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Separation of nucleoside phosphoramidate diastereoisomers by high performance liquid chromatography and capillary electrophoresis☆

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ABSTRACT

Separations of five diastereoisomers of nucleoside phosphoramidate derivatives (pronucleotides) were performed by both HPLC method using derivatized cellulose and amylose chiral stationary phases and CE method using anionic cyclodextrins added in the background electrolyte (BGE). An optimal baseline separation (R_s > 1.5) was readily obtained with all silica-based celluloses and amyloses using in a normal-phase methodology. Capillary electrophoresis was used as an alternative technique to HPLC for the separation of pronucleotides. The diastereoisomers were fully resolved with sulfated cyclodextrins at both BGE pH (2.5 and 6.2). Limits of detection and limits of quantification, calculated for both methods, are up to 200 times higher in CE separations than in HPLC separations. The analytical HPLC method was then applied in a preliminary study for the pronucleotide **1** quantification in cellular extract.

1. Introduction

In an attempt to improve the therapeutic potential of nucleoside analogs, an important class of therapeutic agents for the treatment of virus infections [1,2], various mononucleotide prodrugs (pronucleotides) have been described during the last decade [3–6]. In this area, we previously reported the potentialities of mononucleoside phosphiotriester derivatives of AZT (3′-azido-2′,3′-dideoxythymidine) bearing one *S*-acyl-thioethyl (SATE) group and aryl residue as biolabile phosphate protections [7–10]. In cell culture experiments, such bioconstructs allow the efficient intracellular delivery of the parent 5′-mononucleotide. To improve the oral bioavailability of these potential therapeutic agents, i.e. their intestinal absorption, a new series of compounds incorporating an amino acid residue was synthesized [10–15]. Permeation of these phosphoramidates across the intestinal mucosa could namely be increased by active transport [16].

Due to the presence of a phosphorus atom, these pronucleotides exist as a mixture of two diastereoisomers. As observed in other nucleotide series, configuration at the phosphorus center may have a significant impact on the *in vitro* antiviral activity, enzymatic recognition as well as pharmacokinetic profile [17–21]. Consequently, the development of a rapid and reliable analytical method for the monitoring of diastereoisomeric pronucleotide, especially in biologic media, is needed.

Achiral chromatographic methods using C8 or C18 phase and polar organic eluent can be used for the resolution of diastereoisomers. Nevertheless, methods described in the literature for the separation of phosphotriesters and phosphoramidates containing a phosphorus asymmetric center [19,22–27] lead to poor separative performances. As a result, development of chiral separation method was chosen. Indeed, separation of diastereoisomers can be achieved by chiral HPLC, a well-established method with over 100 different chiral stationary phases (CSP) commercially available or by chiral capillary electrophoresis (CE) using cyclodextrins (CDs) as chiral selector added in the background electrolyte (BGE), as shown in recent reviews on chiral separation [28,29]. The aim of this work was to compare the potential of chiral HPLC and CE for the separation and quantification of pronucleotide diastereoisomers **1**–**5** without pre-derivatization (Fig. 1).

Amoung chiral HPLC methods proposed for the separation of phosphotriesters and phosphoramidates diastereoisomers [30–37], few separations were carried out in reversed-phase mode using immobilised cyclodextrins [34] or polysaccharide type stationary phases [24–27,30–36]. Most proposed methods are based on the use of Pirkle [31–33] or polysaccharide type phases in normal-phase mode [23,34,37], which lead to efficient separation. In previous works [34,35], the direct diastereoisomeric separation of three phosphotriesters was validated with a stereospecific HPLC

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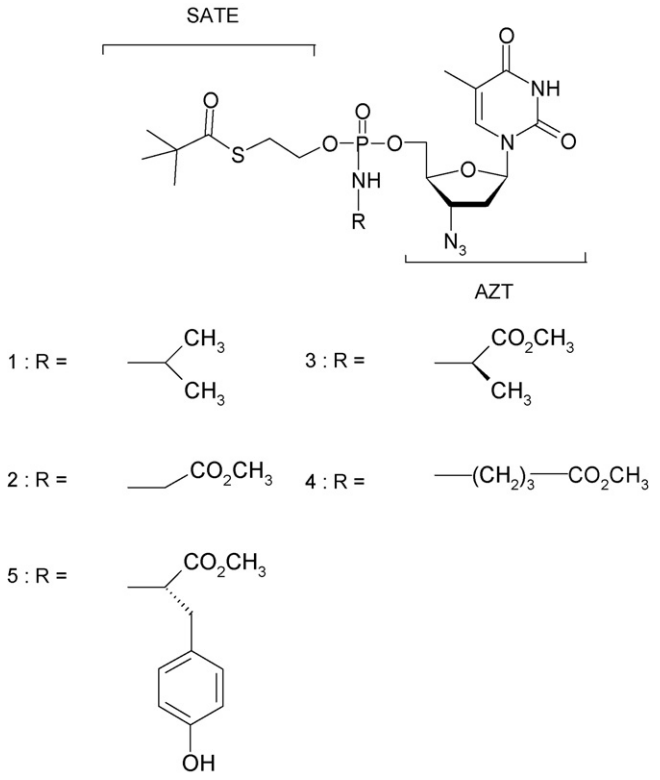

Fig. 1. Structure of the studied pronucleotides of AZT **1**–**5**.

methodology. Moreover, including a solid phase extraction step, the diastereoisomeric resolution of one pronucleotide was achieved in cell extracts [35].

To our knowledge, use of CE for the diastereoisomeric separation of compounds containing an asymmetric phosphorus center is quite limited: Perrin et al. [38] have demonstrated the performance of carboxymethyl-β-CD, used alone or with β-CD in a dual system mode, to resolve phosphotriester diastereoisomers.

