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Enantioseparation of four *cis* and *trans* diastereomers of 2′,3′-didehydro-2′,3′-dideoxythymidine analogs, by high-performance liquid chromatography and capillary electrophoresis

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Abstract

Compounds **1**–**4** are the four stereoisomers of a synthetic new potential antiviral agent (d4T analog) containing two chiral centers and a base (uracil). Both high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) techniques were used to separate and quantify enantiomers with high resolution. The determination of enantiomeric purity of the compounds was developed using both amylose chiral stationary phase by HPLC and anionic cyclodextrins (highly S-CD) as chiral selectors in CE. The HPLC method was found to be superior in sensitivity to the CE method.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Chiral stationary phases, CE; 2′,3′-Didehydro-2′,3′-dideoxythymidine; Nucleosides; Cyclodextrins

1. Introduction

Nowadays, three different classes of anti-HIV chemotherapeutic agents have been developed in the treatment of acquired immuno-deficiency syndrome (AIDS): nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). The NRTIs such as 3′-azido-3′-deoxythymidine (AZT—Zidovudine, Retrovir®), 2′,3′-dideoxyinosine (ddI—Didanosine, Videx®), 2′,3′-dideoxycytidine (ddC) and 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T—Stavudine, Zerit®), possessing β-D configuration and β-L-(−)-2′-deoxy-3′-thiacytidine (3TC—Lamivudine, Epivir®) possesses the β-L configuration. All of these drugs have been approved by the US Food and Drug Administration in human immuno-deficiency virus (HIV) therapy [1–4]. D4T shows selective anti-HIV activity comparable to that of AZT in vitro [5], but is less toxic than AZT [6].

However, the currently used nucleoside drugs are associated with various adverse effects, hence there is a need to search for novel nucleoside analogues with a higher therapeutic index. In an attempt to expand the variety of nucleoside antiviral drugs, a novel range of 2′,3′-di-C-substituted analogues of d4T were synthesized to explore their potential as antiviral drugs [7]. The second generation of such compounds involved the synthesis of their pivaloyl derivatives, namely, (Fig. 1), (1*S*,3*S*)-1-(3-pivaloyloxymethyl-1,3-dihydrobenzo[*c*]furan-1-yl)uracil (**1**), (1*R*,3*R*)-1-(3-pivaloyloxymethyl-1,3-dihydrobenzo[*c*]furan-1-yl)uracil (**2**), (1*R*,3*S*)-1-(3-pivaloyloxymethyl-1,3-dihydrobenzo[*c*]furan-1-yl)uracil (**3**), and (1*S*,3*R*)-1-(3-pivaloyloxymethyl-1,3-dihydrobenzo[*c*]furan-1-yl)uracil (**4**) were synthesized via asymmetric dihydroxylation (AD-mix α and AD-mix β) starting from an achiral non-saccharidic compound: *o*-phthalaldehyde. The chiral centers in these compounds were introduced using the using asymmetric dihydroxylation (AD-mix α and AD-mix β) starting from the achiral non-carbohydrate precursor, *o*-phthalaldehyde [7]. The reaction mixture was composed of the two *trans* enantiomers **1**-(1*S*,3*S*) and **2**-(1*R*,3*R*) in the ratio 4/1 respectively. Sim-

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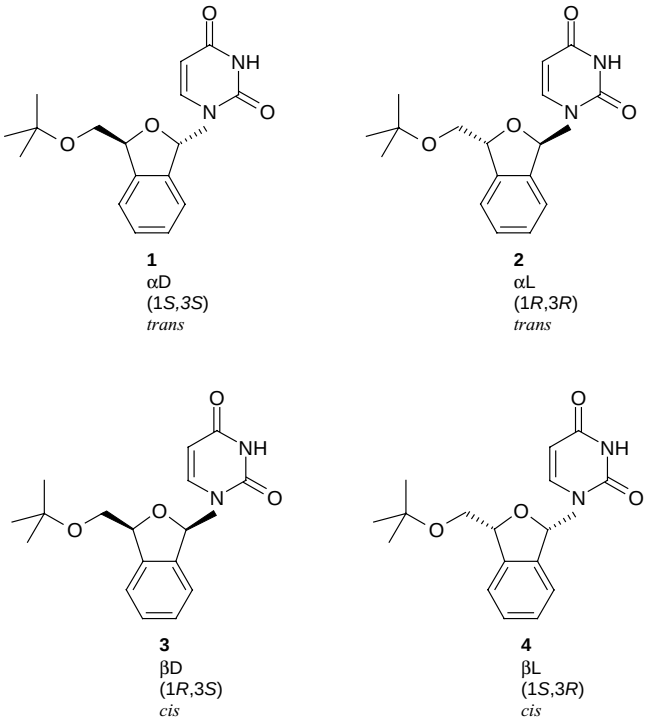

