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# Systematic approach to the treatment of enantiomeric separations in capillary electrophoresis and liquid chromatography III. A binding constant–retention factor relationship and effects of acetonitrile on the chiral separation of tioconazole

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**Abstract**

In previous chiral CE work on the separation of tioconazole enantiomers by β-cyclodextrin, the mode of action of alcohols as organic modifiers has been elucidated. Using acetonitrile, an increase in the organic modifier concentration in the range 0–15% (v/v) is found to cause twice the decrease in the value of the host–guest binding constant in comparison to methanol. With both modifiers, the selectivity ($\alpha = 1.23$) is the same across the full solvent composition range, consistent with action via alteration in solvent affinity for the free analyte. A unified treatment linking binding constants in CE and retention (capacity) factors in LC is developed for the case where 1:1 binding between selector and analyte occurs. This is parameterised for cyclodextrins as mobile phase additives in CE and chiral stationary phase selectors in LC. Reasonable agreement is found between the observed and calculated relationship between LC and CE results for tioconazole binding to β-cyclodextrin in water–organic cosolvent mixtures. This suggests that CE may be used for the optimization of the same separation in LC, allowing method development time in LC to be substantially decreased using data gathered in CE experiments.

*Keywords:* Buffer composition; Enantiomer separation; Mobile phase composition; Binding constants; Retention factors; Tioconazole; Acetonitrile; Cyclodextrins

## 1. Introduction

Free solution capillary electrophoresis (CE) has quickly established a firm foothold in the area of chiral separations which are often difficult to perform using traditional techniques such as high-performance liquid chromatography (HPLC). In CE, chiral separations are normally achieved using mobile phase additives that complex with the charged enantiomeric species to differing degrees, forming a dynamic equilibrium between free analyte and diastereomeric complex. The majority of chiral studies have been performed using cyclodextrins (CDs) in free solution [1–4], though chiral separations using cyclodextrins that have been chemically bound to the capillary surface have also been investigated [5,6]. In the latter case however, there is generally low mass transfer between the bulk solution and the selector which often makes baseline resolution difficult to achieve. Other selectors used for chiral CE separations include crown ethers [7], chiral ligand-ex-

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change complexes [8] and glycopeptide antibiotics [9,10].

For the analysis of chiral neutral species, micellar electrokinetic capillary chromatography has been routinely used with either CDs [11] or chiral bile salts [12] as selectors. Enantioseparation occurs due to partition of the analytes between the solution phase, the micellar pseudo-stationary phase and the cyclodextrin. Recently, negatively charged sulfobutylether cyclodextrin has been employed [13] for the analysis of neutral analytes in CE offering yet another avenue for the analysis of neutral species.

The use of HPLC for the separation of enantiomers has been commonplace since 1981 when Pirkle et al. produced the first chiral stationary phase based on an amino acid packing [14]. Since then many other chiral stationary phases (CSPs) have been employed [15], including cyclodextrins [16], proteins [17] and cellulose phases [18]. Cyclodextrin (inclusion) phases are amongst the most commonly used chiral HPLC packings and may be used in normal-phase, reversed phase and polar-organic modes [19] of operation, allowing analysis of a broad range of compounds in terms of solubility and selectivity.

In developing a rational approach to chiral separations in CE, theoretical models based on binding equilibria have been shown to give an excellent account of the effects of varying the cyclodextrin concentration on the chiral resolution of various cationic analytes by CE [20–22]. When appropriate, organic modifiers can be used to alter the equilibrium position of the host–guest complex so the highest mobility difference between enantiomers may be achieved [22,23]. In cases where the binding constants in aqueous solution are high and the optimum concentration of chiral selector is too low to be analytically useful, addition of methanol can increase this optimum concentration through increasing the affinity of the solvent for the analyte. For example, with the basic drug tioconazole and $\beta$-cyclodextrin the binding constants of the enantiomers were reduced by a factor of six on adding 25% (v/v) methanol to a pH 4.3 aqueous background electrolyte (BGE), while the selectivity was found to remain constant [22].

Though the field of chiral electrophoretic separations is rapidly expanding, there is a much broader store of knowledge in chiral HPLC. The aims of this paper are twofold. Firstly, to establish the effects of using acetonitrile as an organic modifier on the separation of tiaconazole with $\beta$-CD, allowing a systematic comparison of the effects of two organic modifiers in both CE and LC. Secondly, to establish a relationship between binding constants relevant to CE and retention factors [24] (capacity factors) in HPLC, to allow results of method development in one technique to be rapidly transferred to the other.

## 2. Theory

A simple method of expressing the affinity of a species for a selector is through its binding constant, $K$. $K$ is the equilibrium constant for the reaction

$$S + A \rightleftharpoons SA \tag{1}$$

where S is the selector and A is the analyte, and

$$K = \frac{[SA]}{[S][A]} \tag{2}$$

where $K$ has units of $M^{-1}$.

