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Enantioseparation of basic pharmaceutical compounds by capillary electrophoresis using sulfated cyclodextrins Application to E-6006, a novel antidepressant

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Abstract

In this study, a chiral capillary electrophoresis method was optimized and validated for E-6006, a thienylpyrazolylethanamine derivative (pK_a 8.9). Enantioselectivity of neutral and anionic cyclodextrins (CDs) was evaluated at acid pH (3), obtaining cathodic and anodic migration, respectively. Hydroxypropyl-β-CD, carboxymethyl-β-CD and sulfobutyl ether-β-CD led to similar and partial selectivity, whereas sulfate (S)-β-CD produced baseline separation of the enantiomers. Four types of sulfated CDs were compared considering: cavity size (α, β, γ) and random substitution versus unique derivative (S-β-CD, 6-heptakis-S-β-CD). Complete peak separation was obtained in all cases, but with different affinity and binding strength. Some factors that play a role in the complex formation include: position/region/degree of substitution, size of CD cavity and proportion of derivatives in mixtures. Enantioaffinity and enantioselectivity increased with the average of sulfate groups/mol. β Cavity size complexed better, although α and γ cavities did not compromise separation. 6-Heptakis-S-β-CD had less affinity and separation efficiency, attributed to its lower degree and unique position of substitution. The method was optimized with S-β-CD (Aldrich, randomly substituted, 7–11 groups/mol). With this selector, the effect of pH value (3–9) was evaluated. Around pH 7 the cross-over point with change in the direction and order of migration was observed, associated with great enantioselectivity and long migration times. Fine tuning was done by adjusting the CD concentration and the buffer counterion. Definitive conditions were: uncoated silica capillary, 10 m*M* S-β-CD–25 m*M* sodium phosphate, pH 3. Validation parameters are included.

Keywords: Enantiomer separation; Pharmaceutical analysis; E-6006; Antidepressants; Cyclodextrins; Thienylpyrazolylethanamines; Basic drugs

1. Introduction

When a new molecule with a stereogenic center is developed as a pharmaceutical drug, its chiral stereoisomers (enantiomers) should be comparatively considered. Health regulatory authorities recommend marketing the most active/secure enantiomer, instead of the racemic mixture [1,2]. Therefore, the two enantiomers should be obtained, and their stereogenic purity checked with a sensitive and enantioselective method.

In the few last years, capillary electrophoresis (CE) has become the technique of first choice for chiral analysis. High efficiency, as well as a great variety of chiral selectors provide CE with a remark-

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able capability to resolve enantiomers [3–5]. Cyclodextrins (CDs) are the most widely used chiral selectors in CE. Their dual interaction, hydrophobic in the cavity and polar/electrostatic in the rim, besides the diversity of rim functionalities available, afford chiral discrimination for many racemates [3–6].

This paper deals with the optimization and first stage validation of a capillary electrophoresis (CE) method to control the chiral purity of E-6006 (Fig. 1), a drug under development in our company: (±)-5-{α-[2-(dimethylamino)ethoxy]-2-thienylmethyl}-1-methyl-1*H*-pyrazole, E-6006 (***R,S*-1**) [7,8]. E-6006 is an inhibitor of substance P release, which shows good activity as an antidepressant [9].

When approaching the optimization of this method, apart from optimum selectivity, sensitivity, accuracy and precision required, we also took into consideration using a migration buffer (background electrolyte, BGE) as simple as possible, and a cost per analysis as low as possible [10].

E-6006 has two aromatic rings attached to the stereogenic center, that can fit in the CD cavity, and a flexible chain ending with a basic nitrogen, that can interact with the external functional groups of the CD. Moreover, we had previous data of other similar molecules that complex selectively with CDs and are stereogenically resolved by CD-CE [11].

There are many examples in the literature of high selectivity obtained in short migration times, when a charged CD of opposite sign to that of the analyte is used [4,12–16]. Hence, not only neutral CDs (β-CD, heptakis(2,3,6-tri-*O*-methyl)-β-CD, hydroxypropyl-β-CD), but also anionic CDs (carboxymethyl-β-CD, sulfobutyl ether-β-CD and sulfate-β-CD) were tried.