Fig. 1. Chemical structures of d4T analogs **1**–**4**.

ilarly, a mixture of the *cis* enantiomers 80% of **3**-(1*R*,3*S*) and 20% of **4**-(1*S*,3*R*) was also obtained. We firstly developed a method for enantioseparation by high-performance liquid chromatography (HPLC) on different chiral stationary phase (CSP): cyclodextrin and polysaccharides type. In a previous paper [8], enantiomeric separation of similar nucleoside products using several polysaccharide chiral stationary phases showed that both chiral stationary phases: Chiralpak AS, Chiralcel OD-H separated those type of compounds with high ability.

Herein additional studies with Chiralpak AS afford: (i) the determination of the limit of detection (LOD) and the limit of quantification (LOQ); (ii) the determination of the enantiomeric purity of each isomer.

Capillary electrophoresis (CE) is a recent technique, which is especially suitable for enantiomeric separations due to its high efficiency, versatility, short analysis time and low consumption of both sample and chiral selectors. Among different commercial chiral selectors, cyclodextrins (CDs) and their derivatives have a broad range of application [9–13]. Derivatization of the hydroxyl groups change both the selectivity and the solubility of the CD. Negatively charged highly sulfated-CDs have been shown to be particularly effective for the enantiomeric separation of neutral compounds [14–18,19].

In the present paper we report the optimization of experimental conditions for the enantiomeric separation of the four nucleosides **1**–**4**: in CE by using anionic CDs and in HPLC by using polysaccharides CSP. The two separation methods were compared with each other, and the results are presented and discussed below.

2. Materials and methods

2.1. Instrumentation

Chromatographic analyses was performed on a Waters 600 HPLC system equipped with a Waters 996 photodiode array spectrophotometer (compounds were detected at $\lambda = 200$ nm). The sample loop was 20 μl (Rheodyne 7125 injector). Chromatographic data were collected and processed on a computer running with Millennium 2010. The stainless steel columns Chiralcel OD-H (cellulose tris(3,5-dimethylphenylcarbamate); 250 mm × 4.6 mm i.d. 5 μm) and Chiralpak AS column (amylose (tris-(*S*)-1-phenylethylcarbamate); 250 mm × 4.6 mm i.d. 10 μm) were purchased from Daicel Chemical Industries (Tokyo, Japan). The stainless steel columns Cyclobond I 2000 (β-CD); (250 mm × 4.6 mm; i.d. 5 μm) and Cyclobond I 2000 RSP (*R,S* hydroxypropylether of β-CD); (250 mm × 4.6 mm; i.d. 5 μm) were purchased from ASTEC (Whippany, NJ, USA).

Capillary electrophoresis experiments were performed on a Beckman P/ACE MDQ capillary electrophoresis system (Beckman Coulter France S.A., Villepinte, France), including an on-column diode-array UV detector. The whole system was driven by a PC with the 32 Karat software package for system control, data collection and analysis. It was equipped with a 50.2 cm (effective length: 10 cm) × 50 μm i.d. untreated fused silica capillary (Composite Metal Services Ltd., Worcestershire, UK). The capillary was mounted in a cartridge and thermostated at 303 ± 0.1 K unless otherwise stated. An hydrodynamic short-end injection was made with a 5 s injection time at 1.0 psi (1 psi = 6.895 kPa) unless otherwise specified. The applied field was 0.25 kV cm^{-1} unless otherwise specified (normal polarity, cathodic injection). Compounds were detected at $\lambda = 200$ nm.

New capillaries were flushed for 20 min with 0.1 M NaOH ($P = 20$ psi) and 5 min with water $P = 20$ psi). Each day, it was flushed successively with NaOH (5 min, 20 psi), water (1 min, 20 psi), polyethylene oxide (PEO) (1 min, 25 psi), water (25 psi, 1 min) and then with background electrolyte (BGE) (3 min, 25 psi). Between each run, the capillary was treated with water (1 min, 20 psi) and BGE (1 min, 20 psi).

