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Capillary zone electrophoresis for separation of drug enantiomers using cyclodextrins as chiral selectors

Influence of experimental parameters on separation

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ABSTRACT

Enantiomeric separations in cyclodextrin-modified capillary zone electrophoresis (CZE) can be tuned by many experimental conditions, such as the cyclodextrin type, concentration and degree of substitution, pH and separation voltage. The enantiomers of *rac*-terbutaline, *rac*-terbutaline mononsulphate, *rac*-bambuterol, *rac*-propranolol, *rac*-ephedrine and *rac*-brompheniramine were used as model substances to study the effect of the above factors on separation parameters using an uncoated fused-silica capillary. The cyclodextrins used in the experiments were α-cyclodextrin, β-cyclodextrin, dimethyl-β-cyclodextrin and hydroxypropyl-β-cyclodextrin. The results were compared with those predicted by theoretically derived models. The most effective parameters for optimizing resolution were pH and cyclodextrin type and concentration. The applied voltage was the most effective in tuning the separation time. The quality of the cyclodextrins used for the separation is of importance as both the degree of substitution of modified cyclodextrins and the position of substitution were found to affect the separations.

INTRODUCTION

It is well known that drug enantiomers may interact differently with biological components that are able to discriminate between the enantiomers. Examples are enzymes, receptors and plasma proteins, which can lead to differences in absorption, distribution, metabolism, elimination and action at the receptor site. As a consequence, many drug enantiomers show different pharmacological effects [1]. For this reason, analytical methods that allow for the separation and determination of drug enantiomers in biosamples are often needed in drug development [2,3].

Analytical techniques such as column liquid chromatography (CLC) [4,5], gas chromatography [6] and, more recently, supercritical fluid chromatography [7] have all been used for the separation and determination of enantiomers. For the separation and determination of enantiomers in biosamples CLC is probably the most useful technique [8]. For use in bioanalytical work it was shown recently that many bioanalytical problems can be solved by using coupled columns in CLC [9]. Although high selectivity can be obtained in CLC, the efficiency is poor. Much higher efficiency can be obtained in capillary zone electrophoresis (CZE), a technique that seems to have great potential for use in

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pharmaceutical analysis including the separation of enantiomers [10]. However, most applications up to now have involved relatively uncomplicated samples and very little has been published on biosamples [11,12]. Application to biosamples is hampered because of the limited sample volume and because sample pretreatment will often be necessary to be useful for determinations with low concentrations of drugs [13–15].

In CZE, chiral selectivity can be obtained either by adding the chiral selector to the running buffer [13–27] or by immobilization of the chiral selector in the capillary [28,29]. Cyclodextrins have become popular as chiral selectors in CLC [9] and in CZE [13–28]. This popularity arises from the fact that a number of enantiomeric separations can be achieved and there are a number of different cyclodextrins available at reasonable prices, the latter being of minor importance in CZE because of the low volumes required.

Cyclodextrins are cyclic oligosaccharide molecules built up of D-(+)-glucopyranose units via α-(1,4)-linkages. Each glucose unit contributes five chiral centres to the molecule. The structure of a cyclodextrin moiety is unique in that it resembles a truncated cone with both ends open. The primary hydroxyl groups (on C-6) on the glucopyranose units lie on the narrow end of the torus. The secondary hydroxyl groups (on C-2 and C-3) are located on the wider end and have a clockwise direction of rotation. This arrangement makes the interior hydrophobic and the rims hydrophilic. The most commonly used cyclodextrins are α-, β- and γ-cyclodextrin which have six, seven and eight glucopyranose units, respectively, in their structure. Derivatized cyclodextrins have been much used recently for separations of enantiomers in CLC [30], but also in CZE [13–15,17–20,22–24,27]. Fig. 1 shows the structures of β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin and 2,6-dimethyl-β-cyclodextrin.

Cyclodextrins exhibit a broad range of desirable properties useful in both CLC and CZE. For example, cyclodextrins absorb very little in the UV region, are stable over a wide pH range, resistive to light and non-toxic [31]. The solubility is sufficient and can be increased by substitution and by addition of urea [32].

The formation of cyclodextrin complexes with different guest molecules is not well understood [33] and there is as yet no way to predetermine if a certain type of cyclodextrin can be used for the separation of certain enantiomeric pairs or compounds. It remains largely trial and error. However, a growing number of publications reporting on the nature of cyclodextrin host–guest intermolecular interactions aid in the understanding of factors that influence the enantioselectivity of cyclodextrins [34–40].

Cyclodextrins, especially the alkylated forms, are fairly flexible molecules and this facilitates changes in conformation that can increase the stability of the host–guest complex (induced fit). A common understanding seems to be that in order to achieve chiral resolution, inclusion of the enantiomers into the cavity of the cyclodextrin molecule seems to be necessary [41]. Owing to the hydrophobic nature of the interior of the cyclodextrin, hydrophobic interactions with the guest molecule should be favoured. Polar interactions with the polar groups on the rims of the cavity is possible with the polar part of the guest molecule. Interactions on the outside of the cyclodextrin molecule might also be a possibility to obtain enantioselectivity as it has been observed that open-chain polysaccharides can be used to separate chiral molecules [39].

Recent reports on the use of cyclodextrins in CZE show good separations of various drug enantiomers [16–28]. However, very few data have been presented on the effect of experimental parameters on separation parameters such as efficiency, resolution and migration times.

In a previous paper [13] we reported on the separation of the enantiomers of *rac*-terbutaline, *rac*-bambuterol, *rac*-propranolol, *rac*-ephedrine, *rac*-brompheniramine and *rac*-terbutaline monosulphate (the main metabolite of terbutaline) using CZE and various cyclodextrins as chiral selectors. The purpose of this work was to extend our investigations to gain an understanding of how resolution, migration time and efficiency can be affected by the type, degree of substitution and concentration of the cyclodextrin, applied voltage and pH of electrolyte solution, aiming at using this information in extended work on bioanalytical applications [13–15]. The results obtained from our studies are also

