

Enantiomeric separation by capillary electrophoresis using a soluble neutral β -cyclodextrin polymer

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Abstract

Enantiomers of several racemic basic compounds of pharmaceutical interest and three tryptophan derivatives were investigated by capillary electrophoresis employing a soluble β -cyclodextrin polymer and native β -cyclodextrin. The effects of the concentration of the polymer added to the background electrolyte and of the pH of the buffer on the effective mobility and resolution of the studied compounds were examined. The β -cyclodextrin polymer showed a higher stereoselectivity than the parent β -cyclodextrin. Enantioselectivity and resolution were influenced by the concentration of the β -cyclodextrin polymer and the background electrolyte. A pH study, carried out in the pH range 2.7–6, showed that an increase in pH caused a general decrease in both resolution and selectivity. The best results for the enantiomeric separation of the basic compounds studied were obtained at pH 2.7.

1. Introduction

The development of new chiral substances, especially in the pharmaceutical field, places increasing demands on analytical methods for the separation of these kinds of isomers for, e.g., the chiral purity control of drugs and pharmacokinetic studies.

Enantiomers are optical isomers possessing

similar physico-chemical properties and thus difficult to separate from each other. Their separation can be obtained using a chiral environment that interacts with the enantiomers either before or during the separation process forming stable diastereoisomers or labile diastereomeric complexes, respectively.

Analytical methods used so far for the enantiomers separation include high-performance liquid chromatography (HPLC) [1–3], thin-layer chromatography (TLC) [4], gas chromatography (GC) [5] and capillary electrophoresis (CE) [6–17].

CE is a recent separation technique that allows rapid separations to be performed with high resolution and high efficiency, and requiring only

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small amounts of buffer and sample. Among the chiral selectors used for the resolution of enantiomers by CE, buffer additives such as chelator–metal complexes [16], chiral crown ethers [10–12], proteins [8,9,13], bile salts [14,15], cyclodextrins (CDs) and their derivatives [6,7,18,19], etc., have been widely applied.

Native CDs (α , β and γ) and their derivatives (methylated, carboxymethylated, methylamino, hydroxypropylated, etc.) have been successfully utilized in many applications [6,7,18,19]. These chiral selectors have been mainly used in capillary zone electrophoresis (CZE) and in several instances the combination of CDs and micellar electrokinetic chromatography (CD–MEKC) allowed the enantiomeric resolution of neutral compounds.

When CDs or their derivatives are used, the chiral resolution is based on selective complexation with analytes. Hydrophobic interactions between analytes and the CD cavity and hydrogen bonds with hydroxy (or modified) groups on the CD rim can lead to the formation of labile diastereoisomeric complexes with different stability constants. The most stable complex formed moves with a lower effective mobility.

Linear or cross-linked polymers have been mainly used in CE as sieving media for improving the selectivity of the separation of high-molecular-mass compounds of biological origin such as proteins and nucleic acids [20]. Recently we used a chargeable β -cyclodextrin polymer for the enantiomeric resolution of several basic compounds [21] and Nishi et al. [22] studied the enantiomeric resolution of trimetoquinol hydrochloride and related substances using uncharged β -cyclodextrin polymer.

In this work, we investigated the effect of a soluble uncharged β -cyclodextrin polymer for the separation of enantiomers of several basic compounds by CE. The effect of the chiral polymer concentration on the effective mobility, the resolution and the selectivity was investigated. Further, the effects of the pH and of the concentration of the background electrolyte were also studied.

2. Experimental

2.1. Chemicals

Soluble β -cyclodextrin polymer (EP- β -CD) was purchased from Cyclolab (Budapest, Hungary), β -cyclodextrin from Fluka (Buchs, Switzerland), sodium hydroxide, acetic acid, phosphoric acid, sodium dihydrogenphosphate and citric acid from Carlo Erba (Milan, Italy) and standards of analytical-reagent grade from Sigma (St. Louis, MO, USA). Doubly distilled water (Menichelli, Rome, Italy) was used to prepare all solutions.