2.2. Chemicals

Ethanol, 2-propanol, acetonitrile and *n*-hexane were HPLC grade from Merck (Darmstadt, Germany) or Baker (Deventer, The Netherlands). All the solutions were filtered (0.45 μm), degassed with a Waters in-line degasser apparatus. The mobile phases used were A: *n*-hexane/ethanol: 80/20; B: *n*-hexane/ethanol: 90/10; C: *n*-hexane/2-propanol: 80/20; D: *n*-hexane/2-propanol: 90/10; E: ace-

tonitrile/methanol: 90/10; F: acetonitrile/methanol: 95/5; and G: acetonitrile/methanol: 98/2. The flow-rate was 1 ml min^{-1}. The peak of the injection of methanol was considered to be equal to the dead time (t_0) on cyclobond stationary phase. It was about 3.80 min for the Cyclobond I 2000 (β-CD), 3.50 min for the Cyclobond I 2000 RSP (*R*,*S* hydroxypropylether β-CD). The peak of the injection of 1, 3, 5-tri-(*tert*-butyl)benzene (C = 0.5 mM) was considered to be equal to the dead time (t_0) on polysaccharide stationary phase. It was about and 4.10 min for Chiralpak AS and 3.72 min for Chiralcel OD-H.

Compounds were chromatographed by dissolving them in methanol to a concentration of about 0.75 mM (0.28 g l^{-1}) (which corresponds to 15 nmol injected) and passed through a 0.45 μm membrane filter prior to loading the column.

Highly sulfated α-cyclodextrin (S-α-CD), highly sulfated β-cyclodextrin (S-β-CD) and highly sulfated γ-cyclodextrin (S-γ-CD) (aqueous solutions containing 20% of CD; concentrations of S-α-CD (2212.38 g mol^{-1}), S-β-CD (2380.95 g mol^{-1}) and S-γ-CD (2538.07 g mol^{-1}) are 90.4 mM, 84 mM and 78.8 mM respectively), polyethylene oxide (PEO) and sodium hydroxide were purchased from Beckman Coulter France S.A., Villepinte, France. Phosphoric acid (85%, w/w), triethanolamine (TEA), methanol, ethanol, 2-propanol and acetonitrile of analytical grade were obtained from Merck (Nogent-sur-Marne, France). Deionized (DI) water was obtained from Milli-Q system (Millipore, Saint Quentin-en-Yvelines, France). Nucleosides **1**–**4** were prepared as previously described [7]. A 50 mM phosphate buffer was prepared from a H_3PO_4 solution adjusted to pH 2.5 by addition of TEA. Background electrolyte (BGE; 25 mM phosphate buffer, pH 2.5) containing from 1 to 7% (w/v) of CD was made by appropriate dilutions of CD stock solutions and 50 mM phosphate buffer. Stock solutions of samples were prepared in ethanol (2 g l^{-1}) and diluted to 0.075 g l^{-1} with 2.5 mM phosphate buffer.

3. Results and discussion

3.1. Optimization of the HPLC enantioseparation

Table 1 shows the chromatographic data: retention, selectivity and resolution factors for the stereoisomers **1**–**4** using different mobile phase and different stationary phases. Of the different chiral stationary phases which are commercially available, such as Cyclobond I, Cyclobond I RSP, Chiralcel OD-H and Chiralpak AS, the efficiency of the latter in related separations prompted us to use it for the present study. An alcohol modifier can affect the retention of the solute in different ways: (a) by improving solvation in the mobile phase and/or (b) by competing for the H-bonding sites in the stationary phase. We observed an increase in the retention factors k for all of compounds we investigated by chang-

Table 1
HPLC: retention factor of second eluted enantiomer (k_2), selectivity α and resolution R_s

Compound	CSP	Mobile phase	k_2	α	R_s	1st enantiomer
1 + **2** *trans*	Chiralpak AS	A	2.94	1.61	4.72	**2**-(1*R*,3*R*)
		B	7.51	1.57	5.22	**2**-(1*R*,3*R*)
		C	3.45	1.21	2.01	**2**-(1*R*,3*R*)
		D	10.19	1.22	3.00	**2**-(1*R*,3*R*)
	Chiralcel OD-H	D	9.32	1.00	n.r.	–
	Cyclobond I 2000	E	0.37	1.04	<0.5	**2**-(1*R*,3*R*)
		F	0.48	1.03	<0.5	**2**-(1*R*,3*R*)
		G	0.63	1.09	<0.5	**2**-(1*R*,3*R*)
	Cyclobond I 2000 RSP	E	0.27	1.01	<0.5	**2**-(1*R*,3*R*)
		F	0.73	1.01	<0.5	**2**-(1*R*,3*R*)
		G	1.03	1.03	<0.5	**2**-(1*R*,3*R*)
3 + **4** *cis*	Chiralpak AS	A	3.59	1.61	4.95	**4**-(1*S*,3*R*)
		B	8.89	1.58	5.81	**4**-(1*S*,3*R*)
		C	5.92	1.63	5.24	**4**-(1*S*,3*R*)
		D	15.44	1.68	3.94	**4**-(1*S*,3*R*)
	Chiralcel OD-H	D	9.26	1.29	1.66	**4**-(1*S*,3*R*)
	Cyclobond I 2000	E	0.35	1.05	<0.5	**4**-(1*S*,3*R*)
		F	0.46	1.00	n.r.	–
		G	0.59	1.03	<0.5	**4**-(1*S*,3*R*)
	Cyclobond I 2000 RSP	E	0.27	1.05	<0.5	**4**-(1*S*,3*R*)
		F	0.74	1.03	<0.5	**4**-(1*S*,3*R*)
		G	1.02	1.05	<0.5	**4**-(1*S*,3*R*)