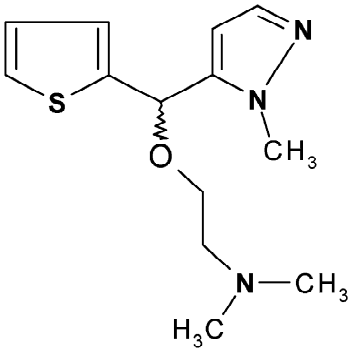

Fig. 1. Structure of E-6006.

Provided that sulfated CD showed the best enantiorecognition capability for (***R,S*-1**), in a second series of experiments different randomly sulfated CD mixtures and a pure derivative were evaluated and compared. With the best selector, the effect of the pH on the migration and the selectivity was studied. Finally, the method was fine tuned by adjusting the CD concentration and the buffer co-ion.

2. Experimental

2.1. CE system

The experiments were performed on an HP^{3D} CE capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany), with an on-column UV diode array detector and Chemstation data handling software. An uncoated barefused-silica capillary of 56 cm (64.5 cm inlet to outlet)×50 μm I.D. was used (Hewlett-Packard). The temperature was 20 °C. UV detection was done at 225 nm. The applied voltage was 20 kV with an initial ramp of 1 kV/s. Injection was performed on the anodic or cathodic side as detailed in the experiments. The sample was introduced into the capillary hydrodynamically, at 50 mbar over 5 s. Daily, and after changes in the buffer composition, the capillary was activated with a 5-bar flush of water (2 min), 0.1 *M* NaOH (5 min), water (2 min), and buffer (5 min).

Between analyses, the capillary was flushed with the migration buffer (3 min). In the definitive method the buffer was changed every 15–20 injections. Working solutions were prepared in methanol–water (15:85, v/v) or in water if sample was a salt (citrate).

2.2. Samples, chemicals, reagents and general procedures

E-6006, (±)-5-{α-[2-(dimethylamino)ethoxy]2-thienylmethyl}-1-methyl-1*H*-pyrazole (***R,S*-1**), (***S*-1**) and (***R*-1**) were synthesized in Laboratorios Dr. Esteve (Barcelona, Spain). Absolute configuration of the enantiomers was determined by X-ray diffraction analysis. pK_a was determined potentiometrically.

$Na_2HPO_4 \cdot 2H_2O$, $NaH_2PO_4 \cdot H_2O$, $Na_2B_4O_7 \cdot$

$10H_2O$, disodium citrate, H_3PO_4 (85%), NaOH, methanol, triethanolamine (TEOA), *p*-toluenesulfonic acid monohydrate, and tris(hydroxymethyl)aminomethane (Tris) were all analytical grade, from Scharlau (Barcelona, Spain), Merck (Darmstadt, Germany), or Aldrich (Milwaukee, WI, USA). Deionized water was obtained with a Milli-Q Plus system (Millipore, Bedford, MA, USA). β-Cyclodextrin (β-CD) was purchased from Fluka (Buchs, Switzerland). Heptakis(2,3,6-tri-*O*-methyl)-β-CD (TM-β-CD, M_r 1430) was from Sigma (St. Louis, MO, USA); hydroxypropyl-β-CD (HP[7]$_{ds}$-β-CD, M_r 1540, mixture of approximate degree of substitution (d.s.) of 7 groups/mol), and sulfate-β-CD sodium salt (S(7–11)$_{sr}$-β-CD, supposed M_r 2053, mixture of substitution range (s.r.) of 7–11 groups/mol and supposed d.s. 9 groups/mol) were from Aldrich; carboxymethyl-β-CD sodium salt (CM[3]$_{ds}$-β-CD, M_r 1309, mixture of d.s. 3 groups/mol) was purchased from Cyclolab (Budapest, Hungary); sulfobutyl ether-β-CD sodium salt (SBE[4]$_{ds}$-β-CD, M_r 1976, mixture of d.s. 4 groups/mol) was from Bioscience Innovation (Lawrence, KS, USA); sulfate-α-CD sodium salt (S[11]$_{ds}$-α-CD, M_r 2095, mixture of d.s. 11 groups/mol) and sulfate-γ-CD sodium salt (S[13]$_{ds}$-γ-CD, M_r 2623, mixture of d.s. 13 groups/mol) were obtained as 20% (w/v) aqueous solutions from Beckman Coulter (Fullerton, CA, USA); and heptakis-6-sulfate-β-CD sodium salt (6-HS-β-CD, M_r 1849) was purchased from Regis Technologies (Morton Grove, IL, USA).

