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Diastereoisomeric resolution of a pronucleotide using solid phase extraction and high performance liquid chromatography: Application to a stereoselective decomposition kinetic in cell extracts

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Abstract

A stereospecific HPLC methodology has been developed for the diastereoisomeric resolution of a mononucleotide prodrug in cell extracts. This method involves the use of solid phase extraction on a C18 cartridge. Diastereoisomers and internal standard resolutions were performed on a cellulose based chiral column (Chiralcel OD-H) used in the normal phase mode. The method was validated in terms of specificity, recovery, linearity (diasteroisomers mixture concentration: 3–60 $\mu mol\,L^{-1}$), precision and accuracy and detection limit (1.67 and 1.33 $\mu mol\,L^{-1}$ for first and second eluted diastereoisomer). This method was applied to the determination of the apparent rate constants of disappearance and half-lives of each stereoisomers. This permits to conclude to the stereoselectivity of the enzymatic activity involved in the decomposition pathway of **2**.

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Keywords: Phosphotriester derivative; SPE; HPLC; Cell extracts; Stereoselective kinetic

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 phosphotriester derivatives bearing one *S*-acyl-2-thioethyl (SATE) group and an aryl residue as biolabile phosphate protections [7–10]. We demonstrated that these mixed pronucleotides allow an efficient *in vitro* intracellular delivery of the parent 5′-mononucleotide. Their decomposition pathway requires successively an esterase activation step followed by a phosphodiesterase hydrolysis (Fig. 1). Among all developed chiral pronucleotides, three phenylphosphotriester derivatives of AZT, **1**–**3** (Fig. 2), were shown to exhibit a pharmacodynamic activity similar to AZT activity (data not shown). This activity decreases when the number of hydroxyl substituents of the tertiobutyl group increases. Nevertheless, an increase in the stability in cellular extracts appears when increasing the number of hydroxyl substituents: half-lifes, $t_{1/2}$, for **2** and **3** are greater than $t_{1/2}$ of **1** (1.2 h) [7]. These two opposite effects lead us to select the monohydroxylated derivative **2** for a preliminary kinetic study. The resulting pronucleotide **2** exists as a mixture of two diastereoisomers produced by the chirality of the phosphorus atom. As observed in other pronucleotide series [11–14], configuration at the phosphorus center may have a significant impact on the *in vitro* antiviral activity, enzymatic recognition as well as transport of each diastereoisomers. Consequently, the development of a rapid and reliable analytical method for the separation of diastereomeric pronucleotide **2** in biological media, especially in cell extracts, is needed.

Diastereoisomeric separation of this pronucleotide was achieved by capillary electrophoresis [15]. Use of an HPLC

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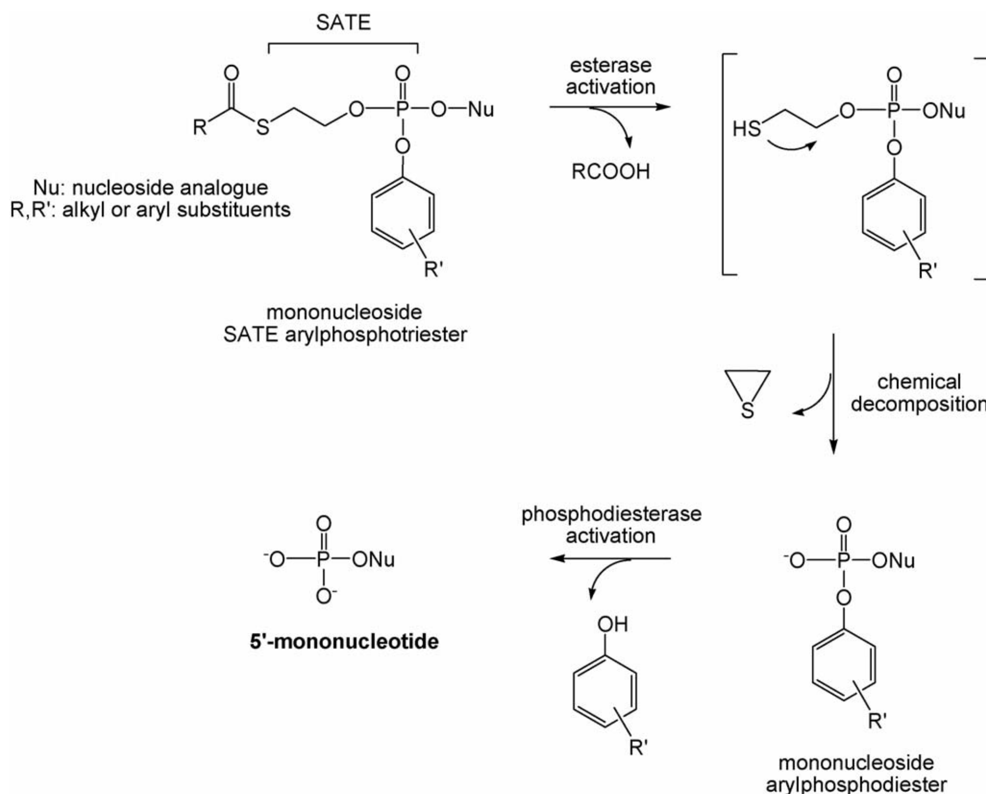