The mobile phases used were A: *n*-hexane/ethanol: 80/20; B: *n*-hexane/ethanol: 90/10; C: *n*-hexane/2-propanol: 80/20; D: *n*-hexane/2-propanol: 90/10; E: acetonitrile/methanol: 90/10; F: acetonitrile/methanol: 95/5; G: acetonitrile/methanol: 98/2. The flow-rate was 1 ml min^{-1}, λ = 200 nm; n.r.: no resolution; C = 0.28 g l^{-1}; $k_i = (t_i – t_0)/t_0$; $\alpha = k_2/k_1$; $R_s = 2(t_2 - t_1)/(\omega_1 + \omega_2)$.

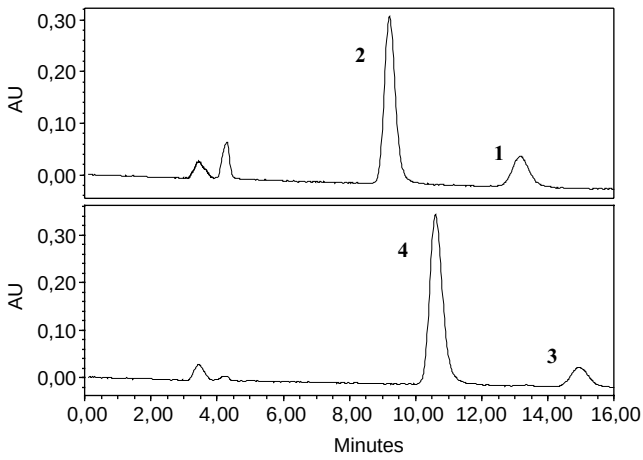

Fig. 2. Stacked chromatograms of *trans* enantiomers (**1** + **2**) and *cis* enantiomers (**3** + **4**), on Chiralpak AS, eluent A, 1 ml min^{-1}, 303 K and $\lambda = 200$ nm.

ing the modifier from ethanol to 2-propanol which is less polar.

When using Chiralpak AS, the type and concentration of alcohol did not influence the elution order. It is noteworthy that the same elution order was observed between the diastereoisomers **2** (first eluted) and **4** (second eluted) corresponding to the (3*R*) series, and between the diastereomers **1** (first eluted) and **3** (second eluted) corresponding to the (3*S*) series, respectively. The same elution order was observed between the enantiomers **2** (first eluted) and **1** (second eluted) corresponding to the *trans* isomers, and between the enantiomers **4** (first eluted) and **3** (second eluted) corresponding to the *cis* isomers (Fig. 2) respectively, in whatever the type of CSP and the kind of mobile phase employed.

After optimization, each enantiomer of the four nucleosides was analyzed using the Chiralpak AS with a mobile phase which consisted of *n*-hexane/ethanol (80/20) (eluent A). The chiral assay for each enantiomer was validated for detection and concentration limits. LOD and LOQ were calculated at a signal-to-noise equal to 3 and 10, respectively (Table 2). The LOD was between 0.36 and 0.63 mg l^{-1} for molecules having a 0.13–0.22% enantiomer impurity level and a major enantiomer target concentration of 0.28 g l^{-1} (0.75 mM). The LOQ was between 1.30 and 2.09 mg l^{-1} (i.e. enantiomer impurity level of 0.43–0.75%, respectively).

Table 2
HPLC: limit of detection and limit of quantification of compounds **1**–**4** on Chiralpak AS CSP (eluent A)

Compound	Configuration	LOD (%)	*C* limit (mg l^{-1})	LOQ (%)	*C* limit (mg l^{-1})
1	(1*S*,3*S*)	0.22	0.60	0.73	2.03
2	(1*R*,3*R*)[a]	0.13	0.36	0.43	1.20
3	(1*R*,3*S*)	0.22	0.63	0.75	2.09
4	(1*S*,3*R*)[a]	0.14	0.39	0.47	1.30

Target concentration: 0.28 g l^{-1} (100%).
[a] First eluted enantiomer.