For chiral CE separations, the selector concentration which gives optimum resolution may be predicted by knowledge of the binding constants of both enantiomers. Binding constants may be calculated by fitting CE data for analyte mobility over a range of selector concentrations using Eq. 3, as previously defined by Penn et al. [21,22],

$$\mu = \frac{(\mu_0 - \mu_\infty)}{(1 + KC)} + \mu_\infty \tag{3}$$

where $\mu$, $\mu_0$, and $\mu_\infty$ are the observed analyte mobility, mobility at zero selector concentration and mobility at infinite selector concentration respectively and $C$ is the concentration of *free* selector. The highest mobility difference between enantiomers occurs at a cyclodextrin concentration equal to the inverse of the average binding constant [20–22]. The optimum concentration for resolution may also be calculated [22], and in the case where there is no EOF present

$$\bar{K}C = \sqrt{\frac{\mu_0}{\mu_\infty}} \tag{4}$$

where $\bar{K}=(K_1K_2)^{0.5}$ with $K_1$ and $K_2$ the binding constants for the individual enantiomers.

For LC using a stationary phase containing a selector which acts through 1:1 binding, we propose the use of an effective binding constant, $K'$, defined as

$$K' = \frac{n_{SA}}{n_A c_S} \quad (5)$$

where $c_S$ is the effective concentration of selector in the column. The ratio of the number of moles of analyte in the stationary phase to the number of moles of analyte in free solution ($n_{SA}/n_A$) is equal to the retention factor of the analyte, $k'$. From this,

$$K' = \frac{k'}{c_S} \quad (6)$$

$c_S$ may be calculated by dividing the total number of moles of selector, $n_S^T$, present in the column by the total solution phase volume of the column, $V$. For typical silica supports with ~240 $m^2 g^{-1}$ surface area and assuming there is 10–100% surface coverage by the selector, $c_S$ values for cyclodextrin selectors are calculated to be in the range of $(1-10)\cdot 10^{-2}$ $M$. From this it follows that

$$K'/M^{-1} \sim (10-100)k' \quad (7)$$

For the ideal case where the support and tether for the selector have no effect both on the degree of ionisation of the analyte and on the binding constant, the equilibrium constant for binding to the supported selector should be the same as that for binding to the selector in free solution, i.e. $K=K'$. In such a case there should be a quantitative link between CE and LC:

$$K = \frac{k'}{c_S} \quad (8)$$

Order of magnitude estimates of retention factor ranges for a given binding constant calculated given the assumptions of Eq. 8 are shown in Table 1. The table suggests an appropriate conversion between $K$ and $k'$ under conditions where Eq. 8 holds. Since the desired working range of $k'$ in LC is 1–10, for direct transfer of conditions from CE to LC a binding constant in CE of ~$10^2$ $M^{-1}$ is desirable.

It must be stressed that Eqs. 5–8 can only be expected to be applicable when there are no competing binding interactions for the analyte on the support. For example, when using cyclodextrin selectors tethered to a silica surface, interactions between basic analytes and surface groups, such as non end-capped silanols, must be avoided. Whilst the numerical values in Table 1 are calculated for cyclodextrins, the treatment is general and $c_S$ values for other selectors operating via 1:1 binding equilibria, for example proteins and antibiotics, could readily be calculated knowing the diameters of the molecules.

Table 1
Capacity factor working ranges for given binding constant values found in free solution

| $K/M^{-1}$ | $k'$ range |
|---|---|
| $10^4$ | 100–1000 |
| $10^3$ | 10–100 |
| $10^2$ | 1–10 |
| $10^1$ | 0.1–1 |

*The higher values in the capacity factor range correspond to 100% coverage while the lower values correspond to 10% surface coverage.*

## 3. Experimental

### 3.1. Materials

Tioconazole hydrochloride and its single enantiomers (Fig. 1) were provided by Pfizer Central Research. β-CD was a gift from Wacker Chemicals (Halifax, UK). Other chemicals used were HPLC grade acetonitrile, methanol, triethylamine and glacial acetic acid (Fisons, Loughborough, UK) and triethanolamine (Aldrich, Gillingham, UK). In CE, BGE solutions were prepared by mixing the relevant amount of organic component (0–20%, v/v) with a

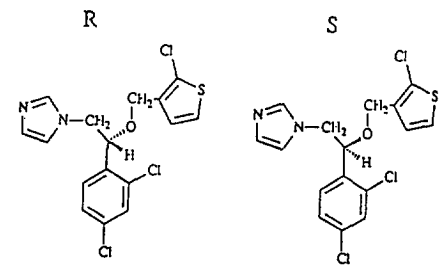


Fig. 1. *R* and *S* structures of tioconazole.

buffer comprising of 0.1 *M* $H_3PO_4$ titrated to pH 3.0 with triethanolamine [25]. In the HPLC investigation, the mobile phases studied were produced by titrating a triethanolamine or triethylamine solution to pH 4.0 with glacial acetic acid and adding the appropriate amount of acetonitrile. Experiments to examine the effects of methanol on the LC separation of tioconazole were performed in the triethanolamine–acetate buffer and a buffer identical to that used in the previous CE study involving methanol [22], a 0.2 *M* $Na_2HPO_4$–0.1 *M* citric acid buffer (pH 4.3). All CE BGEs and HPLC mobile phases were filtered prior to use through 0.2 $\mu$m filters resistant to organic solvent.