Fig. 1. General structure of the studied pronucleotides and their decomposition mechanism into the corresponding 5′-mononucleotide.

method is another alternative for the resolution of the diastereoisomers. An achiral method using a C18 phase was previously described [16,17]. Nevertheless, separation of diastereoisomers can be achieved by chiral HPLC, a well established method with over 100 different chiral stationary phases (CSP) commercially available. Several separations of the enantiomers or diastereoisomers of compounds containing a phosphorus asymmetric center by HPLC have been reported [18–22]. In a previous work [23], the direct diastereoisomeric separation of pronucleotide **2**, without any pre-derivatisation, was studied

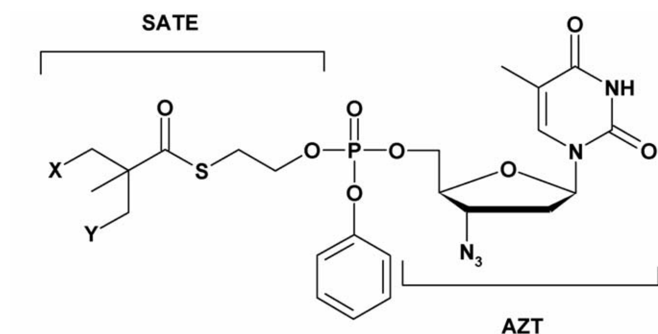

Fig. 2. Chemical structures of the studied pronucleotides **1**–**3**.

using chiral stationary phases such as cellulose (Chiralcel OD-H, OJ, OD-RH and OJ-R) and amylose (Chialpak AS and AD) in normal and reversed phase modes, or immobilized cyclodextrines (Cyclobond I 2000, β-CD and Cyclobond I 2000 RSP, HP-β-CD) in reversed phase and polar organic modes.

Despite the performances of Chiralcel OD-H phase, direct analysis of **2** in cell extracts cannot be envisaged without preliminary sample cleaning, to discard hydrophilic proteins and to obtain a non-aqueous sample. Different techniques could have been used, but solid phase extraction (SPE) appears to be the most performant in terms of time, solvent consumption and selectivity, as illustrated by the extent of its use. Several methods have been developed for the analysis of nucleosides in plasma using SPE coupled to HPLC in reversed phase mode [24–26]. Concerning nucleotides in biological medium, the most reported methods available in the literature deal with HPLC analysis of ionizable organophosphorus compounds. They are based either on the dephosphorylation of nucleotides, followed by a solid phase extraction of the nucleoside analogues [27,28], or on direct sample cleaning using an exchange anion solid phase extraction procedure [29–31]. To our knowledge few methods for the analysis of non-ionizable nucleotide in biological medium are available in the literature. Siccardi et al. [13] proposed a reversed phase HPLC method for the analysis of chiral phosphoramidates in culture medium after proteins precipitation and centrifugation of the sample. Other protocols are proposed for the determination of organophosphorus pesticides in biological medium or in food. They involve a solid phase extraction procedure using C18

[32,33] or hydrophilic–lipophilic balanced copolymer phases [34].

