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Systematic approach to cost- and time-effective method development with a starter kit for chiral separations by capillary electrophoresis[1]

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Abstract

In capillary electrophoresis, the use of cyclodextrins (CDs) as additive in the background electrolyte plays a very important role in achieving enantiomeric resolution. The wide range of available CD derivatives contained in commercial kits provides several possibilities for method development (LC·GC Int., 9 (1996) 88. [1]). The advantage of the large selection of CD derivatives and the application guidelines provided by the kits make method development versatile. A disadvantage, however, is often the difficulty at the starting point to choose which CD or modified CD to use. Most of the guidelines suggest complicated multistep selection methods assuming that all information about the analyte is available. In this paper the Elphodextrin starter kit from Cyclolab, Hungary has been evaluated. An easy, step-by-step protocol is developed to speed up the CD selection procedure and the analytical method development, even if the character of the analyte is unknown. The applicability of the Elphodextrin starter kit for the separation of neutral, acidic and basic enantiomers is demonstrated. © 1997 Elsevier Science B.V.

Keywords: Method development; Starter kits; Enantiomer separations; Buffer composition; Chiral selectors; Cyclodextrins

1. Introduction

In capillary electrophoresis (CE) direct separation of enantiomers can be performed using a chiral selector in the background electrolyte. One of the most widely used chiral selector groups is that of cyclodextrins (CDs). α-, β- and γ-CDs are cyclic oligosaccharides which consist of six, seven, or eight α-1,4-linked D-(+)-glucopyranose subunits, respectively (see Fig. 1). The linkage at C1 atom results in a specific arrangement of the secondary hydroxyl-groups (C2 and C3) on the wider edge of the ring and of the primary hydroxyl-groups (C6) on the other edge (for dimensions and other important properties see Table 1). The torus-shaped CDs have a polar outside and a hydrophobic cavity. These properties and the chiral environment of the D-(+)-glucose enable the CDs to form relatively stable stereoselective complexes in aqueous solutions with a variety of guest molecules [2–4].

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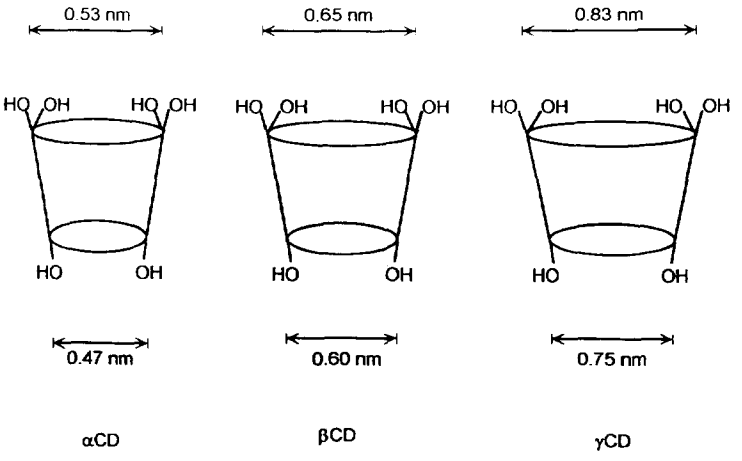

Fig. 1. Molecular structures of α-CD, β-CD and γ-CD.

Besides the three natural CDs, α-, β- and γ-CDs, a variety of CD derivatives have been recently discovered for chiral separations. Substitution or modification of the hydroxyl groups on the CD with methyl-, hydroxypropyl-, carboxymethyl- or monoamino groups, for example were described in many papers [5–8]. Modifying the naturally occurring CDs leads to significant changes in their physicochemical properties, for example, change in type of interactions between analyte and CD derivative (see Table 1), and thus change in complex forming ability. A wide number of CDs and CD derivatives – such as the CDs contained in the Elphodextrin starter kit: α-, β- and γ-CD, (2-hydroxy)propyl-β-cyclodextrin (HPBCD), (2,6-di-O-methyl)-β-cyclodextrin (DIMEB), 6-monodeoxy-6-monoamino-β-cyclodextrin (MoAMBCD) and carboxymethyl-β-cyclodextrin (CMBCD) – can also be successfully used as chiral selector in CE [9–19]. During the electrophoretic process the chiral selector interacts with the enantiomers with relatively weak forces, for example hydrophobic interaction via hydrogen bonds or electrostatic interaction [4,20]. The stability constants of the enantiomer–selector complexes are not the same which results in different electrophoretic mobility of enantiomers, thus they separate [10].

At the beginning of the method development of a chiral separation the selection of the right CD is often done by "trial-and-error" approach. Thus a cost- and time-consuming search may begin. Our aim is to present a systematic method development scheme with advised conditions for enantiomeric separation using CDs and their derivatives (provided in the Elphodextrin starter kit). The experiments, necessary for the method optimization, are limited to an acceptable number. Several examples are also presented to demonstrate the applicability of the suggested scheme.

2. Experimental

2.1. Apparatus

The CE experiments were carried out on a Hewlett-Packard ^{3D}CE system (Hewlett-Packard, Waldbronn, Germany). Uncoated fused-silica capillary 58.5 cm (50 cm effective length)×50 μm I.D.×375 μm O.D (Composite Metal Services, Worcestershire, UK) was used throughout the study. The capillary was washed every morning with 0.1 *M* NaOH for 5 min and rinsed with deionized water for 15 min. Prior to each analysis the capillary was washed for 5 min with the appropriate buffer. After changing the pH of the buffer a voltage of 30 kV was applied for 20 min to equilibrate the capillary surface. Special care was taken when switching to another type of CD; the capillary was washed as follows: deionized water, 1 *M* NaOH, 0.1 *M* NaOH (5 min, respectively), and rinsed with deionized water and the running buffer (15 min with each). Samples and capillary were thermostated at 25°C. Samples were introduced

