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Comparison of native, alkylated and charged cyclodextrins for the chiral separation of labetalol stereoisomers by capillary electrophoresis

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Abstract

A capillary electrophoresis method for the enantioresolution of labetalol was developed using CDs as chiral selectors and uncoated capillaries. Various native (α-, β- and γ-CD), alkylated (hydroxy propyl-β- and γ-CD, methyl-β-CD) and anionic (sulfated-β-CD and sulfobutylether-γ-CD) cyclodextrins were tested and operational parameters such as buffer pH, concentration of CD were investigated. Propranolol was also studied as a model compound. Uncharged γ-CDs were more effective than β-CDs to separate the enantiomers of labetalol but no complete resolution of the four isomers was obtained. The use of charged cyclodextrins led to a combination of hydrophobic inclusion and ion-pairing interaction in the chiral recognition mechanism. Thus, a complete resolution of the four enantiomers of the labetalol was attained using 7.7 g/l sulfated-β-CD in a 30 m*M* phosphate buffer, pH 6.5.

Keywords: Enantiomer separation; Buffer composition; Labetalol; Cyclodextrins; Propranolol; β-Blockers

1. Introduction

Labetalol is an α- and β-adrenergic blocking agent used for its cardiovascular properties in treatment of angina pectoris, cardiac arrhythmias and hypertension [1,2]. As for all β-blockers, the therapeutic action depends on the stereospecificity of molecules, each isomeric form having its own pharmacological effectiveness. In the case of labetalol, there are two asymmetric carbons leading to four stereoisomers (*R*,*R*), (*S*,*S*), (*R*,*S*) and (*S*,*R*). The (*R*,*S*) isomer is the most potent, slightly more active than the racemic and exhibits mainly a β-adrenergic activity while the (*S*,*S*) isomer is the less potent one and possesses rather an α-adrenergic activity [1]. Thus, it is of particular importance in the pharmaceutical industry to develop quality control methods to determine the optical purity of this drug.

Although chiral separation can generally be achieved by HPLC [3–6], GC [7] or SFC [7], capillary electrophoresis leads to attractive results. Indeed, CE provides high separation efficiency and, due to the small volumes of buffer required, low operating costs. Chiral separations using CE can be achieved using two strategies: the first employs chiral additives in the running buffer while the second uses capillaries which have been modified using a chiral selector [8–10]. Although a wide

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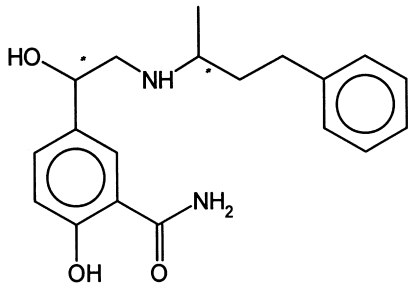
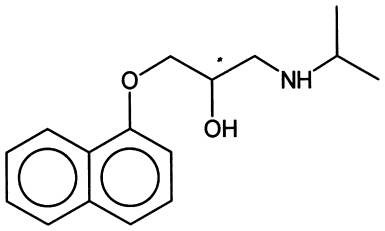
range of chiral selectors have been already reported, e.g. bile acids [11,12], monomeric or polymeric chiral surfactants [13–16], proteins and peptides [17–20], crown ethers [21], polysaccharides [22–27] or alkaloids [28], the most popular among them are CDs. Native α-, β- and γ-cyclodextrins have been used successfully in many applications [29–31]. However, the low solubility of β-CD have limited its usefulness. Attempts to overcome this problem have included the development of derivatized cyclodextrins such as dimethyl- (DM-CD) or trimethyl- (TM-CD) cyclodextrins [33–38] and hydroxypropylcyclodextrins (HP-CD) [9,39–43]. Charged CDs such as carboxymethylated (CM-CD) and carboxyethylated (CE-CD) cyclodextrins [44,45] have offered new possibilities for the separation. Sulfobutylether-β-cyclodextrins (SBE-β-CD) [40,41,43,46–49] and sulfated cyclodextrins [50] have considerably extended the application because of a dramatically improved aqueous solubility. The sulfonic groups provide a derivative which is anionic over the entire pH range accessible to CE experiments and which can thereby lead to ionic interaction in addition to the hydrophobic inclusion.

