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## Enantiomeric separation of a group of chiral dihydropyridines by electrokinetic chromatography

Electrokinetic chromatography (EKC) was employed to achieve the enantiomeric separation of a group of chiral 1,4-dihydropyridines (DHPs) with pharmacological activity. Micelles of bile salts alone or mixed with neutral cyclodextrins, micelles of sodium dodecyl sulfate (SDS) mixed with neutral cyclodextrins, and anionic cyclodextrin derivatives, *i.e.*, carboxymethyl- $\gamma$ -cyclodextrin (CM- $\gamma$ -CD), carboxymethyl- $\beta$ -cyclodextrin (CM- $\beta$ -CD), and succinylated  $\beta$ -cyclodextrin (Succ- $\beta$ -CD), were employed as pseudo-stationary phases. The enantiomeric separation ability of these chiral selectors with respect to DHPs was studied in different experimental conditions. CM- $\beta$ -CD was shown to be the best chiral selector to perform the enantiomeric separation of DHPs by EKC. Next, the influence of the CM- $\beta$ -CD concentration, the pH and nature of the buffer, the temperature, and the applied voltage on the enantiomeric resolution of DHPs was studied. The use of a 50 mM ammonium acetate buffer, pH 6.7, 25 mM in CM- $\beta$ -CD together with an applied voltage of 15 or 20 kV, and a temperature of 15°C enabled the individual enantiomeric separation of twelve DHPs, each one into its two enantiomers, and their separation in multicomponent mixtures of up to six DHPs into all their enantiomers.

**Keywords:** Electrokinetic chromatography / Enantiomeric separation / 1,4-Dihydropyridines

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### 1 Introduction

Enantioseparation of chiral compounds of pharmaceutical interest is an important task owing to the different pharmacological activity that can be exhibited by each enantiomer. Therefore, excellent chiral resolution techniques are required in order to monitor the chiral compounds or their synthetic processes. Because of its high separation efficiency and flexibility, capillary electrophoresis (CE) has experienced enormous growth in the field of chiral separations [1].

If electrically neutral compounds need to be enantiomerically separated by CE, an electrokinetic chromatography (EKC) technique must be employed. EKC [2] is based on the partitioning of the analytes between two phases of dif-

ferent velocities relative to each other. One of these phases is a buffer solution, which migrates with the velocity of the electroosmotic flow (EOF), and the other is a compound distributed homogeneously in the buffer solution that acts as a pseudostationary phase. In order to obtain chiral separations in CE, this compound should be a chiral selector which can recognize both enantiomers stereoselectively, *i.e.*, with different binding constants [1]. Different pseudostationary phases were employed to perform enantiomeric separations, among them: native cyclodextrins [3–6], neutral and charged cyclodextrin derivatives [3, 5, 7], bile salts [4], monomeric and polymeric chiral surfactants [8], crown ethers [3], calixarenes [6], proteins [9], macrocyclic antibiotics [10], polysaccharides [6] or ergot alkaloids [6].

To understand the mechanisms of chiral separations in CE, it is important to know the forces which favor complex formation and control the stereoselectivity in intermolecular selector-selectand interactions. Usually, several forces and mechanisms act jointly, which makes the identification of the main force difficult. The most popular and best studied in this respect are cyclodextrin-type chiral selectors, which form inclusion complexes with several compounds [1].

The 1,4-dihydropyridines (DHPs) studied in this work are Hantzsch esters, which are of a pharmacological interest owing to their calcium antagonist activity [11]. Chemically, this family of compounds contains the pattern structure of

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**Abbreviations:** CM- $\beta$ -CD, carboxymethyl- $\beta$ -cyclodextrin; CM- $\gamma$ -CD, carboxymethyl- $\gamma$ -cyclodextrin; DHPs, 1,4-dihydropyridines; HP- $\beta$ -CD, (2-hydroxy)propyl- $\beta$ -cyclodextrin; SC, cholic acid sodium salt; SDC, deoxycholic acid sodium salt; STC, taurocholic acid sodium salt; STDC, taurodeoxycholic acid sodium salt; Succ- $\beta$ -CD, succinylated- $\beta$ -cyclodextrin.

a benzene or methyl substitute included in position 4 of the tetrasubstituted DHP ring. These compounds contain a chiral carbon atom at this position and have been synthesized as racemic mixtures of enantiomers. A few reports have appeared in the literature in which CE was used to achieve the enantiomeric separation of some chiral DHPs. Native and substituted cyclodextrins (CDs) were employed as chiral selectors in order to compare the ability of high performance liquid chromatography (HPLC) and CE to perform the enantiomeric separation of five DHPs (amlodipine, nitredipine, nimodipine, isradipine, and nisoldipine) [12]. In this work, the use of carboxymethyl- $\beta$ -CD (CM- $\beta$ -CD) in EKC was found to be the most suitable selector. On the other hand, hydroxypropylated  $\gamma$ -CD appeared to be the best chiral selector to separate, by CE, the two enantiomers of one DHP when compared to other CD derivatives [13].

Since it is not yet possible to predict the success of an enantiomeric separation on the basis of the chemical structure of a solute and a chiral selector, it is of primary interest to compare the ability of different chiral selectors for the separation of a particular chiral compound or to compare the separation of different racemic analytes for a particular chiral selector to understand chiral recognition principles [14]. Furthermore, several experimental parameters, such as chiral selector type and concentration, pH, ionic strength and concentration of the background electrolyte, EOF, organic modifier, *etc.* [15] should be taken into account to obtain a successful enantiomeric separation by CE.