Phosphate buffer of pH 3 was prepared by diluting phosphoric acid and adjusting the pH with TEOA or NaOH. Citrate buffer of pH 5 was directly prepared by dissolving disodium citrate in water. Phosphate buffers of pH 6, 7 and 8 were prepared by dissolving in water monosodium and disodium phosphate salts at appropriate concentrations. Borate buffer of pH 9.2 was directly prepared by dissolving disodium tetraborate in water. Cyclodextrins were added after adjusting the pH. Buffers were filtered using a 0.22-μm nylon membrane.

The purity profile of the two batches of S(7–11)$_{sr}$-β-CD used (05208HU, 05706HI) was determined by CE and indirect UV detection using a poly(vinyl alcohol) (PVA) coated capillary 56 cm (64.5 cm inlet to detector)×50 μm I.D. (Hewlett-Packard) and a migration buffer composed of 40 m*M* *p*-toluenesulfonic acid adjusted to pH 8 with Tris [17]. Hydrodynamic injection was done at the cathodic site and 25 kV were applied.

Peak identification was done by spiking the racemic with an enantiomer. For quantitative purposes, area was normalized by migration time [18].

Resolution (R_s) was calculated according to the equation:

$$R_s = 2 \times (t_2 - t_1)/(W_1 + W_2)$$

where t_1 and t_2 are the migration times of the two enantiomers and W_1 and W_2 are their respective peak widths at peak base.

3. Results and discussion

3.1. Method optimization

3.1.1. CD selector

The experiments were done at pH 3.0, acting (**1**) as a cation. A unique peak appeared with β-CD and TM-β-CD. Almost baseline separation was obtained with HP-β-CD (Fig. 2), but no improvement was observed when increasing the CD concentration up to 100 m*M* (results not shown).

At pH 3 CM-β-CD is expected to act as a practically neutral selector (pK_a 4.5) [19], but surprisingly, anodic migration was obtained (Fig. 2), indicating that the residual negative charges of the CD strongly interact with the cationic analyte. However, separation efficiency was not good.

At pH 3 the strongly acidic sulfate groups of SBE-β-CD (pK_a 2) are totally deprotonated [16] and, since the electroosmotic flow (EOF) is minimum, fast anodic migration was observed, although peak separation was only partial. In the same conditions, S-β-CD produced fast and generous resolution of our racemate (R_s 4), with inverse migration order compared to that obtained with SBE-β-CD (Fig. 2).

The properties of the rim substituents are of major importance in chiral recognition. The structural difference between S-β-CD and SBE-β-CD is the alkyl chain between the CD rim and the sulfate group. Nevertheless, we cannot conclude that this is the unique cause of the better separation obtained with S-β-CD, since SBE-β-CD is less substituted

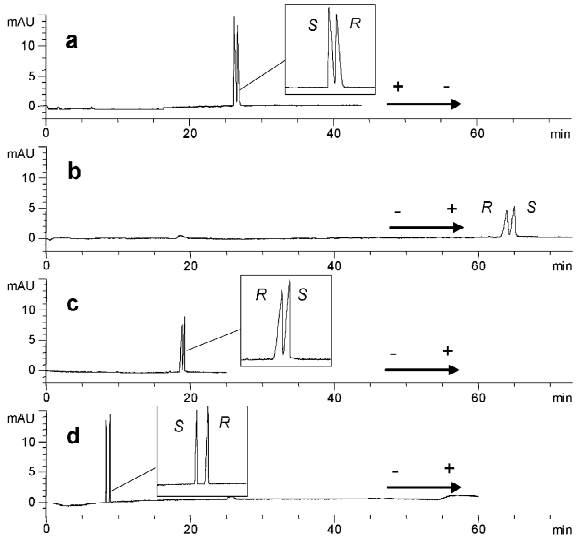

Fig. 2. Effect of CD functional groups. Run buffer: (a) 25 m*M* HP-β-CD–100 m*M* sodium phosphate, pH 2.5, (b) 10 m*M* CM-β-CD–25 m*M* TEOA phosphate, pH 3, (c) 10 m*M* SBE-β-CD–25 m*M* TEOA phosphate, pH 3, (d) 10 m*M* S-β-CD–25 m*M* TEOA phosphate, pH 3. Sample: 200 μg E-6006/ml. Other conditions as in the Experimental section. The arrow indicates the sense of migration.