Table 1
Physicochemical properties of cyclodextrins and cyclodextrin derivatives

	α-CD	β-CD	γ-CD	HPBCD (2-hydroxy)propyl-β-CD	DIMEB heptakis(2,6-di-O-methyl)-β-CD	CMBCD carboxymethylated β-CD	MoAMBCD 6-monodeoxy-6-monoamino-β-CD
Glucose units	6	7	8	7	7	7	7
Internal diameter (nm)	0.47–0.53	0.60–0.65	0.75–0.83	0.60–0.65	0.60–0.65	0.60–0.65	0.60–0.65
Depth of cavity (nm)	0.79	0.79	0.79	0.79	1.00 (apparent)[a]	0.79	0.79
Substituent	–	–	–	(2-hydroxy)propyl	methyl	carboxymethyl	amino
Position of substituents	–	–	–	C(2)O	C(2)O and C(6)O	C(2)O	C(6)
Av. degree of substitution(subst. groups/ring)	–	–	–	4.3	14	3	1
pK_a value	12.3	12.2	12.1	N.A.[b]	N.A.	4.5	11
Isomeric distribution	–	–	–	random	50 (weight %)heptakis(2,6-di-O-methyl)-β-CD	random	99 (molar %)6-monodeoxy-6-monoamino-β-CD
Water solubility(mg/ml) (25°C)	145	18.5	232	>300	>300<10 (100°C)	unlimited	unlimited
Type of interactions							
Hydrophopic interaction	-CD cavity	-CD cavity	-CD cavity	-CD cavity-hydroxypropyl	-CD cavity-methoxy	-CD cavity-methyl	-CD cavity
Hydrogen bond	-glucose OH	-glucose OH	-glucose OH	-glucose OH-hydroxypropyl	-glucose OH	-glucose OH-carboxymethyl	-glucose OH
Electrostatic interaction						-carboxymethyl	-amino

[a] The cavity is deeper than that of β-CD, due to of the methoxy groups in C(2)O and C(6)O position [7].
[b] Not applicable.

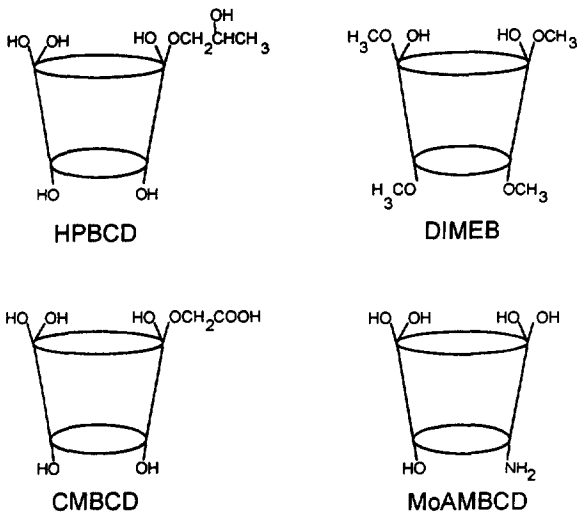

Fig. 2. Molecular structures of HPBCD, DIMEB, CMBCD and MoAMBCD.

to the anode side of the capillary using 150 mbar s. A constant voltage of 30 kV was applied. The analytes were detected at a wavelength of 202 nm, the reference wavelength was 450 nm.

2.2. Chemicals

All CDs used in our experiments were products of Cyclolab (Budapest, Hungary) and were of CE grade. Their structures are shown in Figs. 1 and 2.

Phenprocoumon, warfarin, oxedrine, acenocoumarol and zixoryn were of analytical grade and obtained from Sigma (St. Louis, MO, USA). Their structures are shown in Fig. 3.

Phosphoric acid, boric acid, acetic acid, sodium hydroxide were of analytical grade and obtained from Merck (Darmstadt, Germany). Methanol and acetone – chromatographic grade – were obtained from Merck. Deionized water was prepared using a Milli-Q system (Millipore, Wien, Austria).

2.3. Buffers

A 120 m*M* Britton–Robinson buffer (BRB) (consisting of 40 m*M* acetic acid, 40 m*M* phosphoric acid and 40 m*M* boric acid) – prepared according to Britton [21] and adjusted to the appropriate pH with 0.2 *M* sodium hydroxide solution – was used for control measurements and for preparing the buffers containing the chiral selector. β-CD containing buf-

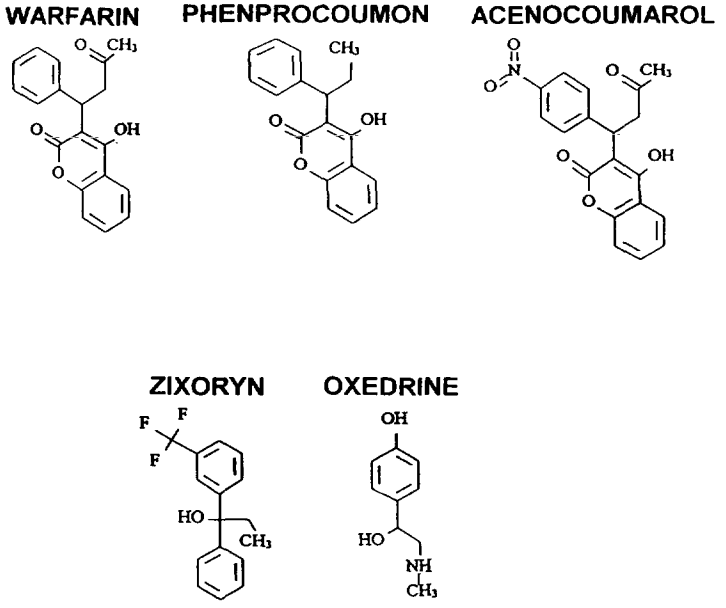

Fig. 3. Molecular structures of test samples.

fer was prepared in a 10 m*M* concentration. HPBCD, DIMEB, α-CD and γ-CD solutions were prepared in a concentration of 15 m*M*. CMBCD and MoAMBCD buffers were prepared in a concentration of 5 m*M*. The CDs were dissolved in 120 m*M* BRB buffer. When using ionic CDs the pH was adjusted after dissolving the CD derivative in the 120 m*M* buffer solution. All buffers were freshly prepared, filtered and sonicated for 10 min prior to analysis.