Although extensive efforts have been directed to the enantiomeric separation of cardiovascular drugs, especially β-blocking agents using CE [38,51,52] little work has been devoted to labetalol whose unique structure is presented in Fig. 1. One of the first enantioseparation of β-blockers was achieved with high concentration of β-CD [36]. Later, alkylated β-cyclodextrins were employed to enhance their inclusion into the hydrophobic cavity larger than for native β-cyclodextrins [39,41,53–55]. Most of the enantiomeric separations were achieved at pH 2.5 and 3 to avoid the detrimental effect of the electroosmotic flow (EOF) which may reduces the enantiomeric resolution. Several authors have reported the use of coated capillaries [44] or the addition of neutral polymers [42] to suppress the EOF and to assist the enantiomeric separation. Quang and Khaledi [32] have proposed the use of charged surfactants to reverse the electroosmotic flow and to improve the enantioseparation of some pharmaceuticals including labetalol. Alternatively, good results have been obtained with anionic β-cyclodextrins. Baseline resolution of the propranolol enantiomers was attained with carboxymethyl-β-cyclodextrins [44,45] and with sulfobutylether-β-cyclodextrin [49]. With some more elaborate systems, enantiomeric separation of racemic propranolol was achieved using either a (*R*)-propranolol-imprinted column or protein-mediated capillary electrochromatography [51].



Labetalol



Propranolol

Fig. 1. Chemical structures of the β-blockers studied.

Aumatell et al. [41] have described a separation of labetalol using 120 m*M* of hydroxypropyl-β-cyclodextrins, in an acidic buffer. Latter Quang and Khaledi [55] have proposed a combination of reversing the EOF and adding hydroxypropyl-β-CD to achieve enantiomeric resolution of labetalol. However, using these conditions, only two peaks were observed pointing out an incomplete separation of the four isomers. Valtcheva et al. [18] have reported a separation of the four isomers of labetalol in 80 min with the addition of cellobiohydrolase to the running buffer at pH 5.1 in a capillary coated with linear polyacrylamide. Recently, Kilar [20] has shown that transferrin was able to interact with labetalol and achieved the separation of three of the four stereo-

isomers of this drug in a coated capillary by applying a transferrin zone to the capillary prior to injecting the dry samples. Very recently, an extensive study performed by Vigh's group [56–58] reported the use of a family of a single isomer heptasulfated CD to achieve enantioseparation of a wide variety of analytes. The enantioseparation of the four diastereoisomers of labetalol was successful using heptakis(2,3-dimethyl-6-sulfato)-β-CD at an acidic pH.

In view of the literature, anionic cyclodextrins are promising chiral selectors in CE to improve enantiomeric resolution of β-blockers, adding ion-pair formation to hydrophobic inclusion. In the present study, we have compared several cyclodextrins: α-, β- and γ-CD, HP-β- and γ-CD, methyl-β-CD, sulfated-β-CD to gain understanding on the nature of the mechanism (hydrophobic inclusion or ion pairing) involved in the enantiomeric separation. For this purpose and in the case of sulfated cyclodextrins, we have investigated the influence of the pH of the running buffer in the neutral and alkaline pH range. Propranolol has been studied simultaneously as a model solute.