The background electrolyte (BGE) containing EP- β -CD was filtered by using nylon filters of 0.45 μm pore size (Lida, Kenasha, WI, USA). Stock solutions of standard samples (10^{-4} M) were prepared and stored at 4°C. The concentration of injected racemic mixtures was 10^{-5} M for propranolol, terbutaline, isoproterenol and ester tryptophan derivatives and that of all other standards was $5 \cdot 10^{-5}$ M. For the electrophoretic experiments performed with the laboratory-made electrophoresis apparatus, 25 mM of the following buffers were used: phosphate, pH 2.7; citrate, pH 3.5; acetate, pH 4.5; and phosphate, pH 6.0.

2.2. Apparatus

Experiments were carried out using a laboratory-made electrophoresis apparatus. The electrode chamber in which the detection end of the capillary was connected to a refilling block were as described previously [17]. The electrophoretic separations were performed at room temperature in untreated fused-silica capillaries obtained from Supelco (Bellefonte, PA, USA), 560 mm \times 50 μm I.D., with an effective length of 360 mm. The apparatus included a Spellman (Rockford, IL, USA) CZE1000R power supply and a Spectra FOCUS variable-wavelength UV detector (Thermo-Separation, San Jose, CA, USA). The detector was operated at 206 nm, and connected to a ChromJet integrator (Thermo-Separation). Before applying the sample, the capillary was

filled with the background electrolyte (BGE) containing the polymer. No chiral polymer was present in the electrode chambers. The injection of the sample was performed hydrodynamically at the anodic end of the capillary for 10 s; the difference between the liquid level in the electrode chamber and the sample vial was set to 20 cm. The applied voltage was 15 kV. The electroosmotic flow was measured injecting benzyl alcohol ($10^{-4}M$).

The experiments for the study of the effect of the concentration of BGE on the resolution of enantiomers of bupivacaine were performed in a P/ACE 2200 capillary electrophoresis system (Beckman, Fullerton, CA, USA) with UV detection at 214 nm. A fused-silica capillary (Microquartz, Munich, Germany), 370 mm \times 0.05 mm I.D., effective length 300 mm, was used for the experiments and the cartridge temperature was 20°C.

3. Results and discussion

Different basic compounds of pharmaceutical interest, namely adrenergic agonists (ephedrine, epinephrine, norepinephrine, isoproterenol and terbutaline), β -adrenergic blockers (atenolol, metoprolol, oxprenolol and propranolol), anaesthetics (ketamine and bupivacaine), an anorexic (norephedrine) and tryptophan methyl, ethyl and butyl esters were selected for the electrophoretic experiments. Their structures are shown in Fig. 1.

The racemic mixtures of the analytes were run in phosphate buffer at pH 2.7 in the absence of chiral additive and owing to the protonation of nitrogen atom they moved towards the cathode; as expected, no enantiomeric separation was obtained.

For the study of enantiomer resolution, the phosphate buffer was supplemented with different amounts of β -cyclodextrin polymer (for the composition, see Table 1).

Under the experimental operating conditions (pH 2.7) the electroosmotic flow was relatively low compared with the effective mobility of the

cationic samples and thus the chiral selector acted as a quasi-stationary phase.

The effective mobility was calculated using the equation

$$\mu_{\text{eff}} = \mu_{\text{app}} - \mu_{\text{eof}} \quad (1)$$

where μ is the mobility and the subscripts eff, app and eof stand for effective, apparent and electroosmotic, respectively.

The selectivity (S) and resolution (R) were calculated using the following equations:

$$S = \Delta\mu/\mu_m \quad (2)$$

$$R = 2(t_2 - t_1)/(w_2 + w_1) \quad (3)$$

where $\Delta\mu$ is the difference in effective mobility, μ_m the median mobility $[(\mu_2 + \mu_1)/2]$, t the migration time, w the peak width at the baseline and subscripts 1 and 2 represent the two enantiomers.