### 3.2. Methods

CE experiments were carried out on an automated instrument (Beckman PACE 2050). Capillaries (Composite Metals, Hallow, UK) used were 57 cm in length (50 cm to the detector)×50 $\mu$m I.D. The following conditions applied; voltage 30 kV; capillary thermostated at 25°C; UV detection at 230 nm; 2 s pressure injection (2 nl) of 0.1 m*M* tioconazole solution dissolved in the BGE. Between injections the capillary was rinsed for 5 min with 0.1 *M* $H_3PO_4$ followed by a 5 min rinse with the run buffer.

Relative viscosities were measured using a capillary viscometer (Townson and Mercer, Croydon, UK) thermostated at 25°C. These values were subsequently fitted to a concentration power series [26] and from this the corrected enantiomer mobilities were calculated using the equation [20–22]

$$\mu = \mu_{obs} \cdot \frac{\eta_c}{\eta_0} \quad (9)$$

where $\mu$ is the corrected analyte mobility, $\mu_{obs}$ the observed mobility, $\eta_c$ and $\eta_0$ are the viscosities of the solutions at cyclodextrin concentration c and 0 respectively. Relative viscosities could not be determined reproducibly from ratio of currents in the CE experiments, due to erratic current baselines thought to be due to organic solvent evaporation upon application of the voltage.

Components in the HPLC system used were; pump (ACS model 352); injector (Rheodyne 7152) with 20 $\mu$l loop; variable wavelength UV detector (ACS 750/12) set at 240 nm; integrator (Trivector Trio). A Cyclobond 1 $\beta$-CD column (250 mm×4.6 mm I.D., Astec, Whippany, NJ, USA) was used throughout the LC experiments and thermostated at 25°C.

All CE and LC experiments were carried out in triplicate.

## 4. Results and discussion

### 4.1. CE studies

#### 4.1.1. Binding constants

The use of a low pH phosphate buffer with triethanolamine as the neutralising basic species has been shown to have the advantageous effect of reducing wall interactions for cationic analytes, with a corresponding improvement in peak symmetry and reduction in peak tailing [25]. There is also a reduction in EOF. All effects may be accounted for by protonated triethanolamine cations binding to negatively-charged free silanol sites on the capillary walls.

Fig. 2 shows the variation in mobility of the tioconazole enantiomers as a function of $\beta$-cyclodextrin concentration, over the normal $\beta$-cyclodextrin solubility range of 0 to 16 m*M*. The migration order of the enantiomers was elucidated by sample spiking with the pure enantiomers and was found to be concordant with previous results using other cyclodextrins [22] i.e. the (−) isomer migrated first followed by the (+) isomer, indicating a preferential binding of the (+) form with $\beta$-CD. Analyte mobilities were calculated from direct measurement of the migration times of the enantiomers as no appreciable EOF was observed using mesityl oxide as a neutral marker after 150 min in either polarity. This sets a limit to the EOF of $< \pm 0.11 \cdot 10^{-8}$ $m^2 V^{-1} s^{-1}$ with this buffer. Other authors have observed a reversal of EOF with a value in the order of $-0.4 \cdot 10^{-8}$ $m^2 V^{-1} s^{-1}$ [27].

The binding curves shown in Fig. 2 are non-linear least squares fits using Eq. 3 [28]. Fig. 3 gives the optimum fit of the data to an expression in [22] with the binding constant difference $\Delta K$ as the variable, $\bar{K}$, $\mu_0$ and $\mu_\infty$ having been calculated from Eq. 3. This plot, using dimensionless reduced concentration and reduced mobility difference, has been shown to