2. Materials and methods

2.1. Apparatus

Capillary zone electrophoresis was performed using a Beckman P/ACE 2100 system equipped with a UV detector (Beckman Instruments, Fullerton, CA, USA). A fused-silica capillary (Beckman) of 47 cm (effective length of 40 cm)×50 μm I.D. was used for the separation. Data acquisition and instrument control were carried out using a Beckman Gold system (version 7.11) software. The samples were introduced into the capillary by hydrodynamic injection for 5 s. The analyses were performed at +15 kV and 30°C. The detection was carried out at the cathode with the wavelength set at 200 nm. Before each analysis, the capillary was equilibrated with the running buffer for 5 min. The capillary was rinsed under pressure after each analysis by the following flushing sequence: water for 2 min, followed by 5 min with methanol–sodium hydroxide (50:50, v/v) and finally water for 2 min. The EOF was monitored by the migration time of the first negative peak corresponding to water.

2.2. Reagents

Buffers and standard solutions were prepared with Milli-Q water (Millipore, Bedford, MA, USA) and were filtered through a 0.22-μm pore size membrane filter (Millex, Millipore, France). Sodium monohydrogenphosphate, potassium dihydrogenphosphate, orthophosphoric acid (85%), sodium hydroxide and methanol were purchased from Prolabo (Paris, France). Boric acid was obtained from Sigma (St. Louis, MO, USA).

Hydroxypropyl-β-CD (HP-β-CD), α- and γ-CD, methyl-β-CD (Me-β-CD) were provided by Wacker (Munich, Germany). β-CD, hydroxypropyl-γ-CD (HP-γ-CD) and sulfated-β-CD (S-β-CD) (S.D. ca 7–11) were obtained from Aldrich (Milwaukee, WI, USA). DL-Propranolol hydrochloride was obtained from Sigma. Labetalol hydrochloride racemate was synthesized by Sicor (Limay, France). The SBE-γ-CD has been prepared at the Institute of Organic and Analytical Chemistry (Orleans, France) as previously described [59]. The substitution pattern of this anionic CD was characterized by CE analysis with indirect UV detection employing Tris–benzoic acid buffer [60]. As the different substitution isomers have not been isolated, the average degree of substitution was estimated from the peak area ratios assuming the same response factor for all the components of the mixture. The degree of substitution was estimated at around 6. However, this value may vary slightly from the one obtained when response factors of the isomers are taken into account, as it has been shown recently by Luna et al [61].

2.3. Procedures

For pH 5.8 to 8.2, the phosphate buffers were prepared by mixing in different proportions 0.06 *M* stock solutions of sodium monohydrogenphosphate and potassium dihydrogenphosphate. For acidic pH, potassium dihydrogenphosphate solution was adjusted to the desired value with 1 *M* phosphoric acid. For basic pH values, 0.06 *M* boric acid solution was adjusted to the desired value with 1 *M* phosphoric acid. Fifty or 100 g/l stock solutions of each CD

were prepared in water. The working electrolyte (30 m*M* phosphate buffer, 0–60 g/l CD) was prepared by appropriate dilutions of buffer and CD stock solutions.

Salts of racemic propranolol and labetalol were dissolved at a concentration of 1.4 g/l in water and diluted sample standard solutions at a concentration of 0.07 g/l.

The electrophoretic mobility m_{ep} ($cm^2\ V^{-1}\ s^{-1}$) of the solute was measured from the electropherogram as:

$$m_{app} = m_{ep} + m_{eof} \text{ and } m_{app} = L_d \cdot l_t / V \cdot t_M$$

where m_{app} is the apparent mobility of the solute, L and l the total and the effective length of the capillary, respectively, V the applied voltage (V) and t_M (min) the migration time of the solute.