2.4. Sample solutions

Sample stock solutions were prepared in methanol in a concentration of 10 mg/ml and stored at 4°C. To obtain a sample concentration of 0.1 mg/ml the stock solution was diluted prior to analysis with the diluted running buffer containing no CD (BRB diluted 1:1 with deionized water). All sample solutions were filtered through a 0.2 μm inert membrane filter and sonicated for 10 min.

3. Practical aspects

3.1. Measurement of the electroosmotic flow

Due to its limited interaction with the majority of the CDs, acetone was used as neutral marker in each run. A 20 μl volume of acetone was added to 1 ml of sample solution. To calculate the electroosmotic flow (EOF) according to Eq. (1) the measured migration time of acetone was used.

$$\mu_{EOF} = l_{eff}L/(t_m V) \quad (1)$$

where l_{eff} is the length of the capillary to the detector, L is the total length of the capillary, t_m is the migration time of acetone and V is the applied voltage.

3.2. Calculation of effective mobility and resolution factor

Effective mobility of the samples was calculated as follows:

$$\mu_{eff} = \mu_{app} - \mu_{EOF} \quad (2)$$

where μ_{app} is the apparent mobility of the sample calculated according to Eq. (3), μ_{EOF} is the electroosmotic flow.

$$\mu_{app} = l_{eff}L/(t_{m(sample)}V) \quad (3)$$

Generally the quality of the separation is characterized with resolution values (R_s) assuming near baseline separations. At the beginning of method development the majority of the electropherograms contain unresolved or poorly resolved peaks. Using resolution factor (R) instead of R_s enables us to also describe these poorly resolved peaks [22].

The modified resolution factor R' is calculated according to Eq. (4):

$$R' = 100(H - H')/H \quad (4)$$

where H is the height of the first peak, H' is the height of the valley between the first and the second peak. $R' = 100$ therefore means baseline resolution [23].

3.3. pH Selection

Since the pH of the running buffer in CE affects the selectivity and the resolution of the chiral separation a careful investigation of the analyte structure is necessary at the beginning of the analysis. If the pK_a value of the analyte is well known, the working pH using neutral, or chargeable CD derivatives can be chosen with regard to the following considerations.

3.3.1. Neutral CDs

Weak acids and bases can be separated either as anions or cations depending on the buffer pH. When fused-silica capillaries are used, however, anionic separation is recommended to avoid solute–wall interactions and to have reasonable migration time; the selected pH should be: $pH > (pK_a + 1)$.

Strong acids can be separated as anions, strong bases as cations using buffers at $pH > (pK_a + 1)$ and $pH < (pK_a - 1)$, respectively. The mobility of the analyte is not strongly affected by the pH changes over a broad pH range, thus the effective mobility values will reflect the pH dependence of the EOF. A working pH with moderate or low EOF is recommended. In cationic separations, however, a careful investigation should be performed to check

possible solute–wall adsorption. If necessary, charged-reversed coating of the capillary could be applied to avoid solute–wall interactions [24].

In cases of multiple-charged compounds the effective mobility of the analyte may vary according to the multiple pK_a values. The pH selection should be performed with careful investigation of this effect (pK_a calculation and control measurements with narrower pH steps is suggested).

3.3.2. Ionic CDs

For the selection of the right pH using ionic CDs the following considerations dependent on the type of analyte are necessary.

Ionic CD derivatives give an apparent charge to the neutral molecules upon complexation. Thus, their mobility will differ from the EOF. Therefore buffer selection is determined by the pK_a values of the charged CD derivatives (see Table 1). In case of cationic derivatives the working pH should be lower than its pK_a value by at least two pH units, whereas when anionic CD derivatives are used the working pH should be two pK_a units above its pK_a value.

Using ionic CD derivatives for the enantioseparation of charged molecules – depending on the pH of the buffer – two different modes of interaction can be utilized [23]:

(1) Formation of ion pairs where the CD derivative and the analyte has opposite charge and thus, the original charge of the analyte becomes masked. The pK_a value of the analyte has to be considered;

(2) Suppression of the ionization of the CD derivatives and thus they can be considered as "neutral" chiral selectors and thus the original charge of the analyte is not altered. For example using CMBCD as additive $pH < pK_a$ is suitable. This method is not applicable for MoAMBCD due to its high pK_a value.

3.4. Determination of experimental parameters

The solubility of the sample and its compatibility with the buffer has to be investigated to avoid precipitation and thus clogging the capillary. The optimum detection wavelength for the sample have to be determined.

The individual steps of the method development are shown in the flow chart in Fig. 4. As is shown in the figure a few control measurements have to be performed prior to the development work. These are the following:

In most cases the pK_a value of the analyte is not known, therefore the determination of the effective mobility of the analyte at various pH values (pH 3, 5, 7 and 9) without CD in the running buffer is recommended to minimize the number of experiments (Fig. 4, Part 2). A reasonable μ_{eff} value indicates the working pH (see Table 2). In case of known pK_a the determination of the effective mobility can be reduced to fewer steps as suggested in Section 3.3.

If the analyte migrates with the EOF ($\mu_{eff}=0$) throughout the whole pH range, the analyte is to be considered as neutral compound. Further method development steps have to be continued according to Fig. 4, Part 6.

If the effective mobility of the analyte changes with the pH ($\mu_{eff}\neq 0$) the existence of one or more chargeable groups in the analyte can be assumed. The suggestions for further development steps (Fig. 4, Part 4) are applicable. The next step in the method development in cases of ionic analytes is the investigation of the enantioselectivity of the buffer with appropriate pH containing neutral CDs.