In the continuity of our work [23], we reported here a study on the direct separation of the diastereoisomers of **2** in cell extracts, by normal phase chiral HPLC on Chiralcel OD-H, after a cleaning step using solid phase extraction in reversed phase mode (C18 phase). In a first step, the method was optimized. Then, it was validated by the study of specificity, linearity, precision, accuracy and recovery, and by calculation of the limits of detection (LOD) and quantification (LOQ) in the determined optimal separation conditions. This method enables stereoselective analysis of both diastereoisomers of **2** and exhibits better performances than electrophoretic method reported by Perrin et al. [15] in terms of resolution and limit of quantification (factor 6).

A kinetic study of decomposition of both diastereoisomers of **2** in cell extracts completes this work.

2. Experimental

2.1. Reagents and chemicals

The hydroxy *t*BuSATE phenylphosphotriester derivative of AZT **2** (Fig. 2) was obtained by extension of a published procedure [7]. The title compound was found to be pure by rigorous HPLC analysis, high-field multinuclear NMR spectroscopy, and high-resolution mass spectroscopy. The diastereomeric ratio (56/44) was determined by ^{31}P NMR. Internal standard, AZT, was purchased from Instel Marsing France. Methanol, ethanol, 2-propanol and *n*-hexane were analytical grade obtained from Merck (Nogent sur Marne, France) or Baker (Noisy le Sec, France). Dimethyl sulfoxide (DMSO), glacial acetic acid and triethylamine were purchased from Acros Organics (Noisy le Grand, France). Deionized (DI) water was obtained from Milli-Q system (Millipore, Saint Quentin en Yvelines). Sep-pak Plus C8 and C18 cartridges containing 400 and 360 mg of stationary phase respectively, were purchased from Waters (Saint Quentin en Yvelines, France). The Vac-Elut vacuum manifold was obtained from Varian Sample Preparation Products (Les Ulis, France). Acetate buffer (pH 6.6; 20 mM) was prepared from acetic acid solution adjusted by addition of triethylamine.

2.2. Preparation of stock and standard solutions

Stock solutions containing 2 mmol L^{-1} of **2** (mixture of two diastereoisomers) and 0.2 mmol L^{-1} of AZT, used as internal standard, were prepared by dissolving appropriate amounts in deionised water–DMSO mixtures (90:10, v/v). They were stored and protected from light at 4 °C. Pronucleotide **2** standard solutions, used for spiking blank cell extracts, were prepared with deionised water–DMSO mixtures (90:10, v/v) from stock solution to yield final concentrations of 0.06, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mmol L^{-1}. Stock and standard solutions were shown to be stable over 6 months.

2.3. Preparation of spiked cell extract samples

Human CEM Leukemia cells were a gift from Dr. A.M. Aubertin (INSERM U74, Strasbourg, France) [35]. Cells were grown at 37 °C in an humidified atmosphere containing 5% CO_2 in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mmol L^{-1}), 1.5 g L^{-1} sodium bicarbonate, penicillin (100 IU mL^{-1}) and streptomycin (20 μg mL^{-1}) (Gibco BRL, France). After centrifugation (10^4 g, 4 min, 4 °C), the cell pellet (about 5×10^7 cells) was resuspended in 2 mL of Tris–HCl buffer (pH 7.4, 10 mmol L^{-1}, KCl 140 mmol L^{-1}) and then sonicated. After lysate centrifugation (10^5 g, 1 h, 4 °C), the supernatant was filtered on Millex GV (Millipore 0.22 μm). The protein concentration was calculated by the method of bicinchoninic acid (BCA) (Interchim, Monluçon, France) using bovine serum albumin as reference standard [36]. Aliquot of homogeneizated cell extracts were stored at −80 °C until use.