3. Results and discussion

Native CDs and chemically modified CDs: α-, β- and γ-CD, HP-β- and -γ-CD, methyl-β-CD, and S-β-CD were tested as chiral additives for the enantiomeric separation of labetalol. Propranolol used as a model compound was analysed simultaneously. The separations were performed at low pH to minimize the EOF. Fig. 2 displays the electrophoretic profiles at the optimum conditions found for labetalol and for each of the tested native cyclodextrins. α-CD were inefficient to separate the isomers of the two β-blocking agents due to its small cavity size (data not shown). In our attempts, we found that native β-CD was better suited for the chiral separation of propranolol while a higher enantioselectivity was obtained with native γ-CD for labetalol. Using the optimized concentration (50 g/l) of γ-CD, the two enantiomeric pairs of labetalol were poorly resolved. As ionic interaction between the uncharged γ-CD and the solute is not possible, the partial resolution observed is probably the result of an interaction which involves inclusion of the molecule in the hydrophobic cavity of the CD. However, with this latter CD, no satisfactory separation between the two enantiomers of propranolol or between the two isomer pairs of labetalol was achieved. Under these experimental conditions, chemically modified β-CD and γ-CD were also

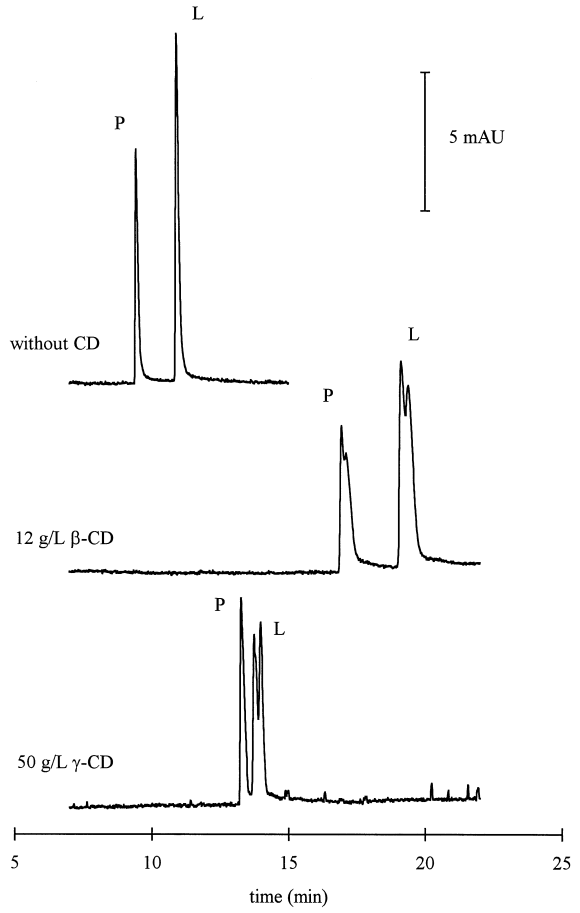


Fig. 2. Electropherograms of racemic labetalol (L) and propranolol (P) using different native CD. Conditions: background electrolyte, 30 m*M* phosphate buffer, pH 3.0, without CD and with either 12 g/l of β-CD or 50 g/l of γ-CD; applied voltage, +15 kV; temperature, 30°C; detection, 200 nm.

tested (Fig. 3). Alkylated β-CD proved to be more powerful chiral selector for the propranolol. This result is in agreement with previous works which reported the use of alkylated CD to achieve its enantioseparation [36,38,39,45,54,55]. But surprisingly the use of alkylated CDs did not improve the enantioseparation of labetalol. As expected from the preliminary results obtained with native CD, the enantiomeric separation of labetalol was slightly better with HP-γ-CD compared to the HP-β-CD. This supports the hypothesis that the cavity size of CD plays a role in the enantioseparation through a