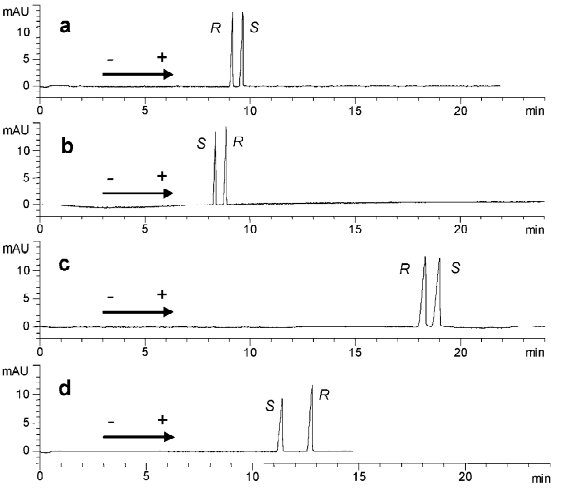

Fig. 3. Electropherograms of E-6006 obtained with different sulfated CDs. Run buffer: 10 m*M* CD–25 m*M* TEOA phosphate, pH 3, (a) S-α-CD, (b) S-β-CD, (c) 6-heptakis-S-β-CD, (d) S-γ-CD. Sample: 200 μg E-6006/ml. Other conditions as in the Experimental section. The arrow indicates the sense of migration.

(d.s. 4) than S-β-CD (d.s. 9) and has a lower electrostatic binding strength.

After these results, different randomly substituted CDs (S-α-CD, S-β-CD, S-γ-CD) and the pure 6-HS-β-CD were evaluated and compared (Fig. 3). With the four sulfated CDs checked, anodic migrations, with complete resolution of the racemic, were produced. The fact that the CD cavity size did not compromise the peak separation proves that the sulfate groups are the main factor of selectivity. However, in the light of the migration times obtained with S-α-CD, S-β-CD and S-γ-CD (similarly substituted), the strongest hydrophobic interactions were presumably produced in the β cavity.

Differences in the migration order of the enantiomers do not have a simple explanation: (***R*-1**) migrates first with S-α-CD and 6-HS-β-CD, and (***S*-1**) migrates first with S-β-CD and S-γ-CD. Many factors contribute to the enantioaffinity: (i) position/proportion of sulfate groups, (ii) hydrophobic cavity size, and (iii) relative abundance of each derivative in the mixtures [20].

Taking into account this multifactorial and unpredictable dependence on the enantiomers' CD affinity, together with the possibility of a lack of homogeneity between batches in randomly substituted mixtures, a unique derivative is conceptually preferable [14,21]. Apart from better reproducibility, better peak shape is also predicted for pure selectors, because only one complex is formed for each enantiomer and fewer electrodispersive interactions are produced [4]. Nevertheless, in this application, 6-HS-β-CD led to longer migration times and had slightly worse peak efficiency. Other examples have been published with similar results [22,23]. This behavior was mainly attributed to the lower number of sulfate groups/mol in the single derivative. Moreover, in this derivative all sulfate groups are in the position 6 of the CD rim, and this can be a negative characteristic with respect to the mixtures that are also partially substituted in positions 2 and 3 [14,17].

Considering the selectivity, S-γ-CD was the best option (R_s 8), whereas with respect to the sensitivity and speed of analysis, S-β-CD was a better choice, with not so great, but optimum separation (R_s 4). On the other hand, when developing the method the eutomer enantiomer was not yet known, and we had no preferences in the elution order, which is opposite with these two CDs. Moreover, S-β-CD was cheaper

(~20 times) than S-γ-CD. Finally, the method was optimized and validated with S-β-CD, and since it is a mixture, a test of CD batch reproducibility was carried out.

The concentration of S-β-CD was optimized in the range of 0.5–15 m*M* (Fig. 4). Even at 0.5 m*M* the migration was anodic and resolution was good, but peaks were asymmetric and wide. In order to achieve a quantitation limit of 0.1% of the non-desired enantiomer, good efficiency of response (tight and high peaks) was required. 10 m*M* was chosen as the lower CD concentration that afforded the required features of this method.