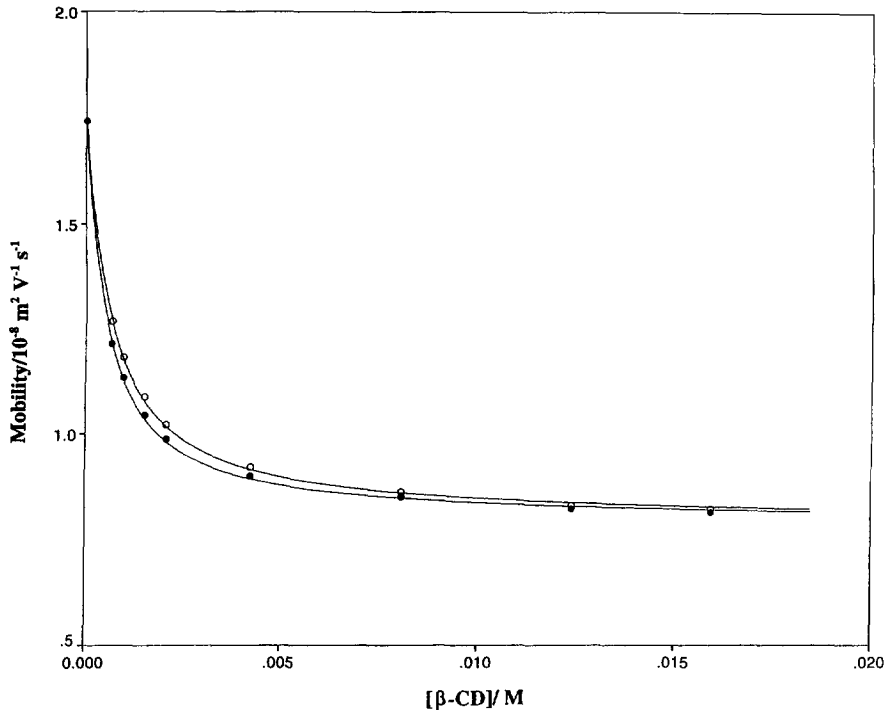


Fig. 2. Mobility of tioconazole enantiomers as a function of $\beta$-cyclodextrin concentration. Data points for (−)-tioconazole (○) and (+)-tioconazole (●) with curves showing best fit to Eq. 3. BGE: 100 m*M* $H_3PO_4$ titrated to pH 3.0 with 7.4 *M* triethanolamine.

provide the most precise method for calculating $\Delta K$ and the selectivity, $\alpha = K_2/K_1$ [22]. The quality of these fits are typical of those found in the of 0–15% (v/v) acetonitrile range.

For tioconazole enantiomer binding to $\beta$-CD, binding constants, selectivity, and mobilities for free and selector-bound analyte, $\mu_0$ and $\mu_\infty$ respectively, are given as a function of volume percentage acetonitrile in Table 2a.

The binding constants calculated without organic modifier are significantly higher than those found in the previous study [22]. This must be due to differences in the BGE, with a pH 4.3 phosphate–citrate BGE (ionic strength $I = 0.04$ mol kg$^{-1}$) in the previous work and a pH 3.0 phosphate–triethanolamine BGE ($I = 0.09$ mol kg$^{-1}$) in the present case. Correction of $K$ values to zero ionic strength [22] does not affect the discrepancy. Both sets of experiments were carried out at pH values greater than two units below the p$K_a$ of tioconazole (p$K_a = 6.5$), thus this basic molecule is fully protonated under all conditions. The explanation is suggested to rest with the cations present in the two BGEs. Competition between analyte binding at the capillary wall and to $\beta$-CD in the BGE would result in a decrease in the observed binding constant. Analyte–wall interactions are reduced using triethanolamine cations, which bind strongly to the silica capillary wall as evidenced by the decrease of the EOF to zero. Sodium ions bind less strongly to the walls, and are also present at lower concentration in the phosphate–citrate BGE.

### *4.1.2. Selectivity*

The selectivity in acetonitrile–water mixtures does not vary with organic modifier content, and is within experimental error equal to that found in a methanol–water mixtures (Table 2b) [22]. This is consistent with the effect of the organic modifier being to increase the affinity of the analyte for the solvent, without affecting its chiral interaction with the cyclodextrin.

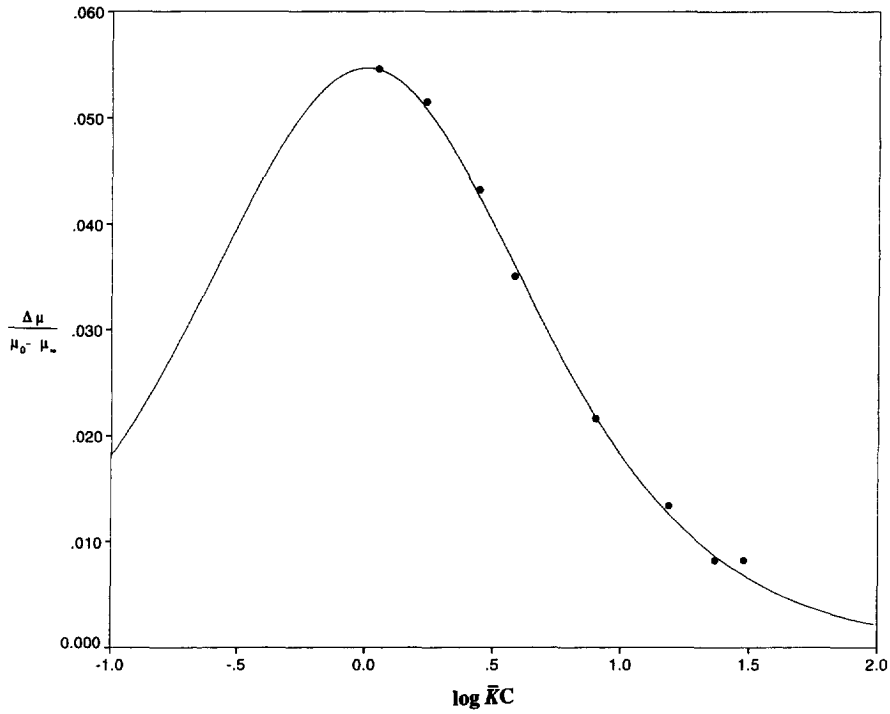


Fig. 3. Enantiomeric mobility difference as a function of the dimensionless variable $\bar{K}C$. Experimental points with optimum fit using $\Delta K$ as the variable $\bar{K}$, $\mu_0$ and $\mu_\infty$ having been calculated from Eq. 3.