Accurately measured standards solutions of **1** (0.06, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mmol L^{-1}, 10 μL each) were each added to 1 mL volumetric tubes followed by the addition of 20 μL of 0.2 mmol L^{-1} internal standard solution and 170 μL of cell extracts (71.4 μg) extemporary. Final concentrations in spiked cell extracts were 3, 10, 20, 30, 40, 50, 60 μmol L^{-1} for **2** respectively and 20 μmol L^{-1} for AZT, the final DMSO percentage being of 1.5%.

2.4. Chromatographic system

Measurements 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 205 and 266 nm. The sample loop was 20 μL (Rheodyne 7125 injector). Chiral chromatography was carried out on a Chiralcel OD-H (Tris-3,5-dimethylphenylcarbamate; 250 mm × 4.6 mm i.d.; 10 μm) (Daicel Chemical Industries, Baker France), with a LiChrospher® 100 Diol 5 μm guard column (Merck, Nogent-sur-Marne, France). The mobile phase consisting of hexane/2-propanol (70:30, v/v) was filtered through membrane (0.45 μm), degassed with a Waters in-line degasser apparatus and delivered at a flow rate of 0.8 mL min^{-1}. All the separations were carried out at 40 °C. The peak of the solvent front was considered to be equal to the dead time (t_0) and was about 4.70 min. For preliminary studies, compounds were chromatographed by dissolving them in ethanol to a concentration of about 0.50 mmol L^{-1} (concentration 100%) and passed through a 0.45 μm membrane filter prior loading the column.

2.5. Sample preparation

Sep-pak Plus C18 cartridges were attached to the vacuum manifold and conditioned with 2 mL of methanol followed by 1 mL of acetate buffer pH 6.6 and careful attention was taken so that the cartridges did not run dry. Hundred microliters of blank or of spiked cell extracts were transferred into the cartridges and a moderate vacuum was applied. After complete aspiration of the sample through the cartridge, it was washed with 2× 2.5 mL of acetate buffer, 1 mL deionised water and then dried under full vacuum for 15 min. Then, the pronucleotide **2** and