Fig. 2 shows the effect of the concentration of β -cyclodextrin polymer added to the BGE at pH 2.7 on the effective mobility of racemic β -adrenergic blockers. An increase in the concentration of the complexing additive leads to a general decrease in the effective mobility for all the compounds studied owing to the complexation with the polymer. The analytes formed labile diastereomers during the electrophoretic runs, causing a decrease in the velocity depending on the stability constant of the complex formed. The complexation power of the modified CD used (10–50 mg/ml) decreased in the order propranolol > metoprolol > atenolol > oxprenolol. Wren and Rowe [23] proposed to relate the interaction between β -blockers and cyclodextrin to the hydrophobicity of the analytes. This means that the most hydrophobic compound has the strongest interaction with the hydrophobic cavity of the CD. The influence of the hydrophobicity of the analytes on the inclusion complexation is also illustrated by our results obtained for methyl, ethyl and butyl esters of tryptophan. Fig. 3 shows the effect of the concentration of β -cyclodextrin polymer on the effective mobility of these tryptophan esters. The complexation was very strong for the most hy-

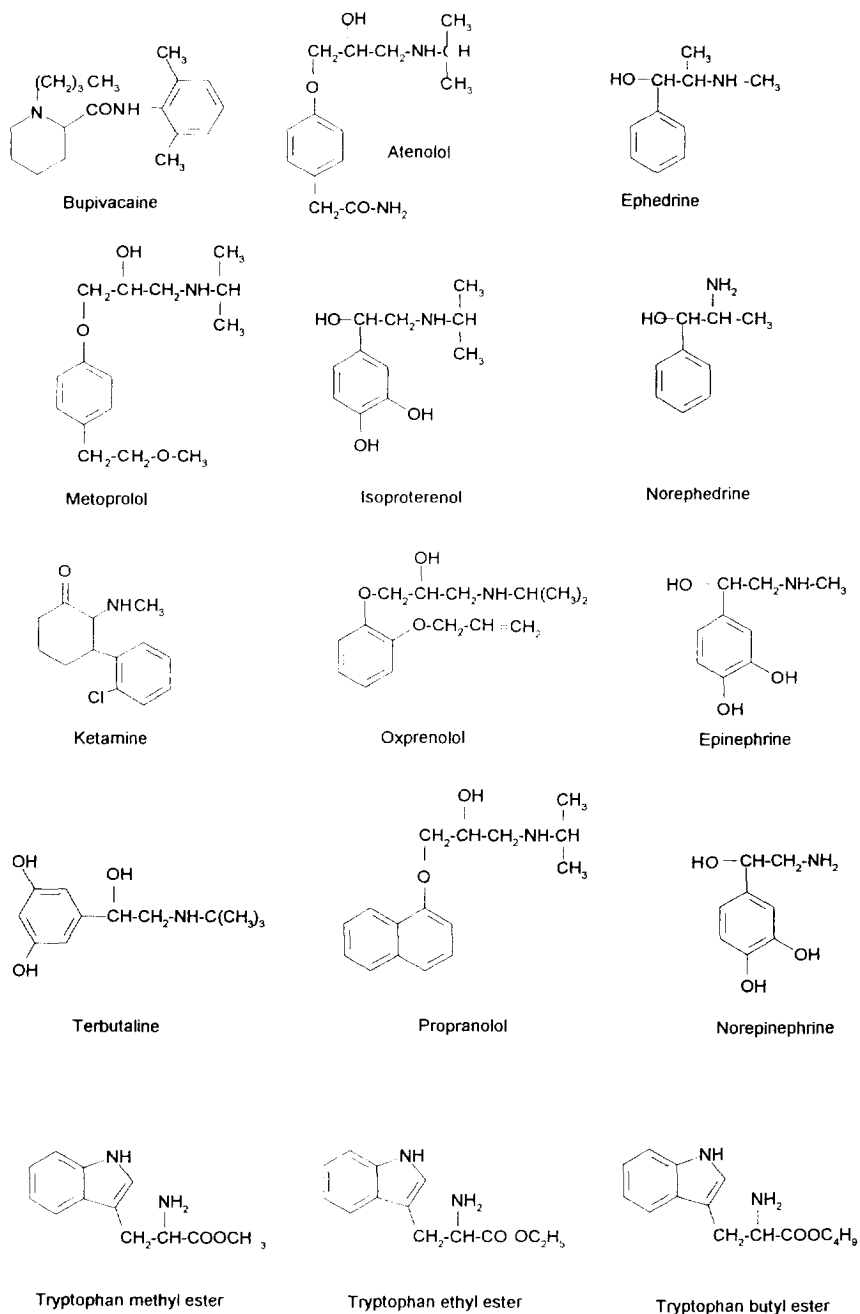


Fig. 1. Structures of the compounds studied.