The aim of this study was the selection of the optimal experimental conditions enabling the enantiomeric separation of a group of DHPs of pharmacological interest. Several chiral selectors, including charged CD derivatives, micelles of bile salts alone or mixed with neutral CDs and micelles of sodium dodecyl sulfate (SDS) mixed with neutral CDs, were evaluated systematically. The influence of other experimental conditions (nature and pH of the buffer, concentration of the chiral selector, temperature, and applied voltage) on the enantiomeric separation of DHPs was studied to obtain the best chiral selector.

## 2 Materials and methods

### 2.1 Chemicals and samples

All reagents were of analytical grade. 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES), taurocholic acid sodium salt (STC) and cholic acid sodium salt (SC) were purchased from Sigma (St. Louis, MO, USA); sodium dihydrogen phosphate, SDS, *N,N*-dimethylformamide (DMF),

and sodium hydroxide were supplied from Merck (Darmstadt, Germany); deoxycholic acid sodium salt (SDC), taurodeoxycholic acid sodium salt (STDC), ammonium acetate,  $\beta$ -CD,  $\gamma$ -CD, and urea were from Fluka (Buchs, Switzerland); (2-hydroxy)propyl- $\beta$ -cyclodextrin (HP- $\beta$ -CD), CM- $\gamma$ -CD, CM- $\beta$ -CD, and succinylated  $\beta$ -cyclodextrin (Succ- $\beta$ -CD) were obtained from Cyclolab (Budapest, Hungary). Water used to prepare solutions was purified through a Milli-Q system from Millipore (Bedford, MA, USA). All solutions were filtered through 45  $\mu$ m pore size disposable nylon filters from Scientific Resources (Eatontown, NJ, USA). The 18 DHPs studied were synthesized at the Department of Organic Chemistry of the University of Alcalá, Spain. Table 1 shows identification numbers (taken from [11]) and structures of these compounds as used throughout this study.

### 2.2 Apparatus

Two CE instruments were used: (i) a Prince model from LauerLabs (Emmen, The Netherlands), equipped with a Lambda 1000 UV detector and acquisition data system Model Star 4.5 from Varian Associates (Sugar Land, TX, USA) when bile salts with or without neutral CDs and SDS with neutral CDs were used; and (ii) an HP<sup>3D</sup> CE system (Hewlett-Packard, Waldbronn, Germany) equipped with an on-column diode array detector (DAD) and HP 3D-CE Chemstation software when charged CD derivatives were used. A 50  $\mu$ m inner diameter (ID) and 375  $\mu$ m outer diameter (OD) fused-silica capillary with an effective length of 50 cm (65 cm total length for the LauerLabs instrument and 58.5 cm for the HP system) was employed (Polymicro Technologies, Phoenix, AZ, USA). The capillary temperature was varied from 15 to 45°C and UV detection was performed at 238 nm [12]. Electrolytic solutions were degassed in an ultrasonic cleaner KM from Raypa (Barcelona, Spain). The pH of the separation buffers was adjusted with a 654 pH meter from Metrohm (Herisau, Switzerland).

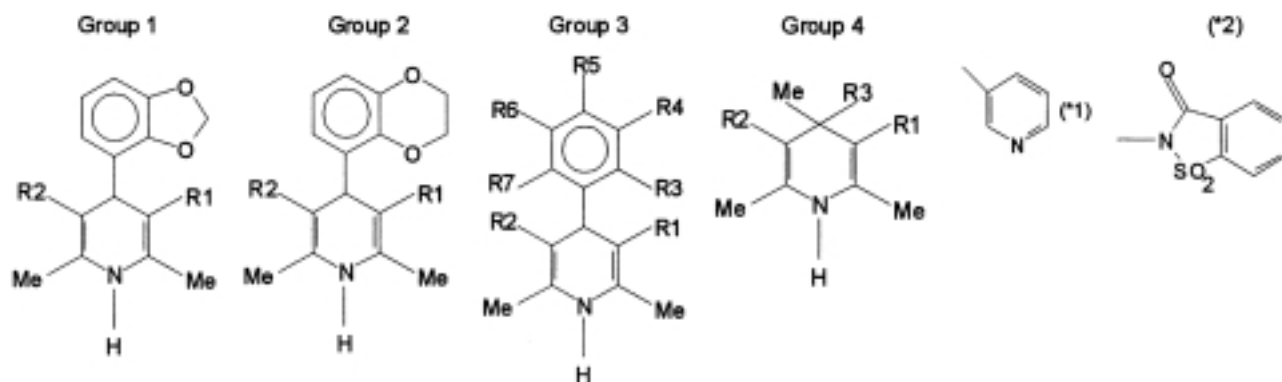
### 2.3 Procedure

When micelles were used in the separation buffer, electrolytic solutions were prepared in two steps: (i) weighing and dissolving the appropriate amount of buffer (CHES) in Milli-Q water to obtain the required concentration and adjusting the pH to the desired value with a concentrated solution of sodium hydroxide, and (ii) adding the adequate volume of this buffer solution (to obtain the desired concentration of the buffer in the final solution) to the appropriate amount of a bile salt, or a bile salt and a neutral CD, or SDS and a neutral CD, plus urea and adding Milli-Q water to 10 mL. When charged CD derivatives were