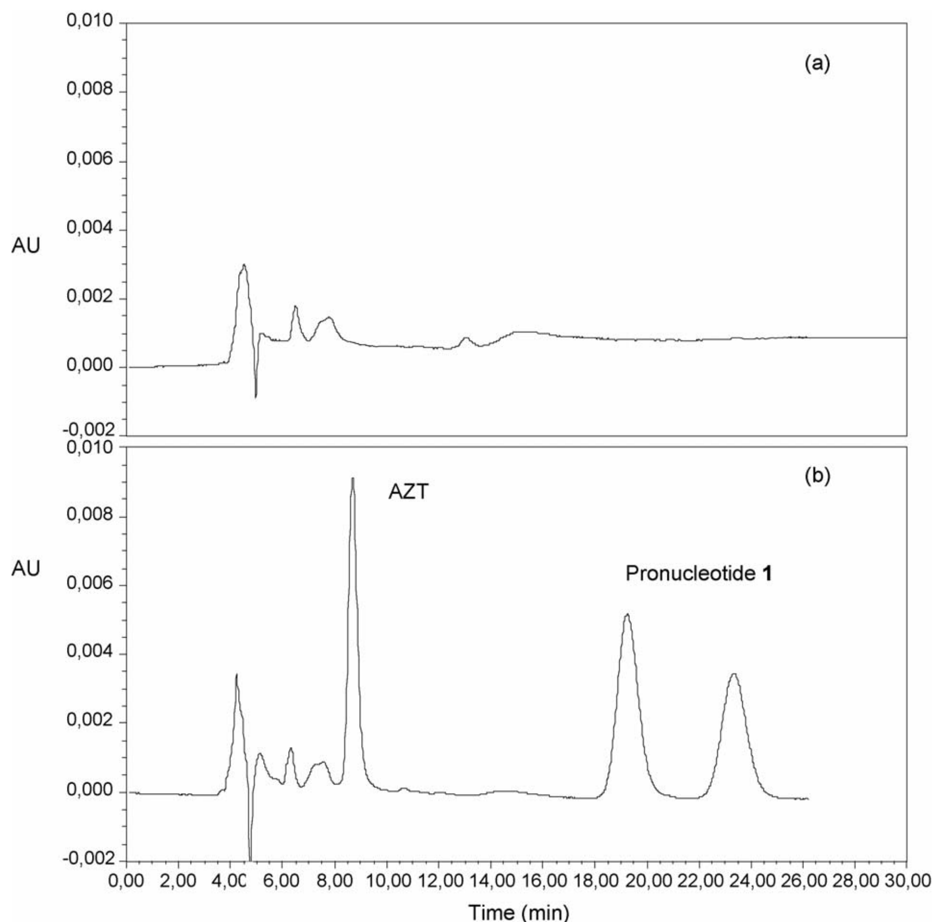

Fig. 3. Chromatograms obtained for (a) the blank cell extracts and (b) spiked cell extracts containing AZT (20 μmol L^{-1}), used as an internal standard, and both diastereoisomers of **2** (60 μmol L^{-1}). Chromatographic conditions were: Chiralcel OD-H at λ = 266 nm, eluent hexane/2-propanol (70:30, v/v), 40 °C, flow rate 0.8 mL min^{-1}.

AZT were eluted with 2 mL of methanol. The corresponding eluate was evaporated to dryness under a nitrogen steam at 40 °C. The residue was dissolved in 100 μL of ethanol; 20 μL of this solution were injected in the chromatographic system.

2.6. Validation of the method

2.6.1. Specificity

The specificity of the method was investigated by observing any interference encountered from the endogeneous cellular components. The assay was checked by analyzing three independent blank cell extracts. The chromatograms of these blank extracts were compared with chromatograms obtained by analyzing cell extracts spiked with **2** and internal standard AZT (Fig. 3).

2.6.2. Recovery

The recoveries of diastereoisomers of **2** and AZT from cell extracts were assessed by using spiked samples at 3, 30 and 60 μmol L^{-1} of **2** (mixture of both diastereoisomers) and 20 μmol L^{-1} of AZT (n = 3). The absolute recoveries were determined by comparison of the extracted analyte peak area with unextracted analyte peak area (standard evaporated, diluted in the corresponding amount of ethanol and directly injected in the HPLC system).

2.6.3. Linearity

Three independent series of spiked cell extracts containing 20 μmol L^{-1} of AZT and the following concentrations of first and second eluted diastereoisomers of **2** (C_1, C_2) in μmol L^{-1}, respectively: (1.67, 1.33), (5.57, 4.43), (11.14, 8.86), (16.71, 13.29), (22.28, 17.72), (27.85, 22.15), (33.42, 26.58), were extracted and analysed in duplicate according to the previously described methods. Calibration curves were plotted as the peak area ratio of the respective compounds to the internal standard versus the concentration. A linear regression using an ANOVA method was used to assess linearity.

2.6.4. Precision and accuracy

Seven replicates of cell extracts spiked with **2** (diastereoisomers mixture) at the concentration level of 3 μmol L^{-1} (limit of quantification), 30 μmol L^{-1} and 60 μmol L^{-1} were extracted and analysed to determine the intra-day precision (repeatability) defined by the relative standard deviation (% R.S.D.) and accuracy defined by the relative error (% error). Inter-day precision (intermediate precision) and accuracy were determined by

assaying the same three cell extracts (3, 30 and 60 μmol L^{-1}) in triplicate over a period of 3 days.

2.6.5. Limit of detection (LOD) and quantification (LOQ)

The limit of detection and quantification were determined as 3 and 10 times baseline noise, respectively [37], from the analysis of the mixture of both diastereoisomers of **2**, spiked in cell extracts.

2.7. Kinetic

The kinetic decomposition of both diastereoisomers of pronucleotide **2** (initial concentration 50 μmol L^{-1}) was studied at 37 °C in total cell extracts from lymphocytes (CEM-SS cells) containing 1.5% of DMSO and 20 μmol L^{-1} of AZT as internal standard. A series of 200 μL of identical assay were incubated between 0 and 1328 min, according to the above developed method [16,17]. For each sampling time, three samples were analysed. Hundred microliters of crude aliquots of incubates obtained between 0 and 1328 min were then analysed by HPLC after a SPE pre-cleaning step.