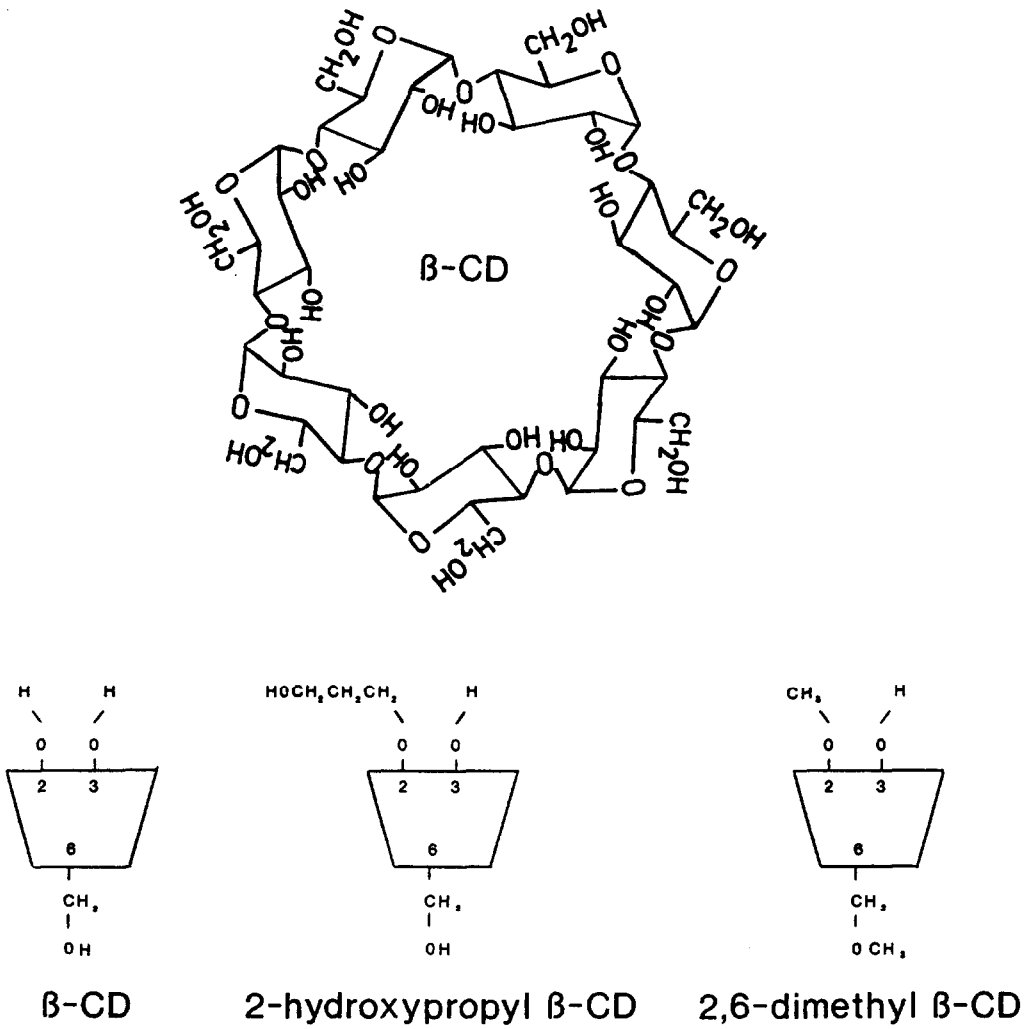


Fig. 1. Structures of β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin and 2,6-dimethyl-β-cyclodextrin.

compared with theoretically derived equations for efficiency and resolution.

EXPERIMENTAL

Apparatus

The CZE separations were carried out on a P/ACE 2000 capillary electrophoresis system (Beckman, Palo Alto, CA, USA). A UV detector equipped with a deuterium lamp was used for detection and operated at 214 nm (bandpass filter). CZE was performed in 50-cm effective length (57-cm total length) Beckman fused-silica capillaries, 75 μm I.D. and 375 μm O.D., with preburned windows. The capillary was cooled in the capillary cartridge by means of fluorocarbon liquid and the temperature was maintained at 25 ± 0.1°C. To minimize any effect due to Joule heating on the observations, the recommendation given by the manufacturer of a limit of 0.05 W/cm of capillary was followed. Pressurized nitrogen was used for injection. Data analysis and collection were accomplished using the Beckman System P/ACE 2000 software, version 1.5.

Chemicals

2,6-Dimethyl-β-cyclodextrin was obtained from AVEBE (Veendam, Netherlands). From Wacker-Chemie (Burghausen, Germany) we ob-

tained 2-hydroxypropyl-β-cyclodextrin with an average degree of substitution of 0.6 and 0.9 and 2,3,6-"di"-methyl-β-cyclodextrin having the 2-, 3- and 6-hydroxyl groups partially substituted with methoxyl groups, the average degree of substitution being 1.8. β-Cyclodextrin, *rac*-brompheniramine (maleate) and *rac*-ephedrine (hydrochloride) were obtained from Sigma (St. Louis, MO, USA), mesityl oxide from Aldrich (Steinheim, Germany), *rac*-terbutaline(sulphate), *rac*-bambuterol (hydrochloride) and terbutaline sulphate conjugate from Astra Draco (Lund, Sweden) and *rac*-propranolol (hydrochloride) from Astra-Hässle (Mölndal, Sweden). All other chemicals were of analytical-reagent grade from Merck (Darmstadt, Germany).

Electrolytes

Phosphate buffers of pH *ca.* 2.5–4.5 were prepared by mixing appropriate concentrations of phosphoric acid and sodium dihydrogenphosphate solutions. Phosphate buffers of pH *ca.* 5–9 were prepared by mixing appropriate concentrations of sodium dihydrogenphosphate and disodium hydrogenphosphate solutions. Phosphate buffers of pH *ca.* 9.5–11.5 were prepared by adjusting the pH of disodium hydrogenphosphate solution, at the appropriate concentration, with 10 *M* sodium hydroxide solution.

All water used was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

Procedures

All separation parameters were calculated for both enantiomers and the mean value was used. All separations were repeated at least twice. To estimate the quality of the observations, repeatability tests were performed on series of separations showing relative standard deviations below 1% for migration time and 2–3% for resolution.

At the beginning of each working day the capillary was washed with 100 column volumes of 0.01 *M* NaOH solution. Before each analysis the capillary was washed with three column volumes each of 0.01 *M* NaOH, water and electrolyte solution. After each working day the capillary was washed with ten volumes each of 0.01 *M* NaOH and water.

All sample components were dissolved in water and sample concentration in all the CZE analysis was *ca.* 0.2 m*M*. Samples were injected for 3 s (*ca.* 15 nl).