3.1.2. The role of pH

The behavior of (***S*-1**) and (***R*-1**) was investigated in the usual pH range in CE, 3–9.2 (Fig. 5). At pH values 3–7 the analytes are totally protonated (pK_a 8.9), whereas at pH values of 8 and 9.2 they are only partially ionizated. Sulfated CD is totally deprotonated in the entire range (pK_a 2).

When increasing the pH in an uncoated silica capillary, the cathodic electroosmotic flow (EOF) rises. The balance between the cathodic mobility of the free cationic analytes plus the EOF, versus the anodic mobility of the anionic CD complexed analytes, determines the direction and speed of migration. Certainly, in this application, the most relevant effect of pH on the mobility and the resolution is due to the EOF dragging strength.

Reversed polarity should be applied at pH values 3–6. At lower pH the migration was faster and the peak shapes were better. It is known that when EOF is low, electrodispersion distortions are reduced and peaks are generally narrower [24]. Although the selectivity was good in the entire acidic pH range, it directly correlates with the pH, being very high at pH 6 (R_s 13), but with long migration time (80 min).

At pH 7, after 60 min, no peak appeared, either in the anodic sense or in the cathodic direction. At this pH the apparent mobility of the two enantiomers was minimum and likely opposite. Hence, this pH is close to the cross-over point, where direction and order of migration change and maximum selectivity is obtained [22,25].

As expected, normal polarity had to be applied at pH values 8 and 9.2, with the stronger binding enantiomer (***S*-1**) migrating later. Faster migration and lower selectivity was obtained at pH 9.2. The

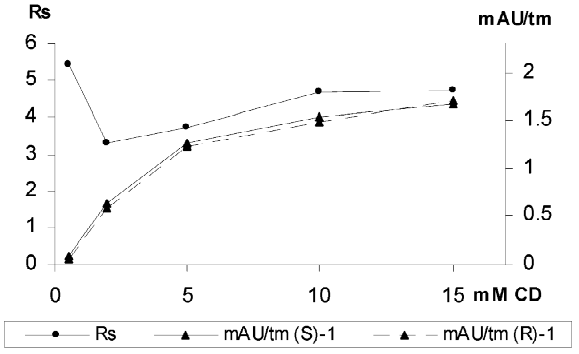

Fig. 4. Effect of S-β-CD concentration. Run buffer: S-β-CD–25 m*M* sodium phosphate, pH 3. Sample: 200 μg E-6006/ml. Other conditions as in the Experimental section.

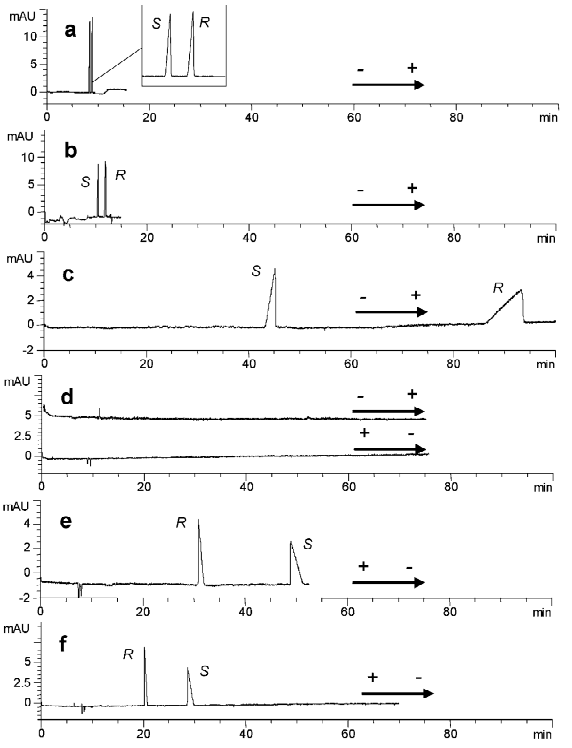

Fig. 5. Effect of the pH. Run buffer: 10 m*M* S-β-CD–50 m*M* buffer, (a) sodium phosphate at pH 3.0, (b) sodium citrate at pH 5.0, (c) sodium phosphate at pH 6.0, (d) sodium phosphate at pH 7.0, (e) sodium phosphate at pH 8.0, (f) sodium borate at pH 9.2. Sample: 200 μg E-6006/ml. Other conditions as in the Experimental section. The arrow indicates the sense of migration.

selectivity was also very high at this pH value, but inversely correlated with the pH.