**Table 1.** Identification numbers and structures of DHPs studied

Group	-R <sub>1</sub>	-R <sub>2</sub>	-R <sub>3</sub>	-R <sub>4</sub>	-R <sub>5</sub>	-R <sub>6</sub>	-R <sub>7</sub>
Group 1							
1	-COOCH <sub>2</sub> -(*1)	-COOCH <sub>3</sub>					
6	-COOCH <sub>2</sub> CH <sub>2</sub> OCH <sub>3</sub>	-COOlsp					
7	-COOlsp	-COOCH <sub>2</sub> CH <sub>3</sub>					
12	-COOCH <sub>2</sub> CH <sub>3</sub>	-COOCH <sub>3</sub>					
17	-COOlsp	-COOCH <sub>3</sub>					
Group 2							
4	-COOCH <sub>2</sub> CH <sub>3</sub>	-COOCH <sub>3</sub>					
Group 3							
8	-COOCH <sub>2</sub> CH <sub>3</sub>	-COOCH <sub>3</sub>	-H	-NO <sub>2</sub>	-H	-H	-H
9	-COOCH <sub>2</sub> CH <sub>3</sub>	-COOCH <sub>3</sub>	-Cl	-Cl	-H	-H	-H
10	-COOCH <sub>2</sub> CH <sub>3</sub>	-COOCH <sub>3</sub>	-OCH <sub>2</sub> OCH <sub>3</sub>	-H	-H	-H	-H
11	-COOCH <sub>2</sub> CH <sub>3</sub>	-COOCH <sub>3</sub>	-H	-OCH <sub>3</sub>	-H	-OCH <sub>3</sub>	-H
13	-COOCH <sub>2</sub> CH <sub>3</sub>	-COOCH <sub>3</sub>	-H	-H	-H	-H	-H
15	-COOCH <sub>2</sub> CH <sub>3</sub>	-COOCH <sub>3</sub>	-H	-OCH <sub>3</sub>	-OCH <sub>3</sub>	-H	-H
16	-COOCH <sub>2</sub> CH <sub>3</sub>	-COOCH <sub>3</sub>	-OCH <sub>3</sub>	-OCH <sub>3</sub>	-H	-H	-H
18	-COOCH <sub>2</sub> CH <sub>3</sub>	-COOCH <sub>3</sub>	-H	-OCH <sub>3</sub>	-OH	-OCH <sub>3</sub>	-H
19	-COOCH <sub>2</sub> CH <sub>3</sub>	-COOCH <sub>3</sub>	-H	-OCH <sub>3</sub>	-OCH <sub>3</sub>	-OCH <sub>3</sub>	-H
25	-COOlsp	-COOCH <sub>2</sub> CH <sub>2</sub> OCH <sub>3</sub>	-H	-NO <sub>2</sub>	-H	-H	-H
Group 4							
22	-COOCH <sub>3</sub>	-COOCH <sub>2</sub> CH <sub>3</sub>	-H				
23	-COOCH <sub>2</sub> CH <sub>2</sub> -(*2)	-COOCH <sub>2</sub> CH <sub>3</sub>	-H				

lsp, isopropyl



used in the separation buffer, solutions were prepared by a different procedure because the buffer pH was modified by adding these CDs with acid properties. The appropriate amount of the buffer and the charged CD was weighed and dissolved in Milli-Q water. Then, the pH was adjusted to the desired value with a concentrated solution of sodium hydroxide for CHES and phosphate buffers and with 1 M or 0.1 M NH<sub>3</sub> solution for the ammonium acetate buffer. Sample solutions were prepared by dissolving each DHP in DMF. In order to obtain good peak shapes and reproducible retention data, the capillary was conditioned at the beginning of the day prior to each analysis, and at the end of the day. The capillary was rinsed at the beginning of the day with Milli-Q water for 2 min, 0.1 M sodium hydroxide for 2 min, and Milli-Q water for 2 min. Between injections, the washing routine consisted of Milli-

Q water for 2 min, 0.1 M sodium hydroxide for 2 min, Milli-Q water for 2 min, and electrolytic solution for 2 min prior to injection. At the end of the day, the capillary was rinsed with Milli-Q water for 2 min, sodium hydroxide for 2 min, Milli-Q water for 2 min, and then stored in water. The injection was made by pressure: 10 or 5 mbar for 0.02 min in the Lauerlabs instrument and 30 mbar for 2 s in the HP CE system. The applied voltage ranged from 15 to 25 kV. Enantiomeric resolution was calculated by the following equation:

$$R_s = 1.18 (t_2 - t_1) / (w_1 + w_2)$$

where  $t_1$  and  $t_2$  are the migration times of the two enantiomers and  $w_1$ ,  $w_2$  correspond to the middle high widths of the corresponding peaks.

### 3 Results and discussion

Eighteen chiral DHPs were injected in a CE system using different chiral selectors in the separation buffer. Bile salts alone or mixed with neutral CDs or mixtures of SDS and neutral CDs were used in micellar EKC (MEKC) and charged CD derivatives were employed in EKC. The nature, concentration, and pH of the buffer, as well as the temperature, applied voltage, and concentration of the chiral selector were varied. All conditions used in this study for the enantiomeric separation of the compounds studied are summarized in Table 2.

#### 3.1 MEKC

The eighteen DHPs were injected in an MEKC system in which a 100 mM CHES buffer (pH 9), 100 mM in SDS, 2 M urea and 50 mM in  $\beta$ -CD or  $\gamma$ -CD was used. Under these conditions, no chiral recognition was observed for DHPs.