In the continuity of our work [34,35] on the separation of biologically active isomers, we report here a study on the direct separation of pronucleotides **1**–**5** by HPLC using polysaccharides CSP (Chiralcel OD-H and OJ, Chiralpak AD and AS) and by CE using anionic cyclodextrins. After optimisation of the separation methods, the limits of detection (LOD) and quantification (LOQ) were determined in the optimal separation conditions obtained both in HPLC and CE. Comparison of resolutive performances and limit of quantification (LOQ) was realised for both methods. Finally, optimal method was applied to the analysis of compound **1** isomers in a cellular extract.

2. Experimental

2.1. Instrumentation and procedure

Chromatographic analyses were carried out using a gradient Waters 600E metering pump model equipped with a Waters 996 photodiode array spectrophotometer. Chromatographic data were collected and processed on a computer running with Millennium 2010. The column eluate was monitored at 266 nm for all compounds. The sample loop was 20 μL (Rheodyne 7125 injector). An inverse phase methodology was performed on a LiCrospher® RP-18 (Merck, 125 mm × 4 mm I.D., 5 μm) thermostated at 308 K ± 0.1 K. Elution was made isocratically using a 50/50 (v/v) mixture of methanol and acetate buffer (20 mM, pH 6.6) and a flow rate of 0.8 mL min^{-1}. A normal phase methodology was performed with two silica-based cellulose Chiralcel OD-H (tris-3,5-dimethylphenylcarbamate; 250 mm × 4.6 mm I.D., 5 μm), and Chiralcel OJ (tris-methylbenzoate, 250 mm × 4.6 mm I.D., 10 μm), or two silica-based amylose Chiralpak AD (tris-3,5-dimethylphenylcarbamate, 250 mm × 4.6 mm I.D., 10 μm), and a Chiralpak AS (tris-(*S*)-1-phenylethylcarbamate, 250 mm × 4.6 mm I.D., 10 μm) (Daicel Chemical Industries, Baker France). The column was thermostated at 298 K ± 0.1 K. Mobile phase elution was made isocratically using *n*-hexane and a modifier (ethanol, 1-propanol or 2-propanol) at various percentage. The flow rate was 0.8 mL min^{-1}. In this separation mode the dead time (t_o) was considered to be equal to the peak of the solvent front and was taken from each particular run. It was about 4.70 min for the Chiralcel OD-H, 4.10 min for the Chiralcel OJ, 4.50 min for the Chiralpak AD, 4.40 min for the Chiralpak AS and was equal to the value obtained by injection of 1,3,5-tri-*tert*-butylbenzene used as a non-retained sample. In all cases retention times were mean values of two replicate determinations.

Capillary electrophoresis experiments were performed on a Beckman P/ACE MDQ Capillary Electrophoresis system (Beckman Coulter France, Villepinte, France), including an on-column diode-array UV-detector. The whole system was driven by a PC with the 32 Karat software (Beckman Coulter France) package for system control, data collection and analysis. It was equipped with a 50.2 cm (effective length: 40.2 cm) × 50 μm ID untreated fused-silica capillary (Composite Metal Services, Worcestershere, UK). The capillary was mounted in a cartridge and thermostated at 288 K ± 0.1 K, unless otherwise specified. A hydrodynamic injection was made with a 5 s injection time at 0.5 psi (anodic injection) unless otherwise specified. The applied field was between 0.20 and 0.40 kV cm^{-1}. All compounds were detected at 259 nm. New capillaries were flushed for 20 min with 0.1 M sodium hydroxide (NaOH) (P = 20 psi) and 5 min with water (P = 20 psi). For the separation at pH 6.2, the capillary was each day flushed successively with NaOH (5 min, 20 psi), water (1 min, 20 psi) and then with BGE (3 min, 20 psi). Between each run, it was treated with 0.1 M sodium hydroxyde (1 min, 20 psi) and BGE (3 min, 20 psi). When using acidic BGE (pH 2.5), the capillary was each day flushed successively with NaOH (5 min, 20 psi), water (1 min, 20 psi), Polyethylene oxide (PEO) (1 min, 20 psi), water (1 min, 20 psi) and then with BGE (3 min, 20 psi). Between each run, it was treated with water (1 min, 20 psi) and BGE (3 min, 20 psi). Electrophoretic parameters presented are averaged values of three replicate determinations.

2.2. Chemicals and reagents

The phosphoramidate derivatives of AZT **1**–**5** (Fig. 1) were synthesized, as diastereoisomeric mixtures, following adapted procedures [11,12,15] leading to mixture of two diastereoisomers.

In HPLC, methanol, ethanol, 1-propanol, 2-propanol and *n*-hexane were HPLC grade and were obtained from Merck (Nogent sur Marne, France) or Baker (Noisy le Sec, France). In reversed phase methodology, acetic acid and ammoniac (28%) used for the preparation of the mobile phase methanol/acetate buffer (20 mM, pH 6.6) 50/50 (v/v) were purchased from Merck (Nogent sur Marne, France). The mobile phases used in normal phase methodology were (A) hexane/ethanol: 90/10, (B) hexane/ethanol: 80/20; (C) hexane/ethanol: 70/30, (D) hexane/ethanol: 60/40, (E) hexane/ethanol: 50/50, (F) hexane/1-propanol: 70/30, (G) hexane/2-propanol: 70/30). Mobile phases were filtered through membrane (0.45 μm) and degassed with a Waters in-line degasser apparatus. Compounds were chromatographed by dissolving them in ethanol to a concentration of about 0.50 mM (concentration 100%) and passed through a 0.45 μm membrane filter prior to loading the column.