drophobic compound (butyl derivative). Also in this case an increase in the concentration of the complexing additive caused a general decrease in

effective mobility for all three esters. A similar effect was obtained for the two anaesthetics studied, where bupivacaine was more complexed

Table 1
Main properties of β -cyclodextrin polymer

Molecular mass	3000–5000
Solubility in water	>40%
CD content	50–60%
Cross-linking	Epichlorohydrin

than ketamine (results not shown). Finally, the electrophoretic study of adrenergic agonists confirmed the importance of the type and concentration of the CD on the inclusion complexation. In fact, the β -cyclodextrin polymer was found to be a good complexing additive for all the compounds studied. Also in this case an increase in concentration of the modified CD caused a decrease in the effective mobility.

The complexation power at 15 mg/ml of EP- β -CD was found to decrease in the order terbutaline >epinephrine >norepinephrine >ephedrine >isoproterenol; an increase in the concentration of the complexing additive caused a reversal of complexing power, e.g., at 50 mg/ml isoproterenol was more complexed than epinephrine but less so than terbutaline.

3.1. Effect of concentration of β -cyclodextrin polymer on selectivity and chiral recognition

For the study of chiral recognition, the ana-

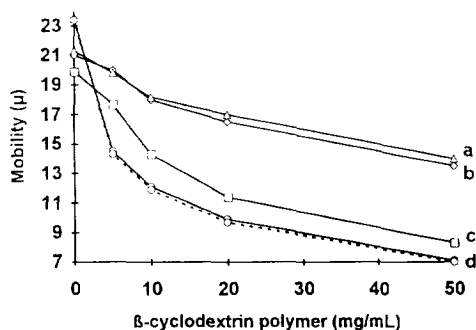


Fig. 2. Effect of β -cyclodextrin polymer concentration on the effective mobility ($\mu \times 10^5 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$) of β -adrenergic blockers: (a) oxprenolol; (b) atenolol; (c) metoprolol; (d) propranolol). Capillary, 560 mm \times 0.05 mm I.D. (effective length 360 mm), uncoated; applied voltage, 12 kV; background electrolyte, 25 mM phosphate buffer (pH 2.7); β -cyclodextrin polymer was present into the capillary only. For other conditions, see text.

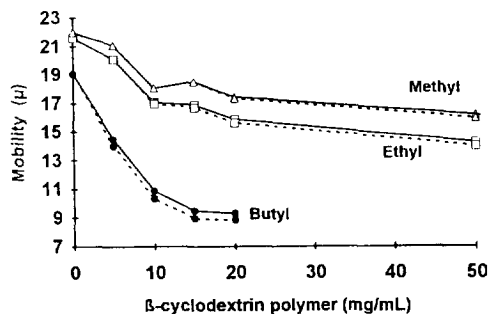


Fig. 3. Dependence of the effective mobility of three tryptophan esters on the β -cyclodextrin polymer concentration. Experimental conditions as in Fig. 2.

lytes were injected using a BGE at pH 2.7 supplemented with different amounts of β -cyclodextrin polymer in the range 0–50 mg/ml. Resolution of the racemic mixtures into their enantiomers was obtained for terbutaline, isoproterenol, epinephrine, propranolol and methyl, ethyl and butyl esters of tryptophan ($R \geq 1$). Norepinephrine was only partly resolved ($R = 0.6$ at 50 mg/ml of chiral additive) and no resolution was recorded in the concentration range of CD studied for ephedrine, norephedrine, ketamine, bupivacaine, atenolol, metoprolol and oxprenolol.

Fig. 4a and b show the effect of the concentration of EP- β -CD on the resolution factor (R) and selectivity (S), respectively, for propranolol and butyl, ethyl and methyl esters of tryptophan.