From the practical point of view, pH 3 is the best choice, because it leads to optimum selectivity in the shortest time.

3.1.3. Buffer concentration and the role of buffer co-ion

The cationic buffer co-ion interacts with the negatively charged CD and competes with the cationic analytes. It has been described that, if the buffer co-ion is small, as Na^+, a mismatching mobility between the buffer co-ion and the analytes occurs and produces electrodispersive migration, which may lead to wide peaks. It seems that, if the co-ion is an organic amine (f.i. triethylamine or triethanolamine) this inconvenience is minimized, because of its closer mobility, with respect to that of the analyte [22,26].

On the basis of this consideration, the experiments to choose the best sulfated CD were done with phosphoric acid, adjusting the pH to 3 with triethanolamine (TEOA). Using this buffer selectivity was satisfactory (Fig. 3). However, a less precise and lower response of the first eluted enantiomer, with respect to the second, was observed. The problem was attributed to the great competition between the amine and the stronger binding enantiomer for the interaction with the most charged CD derivatives, either by ion pair or by complexation [27]. However, migration time and selectivity were not affected, presumably due to the fast migration.

Provided the intended purpose of this method was the determination of the enantiomer purity as relative area percent, an equal response of the two enantiomers, area normalized by migration time, was required. Changing TEOA for NaOH an equivalent response factor for the two enantiomers was obtained, without change in the migration profile (Fig. 6).

3.2. Definitive method validation

The final migration buffer was: 10 m*M* $S(7–11)_{sr}$-β-CD sodium salt in 25 m*M* phosphoric acid at pH 3 adjusted with NaOH, and the injection was done in the cathodic site. In order to achieve a quantitation limit of 0.1% of minor enantiomer, a target concentration of 4 mg E-6006 citrate (2.3 mg base)/ml was defined. The main validation parameters were checked to demonstrate the suitability of this method.

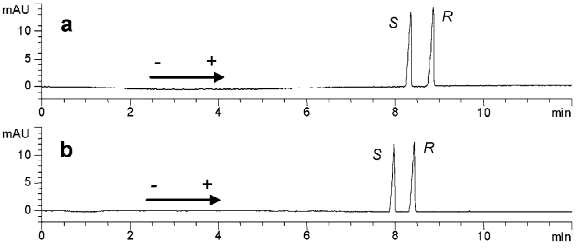

Fig. 6. Effect of buffer counterion. Run buffer: 10 m*M* S-β-CD–25 m*M* phosphate, pH 3, (a) adjusted with TEOA, (b) adjusted with NaOH. Sample 200 μg E-6006/ml. Other conditions as in the Experimental section. The arrow indicates the sense of migration.

3.2.1. Selectivity

Goodness of selectivity for the concentrated target solution, racemic mixture and enantiomers, is shown in Fig. 7.

The ruggedness of the selectivity with respect to the chiral selector variability, was checked using two batches. Migration time and selectivity were compar-

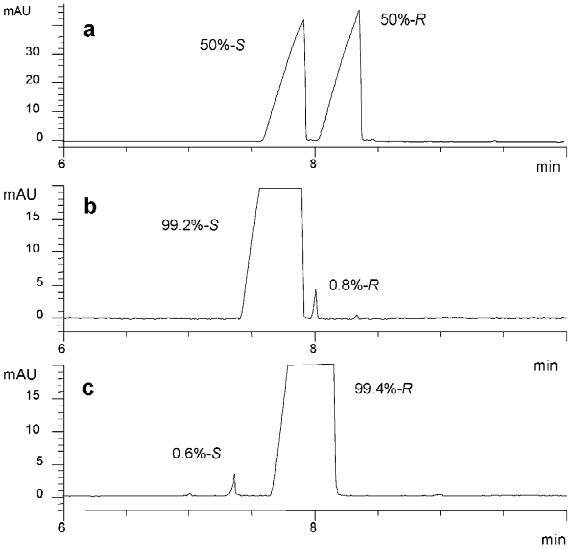

Fig. 7. Electropherograms of (a) E-6006 racemate, (b) (*S*)-enantiomer 99.2%, (c) (*R*)-enantiomer 99.4%. Run buffer as in Fig. 6b. Sample: 2300 μg E-6006/ml. Other conditions as in the Experimental section.