Since mixtures of CDs may originate changes in selectivity, a mixture of 50 mM in  $\beta$ -CD and 50 mM in  $\gamma$ -CD was also used under the same experimental conditions described before but no enantiomeric resolution (of any of the compounds studied) was observed. These results could be explained according to the hypothesis of the competitive interaction of DHPs and surfactant molecules with the CD cavity proposed by Gilar *et al.* [12].

Four bile salts, SC, STC, SDC, and STDC were employed next. Since these micellar systems enable chiral recognition, the addition of a chiral selector to the separation buffer is not needed although it can be used in order to enhance enantioselectivity. First, bile salts were used alone in a 100 mM CHES buffer (pH 9) modified with 2 M urea. A 50 mM concentration of bile salt enabled the partial enantiomeric resolution of DHPs 17 and 25 in the case of SC, and DHP 17 in the case of STC (see Table 2). However, at this concentration, no chiral recognition was

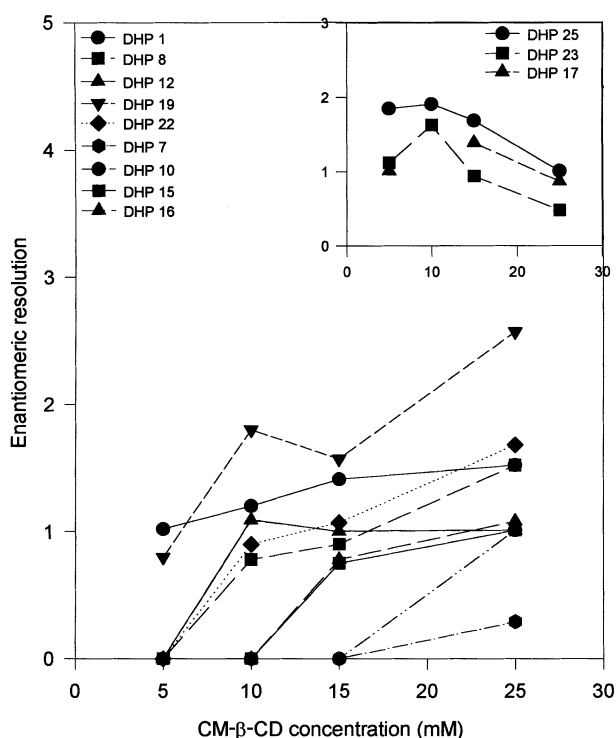
**Table 2.** Experimental conditions for the enantiomeric separation of DHPs studied

Buffer	Additives	Chiral selector	Current intensity ( $\mu$ A)	DHPs enantiomerically separated
100 mM CHES (pH 9)	100 mM SDS + 2 M urea	50 mM $\beta$ -CD	$\approx 18^a$	None
		50 mM $\gamma$ -CD	$\approx 19^a$	None
		50 mM $\beta$ -CD + 50 mM $\gamma$ -CD	$\approx 21^a$	None
100 mM CHES (pH 9)	2 M urea	50 mM SC	$\approx 22^b$	17, 25
		100 mM SC	$\approx 36^b$	17
		150 mM SC	$\approx 54^b$	17
		50 mM STC	$\approx 20^b$	17
		50 mM SDC	$\approx 28^b$	None
		50 mM STDC	$\approx 23^b$	None
		100 mM SC + 25 mM $\beta$ -CD	$\approx 32^b$	17
		100 mM SC + 50 mM $\beta$ -CD	$\approx 31^b$	None
		20 mM CM- $\gamma$ -CD (s.d. <sup>c</sup> $\sim 3.2$ )	$\approx 52^b$	9, 22
100 mM phosphate (pH 6)	–	20 mM CM- $\gamma$ -CD (s.d. $\sim 3.2$ )	$\approx 140^b$	8
50 mM phosphate (pH 6)	–	20 mM CM- $\beta$ -CD (s.d. $\sim 3$ )	$\approx 64^b$	8, 12, 16, 22
50 mM phosphate (pH 6)	–	10 mM CM- $\beta$ -CD (s.d. $\sim 3$ ) + 10 mM CM- $\gamma$ -CD (s.d. $\sim 3.2$ )	$\approx 80^b$	8
50 mM phosphate (pH 6)	–	20 mM CM- $\beta$ -CD (s.d. $\sim 3$ ) + 20 mM HP- $\beta$ -CD (s.d. $\sim 3$ )	$\approx 64^b$	9
50 mM CHES (pH 9)	–	10 mM CM- $\beta$ -CD (s.d. $\sim 3$ ) + 10 mM CM- $\gamma$ -CD (s.d. $\sim 3.2$ )	$\approx 48^b$	25
50 mM CHES (pH 9)	–	18 mM CM- $\beta$ -CD (s.d. $\sim 3$ ) + 20 mM CM- $\gamma$ -CD (s.d. $\sim 3.2$ )	$\approx 130^b$	8, 16, 22, 25
50 mM CHES (pH 9)	–	10 mM CM- $\beta$ -CD (s.d. $\sim 3$ )	$\approx 30^b$	1, 12, 17, 19, 23, 25
50 mM CHES (pH 7.9)	–	10 mM CM- $\beta$ -CD (s.d. $\sim 3$ )	$\approx 30^b$	1, 12, 17, 19, 23, 25
50 mM CHES (pH 6.7)	–	10 mM CM- $\beta$ -CD (s.d. $\sim 3$ )	$\approx 30^b$	12, 17, 19, 23
50 mM ammonium acetate (pH 6.7)	–	10 mM CM- $\beta$ -CD (s.d. $\sim 3$ )	$\approx 60^b$	1, 8, 12, 17, 19, 22, 23, 25
		10 mM CM- $\gamma$ -CD (s.d. $\sim 3.2$ )	$\approx 80^b$	10, 22
		10 mM CM- $\beta$ -CD (s.d. $\sim 3$ ) + 10 mM CM- $\gamma$ -CD (s.d. $\sim 3.2$ )	$\approx 90^b$	17, 19, 22, 25
		10 mM Succ- $\beta$ -CD (s.d. $\sim 3.5$ )	$\approx 55^b$	19