3.2. Optimization of the CE enantioseparation

3.2.1. Selection of a suitable CD

Since uncharged CDs migrate at the same velocity as the electroosmotic flow (EOF), they only allow the separation of charged analytes [19]. According to the pK_a of the heterocyclic moieties [20], the nucleosides **1**–**4** are uncharged compounds at pH 2.5 and are not influenced by an EOF. Therefore, the use of negatively charged CDs, i.e. highly sulfated CDs, would appear to be the most convenient types to achieve effective enantioseparation.

Three types of commercially available highly sulfated S-CDs were investigated: highly sulfated α-CD (S-α-CD), highly sulfated β-CD (S-β-CD) and highly sulfated γ-CD (S-γ-CD).

Results are summarized in Table 3. Fig. 3a shows the electropherograms corresponding to compounds **1** and **2**. Migration times of both *trans* enantiomers decrease by changing from S-α-CD to S-γ-CD and then to S-β-CD. The use of

Table 3
CE: effect of S-CD concentration on migration times of second eluted enantiomer (t_2), selectivity α and resolution R_s

Compound	[S-α-CD] (%)	[S-α-CD] (mol l^{-1})	t'_2 (min)	α	R_s	1st enantiomer
1 + **2** *trans*	1	0.005	26.64	1.07	0.86	**1**-(1*S*,3*S*)
	4	0.022	10.03	1.09	1.39	**1**-(1*S*,3*S*)
	7	0.038	8.31	1.08	1.45	**1**-(1*S*,3*S*)
3 + **4** *cis*	1	0.005	24.96	1.08	1.18	**3**-(1*R*,3*S*)
	4	0.022	11.81	1.07	1.14	**3**-(1*R*,3*S*)
	7	0.038	6.43	1.05	0.76	**3**-(1*R*,3*S*)
	[S-β-CD] (%)	[S-β-CD] (mol l^{-1})				
1 + **2** *trans*	1	0.004	9.63	1.18	2.24	**1**-(1*S*,3*S*)
	3	0.014	4.14	1.17	2.58	**1**-(1*S*,3*S*)
	4	0.019	3.67	1.16	2.48	**1**-(1*S*,3*S*)
	5	0.024	3.33	1.15	2.65	**1**-(1*S*,3*S*)
	6	0.029	3.08	1.15	2.68	**1**-(1*S*,3*S*)
	7	0.034	2.98	1.14	2.70	**1**-(1*S*,3*S*)
3 + **4** *cis*	1	0.004	18.16	1.07	0.80	**3**-(1*R*,3*S*)
	3	0.014	7.27	1.08	1.40	**3**-(1*R*,3*S*)
	4	0.019	6.42	1.09	1.49	**3**-(1*R*,3*S*)
	5	0.024	5.77	1.09	1.47	**3**-(1*R*,3*S*)
	6	0.029	5.24	1.09	1.63	**3**-(1*R*,3*S*)
	7	0.034	5.03	1.09	1.53	**3**-(1*R*,3*S*)
	[S-γ-CD] (%)	[S-γ-CD] (mol l^{-1})				
1 + **2** *trans*	1	0.004	19.17	1.18	2.55	**2**-(1*R*,3*R*)
	4	0.017	6.85	1.15	2.63	**2**-(1*R*,3*R*)
	7	0.030	5.17	1.14	2.87	**2**-(1*R*,3*R*)
3 + **4** *cis*	1	0.004	>28	1.00	n.r.	–
	4	0.017	10.60	1.00	n.r.	–
	7	0.030	8.38	1.00	n.r.	–

Conditions: same as Table 4; n.r.: no resolution; $\alpha = t'_2/t'_1$; $R_s = 2(t_2 - t_1)/(\omega_1 + \omega_2)$ according to reference [12].