Mesityl oxide was used as neutral marker for measurement of electroosmotic flow (EOF).

Calculations

The coefficient of electroosmotic flow, μ_{EOF} in $cm^2\ V^{-1}\ s^{-1}$, was calculated from the equation

$$\mu_{EOF} = L_c L_d / V t_{EOF}$$

where L_d is the distance between the inlet of the capillary and the detector, L_c is the total length of the capillary, V the applied voltage and t_{EOF} the migration time of the neutral marker.

For calculation of resolution (R_s) and number of theoretical plates (N), the following equations were used:

$$R_s = 2(t_2 - t_1)/(t_{w1} + t_{w2})$$

where t is the migration time and t_w is the peak width, and

$$N = (t/\sigma)^2$$

where σ is the peak half-width at 0.6 at the peak height.

RESULTS AND DISCUSSION

Theory

When enantiomers (not involved in any secondary equilibria) of chiral compounds are separated in a CZE system containing cyclodextrins as chiral selectors, the following equilibria exist for each enantiomer [14]:

$$A + CD \overset{K_{A-CD}}{\rightleftharpoons} A\text{–}CD \quad (1)$$

$$B + CD \overset{K_{B-CD}}{\rightleftharpoons} B\text{–}CD \quad (2)$$

where A and B are the enantiomers, CD is the cyclodextrin, A–CD and B–CD are the enantiomer–cyclodextrin complexes and K_{A-CD} and K_{B-CD} are the formation constants. The enantiomers A and B of the enantiomeric pair migrate with the same apparent electrophoretic mobility μ_f in free solution ($\mu_{apparent} = \mu_{effective} + \mu_{EOF}$). The apparent electrophoretic mobilities μ_c of the

enantiomer–cyclodextrin complexes A–CD and B–CD are considered to be identical. If the two enantiomers have different affinities for the chiral selector, the formation constants K_{A-CD} and K_{B-CD} will be different. Chiral resolution will then be possible under the condition that the apparent electrophoretic mobility μ_f of the free enantiomers is different from the apparent electrophoretic mobility μ_c of the enantiomer–cyclodextrin complexes.

For a neutral cyclodextrin, transportation is due only to EOF. The neutral cyclodextrin shields the charge of the guest enantiomer from the electric field. As a consequence, the apparent electrophoretic mobilities of the free enantiomer (charged) and of the enantiomer–cyclodextrin complex will be different. Thus, when the EOF is in the same direction as the effective electrophoretic mobility of the free enantiomer, the apparent electrophoretic mobility of the enantiomer–cyclodextrin complex will lie closer than the apparent electrophoretic mobility of the free enantiomer to the mobility of the EOF.

If the exchange of enantiomers between free and bound forms is very rapid, then the resulting apparent electrophoretic mobilities μ_A and μ_B of the enantiomers will be a function of the proportion of the time when the enantiomer is free and when it is complexed, *i.e.*,

$$\mu_A = \frac{[A]}{[A]+[A\text{–}CD]} \cdot \mu_f + \frac{[A\text{–}CD]}{[A]+[A\text{–}CD]} \cdot \mu_c \quad (3)$$

$$\mu_B = \frac{[B]}{[B]+[B\text{–}CD]} \cdot \mu_f + \frac{[B\text{–}CD]}{[B]+[B\text{–}CD]} \cdot \mu_c \quad (4)$$

$$[A\text{–}CD] = K_{A-CD}[A][CD] \quad (5)$$

$$[B\text{–}CD] = K_{B-CD}[B][CD] \quad (6)$$

and therefore

$$\mu_A = \frac{\mu_f + \mu_c K_{A-CD}[CD]}{1 + K_{A-CD}[CD]} \quad (7)$$

$$\mu_B = \frac{\mu_f + \mu_c K_{B-CD}[CD]}{1 + K_{B-CD}[CD]} \quad (8)$$

The resolution of two zones in CZE can be expressed by the equation [42]

$$R = \tfrac{1}{4} N^{1/2} \cdot \frac{\Delta\mu}{\mu} \quad (9)$$

where $\Delta\mu$ is the difference in apparent electrophoretic mobility between two analytes, μ is the average apparent electrophoretic mobility and N is the average number of theoretical plates, which can be written as [42]

$$N = \frac{\mu V L_d}{2 D L_c} \quad (10)$$

where μ is the average apparent mobility, D is the diffusion coefficient of the solute, L_d is the effective length of the capillary tubing (from injection to detection point), L_c is the total length of the capillary between the two reservoirs and V is the applied voltage.

These same equations should be valid for enantiomeric separations with the aid of cyclodextrins if the equilibrium between the free enantiomer and the enantiomer–cyclodextrin complex is very rapid and does not give rise to band broadening.

Combination of eqns. 7 and 8 with eqn. 9 gives a general expression for the resolution of enantiomers with cyclodextrin complexing agents (see eqn. 11, below), where N is the average number of theoretical plates for the two enantiomers.

Combining eqn. 7 or 8 with eqn. 10 yields the following expression for efficiency:

$$N = \frac{V L_d}{2 D L_c} \cdot \frac{\mu_f + \mu_c K_{X-CD}[CD]}{1 + K_{X-CD}[CD]} \quad (12)$$

$$R = \tfrac{1}{4} N^{1/2} \cdot \frac{[CD](K_{B-CD} - K_{A-CD})(\mu_f - \mu_c)}{\mu_f + \tfrac{1}{2}(K_{A-CD} + K_{B-CD})(\mu_f + \mu_c)[CD] + \mu_c K_{A-CD} K_{B-CD}[CD]^2} \quad (11)$$

From eqns. 11 and 12, the following can be concluded:

(a) Resolution decreases as the difference between the formation constants of the two enantiomer–cyclodextrin complexes decreases. When $K_{A-CD} = K_{B-CD}$ the resolution will be zero.

(b) Resolution decreases as the difference between the apparent electrophoretic mobilities of the free enantiomer μ_f and the enantiomer–cyclodextrin complex μ_c decreases. When the free enantiomer is neutral it moves with the EOF as the cyclodextrins and the resolution will be zero. If it is assumed that effective mobility of the enantiomer–cyclodextrin complex is proportional to the effective mobility of the free enantiomer then resolution should also be favoured by a high charge on the enantiomer ($\mu_f - \mu_c$ larger).

(c) The resolution will be zero if the cyclodextrin concentration is zero and decreases towards zero at very high concentrations. A cyclodextrin concentration giving maximum resolution should therefore exist theoretically.