In CE, phosphoric acid (85%, w/w), triethanolamine (TEA) and acetonitrile (ACN) of analytical grade were purchased from Merck (Nogent-sur-Marne, France). NaOH 0.1 M, PEO (0.4%, M_w = 300,000), highly sulfated α-cyclodextrins (highly S-α-CD, M_w = 2212), highly sulfated β-cyclodextrins (highly S-β-CD, M_w = 2381) and highly sulfated γ-cyclodextrins (highly S-γ-CD, M_w = 2538) (20% (w/v) in a 50 mM phosphate buffer at pH 2.5 which corresponds to 90.4, 84.0 and 78.8 mM, respectively) were purchased from Beckman (Beckman Coulter France, Villepinte, France). Sulfated α-cyclodextrins (S-α-CD, M_w = 1789), sulfated β-cyclodextrins (S-β-CD, M_w = 2053) were obtained from Aldrich (Aldrich, Saint Quentin Fallavier, France). Deionized (DI) water was obtained from Milli-Q system (Millipore, Saint Quentin en Yvelines). BGEs were prepared either by dilution of appropriate volume of highly S-CD solution or by dissolution of appropriate weight of S-CD in a phosphate buffer prepared from a phosphoric acid solution adjusted to pH 2.5 or 6.2 by addition of TEA. Phosphoramidate sample solutions at 0.50 mM in 50 mM phosphate buffer (pH 2.5 or 6.2) were obtained from ethanolic solutions at 3 mM.

2.3. Incubation and extraction of pronucleotide **1** in total CEM-SS cell extracts

Lymphoblastoïd cell line (CEM-SS) cell extracts were kindly prepared by Dr. A. Lansiaux according to published procedures [39]. Pronucleotide **1** (50 μM) was incubated for 10 min at 298 K in this medium. Its extraction was carried out on HPLC on-line cleaning protocol previously described [40]. Crude sample was injected on a pre-column (Hypurity, C_{18}, 10 mm × 2.1 mm, 5 μm, Thermoelectron Corporation). Proteins were eliminated by rinsing the precolumn for 1.5 min with water at flow rate of 3 mL min^{-1} The mobile phase composition was then changed to 100% acetonitrile using linear gradient over 1 min and the flow rate gradually decreased to 0.3 mL min^{-1}. Under these conditions, analytes were eluted. The fraction collected was evaporated under nitrogen.

2.4. Calculation

The HPLC separation parameters were calculated as follows:

$$\text{Retention factor}: \quad k' = \frac{t_r - t_0}{t_0}$$

$$\text{Selectivity}: \quad \alpha = \frac{k'_2}{k'_1}$$

$$\text{Resolution}: \quad R_s = 2\frac{t_{r2} - t_{r1}}{\omega_1 + \omega_2}$$

where t_r is the diastereoisomer elution time, k', α and R_s the retention, selectivity and resolution factors, respectively. ω is the width of the peak at the baseline. The subscripts 1 and 2 refer to the first and second eluting diastereoisomer.

The CE separation parameters were calculated as followed:

$$\text{Apparent mobility}: \quad \mu_{app} = \frac{lL}{Vt}$$

$$\text{Selectivity}: \quad \alpha = \frac{t_{m2}}{t_{m1}}$$

$$\text{Resolution}: \quad R_s = 2\frac{t_{m2} - t_{m1}}{\omega_1 + \omega_2}$$

where μ_{app} is the apparent mobility, L and l the total capillary length and the length to the detector, respectively, V the run voltage, t_m the diastereoisomer migration time, α and R_s the selectivity and resolution, and ω the width of the peak at the baseline. The subscripts 1 and 2 refer to the first and second eluting diastereoisomer.

Table 1a
HPLC resolution on cellulose OJ: retention time (t_r, min) and retention factor (k') of second eluted isomer, selectivity (α) and resolution (R_s)

Compound	CSP	Mobile phase	T (°C)	t_{r2}	k'_2	α	R_s
1	OJ	B	25	14.70	2.59	1.59	2.24
1	OJ	C	25	9.87	1.41	1.60	1.77
1	OJ	G	25	12.14	10.19	1.72	1.09
2	OJ	C	25	34.89	7.51	2.72	5.18
2	OJ	E	25	14.60	2.56	2.67	3.45
2	OJ	G	25	41.52	9.13	2.76	2.65
3	OJ	B	25	26.15	5.38	1.74	3.05
3	OJ	C	25	15.85	2.87	1.74	2.50
3	OJ	G	25	19.18	3.68	1.65	1.16
4	OJ	C	25	41.28	9.07	2.50	4.58
4	OJ	E	25	16.96	3.14	2.44	3.08
4	OJ	G	25	53.71	12.10	2.80	2.30
5	OJ	B	25	36.91	8.00	1.79	1.94
5	OJ	C	25	19.04	3.64	1.73	1.39
5	OJ	G	25	22.98	4.60	2.06	0.52

3. Results and discussion

Compounds **2**, **3** and **4**, which structures are similar, were shown to have identical behavior in HPLC and CE. Illustrations will be made towards results obtained for compound **3**.

3.1. HPLC separation

Tables 1–2 summarized chromatographic data obtained for the separation of diastereoisomeric mixtures **1**–**5** using different mobile phases on polysaccharide-based stationary phases Chiralcel OD-H, OJ and Chiralpak AD and AS.

On all columns, the increase in the percentage of organic modifier (ethanol: eluents A, B, C, D and E) in the mobile phase, leads to a decrease in the measured parameters k' and R_s for the five compounds. This expected phenomenon results from the higher polarity of the mobile phase, improving solubility of the solutes in the mobile phase and decreasing hydrogen bonding occurrence between the solute and the stationary phase.