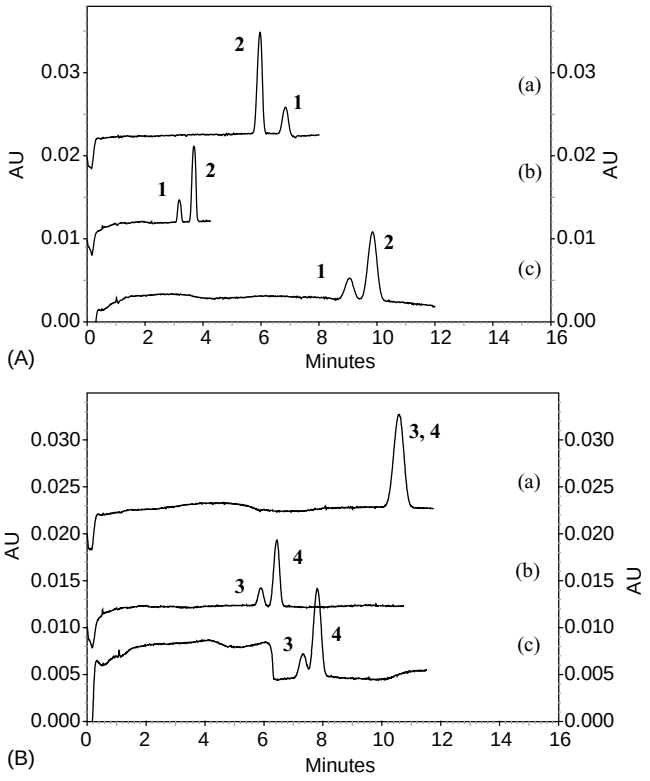

Fig. 3. (A) Overlapped electropherograms of *trans* enantiomers (**1** + **2**) using different types of S-CD. Conditions: fused-silica capillary coated with PEO 50.2 cm (effective length 10 cm) × 50 μm i.d. at 303 K; BGE, 25 mM phosphate buffer pH 2.5 (H_3PO_4 + TEA) with 3% (w/v) (a) S-γ-CD, (b) S-β-CD, and (c) S-α-CD; UV detection at λ = 200 nm; cathodic injection, 1 psi pressure for 5 s of 0.075 g l^{-1} solution; applied voltage, 25 kV. (B) Overlapped electropherograms of *cis* enantiomers (**3** + **4**) using different types of S-CD. Conditions: same as in (A).

S-α-CD and S-γ-CD gave similar ranges of selectivity and resolution but migration times were shorter with the latter CD. Migration times were found to decrease for the *cis* enantiomers, by changing from S-γ-CD to S-α-CD and then to S-β-CD. No resolution occured with S-γ-CD and very low values of resolution were obtained with S-α-CD. These results led to our choosing S-β-CD as the chiral selector for optimizing of the separation.

3.2.2. Effect of the S-β-CD concentration on the separation

CD concentration plays a major role in the optimization of chiral separation, as the complex formation is strongly influenced by this parameter [21–23]. The effect of the S-β-CD concentration, over the range S-β-CD, from 1 to 7% (w/v), on the separation of the enantiomers was examined. The electropherograms obtained are shown in Fig. 4a (*trans* enantiomers). Similar behavior was observed for the *cis* enantiomers (Table 3). An increase in the S-β-CD concentration from 1 to 7% (w/v) was found to enhance the resolution and lead to a decrease in the migration times for the all compounds investigated (Fig. 4b and c). This phenomenon has been reported by Dan et al. [24] using reversed