### 4.1.3. Mobilities

The ratio of $\mu_\infty/\mu_0$ was correlated by Rogan et al. [29] with analyte structures and used to suggest the depth of analyte inclusion into the cyclodextrin cavity. It was postulated that the tighter the fit of the analyte into the cyclodextrin cavity, the smaller the

Table 2
Binding parameters at 25°C for tioconazole enantiomers to β-CD as a function of volume percentage of organic modifier

| (a) % Acetonitrile | 0 | 5 | 10 | 15 | |
|---|---|---|---|---|---|
| $K_{(-)}/M^{-1}$ | 1667±29 | 870±29 | 612±5 | 237±8 | |
| $K_{(+)}/M^{-1}$ | 2091±39 | 1079±38 | 742±6 | 288±9 | |
| $\bar{K}/M^{-1}$ | 1879 | 974 | 677 | 263 | |
| $\Delta K/M^{-1}$ | 411±3 | 204±8 | 128±2 | 52±4 | |
| $\alpha$ | 1.25 | 1.24 | 1.21 | 1.22 | |
| $\mu_0/10^{-8}$ m$^2$ V$^{-1}$ s$^{-1}$ | 1.76 | 1.84 | 1.86 | 1.71 | |
| $\mu_\infty/10^{-8}$ m$^2$ V$^{-1}$ s$^{-1}$ | 0.79 | 0.81 | 0.75 | 0.65 | |
| $\mu_\infty/\mu_0$ | 0.45 | 0.44 | 0.40 | 0.38 | |
| (b) % Methanol | 0 | 1 | 4 | 10 | 25 |
| $K_{(-)}/M^{-1}$ | 1319±28 | 1180±60 | 1090±50 | 580±10 | 240±10 |
| $K_{(+)}/M^{-1}$ | 1595±28 | 1430±80 | 1350±70 | 700±10 | 290±30 |
| $\bar{K}/M^{-1}$ | 1457 | 1305 | 1220 | 640 | 265 |
| $\alpha$ | 1.20 | 1.21 | 1.23 | 1.21 | 1.23 |

Modifier (a) acetonitrile; buffer, 100 m*M* phosphoric acid–triethanolamine pH 3.0 ($I$=0.09 mol kg$^{-1}$) and (b) methanol; buffer, 20 m*M* phosphate–citrate pH 4.3 ($I$=0.04 mol kg$^{-1}$) [22]

Stokes radius of the complex would be. This in turn would lead to a higher $\mu_\infty$ and thus a higher $\mu_\infty/\mu_o$ value when comparing different compounds of similar size. Data in Table 2a, which refers to a single compound, shows that $\mu_\infty/\mu_o$ decreases with increasing acetonitrile concentration. This may indicate that there is a decrease in tightness of binding and an increase in conformational flexibility outside of the cyclodextrin cavity with increasing percentage of organic modifier. This again is concordant with making the bulk buffer more hospitable than the cyclodextrin cavity to the tioconazole analyte, by making the solvent increasingly hydrophobic.

### 4.1.4. Resolution

The concentration for optimum resolution, $R_s$, at the various acetonitrile concentrations (calculated from Eq. 4) are shown in Table 3. Values increase with increasing acetonitrile concentration, consistent with the decrease in binding constant due to the effects discussed above. A representative electropherogram for one of these optimum cyclodextrin concentrations is shown in Fig. 4. The increase in resolution in comparison to that found in a phosphate–citrate BGE [22] provides further evidence for the benefits of using 100 m*M* pH 3.0 triethanolamine–phosphate [25] with basic analytes such as tioconazole.

Table 3 also gives the resolution of the enantiomers measured using the standard chromatographic formula [30]

$$R_s = \frac{1.18\Delta t}{w_{1/2,1} + w_{1/2,2}} \quad (10)$$

where $\Delta t$ is the difference in the migration times of the two peaks and $w_{1/2,1}$ and $w_{1/2,2}$ are their half height widths. The trend in resolution is for an increase with increasing fraction of acetonitrile, the highest resolution being both observed and calculated at 15% v/v acetonitrile. The calculated resolution in Table 3 is seen to be approximately twice as large as the measured resolution. Calculation of the theoretical resolution [22] assumes that diffusion is the principal source of peak variance.

$$R_s = \frac{\Delta\mu}{4\sqrt{2}}\left[\frac{lV}{LD(\bar{\mu} + \mu_{eo})}\right]^{1/2} \quad (11)$$

where $\Delta\mu$ is the analyte mobility difference, $l$ the length of the capillary to the detector, $V$ the applied voltage, $L$ the total length of the capillary, $D$ the average diffusion coefficient, $\mu$ the average analyte mobility and $\mu_{eo}$ the EOF mobility. The variance due to diffusion was calculated using mobility values in Table 2a and the Stokes–Einstein relationship between mobility and diffusion coefficient, and was found to be only around 10% of the total variance measured from peak widths. Calculation of band broadening contributions [31] from diffusion, injection and buffer conductivity differences within the capillary confirmed analyte–wall interaction as the only possible factor to account for the difference between the observed and calculated peak variance. These interactions were found to be present even at this low pH and with the triethanolamine cations providing wall competition with the analyte. This was confirmed by CE runs using the phosphate–