Due to the fact that β-CD and its derivatives are known as universal complexing agents – since nearly 80–90% of the drug molecules interact with them – a fast screening test is suggested using β-CD in the appropriately chosen buffer system. If β-CD fails to differentiate the enantiomers, HPBCD and/or DIMEB can be further tested.

After each measurement a critical investigation of migration time, peak shape and – if applicable – R' is necessary to avoid extended amounts of measurements with the buffer containing CDs. In every case the following should be considered:

(1) Different migration time of the analyte in the CD environment to the control measurement is a sign of analyte–CD interaction.

(2) With an achieved resolution of $R'>50$ further optimization steps – described elsewhere – using the same CD can be started [9].

When none of the non-charged β-CDs leads to success ($R'<10$) careful investigation of the samples structure and selection of proper selector size can lead to the desired resolution. For selecting the

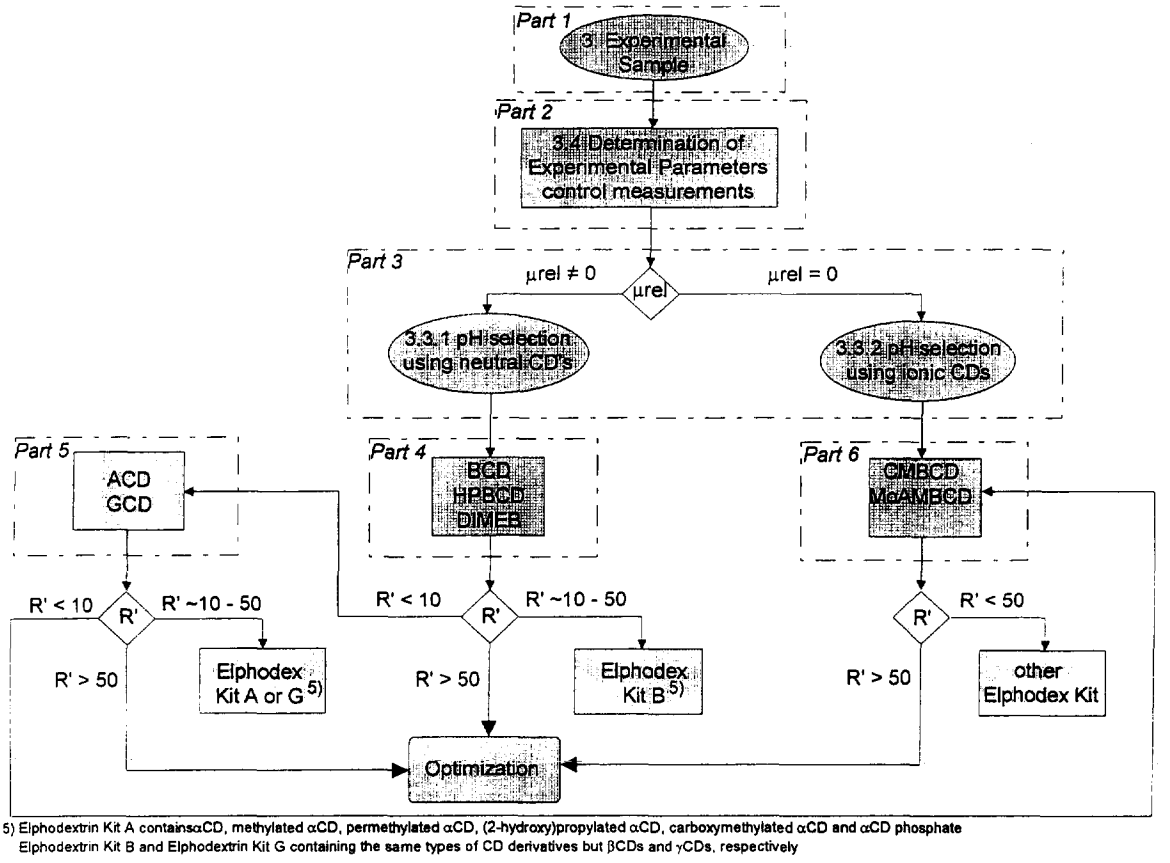

Fig. 4. Flow chart for the method development in CE using cyclodextrins and CD derivatives for enantiomeric separation.

proper selector size, the following guidelines are useful (see Fig. 4, Part 5):

(1) If the molecule has no aromatic ring, or has a long carbon chain, α-CD is suggested as chiral selector [10,25].

(2) When the analyte contains condensed aromatic groups the use of γ-CD can lead to the desired selectivity [26].

If a resolution with $R' > 50$ can be achieved, a further optimization using the same CD can be performed.

If $10 < R' < 50$ the use of other CD derivatives

Table 2
Apparent mobility of samples analyzed without chiral selector

Sample	μ_{app} at pH 3 (10^{-4} cm^2/V s)	μ_{app} at pH 5 (10^{-4} cm^2/V s)	μ_{app} at pH 7 (10^{-4} cm^2/V s)	μ_{app} at pH 9 (10^{-4} cm^2/V s)
Oxedrine	2.3[a]	2.2	2.2	0.8
Phenprocoumon	0	0	−2.1[a]	−2.2
Warfarin	0	−0.8	−2.0[a]	−2.2
Acenocoumarol	−0.1	−0.8	−2.0[a]	−2.1
Zixoryn	0	0	0	0

[a] Indicates the working pH chosen according to the control measurements.

Table 3
Comparison of suggested and measured pH values

Sample	Calculated pK_a value	Suggested pH	Working pH
Oxedrine	9.8	<8.8	3[a], 6, 7
Phenprocoumon	4.7	>5.7	7[a], 9
Warfarin	4.6	>5.6	7[a], 9
Acenocoumarol	4.1	>5.1	7[a], 9
Zixoryn	12.5	–	–

[a]Selected working pH according to Section 3.3.