(d) The apparent electrophoretic mobilities μ_f and μ_c are composed of the EOF and the effective electrophoretic mobility. Increased EOF is favourable for the efficiency part of eqn. 11 but unfavourable for the $\Delta\mu/\mu$ part. The square root on the plate number makes the $\Delta\mu/\mu$ part more dominant and as a consequence the resolution decreases with increasing EOF.

(e) High separation efficiency is favoured by high electrophoretic mobility in the same direction as the EOF, high EOF and high voltages, given the limitation of efficient heat dissipation.

The theoretical expressions given above predict that resolution and efficiency can be affected in cyclodextrin-based enantiomeric separations by changes in the cyclodextrin concentration, applied voltage, or by a change in the environment that alters (i) the difference between the equilibrium constants of the two enantiomer–cyclodextrin complexes, (ii) the EOF or (iii) the effective electrophoretic mobility of the free enantiomer.

The difference between the equilibrium constants of the two enantiomer–cyclodextrin complexes can be affected, for example, by changes in temperature and pH, but it is difficult to predict the effect. A more favourable difference in equilibrium constants might be obtained by using a different type of cyclodextrin.

For ionizable compounds the most straightforward way to affect the effective electrophoretic mobility of the free enantiomer is by varying the pH of the electrolyte solution. Another possibility would be to increase the concentration of the electrolyte solution (ionic strength), which affects the effective electrophoretic mobility of charged molecules [43–45]. The type of electrolyte solution used has also been shown to affect the mobility of ions in CZE [45–47].

The rate of electroosmotic flow in fused-silica capillaries is pH dependent [48–50] but there are several other ways to affect the electroosmotic flow in CZE. Examples are the use of coated capillary columns [51], addition of organic modifiers [49,50], addition of cationic surfactants [52,53], increased concentration of the electrolyte solution [43–45,54,55], different electrolyte solutions [45–47,49] and application of a radial electric field [56].

Attempts to improve enantiomeric separations in cyclodextrin-modified CZE by reducing the EOF can be complicated because modifiers such as surfactants and organic solvents not only interact with the capillary wall to reduce the zeta potential, but can also interact with the cyclodextrin cavity, which could result in decreased enantiomeric resolution [18].

Effect on electroosmotic flow coefficient at different pH and in the presence of various cyclodextrins

Fig. 2 shows the dependence of the EOF coefficient on pH in the electrolyte solution used in this work. The presence of various cyclodextrins in the electrolyte solution at a moderate concentration (12 m*M*) had limited effect on the EOF coefficient. At much higher cyclodextrin concentrations we would expect the EOF to decrease owing to the increase in viscosity [24].

The shape of the curve obtained resembles that obtained by others [48–50]. The EOF coefficient increases strongly between pH 3.5 and 6 as the total number of ionized silanol groups at the

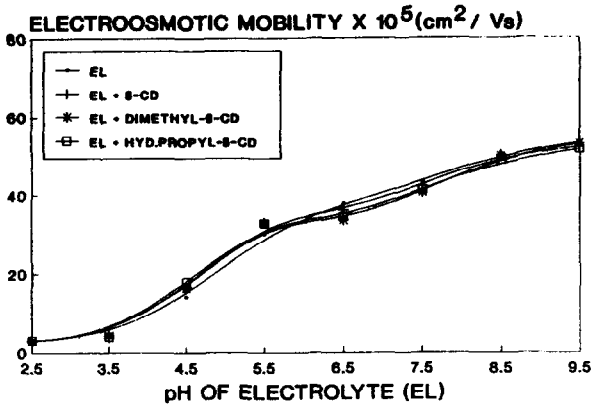


Fig. 2. EOF at different pH values in the presence of various cyclodextrins at 12 m*M* concentration. Conditions: electrolyte solution, 0.05 *M* phosphate buffer; separation voltage, 17 kV; neutral marker, mesityl oxide; $n = 4$ at each pH value for each cyclodextrin.

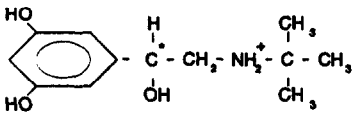
capillary surface increases [49]. At higher pH, the EOF coefficient is still increasing, but more slowly. This behaviour might be explained by a decrease in absorption of cations on the silica surface as the pH increases [49].

At low and high pH the EOF can be controlled with high precision, the R.S.D. of the EOF coefficient being *ca.* 1% ($n = 4$) using the standard capillary conditioning described under *Procedures* between runs. At pH 3.5, 4.5 and 5.5 the mean R.S.D. was found to be 6.1%, 9.8% and 3.0%, respectively. In the pH range 3.5–5.5 the buffer capacity of both the electrolyte and silanol groups is low, which contributes to this behaviour.

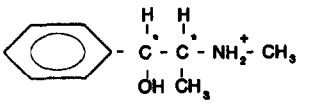
Effect of cyclodextrin type on separation

In this study, α- and β-cyclodextrin, 2,6-dimethyl-β-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin were used as chiral selectors. Fig. 3 shows the structure of the chiral drugs used as model substances. All the separations were carried out at pH 2.5 in 0.05 *M* phosphate buffer, except for *rac*-terbutaline monosulphate, the enantiomers of which were separated at pH 8.5 using 0.20 *M* phosphate buffer.

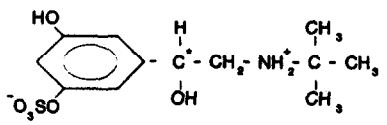
Under these experimental conditions the chemically modified β-cyclodextrins proved to be more powerful chiral selectors than β-cyclodextrin for the chiral drugs studied. None of the drug enantiomers could be resolved using α-cyclodextrin, probably because its small cavity



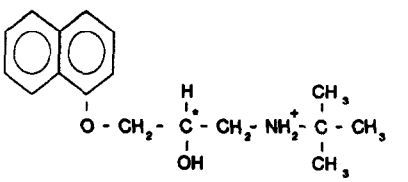
TERBUTALINE



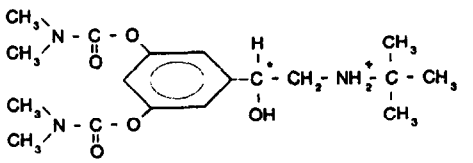
EPHEDRINE



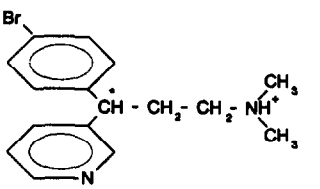
TERBUTALINE MONOSULPHATE CONJUGATE



PROPRANOLOL



BAMBUTEROL



BROMPHENIRAMINE

Fig. 3. Structures of chiral drugs separated in this work.