a) 15 kV

b) 20 kV

c) s.d., substitution degree

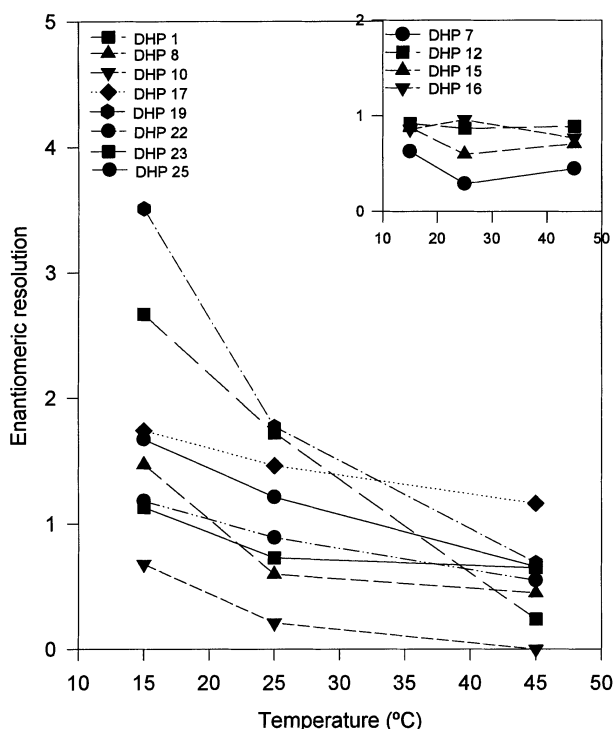


**Figure 1.** Variation of the enantiomeric resolution for twelve DHPs as a function of the CM- $\beta$ -CD concentration. Experimental conditions: 50 mM ammonium acetate (pH 6.7); applied voltage, 20 kV; UV detection, 238 nm; temperature, 25°C; injection by pressure, 30 mbar for 2 s; capillary, 50  $\mu$ m ID, 375  $\mu$ m OD, 58.5 cm length (50 cm to the detector).

obtained when SDC and STDC were used in the separation buffer. Additionally, these two bile salts showed worse peak efficiencies and longer analysis times than SC and STC. Since SC seemed the best chiral micelle to achieve the enantiomeric resolution of DHPs, other concentrations of this bile salt in the separation buffer were tried. However, concentrations of 100 mM and 150 mM enabled only the partial resolution of DHP 17. In order to check if the addition of native  $\beta$ -CD to SC could improve the enantiomeric separations obtained with SC, mixtures of 100 mM SC and 25 or 50 mM  $\beta$ -CD were employed for injecting all the DHPs studied in the MEKC system. Under these conditions, only DHP 17 was partially separated into its two enantiomers, decreasing the resolution with the addition of increasing amounts of  $\beta$ -CD.

### 3.2 EKC with charged CDs

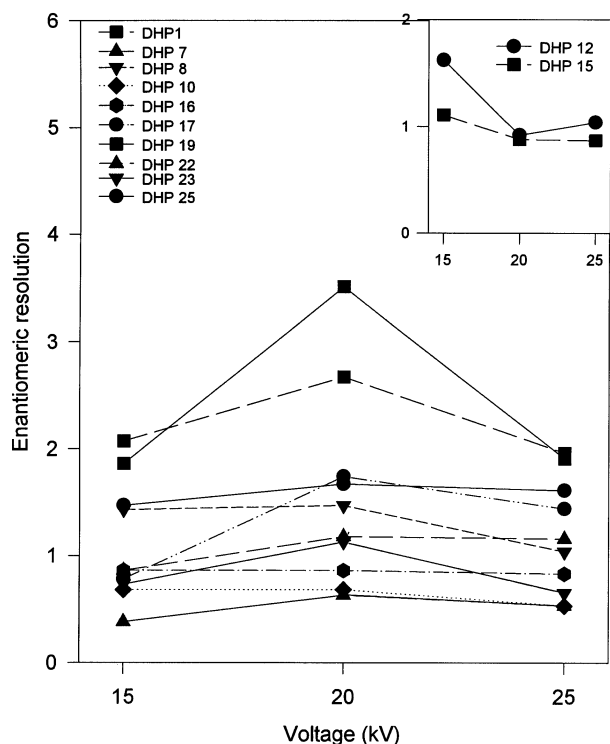
Three anionic CD derivatives (CM- $\beta$ -CD, CM- $\gamma$ -CD and Succ- $\beta$ -CD) were used under different experimental conditions in order to choose the best chiral selector to achieve the enantiomeric separation of DHPs. The follow-



**Figure 2.** Variation of the enantiomeric resolution for twelve DHPs as a function of the temperature. Experimental conditions as in Fig. 1 but with varying temperature; 25 mM CM- $\beta$ -CD.