An increase in the concentration of β -cyclodextrin polymer added to the BGE at pH 2.7 led to a general increase in resolution and selectivity. Among the β -blockers studied, propranolol, the most hydrophobic analyte, was the only to be resolved into its enantiomers even at a relatively low concentration of chiral additive (0.5 mg/ml); Maximum of resolution ($R = 1.8$) and selectivity ($S = 0.0204$) were obtained when 20 mg/ml of chiral agent were used. These data confirm the previous finding [24] and the theoretical model discussed by Wren and Rowe [25] concerning maximum resolution at a certain concentration of chiral selector.

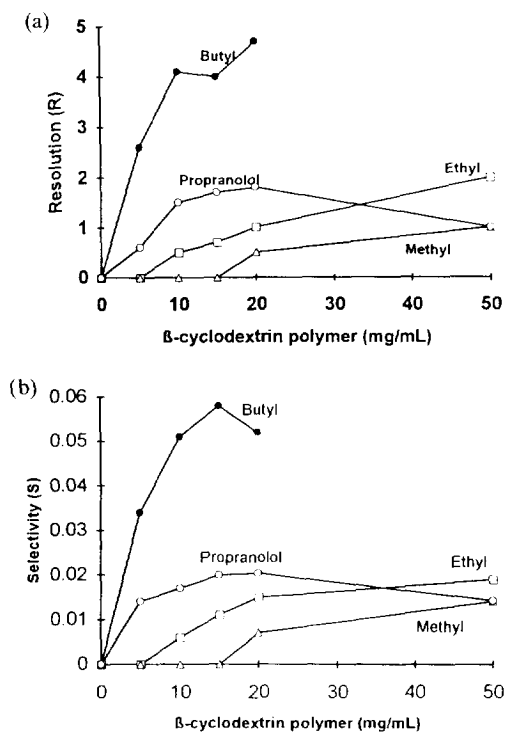


Fig. 4. Effect of the concentration of β -cyclodextrin polymer on (a) resolution, R , and (b) selectivity, S , for three tryptophan esters and propranolol. Experimental conditions as in Fig. 2.

An interesting behaviour was found for tryptophan esters, where the resolving power of β -cyclodextrin polymer decreased in the order butyl > ethyl > methyl. The resolution and selectivity increased with increasing concentration of the chiral selector for ethyl and methyl tryptophan derivatives, whereas for the butyl derivative S increased up to 15 mg/ml and then decreased; the resolution showed similar behaviour to that for the other esters with a slight decrease in R at 15 mg/ml. Fig. 5 shows the electropherograms for the enantiomeric separation of racemic butyl, ethyl and methyl esters of tryptophan.

The length of the ester chain had a strong influence on the enantioselectivity. In order to explain the strong complexation and/or the strong enantioselectivity of the racemic butyl ester with the chiral polymer, we have to consider the hydrophobic interaction of the butyl

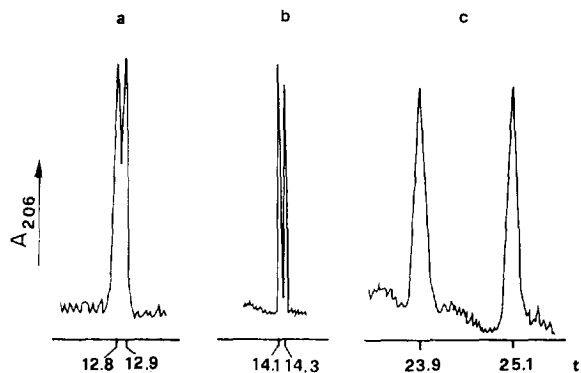


Fig. 5. Electropherograms for the enantiomeric separation of racemic mixtures of (a) butyl, (b) ethyl and (c) methyl esters of tryptophan. Background electrolyte, 25 mM phosphate buffer (pH 2.7) containing 20 mg/ml β -cyclodextrin polymer. Other experimental conditions as in Fig. 2.

chain with the inside and/or the outside of the CD cavity. Further, the inclusion complex formed is stabilized by stereoselective bonds (hydrogen) between the amino groups of the analyte and hydroxyl groups of the CD.