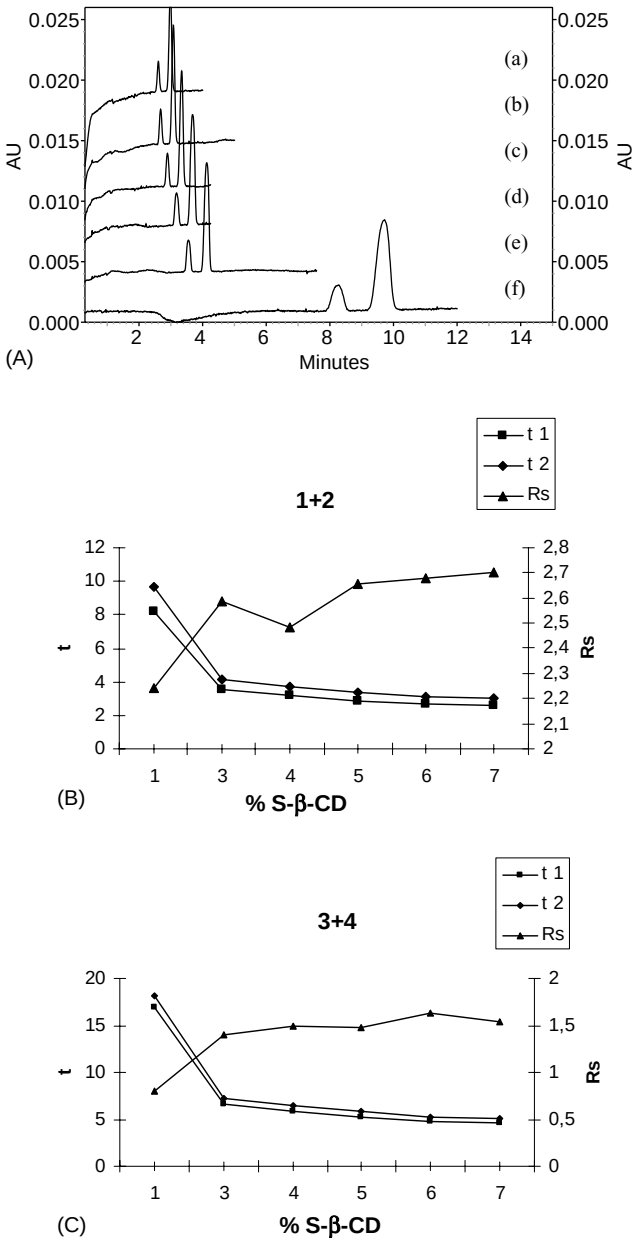

Fig. 4. (A) Overlapped electropherograms of *trans* enantiomers (**1** + **2**) using different concentrations of highly S-β-CD. Conditions: fused-silica capillary coated with PEO 50.2 cm (effective length 10 cm) × 50 μm i.d. at 303 K; BGE,11 25 mM phosphate buffer pH 2.5 (H_3PO_4 + TEA) with S-β-CD (a) 7, (b) 6, (c) 5, (d) 4, (e) 3, and (f) 1% (w/v); UV detection at 200 nm; cathodic injection, 1 psi pressure for 5 s of 0.075 g l^{-1} solution; applied voltage, 25 kV. (B) Effect of S-β-CD on migration times, *t*, and resolution, R_s, of the enantiomers of compounds **1** and **2**. Solid square, migration times of the first eluted enantiomer; open square, migrations times of the latest eluted enantiomer; solid-up triangle, R_s. Conditions: fused-silica capillary coated with PEO 50.2 cm (effective length 10 cm) × 50 μm i.d. at 303 K; BGE, 25 mM phosphate buffer pH 2.5 (H_3PO_4 + TEA); UV detection at λ = 200 nm; cathodic injection, 0.5 psi pressure for 5 s of 0.075 g l^{-1} solution; applied voltage, 25 kV. The concentration of S-β-CD was varied. (C) Effect of S-β-CD on migration times, *t*, and resolution, R_s, of the enantiomers of compounds **3** and **4**. Solid square, migration times of the first eluted enantiomer; open square, migrations times of the latest eluted enantiomer; solid-up triangle, R_s. Conditions: same as in (B).

polarity mode and indicates longer times of the analyte complexed with the CD [19,25].

In order to economize on cyclodextrin, a concentration of 4% (w/v) was selected for LOD and LOQ calculations. Wren and Rowe [21,22] proposed, in a simple mathematical model, the existence of an optimum CD concentration depending on the complex formation constants of the two enantiomers. This optimum decreases with increasing affinity of an analyte for a chiral selector. It is supposed that the diastereomeric complexes of the two enantiomers with the CD have the same electrophoretic mobilities. Separation is achieved if the enantiomers have different affinities for the chiral selector and electrophoretic mobilities of the free and complexed enantiomers are different.

Under our conditions, i.e. at pH = 2.5, as previously described [26,27], it is possible to consider that electroosmotic flow is negligible and that $\mu_{app} = \mu_i$. Moreover, for the analytes being uncharged (**1**–**4**), $\mu_f = 0$ and the following relationship holds:

$$\frac{[CD]}{\mu_{app}} = \frac{1}{\mu_c}[CD] + \frac{1}{\mu_c K} \quad (1)$$

Apparent binding constants *K*, electrophoretic mobilities and μ_c of the complexed solutes were determined using the equation of $[CD]/\mu_{app}$ versus [CD] at 303 K (Table 4). In accordance with Eq. (1), linear plots were obtained for all solutes.

3.2.3. Enantiomer migration order

For the two *trans* enantiomers, the enantiomer **1**-(1*S*,3*S*) was found to migrate first both S-α-CD and S-β-CD. An inversion of migration order occured with S-γ-CD where the enantiomer **2**-(1*R*,3*R*) migrated first. Concerning the *cis* enantiomers, the enantiomer **3**-(1*R*,3*S*) migrated first both S-α-CD and S-β-CD. No inversion of migration was observed for any compounds when the S-CD concentration was increased (Table 3).