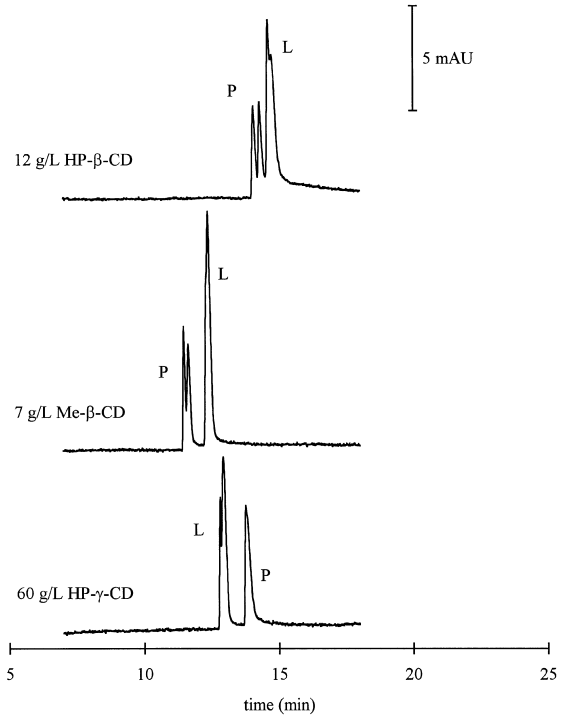


Fig. 3. Electropherograms of racemic labetalol (L) and propranolol (P) using different alkylated CD. Conditions: 30 m*M* phosphate buffer, pH 3.0, containing either 12 g/l of HP-β-CD, 7 g/l of Me-β-CD or 60 g/l of HP-γ-CD. Other conditions as in Fig. 2.

weak inclusion of one portion of the labetalol molecule into the CD cavity.

In a second part of this study, we have tested sulfated β-cyclodextrin to achieve the enantioseparation of labetalol. Indeed S-β-CD is expected to produce also ionic interaction. At acidic pH and in the normal polarity, no peaks were detected: the weak EOF did not compensate for the negative electrophoretic mobility of the charged CD which strongly interacted with the β-blockers. These latter compounds could therefore not reach the cathode, their migration towards the cathode with a normal polarity was possible only for buffer pH above 6. A first separation of the four stereoisomers of the labetalol was obtained at pH 6.5 with 5 g/l S-β-CD, but no separation was observed for the propranolol. These results contrast with the previous observations with the uncharged CD and are probably due to the contribution of ionic interaction between negatively charged sulfated groups and protonated amine group upon labetalol.

We evaluated the effect of the concentration of CD on the resolution of the isomers of the two β-blockers. Fig. 4 shows that the resolution of the four isomers of the labetalol increased as the concentration of S-β-CD raised from 2.6 to 10.3 g/l, a complete resolution of the four peaks being observed at concentrations of 7.7 and 10.3 g/l. However, the shape of the two last peaks was slightly distorted for the highest concentration. A partial separation of the two isomers of the propranolol was observed for