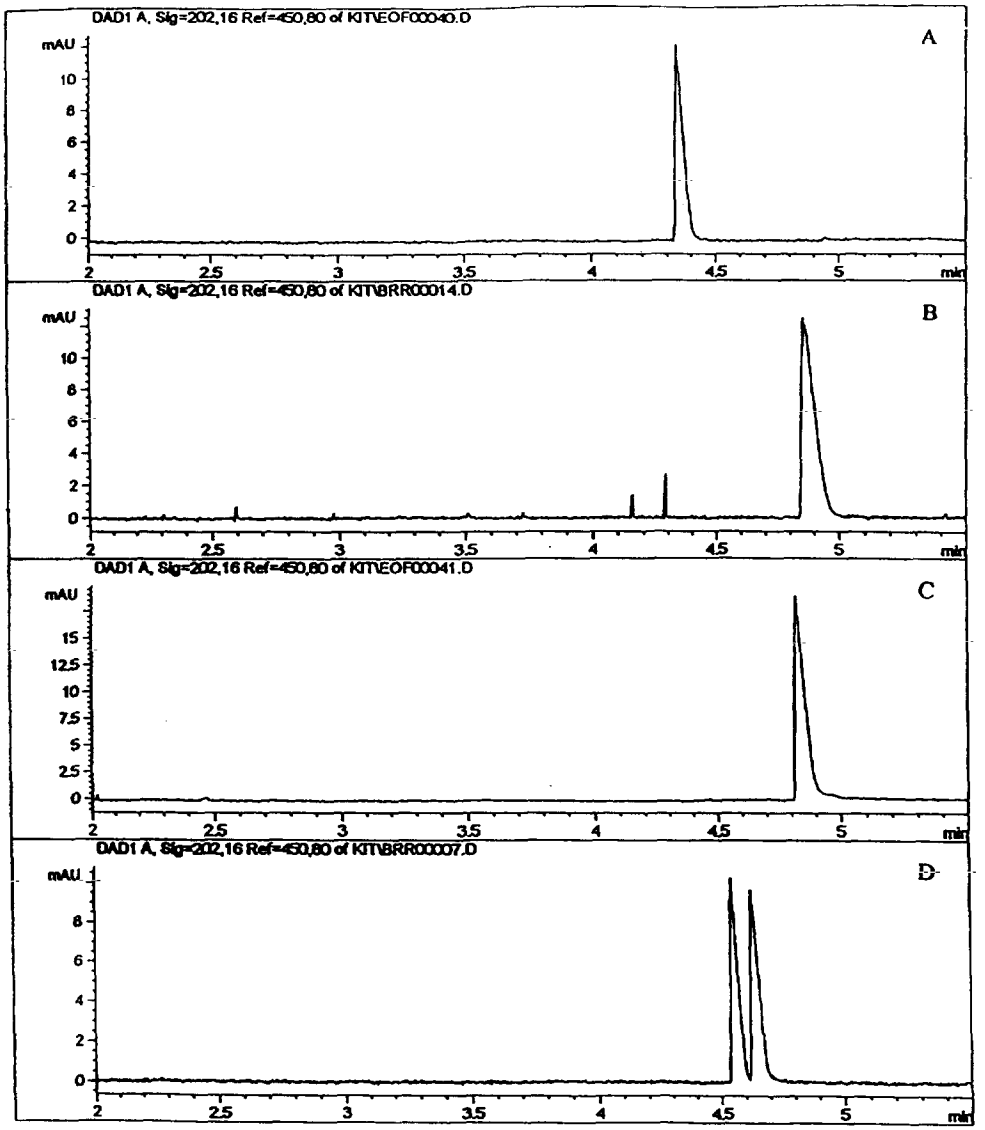

Fig. 5. CE screening of oxedrine: (A) control measurement at pH 3; (B) same as (A) but containing 10 m*M* β-CD; (C) same as (A) but containing 15 m*M* HPBCD; (D) same as (A) but containing 10 m*M* DIMEB. Conditions as described in Section 2.

with the same cavity size is recommended to achieve a better separation (for example, permethylated CD, hydroxypropylated CD, carboxyalkylated CD, phosphated CD, provided in Elphodex kits A, B or G by Cyclolab, see also Fig. 4).

In all other cases when the neutral CDs fail to separate the enantiomers ($R' < 10$) the use of ionic CD selectors such as CMBCD and MoAMBCD are recommended. Ionic β-CDs, however, can also interact with analytes even if the analyte did not show any interaction with the neutral β-CDs (see Fig. 4, Part 5).

4. Results and discussion

To demonstrate the applicability of the above scheme, representative drug samples (oxedrine,