Performances of 1-propanol were evaluated (data not shown): in all cases replacement of ethanol by 1-propanol resulted in a great decrease in resolution, which led us to reject this organic modifier. Moreover, on changing from ethanol (eluent C) to 2-propanol (eluent G), an increase in the retention factor, k′, was observed on

Table 1b
HPLC resolution on cellulose OD-H: retention time (t_r, min) and retention factor (k') of second eluted isomer, selectivity (α) and resolution (R_s)

Compound	CSP	Mobile phase	T (°C)	t_{r2}	k'_2	α	R_s
1	OD-H	A	25	26.59	4.66	1.11	0.99
1	OD-H	C	25	7.49	0.59	1.08	0.49
1	OD-H	G	25	8.76	0.86	1.10	0.87
2	OD-H	A	25	50.20	9.68	1.12	1.15
2	OD-H	C	25	10.07	1.14	1.18	1.13
2	OD-H	G	25	14.82	2.15	1.11	0.62
3	OD-H	A	25	30.18	5.42	1.09	0.83
3	OD-H	C	25	7.73	0.64	1.09	0.45
3	OD-H	G	25	10.28	1.19	1.00	0.00
4	OD-H	A	25	47.66	9.14	1.09	0.84
4	OD-H	C	25	10.00	1.13	1.10	0.62
4	OD-H	G	25	14.96	2.18	1.13	0.76
5	OD-H	A	25	30.22	5.43	1.00	0.00
5	OD-H	C	25	6.94	0.48	1.00	0.00
5	OD-H	G	25	10.37	1.21	1.18	0.65

Table 2a
HPLC resolution on amylose AD: retention time (t_r, min) and retention factor (k') of second eluted isomer, selectivity (α) and resolution (R_s)

Compound	CSP	Mobile phase	T (°C)	t_{r2}	k'_2	α	R_s
1	AD	B	25	20.66	3.59	1.38	2.18
1	AD	C	25	13.44	1.99	1.38	1.82
1	AD	G	25	11.28	1.51	1.00	0.00
2	AD	C	25	32.60	6.24	1.46	3.00
2	AD	D	25	22.43	3.98	1.44	2.74
2	AD	G	25	19.94	3.43	1.00	0.00
3	AD	B	25	33.32	6.40	1.08	0.63
3	AD	C	25	19.54	3.34	1.05	0.48
3	AD	G	25	17.86	2.97	1.03	0.26
4	AD	C	25	26.22	4.83	1.46	2.85
4	AD	D	25	17.51	2.89	1.44	2.50
4	AD	G	25	16.39	2.64	1.00	0.00
5	AD	C	25	38.03	7.45	1.82	3.67
5	AD	D	25	23.39	2.22	1.77	3.25
5	AD	G	25	23.02	4.12	1.81	3.47

Table 2b
HPLC resolution on amylose AS: retention time (t_r, min) and retention factor (k') of second eluted isomer, selectivity (α) and resolution (R_s)

Compound	CSP	Mobile phase	T (°C)	t_{r2}	k'_2	α	R_s
1	AS	A	25	36.49	7.29	1.59	2.84
1	AS	B	25	15.79	2.59	1.58	2.00
2	AS	C	25	36.62	7.32	1.65	2.22
2	AS	D	25	21.42	3.87	1.66	1.61
2	AS	G	25	122.25	26.78	2.70	3.17
3	AS	A	25	45.38	9.31	1.45	2.61
3	AS	B	25	16.41	2.73	1.47	1.82
3	AS	G	25	21.20	3.82	1.00	0.00
4	AS	A	25	85.39	18.41	1.29	1.51
4	AS	C	25	17.17	2.90	1.18	0.78
4	AS	G	25	44.33	9.08	1.14	0.43
5	AS	C	25	23.44	4.33	1.59	0.90
5	AS	D	25	12.78	1.90	1.54	0.71
5	AS	G	25	48.80	10.09	1.00	0.00

both Chiralcel OD-H, OJ and on Chiralpak AS, as expected from the higher polarity of ethanol compared with 2-propanol [41]. Nevertheless, this greater retention leads, in most cases, to a smaller resolution of the diastereoisomers. This suggests that hydrogen-bonding interactions are probably not the predominant type but separation might essentially rely on inclusion into the chiral groove [42] and establishment of dipole–dipole interactions between the solute and the stationary phases. On Chiralpak AD column, use of 2-propanol instead of ethanol leads to a decrease in both retention and resolution. On this column too, the retention mechanism does not lie on hydrogen bonding, inclusion in the chiral cavities, opened by higher alcohol, that can change of geometry and/or size according the kind of organic modifier [43–45], may play an important role [42,43].

Thus, ethanol appears to be the more effective organic modifier for the four stationary phases studied.

Whereas baseline separation is achieved for almost all studied compounds on Chiralcel OJ and on both Chiralpak AD and AS, complete resolution is never obtained using Chiralcel OD-H. As illustrated by shorter retention times, interactions involved with Chiralcel OD-H are weaker in comparison with Chiralcel OJ and lead to an inferior resolution. The same phenomenon can be observed by changing from Chiralpak AD to Chiralcel OD-H. In this case, chiral cavity size and/or geometry of Chiralpak AD seem to be more convenient for the chiral inclusion of the solute that is involved in the retention and in the separation mechanism. Moreover, despite small structural differences between Chiralpak AS and Chiralpak AD, the retention and the resolution observed with Chiralpak AS are smaller (for **5** t_{r2} and R_s are 23.44, 0.90 and 38.03, 3.67 on Chiralpak AS and AD with eluent C, respectively).

In conclusion, for compounds **1**–**4**, best separations (i.e. ratio R_s/t_{r2}) are obtained on Chiralcel OJ, using hexane/ethanol – 50/50, 70/30 and 80/20 for **4** and **2**, for **3** and for **1**, respectively. For compound **5**, Chiralpak AD used with a mobile phase consisting of a hexane/ethanol – 60/40 mixture is the more effective (Fig. 2).