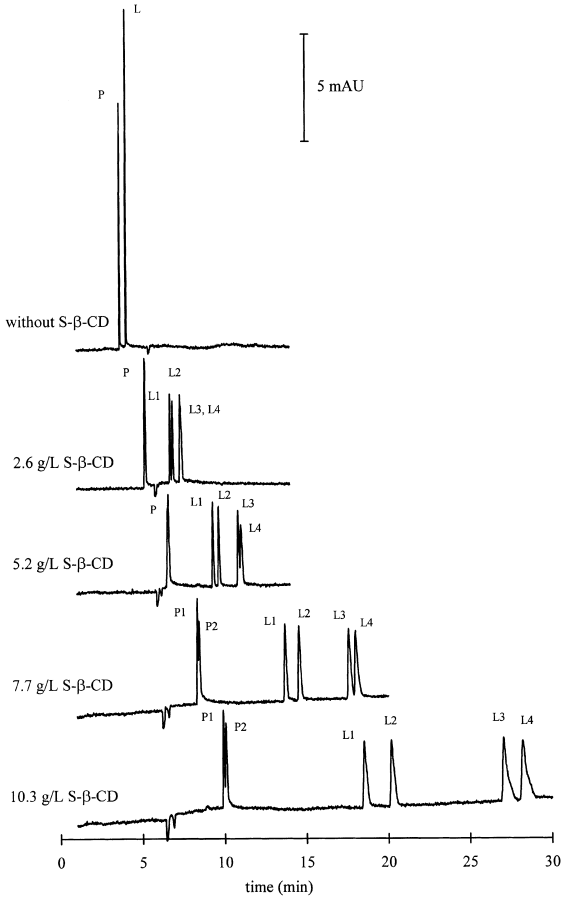


Fig. 4. Effect of S-β-CD concentration on CZE separation of the optical isomers of propranolol (P1, P2) and labetalol (L1, L2, L3, L4). Conditions: background electrolyte, 30 m*M* phosphate buffer, pH 6.5, containing from 0 to 10.3 mg/ml of S-β-CD. Other conditions as in Fig. 2.

concentrations of 7.7 and 10.3 g/l but a complete resolution of the two peaks could not be attained.

The chiral recognition of the β-blockers with the anionic CD can be attributed to two mechanisms: hydrophobic inclusion and ionic interaction. With the aim to evidence these two mechanisms, we have studied the influence of the pH on the separation. The electrophoretic mobilities (m_{ep}) of the two β-blockers, without CD, is expected to vary with the pH. Indeed, labetalol which pK_a value of the secondary amine is 8.7 and which contains also a phenol function with a pK_a of 7.4 is cationic for acidic pH and becomes neutral (zwitterionic) above pH around 8 and then negatively charged for pH above 9. Propranolol, which has a pK_a value of 9.5, is cationic and becomes neutral for pH above 10.5. The influence of the pH (from 5.8 to 8.2) on the migration of the stereoisomers with the addition of 10.3 g/l of S-β-CD was investigated. With the addition of the S-β-CD, the electrophoretic mobilities of each stereoisomer of labetalol and propranolol became negative (Fig. 5). For labetalol isomers, m_{ep} values increase gradually from pH 6 to 8.2, while m_{ep} values of propranolol isomers are poorly affected by the pH of the running buffer.

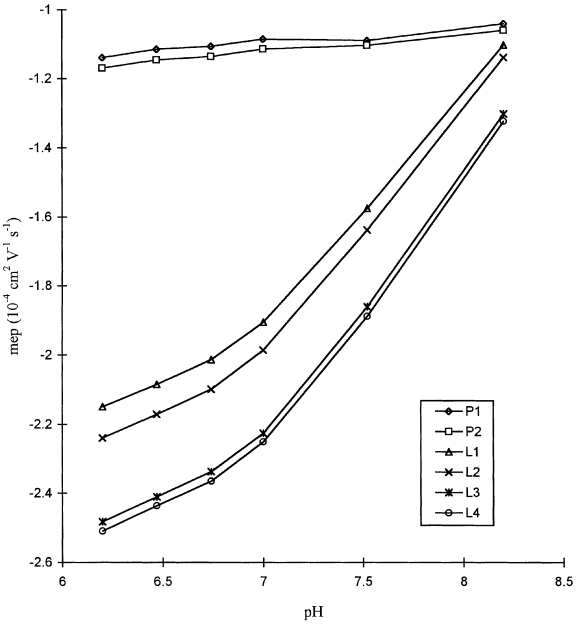


Fig. 5. Plot of the electrophoretic mobilities of the optical isomers of propranolol (P1, P2) and labetalol (L1, L2, L3, L4) against the pH of the running buffer using sulfated CDs. Conditions: running buffer, 30 m*M* phosphate or borate containing 10.3 g/l of S-β-CD. Other conditions as in Fig. 2.