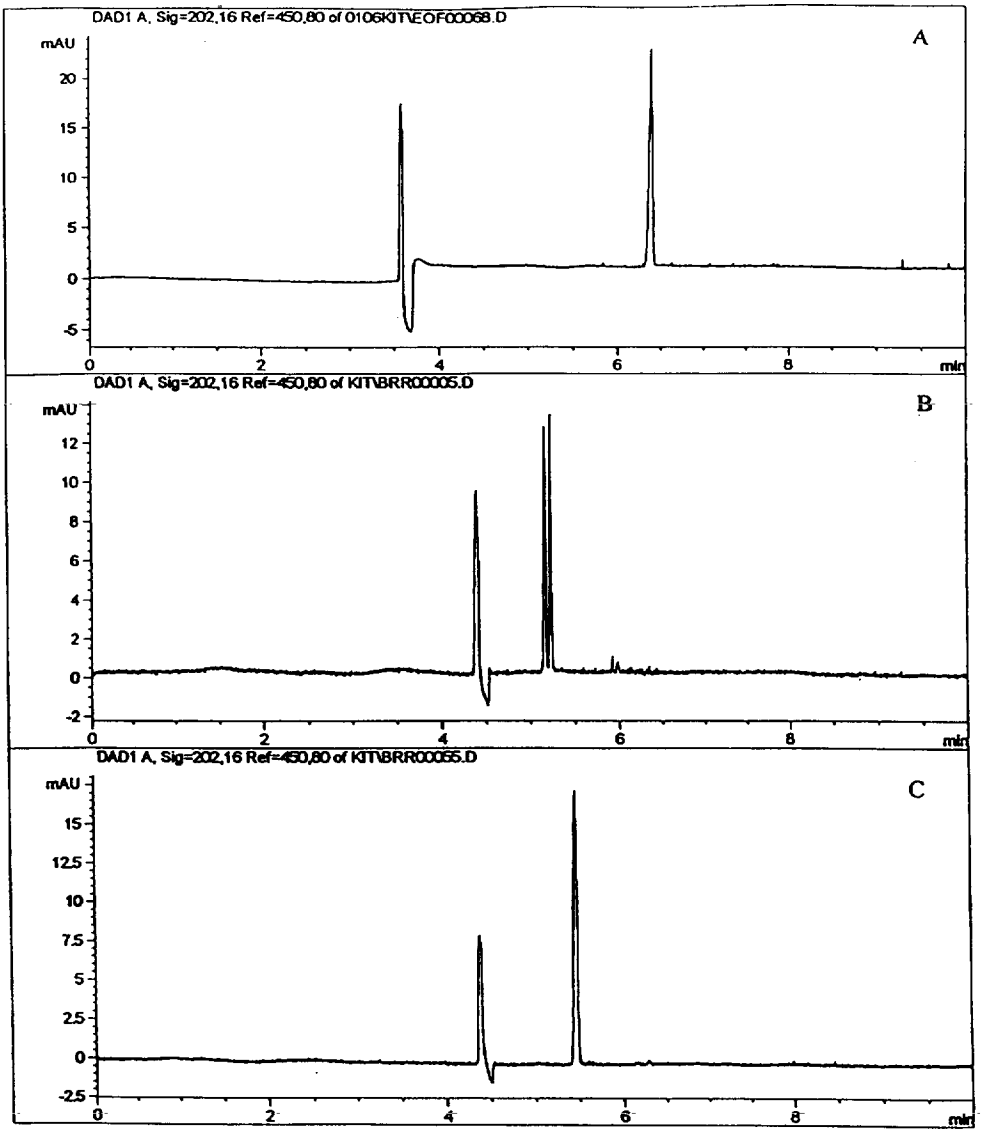

Fig. 6. CE screening of warfarin: (A) control measurement at pH 7; (B) same as (A) but containing 15 m*M* γ-CD; (C) same as (A) but containing 15 m*M* α-CD. Conditions as described in Section 2.

phenprocoumon, warfarin, acenocoumarol and zixoryn) were used.

First the EOF and the migration times of the samples at pH 3, 5, 7 and 9 were measured. In Table 2 the calculated effective mobilities of the analytes in 120 m*M* BRB buffer are listed. The mobility values show maxima for oxedrine, phenprocoumon, warfarin and acenocoumarol at pH 3, 7, 7 and 7, respectively. Zixoryn always moved with the EOF.

The selected working pH values are in good agreement with those suggested for the measurements in Section 3.3. ($pH > pK_a + 1$ or $pH < pK_a - 1$). The pK_a values of drugs were calculated with PCALC 3.1 software; Compudrug Chem., Budapest, Hungary (see Table 3).

Following the flow chart with the ionic drugs, oxedrine, phenprocoumon, warfarin and acenocoumarol, the experiments were carried out with the neutral β-CDs (Fig. 4, Parts 4 and 5). The buffer pHs were selected according to Section 3.4,