After optimization, diastereoisomeric mixtures **1**–**5** were analyzed in the optimal conditions previously determined. The assay was validated for each diastereoisomer for detection and quantification limits. LOD and LOQ were calculated at a signal to noise ratio

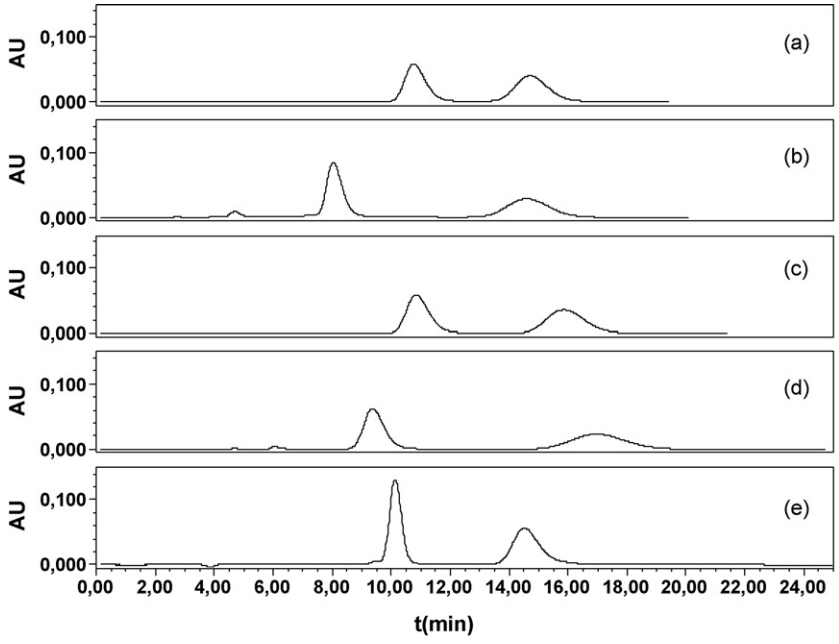

Fig. 2. Chromatograms obtained for the separations, on Chiralcel OJ for (a) compound **1**-Hexane/ethanol – 80/20, (b) compound **2**-hexane/ethanol – 50/50, (c) compound **3**-hexane/ethanol – 70/30, (d) compound **4**-hexane/ethanol – 50/50 and on Chiralpak AD for (e) compound **5**-hexane/ethanol – 60/40 at 0.50 mM in ethanol; flow-rate 0.8 mL min^{-1}, 303 K and λ = 266 nm.

Table 3
Limits of detection and quantification of pronucleotides **1**–**5** using optimal HPLC methods (see Section 3.1)

Compound	CSP	Mobile phase	diastereoisomer	LOD (μM)	LOQ (μM)
1	OJ	B	P1	0.20	0.66
			P2	0.22	0.75
2	OJ	E	P1	0.25	0.84
			P2	0.71	2.36
3	OJ	C	P1	0.30	1.01
			P2	0.46	1.52
4	OJ	E	P1	0.15	0.50
			P2	0.41	1.37
5	AD	D	P1	0.20	0.66
			P2	0.47	1.57

Table 5
Electrophoretic parameters of the separation of the diastereoisomers of the pronucleotides **1**–**5** with S-γ-CD at pH 2.5

Compound	S-γ-CD (mmol L^{-1})	V (kV)	t_{m2}	α	R_s	N
1	20	20	10.23	1.16	1.50	5252
	30	15	10.07	1.13	1.72	7928
	30	20	8.47	1.17	1.65	6410
3	20	20	16.38	1.28	4.81	6466
	30	15	11.82	1.21	4.01	6678
	30	20	8.78	1.19	3.11	6640
5	20	20	8.48	1.28	3.84	4170
	30	15	8.32	1.24	2.61	2431
	30	20	6.53	1.22	2.29	2151

288 K, without organic modifier in the BGE: migration time of second migrating isomer (t_{m2}, min), selectivity (α), resolution (R_s) and efficiency N.

equal to 3 and 10, respectively (Table 3) and were between 0.15 and 0.30 μM and 0.50–1.01 μM, respectively, for the first eluted peaks. They were between 0.22 and 0.71 and 0.75 and 2.36 μM for the second eluted peaks.

3.2. CE separation

pK_a values of pronucleotides **1**–**5** estimated from the ChemAxon's Marvin Program (Budapest, Hungary) were 9.36 and 13.2 for the pyrimidine ring part and the phosphoramide function (N deprotonation), respectively, for compound **5**, additional pK_a value of the phenolic function was 9.9 (O deprotonation). As a result, the diastereoisomers of **1**–**5** pronucleotides are uncharged in acidic and neutral media and negatively charged in alkaline medium. Moreover, hydrolysis of the SATE group in alkaline medium (unpublished data) led us to study the CE separation of the diastereoisomers both in acidic and neutral media, under pH 7.2 where these compounds are neutral.

Since uncharged CDs migrate at the same velocity as the electroosomotic flow (EOF), they only allow the separation of charged analytes. Therefore, use of charged CD is the only way in electrokinetic chromatography to achieve the separation of **1**–**5** diastereoisomers. In the first step, performances of carboxymethyl-β-CD, alone or in mixture with β-CD, were evaluated at pH 6.2 according to the method proposed by Perrin et al. [38], by injecting the sample at the anodic side. Lack of selectivity with CM-β-CD ($\alpha = 1$) led us to study the performances of two batches of different sulfated cyclodextrins at pH 6.2 and at pH 2.5 (manufacture recommendations). Results are summarized in Tables 4–7.

At pH 6.2, for all pronucleotides studied, first analysis using S-α-CD in 50 mM phosphate buffer at 288 K for an applied voltage of 10 kV, were not successful: whatever the concentration of S-α-CD used in the range 5–60 mM in 50 mM phosphate buffer (pH 6.2), an increase in the migration time from 5 to about 10 min is observed, without improvement of the resolution.