prevents the enantiomers from entering the cavity. The terbutaline and brompheniramine enantiomers were resolved by β-cyclodextrin but a better resolution was obtained by using chemically modified β-cyclodextrin. The enantiomers of *rac*-bambuterol, *rac*-terbutaline monosulphate conjugate, *rac*-propranolol and *rac*-ephedrine were only resolved using modified β-cyclodextrin. Fig. 4 shows the effect of cyclodextrin type on the resolution of *rac*-terbutaline at pH 2.5. At the given cyclodextrin concentration maximum resolution was obtained: the resolution was 4.6 for 2,6-dimethyl-β-cyclodextrin (5 m*M*), 3.7 for 2-hydroxypropyl-β-cyclodextrin (25 m*M*) and 2.0 for β-cyclodextrin (15 m*M*).

Chemical modification of the cyclodextrin rim (Fig. 1) clearly affects the enantioselectivity of the guest–host complex formation. Alkylation with methyl groups has been shown to result in a change in the cyclodextrin conformation [36]. The small opening (C-6 side, Fig. 1) becomes smaller whereas the other opening becomes wider. This stretching of the wider opening of the cyclodextrin cavity probably makes it easier for some molecules to enter the cyclodextrin cavity, which could facilitate enantiomeric separation. A decrease in the size of the small opening (C-6) of the cyclodextrin could aid or diminish the enantiomeric recognition of chiral molecules [36]. When the guest molecules are prevented from falling deep into the cyclodextrin cavity, interactions between the rim of the cyclodextrin and the guest molecule could become more favourable or less favourable. Modification of the cyclodextrin rim is also critical for the type of interactions possible (*e.g.*, hydrogen bonding) between the cyclodextrin rim and the guest molecule.

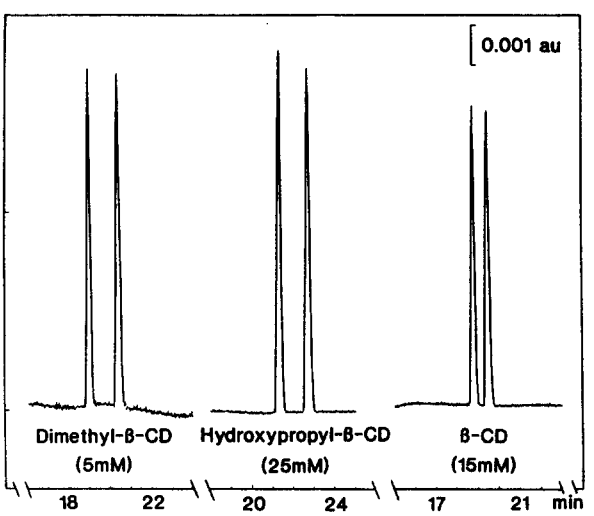


Fig. 4. Resolution of terbutaline enantiomers. Conditions: electrolyte, 0.05 *M* phosphate buffer (pH 2.5); separation voltage, 17 kV; cyclodextrin (CD) concentrations, 2,6-dimethyl-β-CD 5 m*M*, 2-hydroxypropyl-β-CD 25 m*M*, β CD 15 m*M*.

Effect of cyclodextrin concentration on separation

The effect of cyclodextrin concentration on the separation parameters was investigated for all the drugs over the concentration range 5–30 m*M* for the modified cyclodextrins and 5–15 m*M* for β-cyclodextrin.

Fig. 5 shows the effect of cyclodextrin concentration on resolution, migration time and plate number for the enantiomeric resolution of *rac*-terbutaline for various cyclodextrins. The enantiomeric resolution depends on the concentration of the cyclodextrin in the electrolyte solution. The resolution generally increases with increasing cyclodextrin concentration up to a certain point where a maximum is reached. The concentration of the cyclodextrin at maximum resolution is dependent on the chiral compound being separated and on the type of cyclodextrin being used. Further increase in the cyclodextrin concentration can result in a slow or rapid decrease in resolution, again depending on the chiral compound and cyclodextrin type. The above type of behaviour has also been observed by other workers [24] and is qualitatively in agreement with what is predicted by eqn. 11.

The mean migration time of the enantiomeric pairs is also dependent on the cyclodextrin concentration. As the CD concentration increases the mean migration time becomes longer. Almost a linear relationship was observed for all the chiral compounds. When the cyclodextrin concentration is increased to improve the enantiomeric resolution, the trade-off is always longer migration times.

The number of theoretical plates was only

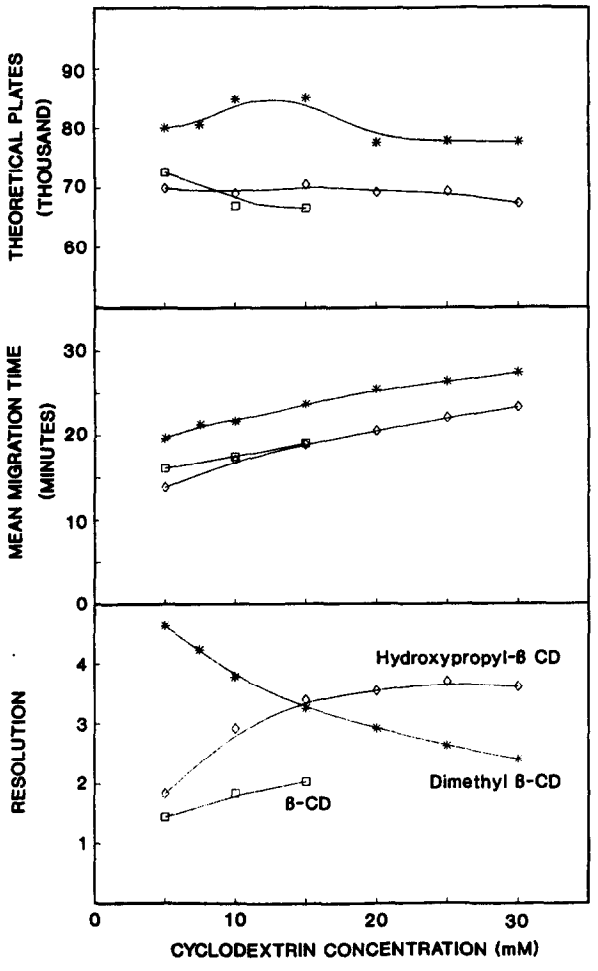


Fig. 5. Effect of cyclodextrin concentration on resolution, mean migration time and theoretical plate number for the separation of terbutaline enantiomers. * = 2,6-Dimethyl-β-CD; ◇ = 2-hydroxypropyl-β-CD; □ = β-CD. Conditions as in Fig. 4.

slightly affected by a change in cyclodextrin concentration.