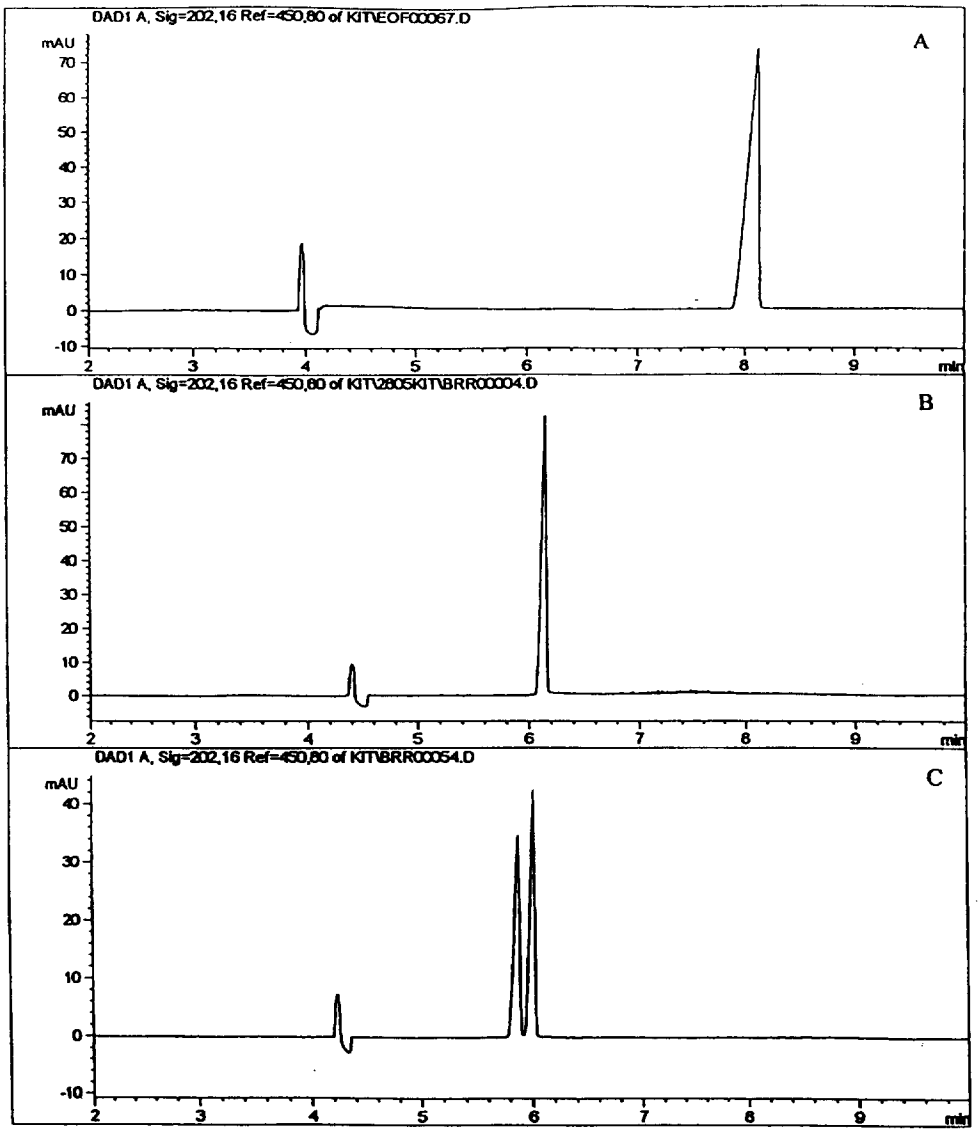

Fig. 7. CE screening of phenprocoumon: (A) control measurement at pH 7; (B) same as (A) but containing 15 m*M* γ-CD; (C) same as (A) but containing 15 m*M* α-CD. Conditions as described in Section 2.

namely pH 3, 7, 7 and 7 were chosen, respectively.

Oxedrine was analyzed using β-CD, HPBCD and finally DIMEB. With DIMEB as chiral selector a separation with $R' \sim 100$ was achieved (Fig. 5). Depending on the resolution needed further optimization by using different DIMEB concentration, variation of capillary temperature, addition of organic modifiers (type and concentration) etc. can performed [8].

Phenprocoumon, warfarin and acenocoumarol were analyzed using β-CD, HPBCD and DIMEB. Compared to the control measurements the peak shape and migration time did not show a significant change when these CDs were used. Thus it is suggested that the drug has no selective interaction with the β-CDs. Phenprocoumon, warfarin and acenocoumarol have a condensed ring system with an aromatic group in the side-chain with nearly

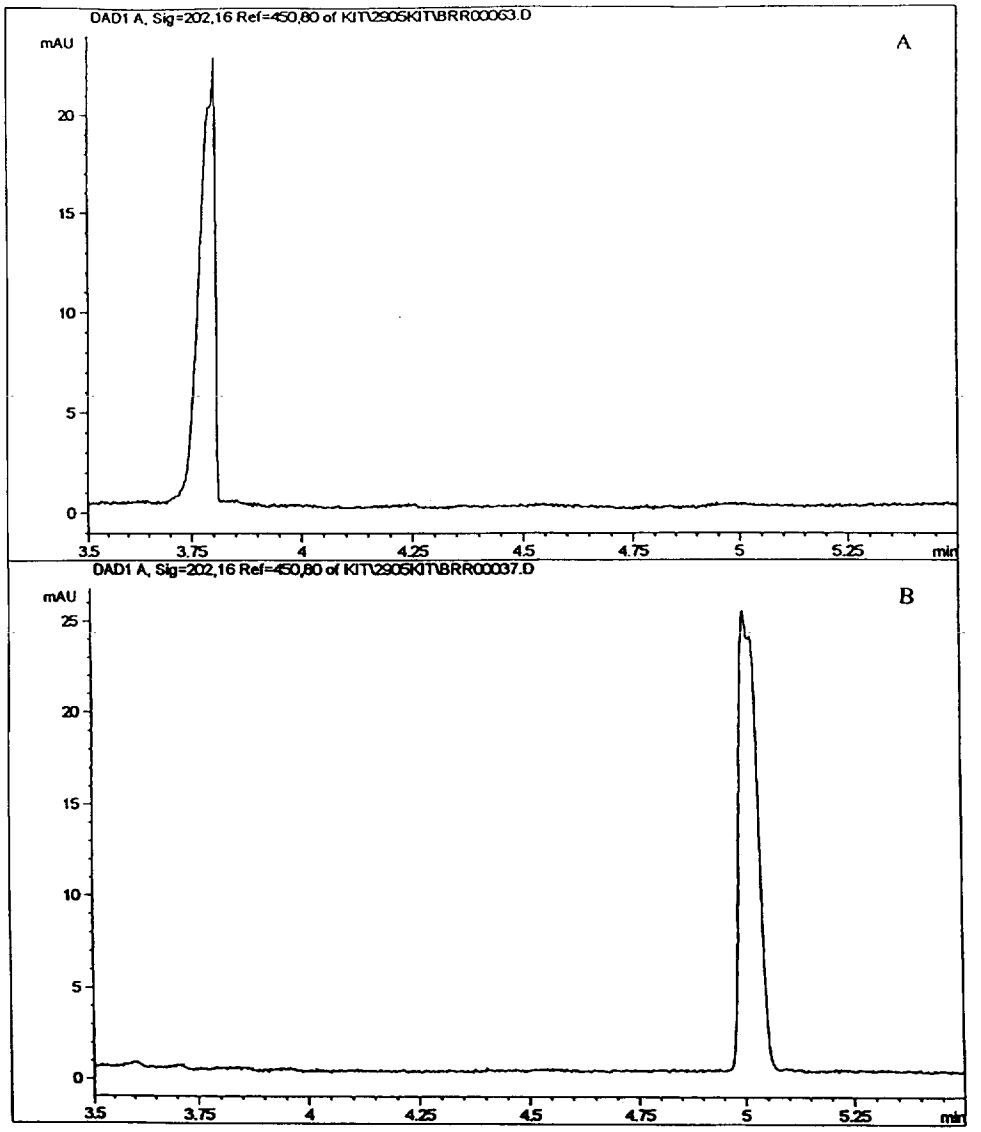

Fig. 8. CE screening of acenocoumarol: (A) measurement at pH 9 containing 5 m*M* MoAMBCD; (B) measurement at pH 9 containing 5 m*M* CMBCD. Conditions as described in Section 2.

similar molecular structure (see Fig. 3). Therefore one can suggest that γ-CD could be the useful selector for all the molecules. However, with 15 m*M* γ-CD only warfarin showed near baseline separation (Fig. 6). Phenprocoumon did not separate at all using γ-CD, while, the use of 15 m*M* α-CD, led to baseline separation (Fig. 7). In this case probably the side-chain of phenprocoumon interacts with the α-CD cavity. Acenocoumarol did not separate either with α-CD or γ-CD (no figures shown). These examples show that the investigation of the molecular structure does not lead in all cases to the selection of the best chiral discriminator. Since acenocoumarol was not separated using neutral CD derivatives, the method development scheme was further executed. The next suggested step is the use of ionic CD derivatives. Performing the experiments with acenocoumarol a separation with $R' \sim 15\%$ was achieved using 5 m*M* CMBCD and MoAMBCD at pH 9 (Fig. 8). With further optimization steps better resolution can be achieved.