Use of S-β-CD in a concentration range between 20 and 40 mM (Table 4), in the same experimental conditions described before, results in higher migration times ($t_{m2} > 19$ min) for S-β-CD than with S-α-CD ($t_{m2} < 6$ min) and provides diastereoselectivity for **1** and **5** with resolutions greater than 0.8. The highest migration times are associated to highest analyte-CDs complexation; the α-CD cavity seems less favorable to the inclusion complexation than the β-CD cavity. In addition, the highest S-β-CD concentration tested leads to the highest migration times, due to greater analyte-CD complexation and results in the best resolution: an increase in the S-β-CD concentration from 20 to 40 mM results in two times higher resolutions for all compounds. Moreover, influence of the addition of organic modifier in the BGE on the separation was evaluated. ACN and ethanol leads to a decrease of both migration times and resolution. Surprisingly, as shown in Table 4, use of 10% of 2-propanol added in the BGE leads to a great decrease in the migration times, without major change in the resolution, and to a great increase in the efficiencies: for example, for **1**, presence of 10% of 2-propanol in a BGE containing 30 mM of S-β-CD leads to a 25% and 8% decrease of t_{m2} and R_s, respectively. The migration times decreases with organic modifier can be explain by the relative weak complexation of the analytes by the CD, resulting from the competition between the analytes and the organic solvent for the CD cavity and from the

Table 4
Electrophoretic parameters of the separation of the diastereoisomers of the pronucleotides **1**, **3** and **5** with S-β-CD and S-γ-CD at pH 6.2, 288 K: migration time of second migrating isomer (t_{m2}, min), selectivity (α), resolution (R_s) and efficiency N

Compound	CD	S-CD (mmol L^{-1})	% of organic modifier	V (kV)	t_{m2}	α	R_s	N
1	S-β-CD	20	0	10	19.68	1.05	1.23	8366
	S-β-CD	30	0	10	25.81	1.05	1.69	6668
	S-β-CD	30	10	10	19.16	1.02	1.54	106592
	S-β-CD	40	0	10	34.46	1.07	2.2	6545
	S-γ-CD	30	10	15	27.98	1.04	1.40	73895
3	S-β-CD	20	0	10	20.33	1.05	<0.8	7210
	S-β-CD	30	0	10	27.27	1.05	1.65	7026
	S-β-CD	30	10	10	20.25	1.02	1.50	112429
	S-β-CD	40	0	10	35.35	1.06	1.91	6057
	S-γ-CD	30	10	15	25.62	1.03	2.44	92951
5	S-β-CD	20	0	10	20.40	1.17	4.58	20303
	S-β-CD	30	0	10	26.35	1.20	6.33	18927
	S-β-CD	30	10	10	17.57	1.07	5.98	121539
	S-β-CD	40	0	10	32.55	1.22	7.49	15870
	S-γ-CD	30	10	15	28.40	1.12	6.24	46355

Table 6
Electrophoretic parameters of the separation of the diastereoisomers of the pronucleotides **1**, **3** and **5** with highly S-γ-CD at pH 2.5

Compound	Highly S-γ-CD (mmol L^{-1})	% of isopropanol	V (kV)	t_{m2}	α	R_s	N
1	20	0	20	17.48	1.07	0.95	3642
	30	0	20	16.72	1.10	1.84	6018
	30	5	20	23.88	1.06	1.12	5364
3	20	0	20	24.6	1.13	2.51	8802
	30	0	20	21.60	1.16	2.65	5064
	30	5	20	29.20	1.10	1.74	4712
5	10	0	30	15.53	1.31	5.51	6958
	20	0	30	10.48	1.27	5.01	7836
	30	0	20	14.97	1.34	6.73	9596
	30	0	30	10.02	1.25	5.03	7642
	30	5	20	22.5	1.34	4.73	4952
	30	5	30	17.55	1.26	3.67	4329

288 K: migration time of second migrating isomer (t_{m2}, min), selectivity (α), resolution (R_s) and efficiency N.

higher solubility of the analytes in the BGE. With 2-propanol, concerning resolution, lack of variation can be explained by the great increase in the efficiencies (increase in efficiency of 1500% with 2-propanol). As described by Perrin et al. [38] with phosphotriesters analogues, separation performances of dual system, consisting of an anionic and neutral CDs mixture (S-β-CD and natives CDs), were studied in our experimental conditions for the separation of the phosphoramidates series. Unfortunately, no improvement of resolutive performances was observed.

Separative performances of S-γ-CD were then evaluated using a concentration of 30 mM and 10% of 2-propanol in the BGE to keep a well solubility of the compounds. The first experiments done at 10 KV did not allow migration of the analytes in less than 30 min. With an applied voltage of 15 kV (Table 4), S-γ-CD leads to the resolution of the diastereoisomers of all pronucleotides with migration times of about 28 min and resolutions from 1.40 to 6.24. For example, migration times and resolution of both diastereoisomers of **3** are 24.80, 25.62 and 2.44 min, respectively. Voltage of 20 kV leads to peaks broadening and degradation of the baseline.

In conclusion, at pH 6.2, the best separations (Fig. 3) are observed using a BGE containing 10% of 2-propanol and 30 mM of S-β-CD for the pronucleotides **1**, **4** and **5** and 30 mM of S-γ-CD for the pronucleotides **2** and **3**, respectively.

At the recommended pH 2.5, two batches of S-CD were tested (S-CD from Sigma–Aldrich and highly S-CD from Beckman Coulter). Their performances were evaluated using cathodic injection, the electroosmotic flow being negligible, in normal polarity mode ("short-end" mode) to detect compounds in short migration times (t_m < 30 min).

S-α-CD and S-β-CD and highly S-CD were shown to be unable to resolve the couple of diastereoisomers, whatever the concentration used in the range 10–30 mM.

Table 7
Limits of detection and quantification of pronucleotides **1**–**5** obtained with a BGE at pH 6.2 and 2.5 in CE (experimental conditions see Section 3.2)

Compound	pH	LOD (μM)	LOQ (μM)
1	6.2	35	117
	2.5	48	123
2	6.2	49	163
	2.5	59	198
3	6.2	59	197
	2.5	50	166
4	6.2	30	100
	2.5	47	157
5	6.2	14	47
	2.5	70	233