3. Results and discussion

3.1. Optimization of the chromatographic conditions and solid phase extraction method

3.1.1. Chromatographic method

Separations of the diastereoisomers of several compounds containing a phosphorus asymmetric center by HPLC have been reported, especially on polysaccharide chiral stationary phases using normal phase [19] or reversed phase mode [20,21]. The separation of the diastereoisomers of **2** was investigated using chiral stationary phases including cellulose (Chiralcel OD-H, OJ, OD-RH and OJ-R) and amylose (Chiralpak AS and AD) in normal phase mode and reversed phase mode. Since stationary phases with immobilized cyclodextrines (Cyclobond I 2000, β-CD and Cyclobond I 2000 RSP, HP-β-CD) are powerful tools for chiral separation [38,39], they were used in reversed phase and polar organic modes, to achieve the separation of the nucleotide diastereoisomers. In all cases, the influence of the mobile phase composition and temperature were investigated. Comparison of the phase nature and operating mode, indicated that better baseline separation and lower retention times were markedly obtained with cellulose and amylose in the normal phase mode. Use of Chiralcel OD-H with hexane/2-propanol (60:40, v/v) and (70:30, v/v) at 40 °C leads to the best results in terms of resolution *per* time unit:retention time of the first eluted diastereoisomer were 12.42 and 19.27 min and R_s were 1.67 and 2.45, respectively. The exact composition of the diastereoisomers mixture was determined by internal normalization. It contains 55.7% of first eluted diastereoisomer and 44.3% of second eluted diastereoisomer, which is in accordance with ^{31}P NMR results (unpublished data). The analysis of **2** using the previously reported HPLC achiral method [16,17], leads to worse results as retention times are superior to 50 min for a resolution of 1.37 (data not shown).

Table 1
Chromatographic parameters (mean ± S.D., $n = 3$) for pronucleotide **2** and the internal standard AZT, in spiked cell extracts

Analyte	t_r (min)	k	α	R_s	N
AZT	8.69 ± 0.05	0.85 ± 0.01			2982 ± 25
			3.65	9.69	
P_1	19.25 ± 0.07	3.10 ± 0.02			2598 ± 32
			1.28	2.45	
P_2	23.34 ± 0.08	3.97 ± 0.02			2706 ± 34

In order to improve the precision of the method, AZT was implemented as an internal standard. Indeed, during the stability studies in cell extracts, this nucleoside analogue was not observed as a product of the decomposition pathway of **2** [7]. This point was verified by incubating **2** (60 μM) at 37 °C in a total cell extract for a period of 1200 min without addition of internal standard. Analysis of this sample leads to a chromatogram without peak corresponding to AZT.

In addition, compared to the pronucleotide **2**, AZT exhibits closely UV properties and its lipophilic character would lead to similar chromatographic behaviour (on Sep-Pak Plus C8, C18 and OD-H). Two mobile phase compositions were tested: hexane/2-propanol 60/40 and 70/30. When hexane/ethanol 60/40 is used, coelution of AZT and components extracted from cell extracts lead us to use the less eluent mobile phase, i.e. hexane/2-propanol 70/30. High recovery from cell extracts during cleaning step on Sep-Pak Plus C18 (87%), and suitable retention time (8.69 min) obtained for AZT, associated with a resolution R_s of 9.69 between the first eluted diastereoisomer of **2** and AZT in these HPLC conditions (Table 1), lead us to select this nucleoside analogue as an internal standard.

3.1.2. Application to spiked cell extracts

In the course of developing a solid phase extraction procedure for cell extracts cleanup, Sep-pak Plus C8 and C18 were investigated. In both cases, the volume of acetate buffer to be used during the washing step of the extraction process was varied in the range 1–6 mL. When the acetate buffer volume used was less than 5 mL, a chromatographic peak corresponding to an impurity (neither phosphodiester, nor nucleotide monophosphate) was observed at 14.5 min. Its surface decreased when the washing volume increased. For these acetate buffer volumes (<5 mL), this impurity and the first eluted diastereoisomer were not totally resolved. When acetate buffer volume was greater than 5 mL, a decrease of the diastereoisomer recovery was observed. The best results were then obtained using 5 mL of acetate buffer (i.e. 2× 2.5 mL). For both SPE columns, no interference was observed in blank cell extracts at retention times for pronucleotide **2** diastereoisomers and AZT. The octyl (C8) SPE column was found to be unacceptable due to low recoveries (<50%) for both diastereoisomers, whereas the octadecyl (C18) SPE column gave high recoveries for diastereoisomers of **2** and AZT (minimum recoveries obtained at the three concentration levels studied for the first and second eluted diastereoisomers: 75.3 and 76.5%; for