ing experimental conditions were varied: the nature and concentration of the buffer and its pH, and the concentration of the chiral selector; for results, see Table 2. When 50 or 100 mM phosphate buffers at pH 6 were used, a concentration of 20 mM CM- $\beta$ -CD enabled the separation of a higher number of DHPs than CM- $\gamma$ -CD alone at the same concentration, or CM- $\beta$ -CD mixed with CM- $\gamma$ -CD or with a neutral CD derivative (HP- $\beta$ -CD). In fact, in the latter three cases only one DHP was enantiomerically separated (DHP 8 or 9) while in the former (20 mM CM- $\beta$ -CD alone) four DHPs (numbers 8, 12, 16, and 22) were enantiomerically separated, each one in its two enantiomers. Then, the use of a mixture of CM- $\beta$ -CD and CM- $\gamma$ -CD decreased the number of DHPs enantiomerically separated (only DHP 8) with respect to the use of CM- $\beta$ -CD alone, whereas the mixture of CM- $\beta$ -CD and HP- $\beta$ -CD, although also decreasing the number of DHPs separated, enabled the enantiomeric separation of a new DHP (number 9) which could not be separated under any of the other conditions tried with phosphate buffer.

When 100 mM or 50 mM CHES buffers were used at pH 9, CM- $\gamma$ -CD at a concentration of 20 mM and modified with 2 M urea enabled the enantiomeric separation of only two DHPs (numbers 9 and 22) while a concentration of 10 mM



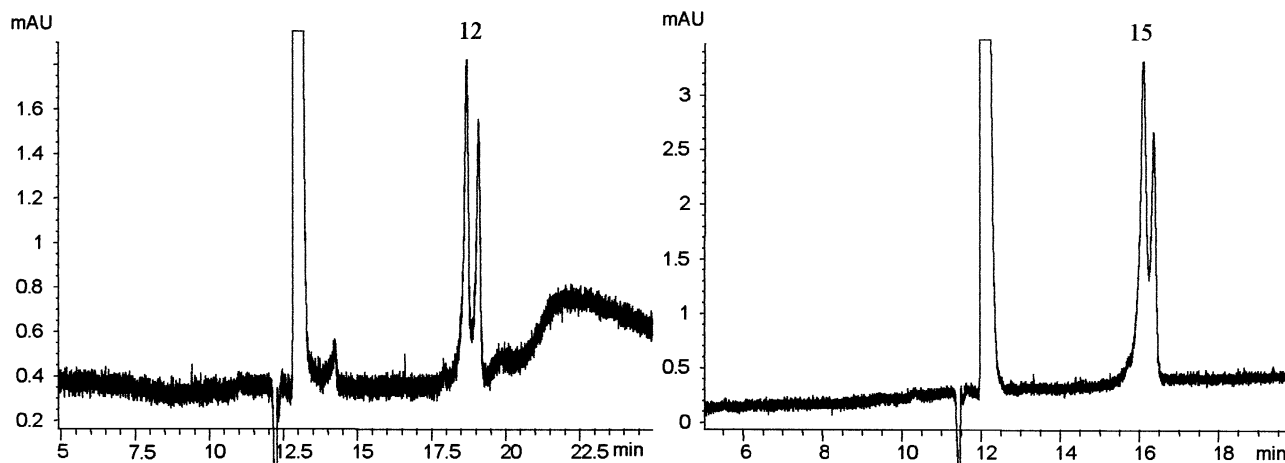
**Figure 3.** Variation of the enantiomeric resolution for twelve DHPs as a function of the applied voltage. Experimental conditions as in Fig. 2; temperature, 15°C; varying voltages.

CM-β-CD enabled the separation of six DHPs (numbers 1, 12, 17, 19, 23, and 25). However, the number of DHPs enantiomerically separated with CM-β-CD decreased when 10 mM CM-γ-CD was added to 10 mM CM-β-CD (one DHP separated) or when 20 mM CM-γ-CD was added to 18 mM CM-β-CD (four DHP enantiomerically separated). In addition, an interesting change in selectiv-

