, more complex binding equilibria are revealed in the LC situations.

In future work we plan to use CE to define mobile phase equilibria and develop a rational strategy for selector choice and concentration to optimise a separation which can be transferred to

LC, where both mobile and stationary phases are involved, and several binding modes have to be considered.

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