Table 4
CE: apparent binding constants and electrophoretic mobilities of the complex formed between compounds **1**–**4** and S-CD

Compound	K_1 ($l\,mol^{-1}$)	K_2 ($l\,mol^{-1}$)	μ_{c1} ($cm^2\,V^{-1}\,s^{-1}$)	μ_{c2} ($cm^2\,V^{-1}\,s^{-1}$)
S-α-CD				
1[a] + **2**	45.87	46.94	0.0696×10^{-3}	0.0635×10^{-3}
3[a] + **4**	30.05	30.25	0.1021×10^{-3}	0.1001×10^{-3}
S-β-CD				
1[a] + **2**	57.64	53.97	0.1983×10^{-3}	0.1766×10^{-3}
3[a] + **4**	41.18	42.11	0.1273×10^{-3}	0.1155×10^{-3}
S-γ-CD				
1 + **2**[a]	44.26	40.61	0.1288×10^{-3}	0.1178×10^{-3}
3 + **4**	67.53	67.53	0.0586×10^{-3}	0.0586×10^{-3}

Conditions: fused-silica capillary dynamically coated with PEO. 50.2 cm (effective length 10 cm) × 50 μm i.d. at 303 K; BGE, 25 mM phosphate buffer pH 2.5 (H_3PO_4 + TEA); UV detection at λ = 200 nm; cathodic injection, 0.5 psi pressure for 5 s of 75 mg l^{-1} solution; applied voltage 25 kV.
[a] First migrating enantiomer.

Table 5
CE: limit of detection and limit of quantification of compounds **1**–**4**

Compound	Configuration	LOD (%)	*C* limit (mg l^{-1})	LOQ (%)	*C* limit (mg l^{-1})
1	(1*S*,3*S*)[a]	3.96	1.48	13.22	4.95
2	(1*R*,3*R*)	4.44	1.66	14.81	5.55
3	(1*R*,3*S*)[a]	5.00	1.87	16.66	6.25
4	(1*S*,3*R*)	6.31	2.36	21.05	7.89

Target concentration: 0.0375 g l^{-1} (100%); Conditions: fused-silica capillary coated with PEO. 50.2 cm (effective length 10 cm) × 50 μm i.d. at 303 K; BGE, 25 mM phosphate buffer pH 2.5 (H_3PO_4 + TEA); UV detection at λ = 200 nm; cathodic injection, 0.5 psi pressure for 5 s of 75 mg l^{-1} solution; applied voltage 25 kV.
[a] First migrating enantiomer.

3.3. Validation of the method

After systematic optimization of the method, the final conditions were found to be as follows: a BGE of 25 mM phosphate buffer pH 2.5 (H_3PO_4 + TEA), with 4% (w/v) S-β-CD (**1**–**4**), an applied field of 25 kV and a fused capillary temperature control of 30 ± 0.1 °C (typical current approximately 70 μA).

3.3.1. Repeatability

To investigate the repeatability of the migration times and peak areas, the racemic mixtures (0.0375 g l^{-1}) **1** + **2** and **3**+**4** were each injected seven times. In order to compensate for the differences in the residence time in the detector, corrected peak area, i.e. peak area per migration time (*S*/*t*), was taken into account, instead of peak area *S* [28].

The results reported in this paper show variation coefficients (CV%) for: (i) migration times (*t*) lower than 0.80%; (ii) corrected peak areas (*S*/*t*), used for the quantitative determination of the relative amount of individual enantiomer, 2.20%). The deviation in the normalized area $\{(S_1/t_1)/(S_2/t_2)\}$ of one enantiomer is smaller when the second enantiomer is used as internal standard (CV < 1.20%), to prevent from injection variations.

3.3.2. Limits of detection and quantification

The chiral assay for each enantiomer was validated for detection and quantification limits. The LOD values were calculated at a signal-to-noise ratio equal to 3 [29] were between 3.96 and 5% corresponding to 1.48 and 1.87 mg l^{-1} for the first enantiomer. The results are summarized in Table 5.

4. Concluding remarks

The HPLC method developed, using Chiralpak AS as CSP with *n*-hexane/ethanol 80/20 as mobile phase, allowed us to separate enantiomers and determine enantiomeric purities with very low LOD and LOQ values. The analytical resolution of the four d4T analogs was also achieved by CE using S-β-CD, and gave high resolution values in short migration

times. Although the latter technique was the more accurate and rapid, the calculated LOD and LOQ values were higher than those found by HPLC. Such values, demonstrate that the limitations of the CE method due poorer sensitivity. Nevertheless, CE can be regarded as a viable alternative to the HPLC technique as a means of effecting enantiomeric separations.

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