The effect of the concentration of EP- β -CD on resolution and selectivity is also demonstrated for the enantiomeric separation of adrenergic agonists and anaesthetic compounds. Fig. 6a and b show the effect of the concentration of EP- β -CD on the resolution of norephedrine, nor-epinephrine, epinephrine, terbutaline and isoproterenol and the selectivity for terbutaline and propranolol, respectively. Both parameters increased with increase in the concentration of the chiral selector added to the BGE, except for norephedrine, which was not resolved at all under the operating conditions (0.50 mg/ml of polymer). The resolving power of EP- β -CD was found to decrease in the order terbutaline > isoproterenol > epinephrine > norepinephrine > norephedrine.

As an example, the electropherograms in Fig. 7 demonstrate the different resolving power of EP- β -CD on the enantiomeric separation of racemic nor-epinephrine, isoproterenol and propranolol; the first was spiked with the (S)-(-)-isomer.

In order to explain the high resolving power of the chiral polymer towards terbutaline, we could

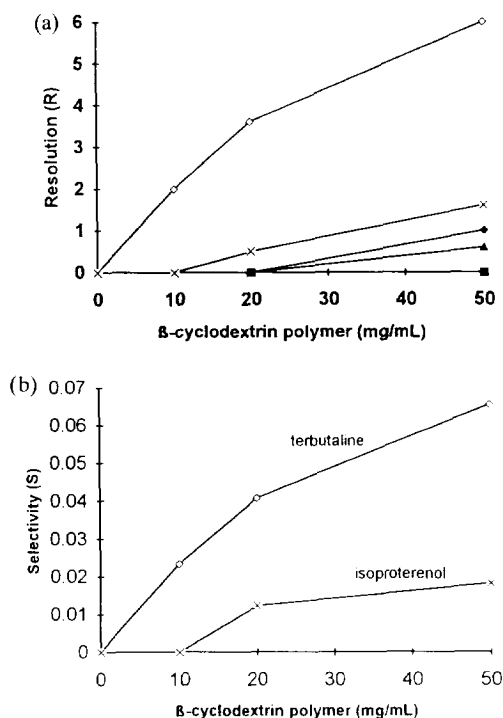


Fig. 6. Dependence of (a) resolution, R , of (■) norepinephrine, (▲) norepinephrine, (◆) epinephrine, (×) isoproterenol and (◇) terbutaline and (b) selectivity, S , of terbutaline and isoproterenol on the concentration of β -cyclodextrin polymer. Experimental conditions as in Fig. 2.

remark that the substituent groups on the asymmetric carbons of terbutaline had a strong influence on the enantioselective mechanism.

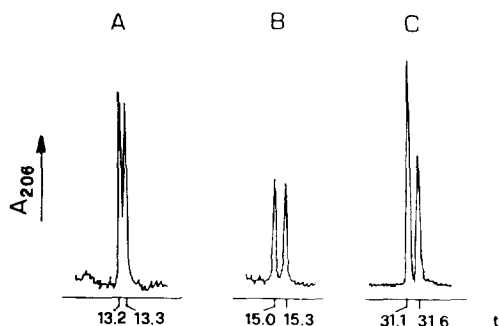


Fig. 7. Electropherograms for the enantiomeric separation of (A) norepinephrine, (B) isoproterenol and (C) propranolol [spiked with (S)-(-)-isomer]. Background electrolyte, 25 mM phosphate buffer (pH 2.7) containing 50 mg/ml β -cyclodextrin polymer. Other experimental conditions as in Fig. 2.

Even though the chiral polymer proved to be a good complexing agent towards the two anaesthetics studied, no enantiomeric resolution was obtained for ketamine and bupivacaine at EP- β -CD concentrations ≤ 50 mg/ml.

Considering the high solubility ($>40\%$, w/v) of the chiral polymer and the effect of its concentration on resolution, we tried to increase its content to 100, 150 and 200 mg/ml for the enantiomeric separation of those analytes not or poorly resolved in this study. No resolution of metoprolol, atenolol and ephedrine was obtained, norephedrine showed $R = 0.5$ at 100 mg/ml and no improvement in resolution was found at higher concentrations of the chiral selector. Bupivacaine was partly resolved into its enantiomers only at 200 mg/ml of EP- β -CD with $R = 0.5$. Good enantiomeric separation was obtained for ketamine and norepinephrine at 100 mg/ml ($R = 1$).