Table 2
The precision (% R.S.D.) and accuracy (% error) for pronucleotide **2** in cell extracts based on $n = 7$ (intra-day) and $n = 9$ (inter-day)

Diastereoisomer	Added concentration (μmol L^{-1})	Intra-day			Inter-day		
		R.S.D. (%)	Found concentration (μmol L^{-1})	Error (%)	R.S.D. (%)	Found concentration (μmol L^{-1})	Error (%)
	1.67	3.7	1.77	+6.1	6.1	1.79	+6.9
P_1	16.70	3.2	15.63	−6.4	6.6	17.45	+4.5
	33.40	2.9	32.70	−2.1	3.4	32.8	−1.8
	1.33	3.6	1.26	−5.4	6.2	1.41	+6.3
P_2	13.3	2.8	13.85	+4.1	4.2	12.79	−3.8
	26.6	1.8	26.09	−1.9	2.5	26.2	−1.5

the internal standard: 87%). It was then selected for the cleanup procedure. Fig. 3 shows the chromatograms of blank cell extracts and spiked cell extracts containing AZT and both diastereoisomers of **2**.

3.2. Assessment of performance characteristics

3.2.1. Linearity

Statistical analysis was carried out as follows: variance homogeneity was assessed on recovery data to verify that no systematic day-to-day effect on the results was involved. This effect was not observed. A common linear regression analysis ($1X - 6Y$) for each diastereoisomers of **2** was constructed by plotting the peak area ratio of each diastereoisomer to the internal standard (int.st.) (Y) versus the analyte nominal concentration in the standards (spiked cell extracts) (X) in the range 1.67–33.40 and 1.33–26.60 μmol L^{-1} for the first and second eluted diastereoisomers, respectively. For the first and second eluted diastereoisomers noted with the subscripts 1 and 2, respectively, the regression line calculated using least squares method were:

$$\frac{A_1}{A_{\text{int.st.}}} = -0.0068 \pm 0.015 + (0.0372 \pm 0.0007)C_1,$$

A = peak area

$$\frac{A_2}{A_{\text{int.st.}}} = -0.0059 \pm 0.011 + (0.0377 \pm 0.0006)C_2,$$

C = concentration (mmol L^{-1})

with the confidence intervals calculated at $P = 0.05$.

The determination coefficient r^2 were 0.9937 and 0.9952 for first and second eluted diastereoisomers, respectively. An ANOVA method was used to confirm the linearity which was highly significant (Snedecor test: $F_1 = 1023$ and $F_2 = 1362 > F_{(0.05;1;34)} = 4.17$). The y-intercepts were not significantly different from zero and both regression lines have identical slope at the level $\alpha = 5\%$.

3.2.2. Precision and accuracy

A summary of the precision and accuracy results is given in Table 2. In the range 3–60 μM (diastereoisomers mixture) of pronucleotide **2**, the data indicated that intra-day precision and accuracy ($n = 7$) as expressed by % R.S.D. and % error were in the range 1.8–3.7% and 1.9–6.1%, respectively, for both diastereoisomers. At these concentrations, the inter-day precision (% R.S.D.) and accuracy (% error) ($n = 9$) were in the range 2.5–6.6% and 1.5–6.9%, respectively. Moreover, intra-day and inter-day precision and accuracy were determined at the limit of quantification (*vide infra*). These values are acceptable according to the common acceptance criteria of the FDA [40].