Table 3
Tioconazole resolution at optimum cyclodextrin concentration as a function of volume percentage acetonitrile

| % Acetonitrile | 0 | 5 | 10 | 15 |
|---|---|---|---|---|
| $[CD]_{opt}$/m*M* | 0.8 | 1.6 | 2.3 | 6.2 |
| Calculated $R_s$ | 6.8 | 7.8 | 7.9 | 8.8 |
| Measured $R_s$ | 2.9 | 3.8 | 3.3 | 4.5 |

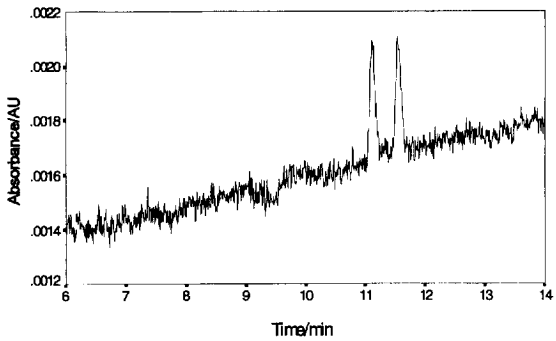


Fig. 4. Typical electropherogram for tioconazole separation at 0.8 m*M* β-cyclodextrin. The (−) enantiomer migrates first followed by the (+). Capillary: 57 cm (50 cm to detector)×50 μm I.D. thermostated at 25°C. BGE: 100 m*M* $H_3PO_4$ titrated to pH 3.0 with 7.4 *M* triethanolamine. Conditions: 30 kV voltage, UV detection at 230 nm, 2 s pressure injection (2 nl) of 0.1 m*M* tioconazole.

triethanolamine BGE solution diluted by a factor five to 20 mM. A shift in migration time, increased peak broadening and peak tailing of the enantiomers was observed.

*4.1.5. Solvent effects*

The variation of binding constants with percentage organic modifier may be correlated as shown in Fig. 5. Assuming that log $K$ varies linearly with percentage organic modifier [32], the average slope and 95% confidence limits of the lines for the (−)- and (+)-enantiomers using acetonitrile as modifier is $-0.054\pm0.029$. This may be compared to a similar plot for the effect of methanol on the binding constants for (±)-tioconazole [22] which gives a slope of $-0.030\pm0.006$. This suggests that acetonitrile is nearly twice as effective as a solvent modifier than methanol for this CE separation.

Solvent effects measured in these experiments for tioconazole correlate with those observed in other binding studies. Spectrophotometric results on the displacement of azo dyes from β-CD have been analysed assuming competitive binding. The apparent binding constant for acetonitrile to β-CD, 1.00 $M^{-1}$ [33] was a factor 2–3 greater than that for methanol (0.32 $M^{-1}$ [34] and 0.40 $M^{-1}$ [33]).

Analysing results assuming 1:1 binding of acetonitrile with the binding constant above and competitive displacement of tioconazole gives values of $K_1$ at 0, 5, 10 and 15% acetonitrile of 1667, 1702, 1783 and 917 $M^{-1}$, respectively. The dramatic fall-off in $K$ at 15% acetonitrile parallels that in 25% methanol [22] and supports the idea that a simple 1:1 competitive displacement is less satisfactory than a general solvent effect description [22].

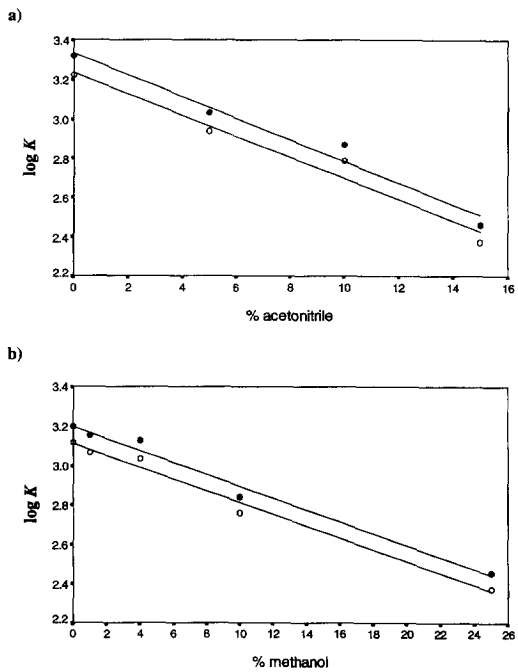


Fig. 5. Log binding constants of tioconazole enantiomers to β-CD as a function of percentage organic modifier in the background electrolyte for (a) acetonitrile and (b) methanol [22]. (○), (−)-isomer (●), (+)-isomer. Buffer (a): as Fig. 4, buffer (b): 20 m*M* phosphate–citrate pH 4.3.