The migration order of enantiomers was verified for propranolol, epinephrine, norepinephrine, isoproterenol and methyl and ethyl esters of tryptophan by spiking the racemic mixtures with the separated optical isomers (commercially available) and performing electrophoresis experiments. The L-isomers of tryptophan esters moved faster than the D-forms. For the other analytes, the (-)-enantiomers moved faster than the (+)-enantiomers, indicating that the (+)-antipodes formed complexes with higher stability constants than their isomers.

The enantiomeric separation power of EP- β -CD was compared with that of native β -CD. The amount of cyclodextrins units per milligram of polymer was calculated according to the specification of the manufacturer (CD content 58.2%, w/w) in order to compare the concentration of EP- β -CD with that of the parent β -CD. Consequently, we compared the resolution of propranolol, terbutaline, butyl ester of tryptophan and epinephrine obtained at 2.5, 5, 10 and 20 mM β -CD with the resolution obtained at 5, 10, 20 and 50 mg/ml [50 mg/ml of EP- β -CD should be compared with 25 mM β -CD (aqueous solutions of <20 mM can be prepared)].

Propranolol and the butyl ester of tryptophan were not resolved at any concentration of β -CD,

whereas epinephrine showed $R = 0.3$ at 20 mM. The three analytes were baseline separated into their enantiomers with a polymer containing less β -CD (propranolol, butyl ester of tryptophan and epinephrine at 10, 5 and 50 mg/ml). Only for terbutaline was the resolving power of native CD greater than that of the polymer; at 2.5 mM β -CD R was 1.26 whereas 5 mg/ml EP- β -CD gave $R = 0.85$. The higher resolving power of the chiral polymer can also be supported by previous results obtained for the methyl ester of tryptophan, which was not resolved with β -CD [26].

The high resolution capability of EP- β -CD toward the compounds studied cannot be interpreted by considering only parameters such as the concentration of CD, hydrophobicity and structure of the analytes; the structure of the chiral selector also has to be taken into account. In fact, the polymerization changes the properties of β -CD units, producing a more rigid and different conformation in comparison with the native CD. Further, the aliphatic chain (due to epichlorohydrin) is probably a source of hydrophobic interactions with the analytes and improves the chiral recognition. Finally, we must also consider the cooperation of two CD moieties of the polymer for inclusion complexation with analytes possessing more than one guest part in their structure [27].

3.2. Effect of pH of BGE on resolution

The effect of the BGE pH on the resolution was investigated using for the electrophoretic experiments four different buffers at pH 2.7, 3.5, 4.5 and 6.0 and containing 10 mg/ml of EP- β -CD. Fig. 8 shows the effect of the pH of the BGE on the resolution of the butyl ester of tryptophan, terbutaline and propranolol. An increase in buffer pH led to a decrease in the resolution factor for all the compounds studied, except for terbutaline, which showed a maximum of R at pH 3.5. One explanation for the decrease in resolution with increasing pH of the BGE is probably the increase in the electroosmotic flow causing a higher apparent mobility of the basic analytes and thus a shorter time of interaction with the chiral selector [27]. It is noteworthy that

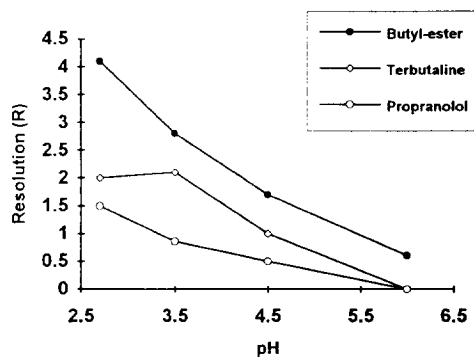


Fig. 8. Influence of the pH of the background electrolyte on the resolution of (●) the butyl ester of tryptophan (◇) terbutaline and (○) propranolol.

at pH 6 the polymer showed interactions with the capillary wall; in some instances the capillary was blocked and the problem could be eliminated by washing with methanol.