3.2.3. Limit of detection and quantification

The limit of detection for the first and second eluted diastereoisomers were 0.50 and 0.39 μmol L^{-1}, respectively. The limits of quantification, which corresponds to the first point in the calibration graph, were 1.67 and 1.33 μmol L^{-1} for the first and second eluted diastereoisomers, respectively. In both cases, they are smaller than quantification limits obtained by Perrin et al. [15] using chiral capillary electrophoresis (10 μmol L^{-1} for each diastereoisomer).

3.3. Stability of the pronucleotide in cell extracts

The diastereoisomeric mixture of **2** (50 μmol L^{-1}) was incubated in triplicate in total CEM-SS cell extracts to mimic the behavior of the pronucleotide inside cells. According to previously reported studies in the mononucleoside SATE arylphosphotriester series [7,9,10,41], pronucleotide **2** is bioconverted into its corresponding phenylphosphodiester (Fig. 1) through an esterase-mediated activation. The concentration time profile of both diastereoisomers of **2** was studied (Fig. 4) using

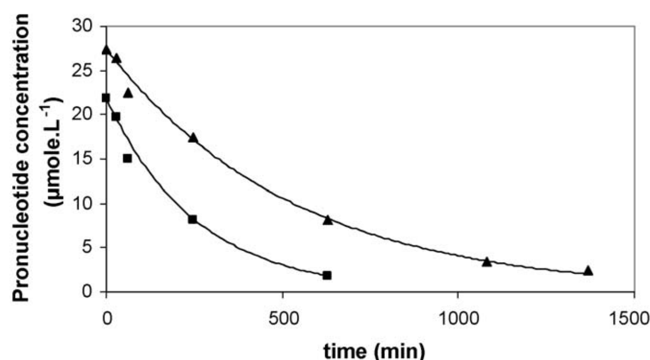

Fig. 4. Kinetic data of each diastereoisomer decomposition in cell extracts: first eluted diastereoisomer (▲) and second eluted diastereoisomer (■).

Table 3
Calculated half-lives ($t_{1/2}$) for the diastereoisomers of pronucleotide **2** in cell extracts and corresponding apparent pseudo-first-order rate constants (k)

Diastereoisomer	k (min^{-1})	$t_{1/2}$ (h)	r^2
P_1	1.90×10^{-3}	6.06	0.992
P_2	3.27×10^{-3}	3.50	0.997

r^2 = determination coefficient obtained for linear regression of $\ln C$ vs. t (C = diastereoisomer concentration (μmol L^{-1}) at t).

the developed method. For the second eluted diastereoisomer, concentrations obtained at 1080 and 1328 min could not be determined as they were under the limit of quantification. Kinetic data are treated according to the reciprocal competitive inhibition equation using Michaëlis-Menten model [42]. In this study, the decomposition of each diastereoisomer can be considered to be independent. The apparent rate constant of disappearance, k, and the half-life, $t_{1/2}$, can be determined according to the pseudo first-order kinetic model [16]. The convenience of the model used is attested by the linearity of $\ln C$ versus t ($r^2 > 0.99$). Data (Table 3) show significant differences in the decomposition kinetics of each diastereoisomer. The first eluted pronucleotide is about twice slower hydrolyzed than the second one ($k_1 = 1.90 \times 10^{-3}$ min^{-1}; $k_2 = 3.93 \times 10^{-3}$ min^{-1}). These results indicate that the enzymatic system (i.e. esterases) involved in the decomposition pathway of such pronucleotides seems to be able to discriminate between each diastereoisomer as previously observed in other pronucleotides series [11,43,44].

4. Conclusion

A rapid and specific isocratic chiral HPLC method, including a solid phase extraction cleaning step, has been developed for the study of pronucleotides in biological media. Their performances in terms of retention times and resolution are greater than previously reported HPLC achiral method. The method shows good characteristics of specificity, recovery, linearity, precision, accuracy and limit of quantification. This method can be applied to the stability study of a diastereoisomeric mixture of a SATE arylphosphotriester prodrug of AZT, contrary to the previously reported CE method for which limits of quantification are to high for this purpose. Thus, we have shown that decomposition kinetic of each diastereoisomer is different, which illustrates the stereoselectivity of the decomposition process mediated by an esterase activity.

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