Zixoryn always moved with the EOF showing no

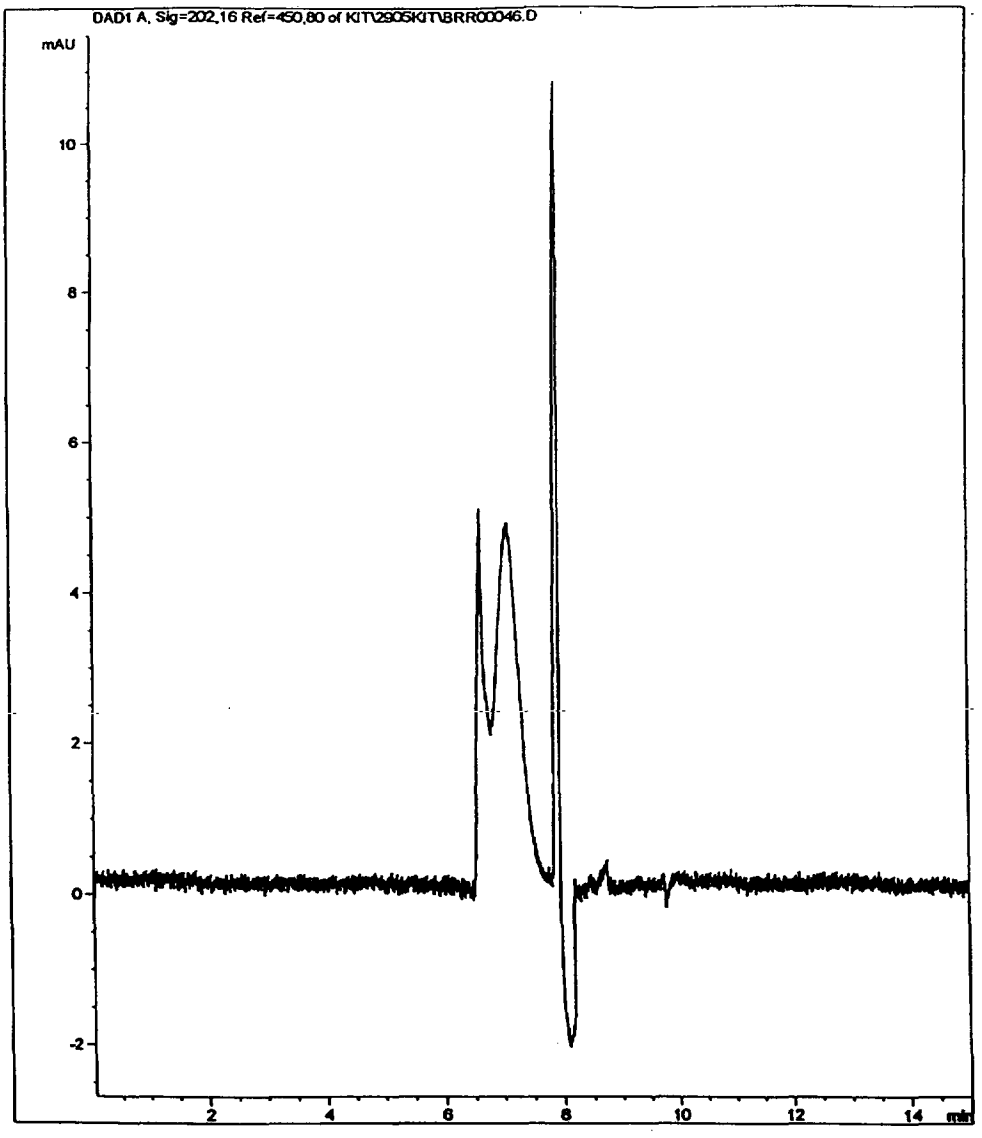

Fig. 9. CE screening of zixoryn at pH 3 with 5 m*M* MoAMBCD. Conditions as described in Section 2.

effective mobility in the studied pH range. Therefore the use of ionic β-CD derivatives, namely CMBCD and MoAMBCD is suggested (Fig. 4, Part 6). A successful separation of zixoryn enantiomers with $R' \sim 50\%$ was achieved using 5 m*M* MoAMBCD pH 3 (Fig. 9).

Following the development scheme for different drug samples (oxedrine, phenprocoumon, warfarin, acenocoumarol and zixoryn) the maximal number of experiments were equal or less than 13 (7, 9, 8, 13, 8, respectively). Thirteen is the maximal number of experiments if all suggested measurements were carried out.

5. Conclusion

A systematic, general approach is presented for the method development of enantiomeric separation by CE using CDs and derivatives provided in the Elphodextrin starter kit. Following the suggested flow chart the suitable CD can be selected using a reasonable number of steps thus saving costs and time in method development.

In order to test the feasibility of the flow chart several chiral pharmaceuticals were analyzed according to the suggestions presented here. In most cases the right chiral selector could be found with a resulting R' of 10–50% in less than ten steps (including control measurements). With the selected CD the method can be optimized by using different CD concentration, variation of capillary temperature, addition of organic modifiers (type and concentration) etc.

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