3.3. Effect of buffer concentration on resolution of enantiomers

The experiments for the study of the effect of buffer concentration on enantiomeric resolution were carried out on bupivacaine using a thermostated capillary cartridge at 20°C in order to reduce the Joule heat, which can strongly in-

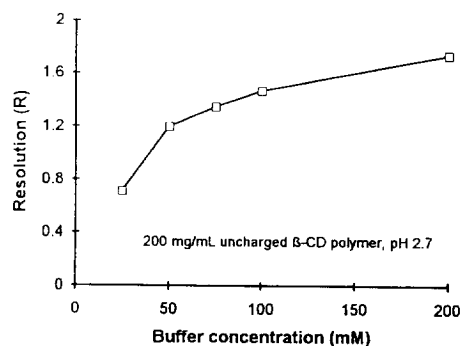


Fig. 9. Dependence of the resolution, R , of bupivacaine on the concentration of background electrolyte. Apparatus, P/ACE 2200; capillary, 370 (300) mm \times 0.05 mm I.D.; applied voltage, 15 kV; temperature of cartridge, 20°C; background electrolyte, phosphate buffer (pH 2.7) containing 200 mg/ml β -cyclodextrin polymer; pressure injection at 0.5 p.s.i. $\times 10^5$ s of 5×10^{-5} M racemic bupivacaine.

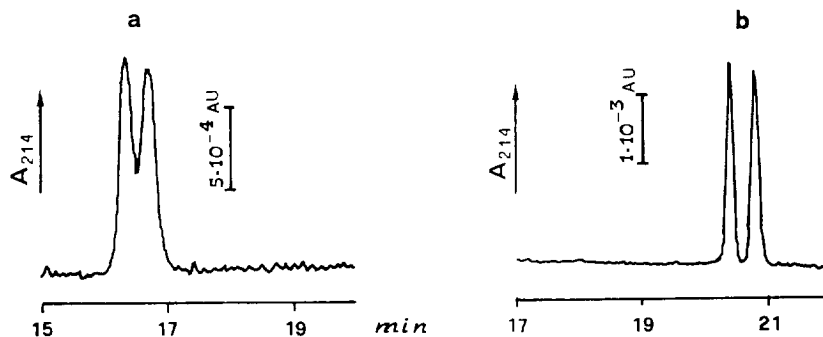


Fig. 10. Electropherograms for racemic bupivacaine at two different concentrations of the background electrolyte. Background electrolyte, phosphate buffer (pH 2.7). (a) 25 and (b) 200 mM, containing 200 mg/ml β -cyclodextrin polymer. Other experimental conditions as in Fig. 9.

fluence the efficiency of the separation. An increase in the buffer concentration caused an increase in the migration time of bupivacaine owing to the influence of the higher ionic strength of the electrophoretic media.

Fig. 9 shows the effect of the buffer concentration, containing 200 mg/ml of β -CD polymer on the resolution of racemic bupivacaine into its enantiomers. Baseline separation of the two antipodes ($R = 1.2$) was obtained at 50 mM phosphate buffer and increased to 1.76 when the buffer concentration was 200 mM. Fig. 10 shows the electropherograms of the enantiomeric separation of bupivacaine at 25 and 200 mM phosphate buffer containing 200 mg/ml β -cyclodextrin polymer.

4. Conclusions

The use of uncharged β -cyclodextrin polymer as a chiral selector in CE allows the enantio-separation of several classes of compounds (β -blockers, α -adrenergic agonists, β -adrenergic agonists, tryptophan esters and anaesthetics).

The complexation, resolution and selectivity are all influenced by the concentration of the β -cyclodextrin polymer added to the BGE. Generally, the higher the concentration of the chiral additive, the greater is the decrease in mobility and the increase in selectivity and resolution, except for propranolol, which shows a maximum of *S* and *R* at 20 mg/ml EP- β -CD.

The solubility of the chiral polymer in water–buffer mixtures is relatively high in comparison with that of the native β -CD. The method is cheap because only a few microlitres of chiral polymer solution are used for the electrophoretic experiments (the chiral additive was present only in the capillary).

Further studies will be carried out in order to verify the usefulness of the uncharged chiral polymer for the resolution of other racemic mixtures into their enantiomers and the influence of organic modifiers on the enantioselectivity.

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