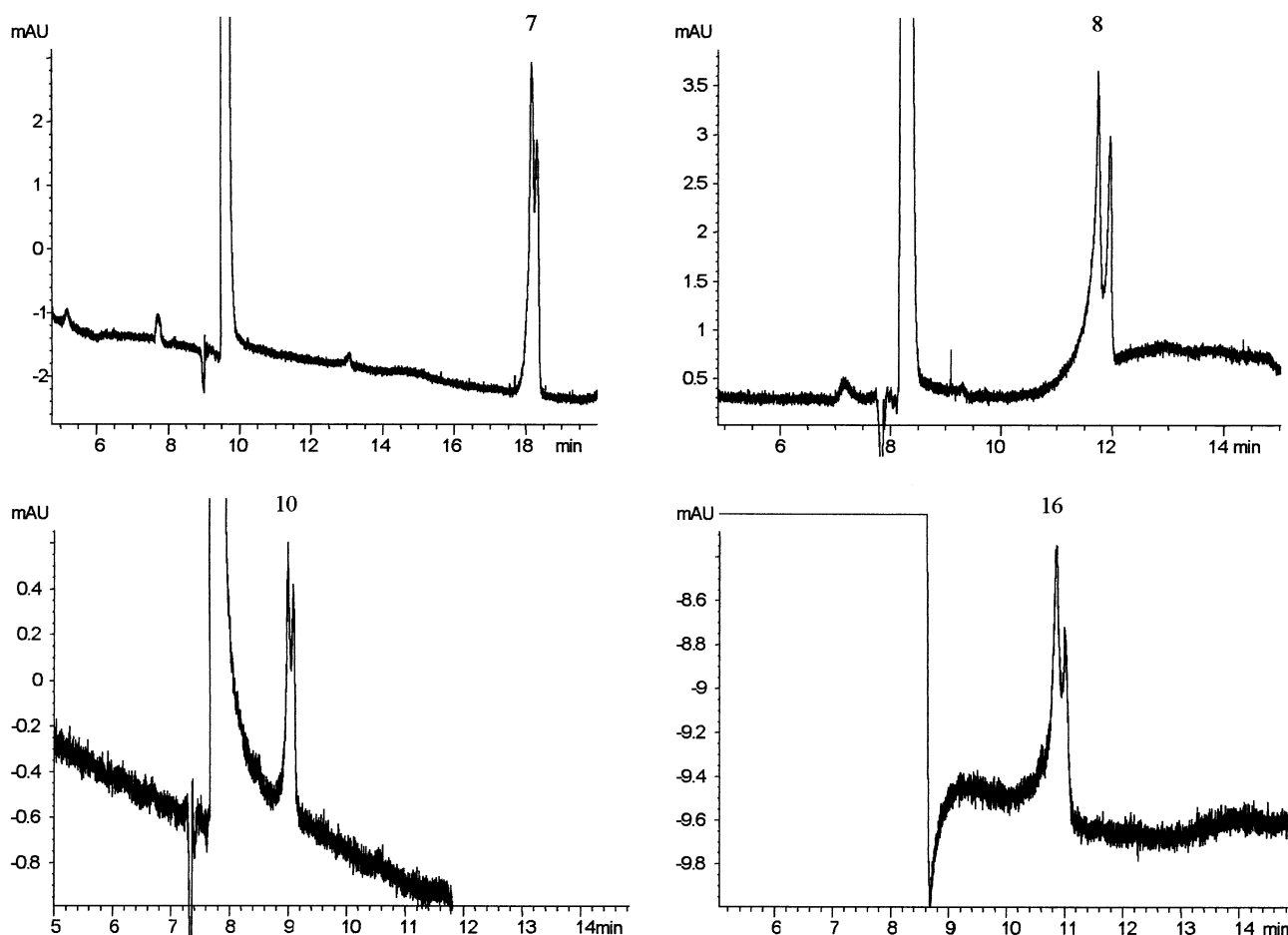
ity occurs when using mixtures of the anionic CD derivatives. In fact, DHPs 8 and 16 were enantiomerically separated with a mixture of 18 mM CM-β-CD and 20 mM CM-γ-CD and they could not be separated with any of the chiral selectors alone or with the other mixture investigated (10 mM CM-β-CD and 10 mM CM-γ-CD). On the other hand, when 10 mM CM-β-CD was used as chiral selector with 50 mM CHES buffers at pH 7.9 and 6.7, no changes in the results were observed at pH 7.9 with respect to pH 9 and a decrease in the number of DHPs enantiomerically separated was obtained at pH 6.7 (DHPs 12, 17, 19, and 23).

Although in the case of CHES buffer, a pH value of 6.7 gave poorer results than a higher pH, Table 2 shows that when 50 mM ammonium acetate (pH 6.7) was used, a concentration of 10 mM CM-β-CD enabled the enantiomeric separation of a higher number of DHPs (eight DHPs numbers: 1, 8, 12, 17, 19, 22, 23, and 25). However, the use of the other two anionic CD derivatives, CM-γ-CD and Succ-β-CD at the same concentration (10 mM) only allowed the chiral separation of two or one DHPs, respectively. While the DHP separated with Succ-β-CD (number 19) was also separated with CM-β-CD, only one of the two DHPs separated with CM-γ-CD (DHP 22) was also separated with CM-β-CD, enabling the use of CM-γ-CD for the chiral separation of DHP 10, which could not be separated when 10 mM CM-β-CD was used. Finally, the mixture of 10 mM CM-β-CD and 10 mM CM-γ-CD enabled the enantiomeric separation of four DHPs that had also been separated when 10 mM CM-β-CD alone was employed in the separation buffer.

The results presented in Table 2 show that the use of a 50 mM ammonium acetate buffer (pH 6.7) with CM-β-CD alone as chiral selector seemed to be the best condition



**Figure 4.** Enantiomeric separation of DHPs 12 and 15 by EKC. Experimental conditions as in Fig. 3; applied voltage, 15 kV.



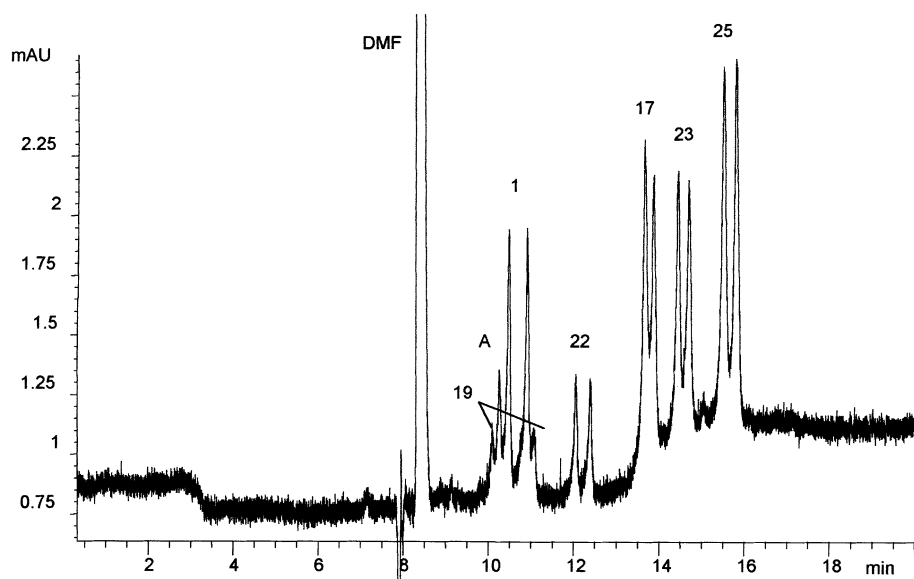
**Figure 5.** Enantiomeric separation of DHPs 7, 8, 10, and 16 by EKC. Experimental conditions as in Fig. 4; applied voltage, 20 kV.