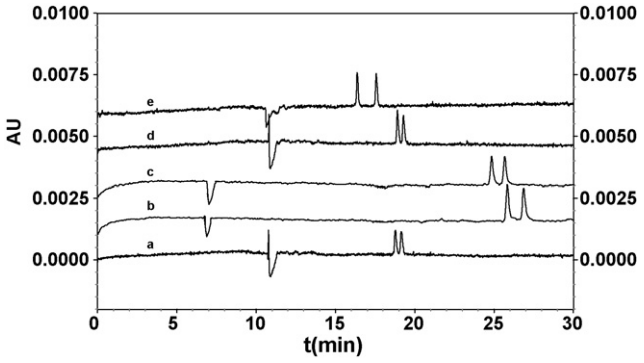

Fig. 3. Electropherograms obtained at pH 6.2 for the separations of diastereoisomers of (a) compound **1**, (b) compound **2**, (c) compound **3**, (d) compound **4** and (e) compound **5**. Conditions: fused-silica capillary 50.2 cm (effective length 40.2 cm) × 50 μm I.D. at 288 K; BGE, 50 mM phosphate buffer pH 6.2 (H_3PO_4 + TEA) containing 10% of 2-propanol and 30 mM of S-β-CD for the separation of **1**,**4** and **5** or 30 mM of S-γ-CD for the separation of **2** and **3**; UV detection at λ = 266 nm; anodic injection, 0.5 psi pressure for 5 s of 0.5 mM solution; applied voltage 10 kV using S-β-CD (thin) and 15 kV using S-γ-CD (bold).

Finally, S-γ-CD and highly S-γ-CD were proven to be the most adequate CD as they lead to the separation of all diastereoisomer pairs. Results obtained with these both CD in the 10–30 mM concentration range, with an applied voltage of 15 or 20 kV, are summarized in Tables 5 and 6.

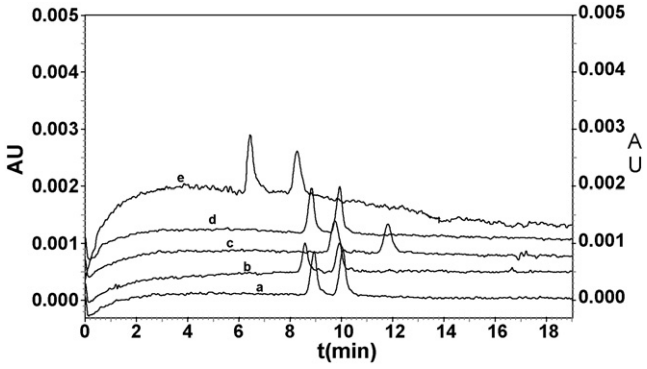

Fig. 4. Electropherograms obtained at pH 2.5 for the separations of diastereoisomers of (a) compound **1**, (b) compound **2**, (c) compound **3**, (d) compound **4** and (e) compound **5**. Conditions: fused-silica capillary 50.2 cm (effective length 10 cm) × 50 μm I.D. at 288 K; BGE, 50 mM phosphate buffer pH 2.5 (H_3PO_4 + TEA) containing 30 mM of S-γ-CD for the separation of **1**–**4** and 20 Mm of S-γ-CD for the separation of **5**; UV detection at λ = 266 nm; cathodic injection, 0.5 psi pressure for 5 s of 0.5 mM solution; applied voltage, 15 kV for **1**–**4** and 20 kV for **5**.

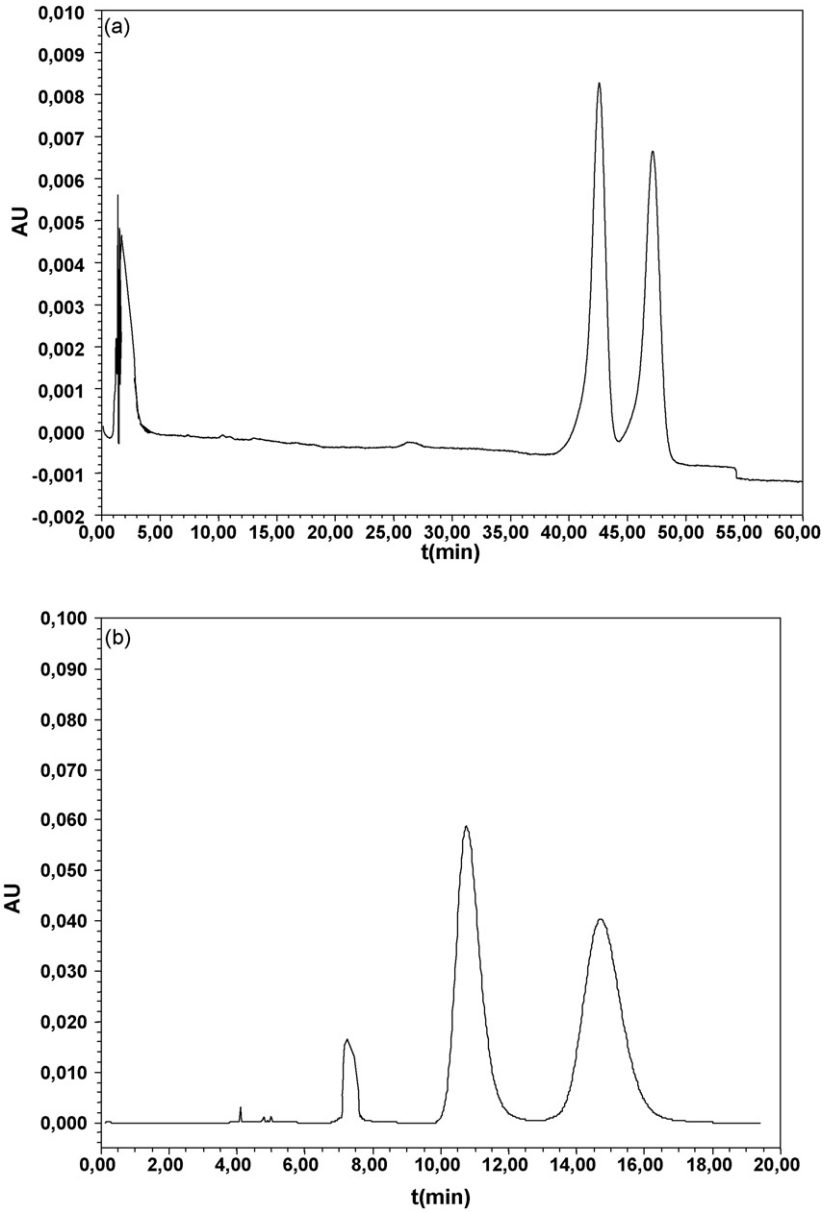

Fig. 5. Chromatogram obtained for the separation of **1** (50 μM) after cleaning from cellular extract. Chromatographic conditions: (a) LiCrospher® RP-18, eluent: methanol/acetate buffer (20 mM, pH 6.6) 50/50 (v/v), 0.8 mL min^{-1}, 308 K; (b) Chiralcel OJ, eluent B, 0.8 mL min^{-1}, 298 K. Detection: λ = 266 nm.