Effects of methyl group positions in dimethyl-β-cyclodextrin on separation

The separation was affected by the position of the methyl groups in dimethyl-β-cyclodextrin. Fig. 6 shows the separation of the enantiomers of *rac*-terbutaline, *rac*-ephedrine and *rac*-bambuterol in the presence of dimethyl-β-cyclodextrin at similar conditions using the cyclodextrin concentration giving the maximum difference in resolution. The separations labelled a were obtained using dimethyl-β-cyclodextrin which, according to the manufacturer, had the 2-, 3- and 6-hydroxyl groups partially substituted with methoxyl groups. The separations labelled b were obtained with dimethyl-β-cyclodextrin having only the 2- and 6-hydroxyl groups substituted with methoxyl groups. 2,6-Dimethyl-β-cyclodextrin gave a better resolution in all instances at the expense of longer migration times. The cyclodextrin concentration giving the maximum resolution varied between the two cyclodextrins, but the highest resolution was always obtained with 2,6-dimethyl-β-cyclodextrin. An unsubstituted hydroxyl group on the wider rim of the cyclodextrin could therefore be critical for the successful enantiomeric separations of these compounds.

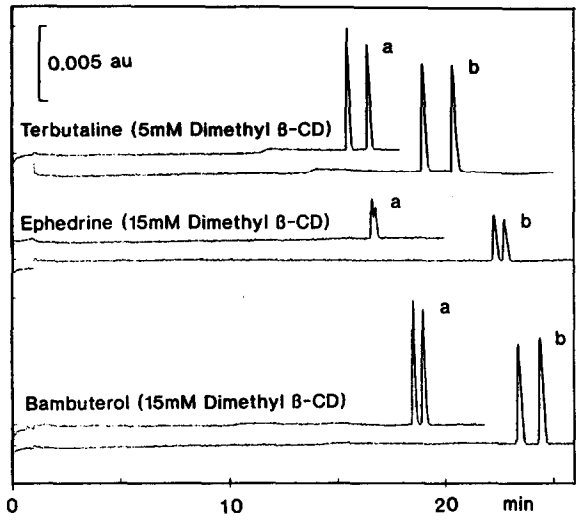


Fig. 6. Comparison between the separation powers of (a) 2,3,6-"di"methyl-β-CD and (b) 2,6-dimethyl-β-CD. Conditions: electrolyte, 0.05 *M* phosphate buffer (pH 2.5); *rac*-terbutaline, CD concentration 5 m*M* and separation voltage 17 kV; *rac*-ephedrine, CD concentration 15 m*M* and separation voltage 11 kV; *rac*-bambuterol, CD concentration 15 m*M* and separation voltage 17 kV.

Effect of degree of substitution on separation

The enantiomeric separations of *rac*-terbutaline, *rac*-propranolol and *rac*-brompheniramine were carried out in the presence of 2-hydroxypropyl-β-cyclodextrin with degrees of substitution of 0.6 and 0.9. It was observed that a higher degree of substitution improved the

resolution for *rac*-terbutaline and *rac*-propranolol without affecting the migration time or plate number. No effect on resolution was seen for *rac*-brompheniramine, the bulkiest of the three compounds. However, a higher efficiency and shorter migration time were obtained.

Quality of cyclodextrins

A complication when studying the effects on chiral separation using commercially available cyclodextrins is that the purity, degree of substitution and exact position of substituents are often not declared by the manufacturer. The above experiments clearly show the importance of having these factors under control to obtain reliable data. The above studies on the effect on separation parameters using differently substituted cyclodextrins should therefore be considered as only qualitative.

Effect of pH on separation

Investigations were made on the effect of pH on the EOF and effective electrophoretic mobility for the enantiomeric separations of *rac*-terbutaline and *rac*-terbutaline monosulphate.

Fig. 7 demonstrates the effect on EOF and the effective electrophoretic mobility for terbutaline and terbutaline monosulphate conjugate with variations in pH. Unexpectedly, the mobility of terbutaline increased slightly on going from pH 2.5 to 5.5 [pK_{a_1} (phenol) = 8.8, pK_{a_2} (amine) = 10.1, pK_{a_3} (phenol) = 11.2] but then, as expected, started to decrease and was zero at pH 9.5, where terbutaline is uncharged (isoelectric point). The effective mobility of terbutaline monosulphate towards the anode increases from pH 7.5 to 10.5 as the negative charge on the molecule increases. At lower pH terbutaline monosulphate is zwitterionic and the effective electrophoretic mobility is zero. At pH >9.5 terbutaline is an anion and the effective migration velocity towards the anode increases with pH (not shown).

From eqn. 11, we concluded that a high effective mobility of the uncomplexed enantiomers and a low EOF were favourable for the enantiomer resolution. Our observations are in an agreement with this. Fig. 8 shows the effect of pH on the separation of terbutaline enantiomers

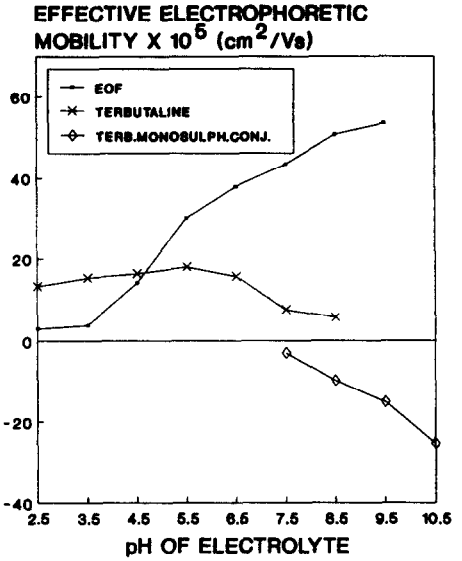


Fig. 7. Change in electrophoretic mobility of *rac*-terbutaline and terbutaline monosulphate conjugate with pH. Also shown is the EOF at pH 2.5–9.5. Conditions: electrolyte, 0.05 *M* phosphate buffer (pH 2.5); separation voltage, *rac*-terbutaline, 17 kV, *rac*-terbutaline monosulphate conjugate 10 kV and EOF 17 kV.