Table 1
Comparison of two S-β-CD batches

Parameter	Batch 05208HU TU		Batch 05706HI KO	
	Mean±SD	RSD (%)	Mean±SD	RSD (%)
μ_{app}, ***S*-1**	0.3762±0.0025	0.66	0.3731±0.0014	0.37
μ_{app}, ***R*-1**	0.3567±0.0027	0.76	0.3546±0.0016	0.45
R_s	4.039±0.078	1.92	3.902±0.032	0.81

$n=5$; μ_{app}, $cm^2\ V^{-1}\ s^{-1}$. Conditions are described in the Experimental section

able (Table 1). Moreover, the two batches were analyzed by CE with UV indirect detection (see details in the Experimental section) with similar profiles (results not shown). Thus, there is no evidence that the applicability of the method could be affected by the change in the selector batch.

3.2.2. Quantitative parameters

Calibration curves (normalized area vs. concentration) were determined for the two enantiomers (2.3–2300 μg base/ml). The straight lines obtained had good correlation coefficient and standard error, without significant differences in the slopes (Table 2).

The limit of detection (LOD), determined at a signal/noise ratio of 3 was 0.7 μg/ml for each enantiomer (0.03% of target concentration). The limit of quantitation (LOQ) determined at a signal/noise ratio of 10 was 2.3 μg/ml for each enantiomer (0.1% of target concentration). Since real samples of this degree of chiral purity were not available, these values were determined with diluted solutions.

Repeatability was determined for the racemic mixture (target and LOQ concentrations), and for the purest enantiomers produced. Within-day variation of migration time was equal to or less than 0.6%. Relative standard variation of normalized area was equal to or less than 3.3% for major enantiomer and not more than 9% for minor enantiomer at LOQ level (Table 3).

Table 2
Linearity

Parameter	Compound	
	***S*-1**	***R*-1**
Range (μg/ml)	2.3–2300	2.3–2300
Slope (mean±SD)	0.8290±0.0110	0.8316±0.0118
Intercept (mean±SD)	0.0127±0.0114	0.0176±0.0122
SE_y	0.0362	0.0387
Correlation coefficient (r^2)	0.99719	0.99680

$n=18$, $k=9$; *Y*, area/migration time; *X*, μg analyte/ml.

4. Concluding remarks

(1) Charged cyclodextrins are a potent tool for chiral separations in CE. Among them sulfated CDs are very good selectors.

(2) Pure sulfated CD derivatives are preferable than mixtures. However, only the sulfated derivative in position 6 is commercially available, whereas some commercialized standardized mixtures are more substituted and also have sulfate groups in positions 2 and 3, providing better enantioselectivity in some cases. E-6006 enantioseparation is an example.

(3) By changing the pH in the range of 3–9 in this application, no significant variations, either in the ionic state of the selector or in the analytes, occurred. Differences in migration and separation were mainly modulated by EOF and did not compromise selectivity.

Table 3
Within-day precision

Compound	Enantiomer ratio		RSD (%)	
	Mean±SD	RSD	Area/t_m	t_m
***S*-1**	99.15±0.01	0.01	3.3	0.4
	49.42±0.10	0.2	0.5	0.4
	0.60±0.04	6.3	8.5	0.3
	0.10[a]	–	7.0[a]	0.3[a]
***R*-1**	99.40±0.04	0.04	2.0	0.6
	50.58±0.10	0.2	0.8	0.5
	0.85±0.01	1.2	4.0	0.5
	0.10[a]	–	8.7[a]	0.3[a]

Sample concentration 2300 μg/ml; $n=6$; t_m, migration time.
[a] Sample concentration 2.3 μg/ml.

(4) Buffer cation interacts with the negatively charged CD and can interfere with the migration and/or the quantitative response of cationic analytes. Thus, it is important to choose carefully not only the buffer but also the co-ion.

(5) The optimized method has good selectivity, linearity, quantitation limit and precision for any proportion of E-6006 enantiomers, from racemic mixture to pure isomer.

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