The variation of the ionic strength related to the modification of the pH (from 36 m*M* at pH 5.9 to 88 m*M* at pH 8.2) cannot explain alone the variation of electrophoretic mobility of labetalol as a two-fold increase in the buffer ionic strength is expected to induce a weak increase (decrease of the absolute value) of the electrophoretic mobility (8%) [62]. The marked increase of labetalol m_{ep} value in the 6–8.5 pH range may be interpreted by a probable diminution of the interaction between labetalol molecule and the anionic cyclodextrins and consequently a weaker chiral recognition. This could be attributed to a decrease in a probable ion-pairing recognition between the CD and labetalol resulting from the diminution of the ionization of the secondary amine. In addition, the progressive ionization of the phenol moiety may also be responsible for an ionic repulsion between the negatively charged phenol and the sulfated groups of the S-β-CD. An additional assay has been carried out at pH 9.5: for labetalol two peaks are obtained. Without being as efficient as observed for lower pH, the chiral recognition still exists and, at this alkaline pH, should involve rather hydrophobic inclusion.

For the propranolol, in the range of pH studied, a slight decrease of m_{ep} is observed for pH below 7 and also for pH above 8.2 but these variations are not significant. They may be rather attributed to the decrease in the ionic strength of the buffer at acidic pH. As the apparent charge of the propranolol is not significantly affected in the range of pH studied, it is difficult to speculate on what mechanism (ion-pairing or hydrophobic inclusion) is rather involved in the chiral recognition of this β-blocker with the S-β-CD.

However, the results observed for labetalol indicate that both hydrophobic interaction and ion-pairing may be involved in its stereospecific recognition with S-β-CD. The relative contribution of each mechanism is probably dependent on the pH of the medium and cannot be evaluated very easily. The resolution between each isomer decreases markedly for the two compounds with the rising of the buffer pH (Fig. 6). This can be attributed to the detrimental effect of the EOF which increases significantly in

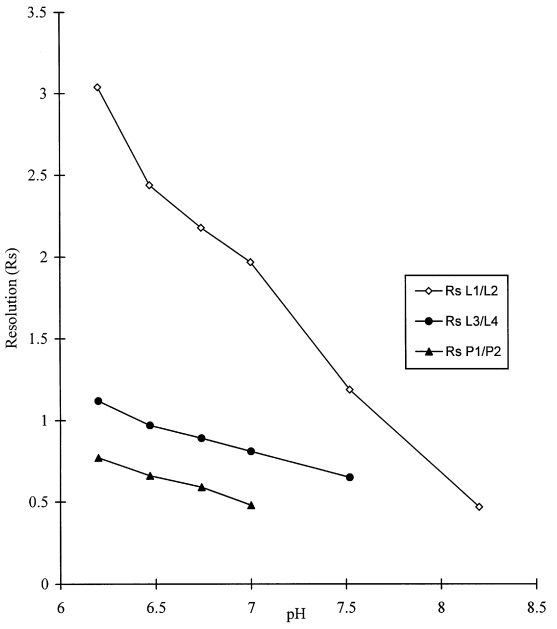


Fig. 6. Effect of pH buffer on stereoisomeric resolutions of racemic propranolol (P1, P2) and labetalol (L1, L2, L3, L4) in presence of 10.3 g/l of S-β-CD in 30 m*M* phosphate or borate buffer. Values less than 0.5 have been neglected. Other conditions as in Fig. 2.

this pH range and leads to a decrease of the analyte/CD interaction time.

As shown in Fig. 4, labetalol showed an optimal enantioseparation at 7.7 g/l of S-β-CD (R_s values are 3.1 and 1.2 for L_1L_2 and L_3L_4, respectively). In these conditions, the efficiency evaluated as the number of theoretical plates ranged from 55 000 to 75 000 for the four labetalol peaks.