*4.2. HPLC studies*

*4.2.1. Retention factors and resolution*

In a recent report [35], it was found that 3.5% (v/v) triethylamine in the mobile phase produced the best separation of tioconazole enantiomers on an Ultron ES-CD β-cyclodextrin column. In the present work using an Astec Cyclobond I β-CD column, the initial mobile phase was prepared by titrating a 3.2% (v/v) triethanolamine solution (the molar equivalent of 3.5%, v/v, triethylamine) to pH 4.0 with glacial acetic acid and adding the relevant fraction of acetonitrile (0–20%, v/v), see Table 4. Ideally an identical buffer to that used in the CE experiments

Table 4
Retention factors, selectivity and resolution for tioconazole enantiomers as a function of volume percentage acetonitrile

| % Acetonitrile | 0 | 5 | 10 | 15 | 20 |
|---|---|---|---|---|---|
| (a) | | | | | |
| $k'_{(-)}$ | >18 | 6.61 | 3.28 | 1.72 | 0.87 |
| $k'_{(+)}$ | >18 | 7.27 | 3.57 | 1.87 | 0.95 |
| $\alpha$ | – | 1.10 | 1.09 | 1.09 | 1.09 |
| $R_s$ | – | ~0.6 | 1.08 | 0.58 | ~0.2 |
| (b) | | | | | |
| $k'_{(-)}$ | 8.98 | 4.39 | 2.23 | 1.04 | 0.56 |
| $k'_{(-)}$ | 9.93 | 4.90 | 2.47 | 1.14 | 0.61 |
| $\alpha$ | 1.11 | 1.12 | 1.11 | 1.09 | 1.08 |
| $R_s$ | 0.83 | 1.03 | 0.93 | 0.60 | ~0.2 |

(a) a 3.2% (v/v) triethanolamine in acetic acid mobile phase (pH 4.0) and (b) a 3.5% triethylamine in acetic acid mobile phase (pH 4.0). Other conditions; column 250 mm×4.6 mm I.D. Cyclobond I β-CD column; temperature 25°C; flow-rate 1.0 ml min$^{-1}$; detection 240 nm

would have been used in the HPLC work, but the cyclodextrin column is limited to a working pH range of 3.5–7.0 for stability of the stationary phase.

The peak shape and resolution were generally found to be poor with protonated triethanolamine as the cationic component of the buffer. To attempt to overcome this, triethylamine–acetate (TEAA) buffers were used. These were prepared by taking a 3.5% triethylamine solution, titrating to pH 4.0 with glacial acetic acid and adding the relevant volume percentage of organic modifier.

Results of analyses using triethanolamine and TEA in the mobile phase are shown in Table 4a and b. Resolution is seen to increase with decreasing acetonitrile percentage but is generally higher using the TEAA buffer (see Fig. 6). Also, retention factor values are lower for a given volume percentage acetonitrile using TEAA.

A central composite study of the effects on enantiomeric resolution of tioconazole was carried out by varying the pH (in the range 4.0–4.5), triethanolamine (2.5–5.5% of the aqueous component of the buffer) and acetonitrile concentration (0–20%, v/v). The major factor influencing resolution was found to be the organic modifier concentration, with the pH and triethanolamine concentration having a roughly equal effect. The lower the acetonitrile content of the mobile phase the better the resolution (as was also found with the percentage triethanolamine in the mobile phase), though this was at the expense of increasing retention time. This is in accord with the results determined from the experimental work presented here.

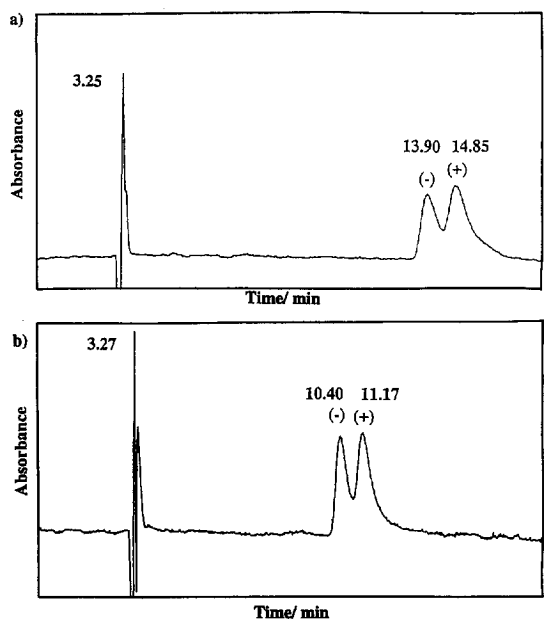


Fig. 6. Chiral HPLC separation of tioconazole enantiomers with 10% acetonitrile and (a) 3.2% triethanolamine, (b) 3.5% triethylamine in the mobile phase. Column: 250 mm×4.6 mm I.D. Astec Cyclobond I ($\beta$-cyclodextrin) thermostated at 25°C. Mobile phase: Amine solution titrated to pH 4.0 with glacial acetic acid. Conditions: Flow-rate 1.0 ml $min^{-1}$; detection 240 nm; 20 $\mu$l injection of 0.1 m*M* tioconazole solution.