in the presence of 2,3,6-"di"methyl-β-cyclodextrin. Maximum resolution of almost 5.5 was reached at pH 3.5. The resolution then decreased rapidly with increasing pH and was zero at pH 8.5. On going from pH 2.5 to 3.5 the EOF remains almost constant. The slight increase in the effective mobility (Fig. 7) of terbutaline might explain the slightly increased resolution found. Between pH 3.5 and 5.5 the effective mobility of terbutaline increases slightly but the EOF increases rapidly (Fig. 7) and seemingly becomes the dominating factor, leading to a decrease in resolution. At pH 5.5–8.5 both the increase in the EOF and the decrease in effective mobility of terbutaline could contribute to the decrease in resolution.

Eqn. 12 predicts that a high EOF and a high effective mobility of the free terbutaline enantiomers in the same direction as the EOF are favourable for high efficiency. The efficiency of the terbutaline enantiomer separation increased rapidly from pH 2.5 ($N \approx 90\,000$) to pH 5.5 ($N \approx 220\,000$) when both the EOF and the effective electrophoretic mobility of the free enantio-

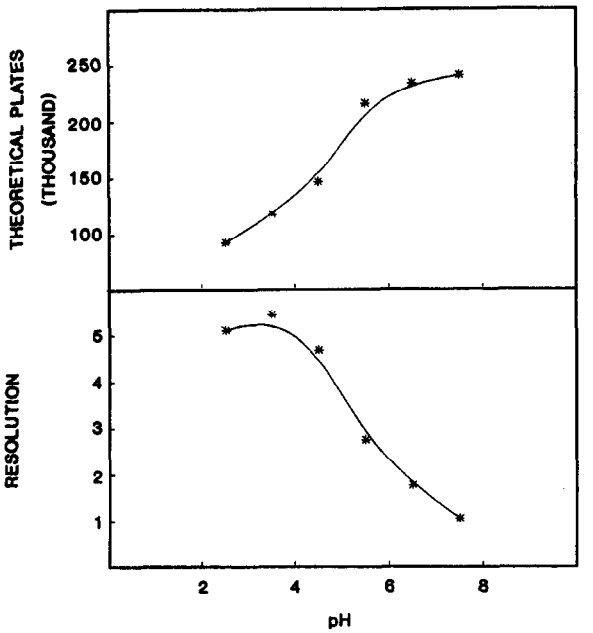


Fig. 8. Separation of terbutaline enantiomers at pH 2.5–8.5 in the presence of 12 m*M* 2,3,6-"di"methyl-β-CD. Electrolyte solutions, 0.05 *M* phosphate buffer at pH 2.5–8.5. Separation voltages: pH 2.5, 17 kV; pH 3.5, 16.8 kV; pH 4.5, 13 kV; pH 5.5, 7 kV; pH 6.5, 6 kV; pH 7.5, 5.7 kV; pH 8.5, 5.4 kV.

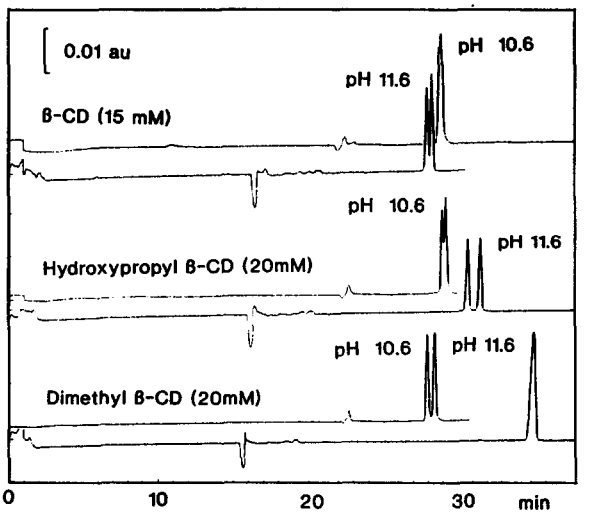


Fig. 9. Separation of terbutaline enantiomers at pH 10.6 and 11.6 in the presence of 20 m*M* 2-hydroxypropyl-β-CD, 20 m*M* 2,3,6-"di"methyl-β-CD and 15 m*M* β-CD. Conditions: electrolyte, 0.05 *M* phosphate buffer at pH 10.6 and 11.6; separation voltage, 5 kV.

mers increased. At pH 5.5–8.5 the efficiency increased more slowly, probably owing to the decrease in the effective electrophoretic mobility of the free terbutaline enantiomers (Fig. 7).

Similar effects on resolution and efficiency were observed for separation of the terbutaline enantiomers in the presence of hydroxypropyl-β-cyclodextrin and β-cyclodextrin at pH 2.5–9.5. Fig. 9 shows the separation of terbutaline enantiomers in the presence of 2,3,6-"di"methyl-β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin and β-cyclodextrin at pH 10.6 and 11.6. At pH 10.6 the terbutaline enantiomers are baseline separated in the presence of dimethyl-β-cyclodextrin but only a very low resolution is obtained in the presence of 2-hydroxypropyl-β-cyclodextrin and β-cyclodextrin. On going from pH 10.6 to 11.6 the EOF increases but the charge on the free terbutaline enantiomers increases from −1 to −2 and seemingly has a dominant effect on the separation. The large increase in the effective mobility of the terbutaline enantiomers in the presence of hydroxypropyl-β-cyclodextrin and β-cyclodextrin results in a better resolution at pH 11.6 than at pH 10.6. On the other hand, we lose the resolution of the terbutaline enantiomers in the presence of 2,6-dimethyl-β-cyclodextrin. Thus a negative charge on both substituents of the aromatic ring does not allow for enantiomeric resolution using dimethyl-β-cyclodextrin as a chiral selector.

The efficiencies ($N \approx 100\,000$–$150\,000$) reached for the enantiomeric separations of terbutaline at high pH were not as high as those observed at pH 7.5 and 8.5. This might be explained by the fact that the effective mobility of the free enantiomers is now in the opposite direction to the EOF. A high effective mobility of the terbutaline anion is now unfavourable for the efficiency reached even though it is favourable for the resolution.