The linearity of the method was investigated using a racemic solution of labetalol for concentrations ranging from 0.0142 to 0.142 g/l and using optimum conditions found with the S-β-CD (7.7 g/l). Linear regression analysis, plotting the peak area of the four stereoisomers L1, L2, L3 and L4 of labetalol (y) versus the concentration in g/l (x) gave the following equations: $y=7.2481x+0.0057$ (L1), $y=7.5792x+0.0146$ (L2), $y=8.4415x+0.0234$ (L3) and $y=8.7128x+0.0227$ (L4), respectively. The determination coefficients were satisfactory with values from 0.998 for L3 to 0.999 for L1, L2 and L4. Limits of detection corresponding to a signal-to-noise ratio of 3, estimated by plotting the analyte peak height versus the concentration, were 1 mg/l for the four diastereoisomers. The reproducibility of the analyses was studied through seven injections of the same standard solution. The relative standard deviations (R.S.D.s) of the electrophoretic mobilities and peak areas obtained for the four compounds were less than 1.6 and 1.7%, respectively.

To extend our investigation, we have tested the ability of another anionic cyclodextrin (SBE-γ-CD) to achieve enantioseparation of labetalol. The electrophoretic profiles obtained at the optimum concentrations found for SBE-γ-CD or for S-β-CD are compared in Fig. 7. The differences observed in the two profiles indicate that ionic interaction between the CD and labetalol is probably not the only mechanism responsible for their interaction. Interestingly, the migration order of the two β-blockers

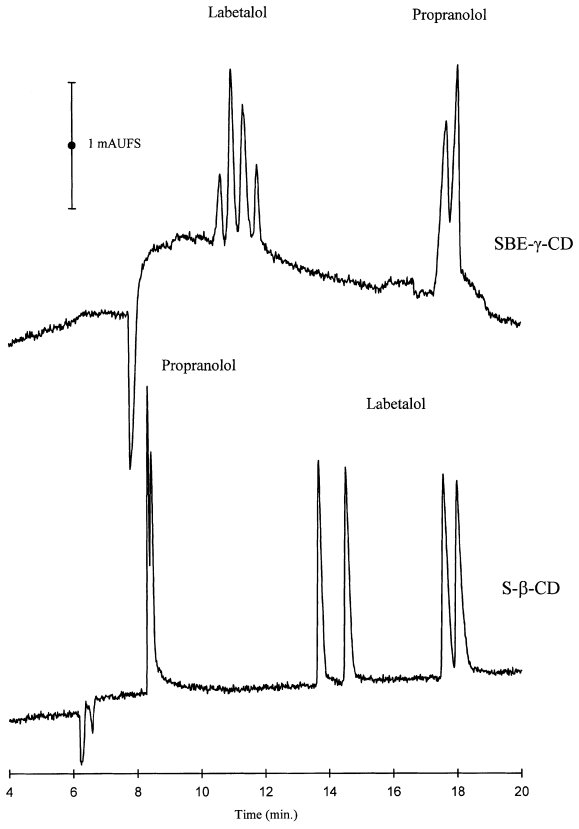


Fig. 7. Electropherogram of racemic labetalol (L) and propranolol (P) using 30 m*M* phosphate buffer containing either 20 g/l of SBE-γ-CD or 7.7 g/l of S-β-CD. Other conditions as in Fig. 6, except pH 5.3 for the phosphate buffer containing the SBE-γ-CD.

(labetalol and propranolol) was reversed when S-β-CD was replaced by SBE-γ-CD. For the SBE-γ-CD, although the separation was carried out at a pH which favours the ion pairing interaction (pH 5.3) between the fully positively charged labetalol and the anionic CD, the selectivity between the labetalol stereoisomers remained lower than the one obtained with S-β-CD due to its lower degree of substitution. Surprisingly, the area ratios of the labetalol peaks are quite inconsistent between the two conditions and further experiments will be needed to explain this phenomenon.

4. Conclusion

CE can be successfully applied to the separation of the four stereoisomers of labetalol. S-β-CD or SBE-γ-CD showed a strong resolving power towards the two cationic β-blockers studied. Different kinds of modified and anionic β- or γ-CD may provide further improvements for chiral resolution. The described method was validated in terms of linearity, precision and limits of detection and it may be used as a routine control method to assess the optical purity of labetalol with a relatively short analysis time.

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