Migration times observed with these CD with respect to their β-CD analogues, in same experimental conditions, are much greater. However, the highest enantioselectivities are obtained with anionic γ-CD, as previously observed at pH 6.2. As expected, for both CD and for all pronucleotides, an increase in the concentration of the CD leads to a decrease in the migration times, resulting from a higher complexation, and to decrease in the resolution. In order to increase solubility of the analyte in the BGE and then the efficiency and resolution, addition of 2-propanol at a 5% level in the BGE was realized using highly S-γ-CD. In all case, an increase in the migration time associated with a decrease in the resolution was observed. This phenomenon might be explained by the competition between this organic modifier and the analyte for the CD cavity, predominant with respect to the increase of the solute solubility in the BGE. Use of organic modifier was then dismissed. For pronucleotides **1**–**4**, resolutions per time unit are smaller with highly S-γ-CD than with S-γ-CD, whereas for **5** they are of the same magnitude.

At pH 2.5, S-γ-CD was then selected for the resolution of all pronucleotides pairs. Concentration of S-γ-CD and voltage chosen for **1**–**4** and **5** were 30 mM, 15 kV and 20 mM, 20 kV, respectively. Electropherogramms obtained in these conditions are presented in Fig. 4.

In conclusion, with regard to the results obtained at pH 2.5 and 6.2, it appears that best separations and shortest migration times are obtained at pH 2.5 using S-γ-CD. Nevertheless, efficiencies are better at pH 6.2 because of the influence of the appropriate use of 2-propanol. Comparison of the limit of detection and quantification must then be taken into account before to choose optimal experimental conditions for further cell extract selective dosage.

After optimization, diastereoisomeric mixtures **1**–**5** were analyzed in the optimal conditions previously determined at pH 6.2 and 2.5. The assay was validated for each diastereoisomer for detection and quantification limits. LOD and LOQ calculated at a signal to noise ratio equal to 3 and 10, respectively, were identical for both

diastereoisomers (Table 7). At pH 6.2, they were between 14 and 35 μM and 47 and 197 μM, respectively, whereas at pH 2.5 they were between 47 and 70 μM and 157 and 233 μM, respectively. Despite longer migration times, best results in term of detection and quantification are obtained at pH 6.2, especially for compound **5**. This might be explained by high efficiencies resulting from the use of 2-propanol.

Capillary electrophoretic separation methods developed at pH 6.2 are more suitable than methods developed at pH 2.5 for the quantitative analysis of pronucleotide diastereomeric mixtures.

3.3. Application of the optimal separation method for analysis in cell extracts

For **1**–**4**, the HPLC methods developed, using Chiralcel OJ with hexane/ethanol mobile phases, allowed the separation of diastereoisomers in shorter times than CE methods developed at pH 6.2 using sulphated CDs. For **5**, CE method developed at pH 6.2, using γ-SCD, leads to a better resolution in a shorter time. Nevertheless, as for **1**–**4**, limits of detection and quantification obtained using CE for **5**, much greater than using HPLC (factor 100–200), lead us to choose the HPLC methods for the further analyzes of these compounds in biologic media.

Compound **1** (50 μM) was incubated in total cell extract from lymphocytes (CEM-SS cells) to mimic the behavior of the nucleotide inside the targeted cells. An analysis method including an on line SPE cleaning coupled to an achiral HPLC separation with a C18 type phase was described [39,40]. The recoveries obtained for **1**–**5** were between 81.2% and 97.3%. In the case of these nucleotide diastereoisomers series, no baseline separation is obtained with retention times inferior to 30 min, which is a limitation of the method. Thus we proposed here an alternative method with normal chiral-phase mode. By using the cleaning method, the collected fraction of interest compound containing water in the mobile phase (not compatible in this separation mode) was lyophilized and the residue was dissolved in adequate anhydrous solvent (ethanol). Typical chromatograms for the two eluted peaks of pronucleotide **1** on the LiCrospher® RP-18 and Chiralcel OJ columns, after precolumn purification of cellular extract containing samples, are shown in Fig. 5a and b, respectively. The achiral separation method using LiCrospher® RP-18 does not lead to the baseline separation of the diastereoisomers, despite high analysis times (Fig. 5a). The chiral method using Chiralcel OJ permits to resolve both diastereoisomers and the residual proteins (Fig. 5b). In these conditions, LOD are 0.24 and 0.26 μM for first and second eluted diastereoisomers, respectively.

4. Conclusion

Separation of diastereoisomers of five phosphoramidate derivatives was investigated both in HPLC and in CE. The HPLC methods developed using Chiralcel OJ and Chiralpak AD with mixtures of *n*-hexane/ethanol as mobile phases, allowed baseline separation of all pronucleotides in less than 20 min with resolution between 2.24 and 3.45. Electrophoretic methods developed at pH 6.2 and 2.5 leads to the baseline resolution of all compounds in less than 27 and 12 min, respectively, with resolution per time units between 1.5 and 6. Nevertheless evaluation of limit of detection and quantification, up to 200 times higher for the CE separations than for the HPLC separations, leads us to retain HPLC methods for the further analyze of pronucleotide in biological medium. Finally, in a preliminary study, the chiral HPLC separative method was successful applied to the analysis of both diastereoisomers of compound **1** in a cellular extract after protein cleaning.

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