For terbutaline monosulphate, which is neutral at low pH (Fig. 3) but an anion above *ca.* pH 7, the enantiomer resolution in the presence of dimethyl-β-cyclodextrin was found to increase as the pH increased from 7.0 to 10, as shown in Fig. 10. This is probably due to a higher effective mobility. The highest efficiencies were reached at pH 8 and 9 ($N \approx 140\,000$).

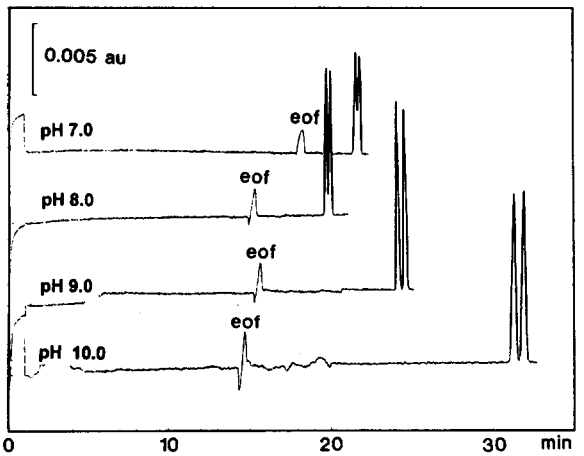


Fig. 10. Separation of the enantiomers of *rac*-terbutaline monosulphate conjugate at pH 7.0–10.0 in the presence of 10 m*M* 2,3,6-"di"methyl-β-CD. Conditions: electrolyte, 0.20 *M* phosphate buffer; separation voltage, 10 kV.

At pH 11 no resolution was obtained, which implies that a negative charge on both substituents of the aromatic ring does not allow for a favourable complex with the dimethyl-β-cyclodextrin.

The above discussion, where comparisons are made with experimental findings and eqns. 11 and 12, is only qualitative. The equations do not involve any secondary equilibria, which for terbutaline will have an influence at pH values above 6–7. The observed effects on resolution and efficiency can also be due to changes in formation constants generated by changes in pH and ionic strength.

Effect of applied voltage on separation parameters

Fig. 11 shows the effect of applied voltage on resolution, migration time and efficiency for the separation of terbutaline enantiomers at pH 2.5, 6.0 and 7.5 in the presence of dimethyl-β-cyclodextrin and 0.05 *M* phosphate buffer.

Higher field strengths should, according to eqn. 12, yield higher efficiency. Fig. 11 shows that the efficiency for the separation of the terbutaline enantiomers increases at higher voltages up to a certain level where a slight decrease or a levelling off is observed. These effects are probably indicative of insufficient cooling of the capillary causing radial temperature gradients

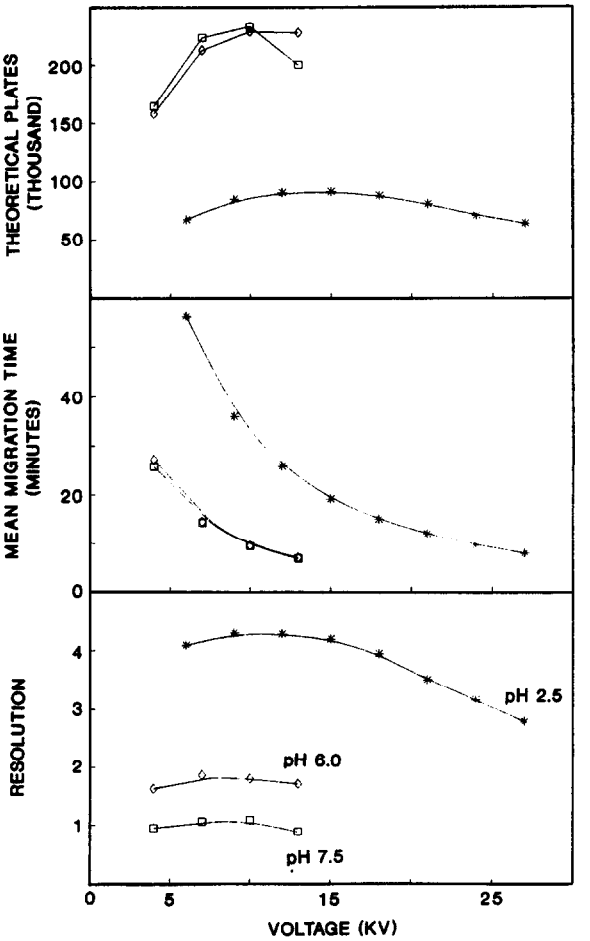


Fig. 11. Effect of separation voltage on the resolution, mean migration time and efficiency for the separation of terbutaline enantiomers at pH 2.5, 6.0 and 7.5 in the presence of 10 m*M* 2,3,6-"di"methyl-β CD. Electrolyte, 0.05 *M* phosphate buffer. * = pH 2.5; ◇ = pH 6; □ = pH 7.5.

that start to affect the separation by broadening of the bands. Owing to the low EOF at pH 2.5, the efficiency is low and hence the above effects are less pronounced.

Fig. 11 also shows that the resolution increases at higher voltages, a performance predicted by eqn. 11. The increase is not as pronounced as for the efficiency, however. This relative behaviour is in accordance with eqn. 11, which predicts that it is only the square root of the efficiency that contributes to the resolution. As for the efficiency, band broadening due to thermal effects results in a decrease in resolution at higher

voltages. As is obvious from the above experiments, the applied voltage has little effect on resolution. More important is the effect on migration time, which decreases dramatically and thus the applied voltage is important for speeding up the separation.

CONCLUSIONS

As experienced in this laboratory and by many other workers, cyclodextrins can be used as chiral selectors to separate a number of drug enantiomers in CZE. This work demonstrates the importance of the experimental conditions, which can be used to improve the chiral separation. Depending on the chemistry of the analyte, experimental conditions such as pH can have a dramatic effect on the chiral separation (compound dependent). A simple theoretical model predicts the effect of several parameters on resolution and efficiency. This model can be of help when experimental conditions for these enantiomer separations are selected. The above findings could also be of importance in bioanalytical work. If enantiomer separations can be obtained even though pH is changed dramatically, as is shown here, the selectivity towards matrix components will also change.

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