*4.2.2. Selectivity*

Table 4a and b show that the enantioselectivity $\alpha$, defined as $k_2'/k_1'$ in HPLC, is the same using both triethanolamine–acetic acid and triethylamine–acetic acid buffers, and that $\alpha$ does not change (within experimental error) over the organic modifier concentration range. This parallels the findings from CE, where the selectivity remained constant throughout the organic modifier range. The elution order of the tioconazole enantiomers is also the same as in the CE experiments. However, the value of $\alpha$, ~1.10, is much less than the value of $\alpha$ ~1.23 from CE. Reasons for this difference could include: (i) inadequate capping of the silica particles allowing for competitive, achiral binding by the analyte with the silica stationary phase; (ii) the unknown chiral nature of the tether connecting the cyclodextrin to the silica support; (iii) the bound cyclodextrin having less rotational degrees of freedom than the free cyclodextrin.

*4.2.3. Solvent effects*

Plots of log $k'$ vs percentage organic modifier for acetonitrile and methanol using the triethanolamine–acetic acid buffer gave average gradients of $-0.059\pm0.003$ and $-0.034\pm0.056$ respectively, within experimental error equal to the results found from CE in Section 4.1 of $-0.054\pm0.029$ and $-0.030\pm0.006$. This shows that acetonitrile is almost twice as effective as methanol as a solvent modifier when using cyclodextrins in both CE and LC. Averaging the CE and LC results suggests that to get identical $k'$ values, a volume percentage acetonitrile equal to 0.57× the volume percentage methanol should be chosen. It is interesting that the

ratio of the effects of the two modifiers, 0.57, is identical to that found by Schoenmakers in reversed-phase HPLC [36] at low modifier concentrations. In so far as the effects are the same in non-specific binding on ODS phases and in specific binding on a cyclodextrin phase, this points to acetonitrile and methanol working through modification of the affinity of the mobile phase rather than through specific binding effects with the cyclodextrin cavity.

### *4.3. Linking binding constants in CE and retention factors in LC*

Table 5 gives the average CE binding constants and average LC retention factors as a function of percentage volume acetonitrile. Without organic modifier in the triethanolamine-based mobile phase, the enantiomers were not eluted after a period of 1 h. This is consistent with the corresponding value of the binding constant in free solution, which by reference to Table 1 explains why the analytes are outside of the desired capacity factor range of 1–10.

Using elemental analysis data of the Cyclobond I column together with data on capacity factors of nitroanilines as reference compounds [15], the effective surface concentration of cyclodextrin was calculated to be $c_S \sim 0.01$ mol dm$^{-3}$. Using Table 1, we predict that $K' \sim 100k'$ in the present work. The data presented in Table 5 using the triethanolamine buffer over the range 5–15% acetonitrile in both CE and LC gives $\bar{K} = (160 \pm 40)\bar{k}'$. A comparison of LC results using the triethanolamine buffer in 25% methanol with previously published CE data in 25% methanol [22] gives $\bar{K} \sim 100\bar{k}'$. These theoretical and experimental results relating $K$ and $k'$ show reasonable agreement considering the assumptions made (Eq. 8) for a quantitative link to be obtained between LC and CE.

Table 5
Average binding constants and capacity factors with the triethanolamine and TEA buffers as a function of percentage volume acetonitrile

| % Acetonitrile | 0 | 5 | 10 | 15 | 20 |
|---|---|---|---|---|---|
| $\bar{K}/M^{-1}$ | 1879 | 974 | 677 | 263 | * |
| $\bar{k}'_{\text{triethanolamine}}$ | >18 | 6.94 | 3.43 | 1.79 | 0.91 |
| $\bar{k}'_{\text{triethylamine}}$ | 9.96 | 4.65 | 2.35 | 1.09 | 0.59 |

* Denotes value outside the working range of the technique.

## 5. Conclusions

The CE and LC results with predicted and observed relationships between $K$ and $k'$ are in reasonable agreement, consistent with our unified theory linking binding constants in CE and retention (capacity) factors in LC. These investigations quantify the effects of acetonitrile and methanol in separations involving a model basic analyte and cyclodextrins over the solvent composition range 0–15% organic modifier. Our work suggests that CE may be used as a rapid scouting technique for the optimization of the same separation in HPLC. This could allow method development time in HPLC, where there are many more variables to optimize than in CE, to be substantially decreased by using data gathered in CE experiments.

For the chiral separation of tioconazole with $\beta$-CD and acetonitrile as an organic modifier it has been shown that acetonitrile is approximately twice as effective as methanol in reducing the affinity of tioconazole as an analyte for $\beta$-cyclodextrin. This is not a binding phenomenon but one of solvent interaction whereby the analyte preferentially stays in the bulk solvent rather than occupying the cyclodextrin cavity. Enantioselectivity is independent of mobile phase composition, but the values in CE and LC differ significantly, and further work is being carried out to address this issue.

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