to perform the enantiomeric separation of the group of DHPs studied. Next, the influence of several experimental parameters such as the concentration of the chiral selector, the temperature, and the applied voltage on the enantiomeric separation of the DHPs was investigated under the above-mentioned conditions.

Figure 1 shows the variation of the enantiomeric resolution obtained for a group of DHPs as a function of the CM- $\beta$ -CD concentration in a 50 mM ammonium acetate buffer (pH 6.7). DHPs that were not enantiomerically separated at any of the concentrations of CM- $\beta$ -CD employed have not been included in this figure. When the concentration of chiral selector was increased from 5 to 25 mM, an increase in the number of DHPs enantiomerically separated, as well as in the enantiomeric resolution, was generally obtained. However, there are some DHPs for which a maximum in resolution was observed at an intermediate concentration of CM- $\beta$ -CD. For example, the enantiomeric resolution for DHPs 23 and 25 was greater at 10 mM CM- $\beta$ -CD than at a higher concentration.

Although the increase in the CM- $\beta$ -CD concentration in the separation buffer up to 25 mM enabled the chiral recognition of twelve DHPs, for some of them only a partial resolution could be obtained (see Fig. 1). For this reason, and with the aim of increasing the enantiomeric resolution of the compounds studied, the effect of the temperature was investigated. The variation of the enantiomeric resolution of the twelve DHPs chirally separated at 25 mM CM- $\beta$ -CD as a function of the temperature is shown in Fig. 2. An increase in the enantiomeric resolution was obtained for most of the DHPs studied when the temperature was decreased. This fact could be due to the increase in the stability of selector-selectand complexes that takes place when the temperature is decreased [1]. From these results, it can be concluded that the use of a 50 mM ammonium acetate buffer (pH 6.7), 25 mM in CM- $\beta$ -CD at a temperature of 15°C, enabled the enantiomeric resolution of twelve DHPs although some of them were only partially resolved.

Since all the experiments described above were carried out at a constant applied voltage of 20 kV, the influence of this variable on the enantiomeric resolution of the twelve



**Figure 6.** Separation of a mixture of six chiral DHPs into their twelve enantiomers by EKC. Experimental conditions as in Fig. 5. A, unknown peak.

DHPs separated was also investigated in order to see if a variation of the applied voltage could enhance the chiral separation of those DHPs that had been only partially resolved up to that moment. Figure 3 shows the variation of the enantiomeric resolution of twelve DHPs as a function of the applied voltage, which was varied from 15 to 25 kV. In these experiments, a 50 mM ammonium acetate buffer, pH 6.7, 25 mM CM- $\beta$ -CD at 15°C, was used. For most DHPs a maximum in the enantiomeric resolution was obtained at an applied voltage of 20 kV. For DHPs 12 and 15, however, the contrary was observed, that is, a minimum in the enantiomeric resolution was obtained at 20 kV, 15 kV being the voltage that enabled the best chiral separation for these compounds. Figure 4 shows the enantiomeric separation of DHPs 12 and 15 using a 50 mM ammonium acetate buffer, pH 6.7, 25 mM CM- $\beta$ -CD at 15°C and an applied voltage of 15 kV. For the remaining DHPs, the enantiomeric separation was performed under the same conditions but at an applied voltage of 20 kV. Although under these conditions some DHPs were not baseline resolved, as shown in Fig. 5, other DHPs could be enantiomerically separated in multicomponent mixtures as shown in Fig. 6.

#### 4 Concluding remarks

An adequate selection of chiral additive to the separation buffer was a crucial factor to achieve the enantiomeric separation of DHP derivatives. The use of charged (anionic) CD derivatives presented the best enantioselectivity with respect to the compounds studied. The results show that DHPs can be enantiomerically better separated with CM- $\beta$ -CD than with CM- $\gamma$ -CD or Succ- $\beta$ -CD, perhaps due to the more stable inclusion complexes formed be-

tween DHPs and this CD. The use of mixtures of CD derivatives did not enable the enhancement of the enantiomeric resolution although interesting changes in selectivity were observed with respect to the use of each CD derivative alone in the separation buffer. In addition, the enantiomeric resolution obtained with the best chiral selector for DHPs (CM- $\beta$ -CD) was highly influenced by its concentration in the separation buffer, the buffer nature and pH, the temperature, and the applied voltage. For most DHPs, an increase in the enantiomeric resolution was obtained upon increasing the CM- $\beta$ -CD concentration in the separation buffer and decreasing the temperature, according to the decrease in the stability of selector-selectand complexes when increasing the temperature. The use of a 50 mM ammonium acetate buffer, pH 6.7, 25 mM CM- $\beta$ -CD at a temperature of 15°C and an applied voltage of 15 or 20 kV enabled the enantiomeric resolution of twelve of the DHPs studied as well as their enantiomeric separation in multicomponent mixtures of up to six DHPs into all their enantiomers.

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