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Separation of diastereoisomers of Ara-C phosphotriesters using solid phase extraction and HPLC for the study of their decomposition kinetic in cell extracts

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ABSTRACT

Separations of the diastereoisomers of three nucleoside 5′-phosphotriester derivatives of Ara-C (*t*BuSATE, hydroxy *t*BuSATE and bishydroxy *t*BuSATE phenylphosphotriester derivatives; pronucleotides) were performed by HPLC using derivatized cellulose and amylose chiral stationary phases. An optimal baseline separation (*Rs* > 1.5) was readily obtained with an amylose based chiral column (AD-H) used in normal phase mode. This stereospecific HPLC method has been associated to a solid phase extraction step using a C18 cartridge and an internal standard for the quantification of one nucleoside 5′-phosphotriester derivative in cell extracts. After optimization, this method was validated in terms of specificity, recovery, linearity, precision and accuracy and detection limit. It was applied to the determination of the apparent rate constants of disappearance and half-lives of each diastereoisomer. This enabled us to conclude that the enzymatic activity involved in the first step of the decomposition pathway of the hydroxyl *t*BuSATE phenylphosphotriester of Ara-C is stereoselective and is related to the nature of the pyrimidic base.

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1. Introduction

Ara-C (1-β-D-Arabinofuranosylcytosine) or cytarabine is a well-known nucleoside analogue used as a chemotherapeutic agent for the treatment of acute lymphoblastic and myelogenous leukaemia [1]. In an attempt to improve the therapeutic potential of this nucleoside analogue, especially to overcome cellular resistance mechanisms observed during long term chemotherapy with Ara-C, various mononucleoside prodrugs (pronucleotides) have been described during the last decade [2–6]. In this area, we previously reported the potential of a 5′-mononucleoside phosphotriesters derivative of Ara-C bearing two S-acyl-2-thioethyl (SATE) groups as biolabile phosphate protection (Fig. 1) [7]. In cell culture experiments, such bioconstruct allows the efficient intracellular delivery of the parent 5′-mononucleotide through an esterase-mediated activation [3,5] (Fig. 2). In a continuation of this work mixed phosphotriesters derivatives of Ara-C bearing a SATE group and an aryl residue have been developed (Fig. 1).

Owing to the presence of an asymmetric phosphorus atom, these pronucleotides exist as a mixture of two diastereoisomers. As observed in other nucleotide series, configuration at the phosphorus centre may have a significant impact on the in vitro antiviral activity [8–10], enzymatic recognition as well as the pharmacokinetic profile [11–15]. Consequently, the development of a rapid and reliable analytical method for the monitoring of diastereoisomeric pronucleotides is needed, especially in biological media.

Although non-chiral liquid chromatographic method using C8 or C18 phases and a polar organic eluent can be used for the resolution of diastereoisomers, methods described in the literature for phosphotriesters and phosphoramidates containing an asymmetric phosphorus centre [9,13,14,16–19] lead to poor separation. Separation of diastereoisomers can be achieved by chiral HPLC. Therefore, several chiral separation methods of mixed phosphotriesters or phosphoramidate diesters diastereoisomers have been proposed [11,15,18–25]. Most frequently used stationary phases are of a polysaccharide type whose separation efficiency has been particularly demonstrated for normal phase mode methods [15,19–21,25]. Use of these phases in normal and in reversed phase mode was then selected for development of an analytical method for the monitoring of both diastereoisomers of pronucleotides **1**–**3** (Fig. 1).

Analysis of these pronucleotides in cell extracts requires a preliminary sample cleanup in order to remove macromolecular compounds such as proteins and to obtain a non-aqueous sample. Among different techniques available, solid phase extraction (SPE) appears to be the most effective in terms of time, solvent consumption and selectivity, as illustrated by the extent of its use. The choice of SPE stationary phase to use is directly linked to the ionisation state (capacity) of the target compound and to the nature of the sample. As a result methods available in the literature for the extraction of anionic nucleotides (5′-mono or 5′-

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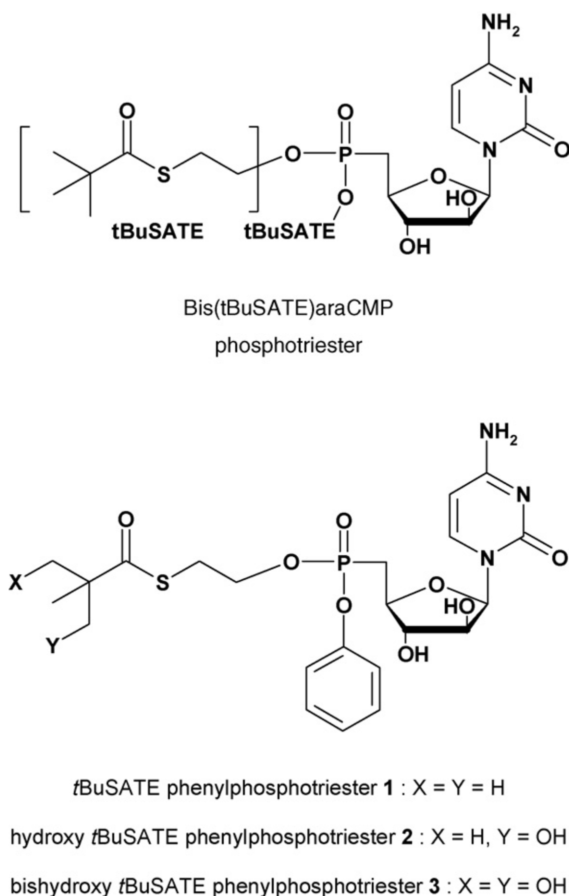

Fig. 1. Structure of Bis(*t*BuSATE)-AraCMP and of the studied mixed pronucleotides of Ara-C **1**–**3**.

triphosphate nucleosides) in biological media use anion exchange solid phases [26–28], whereas those for the extraction of non-ionized nucleotides essentially use a reversed phase extraction protocol with C18 SPE phases [15,29,30,31,32]. Because of the neutral nature of the target compounds in a wide pH range (between 4.28 and 12.96, pK_a: see Section 2.5.1.) and their hydrophobic character, SPE reversed phase methodologies were evaluated here.

According to a previous study relative to the pharmacodynamic activity of analogue phenylphosphotriester derivatives of AZT [33] and to their stability in cellular extracts, monohydroxylated derivative **2** (Fig. 1) was the lead compound in this series. We report herein a study of the direct separation of the diasteroisomers of phosphotriester **2** in cell extracts by HPLC using a polysaccharide CSP (Chiralpak AD-H) in normal phase mode, after a clean-up step using solid phase extraction in reversed phase mode (C18 phase). In a first step, an optimization of the HPLC procedure using polysaccharides CSP (Chiralcel OD-H and OJ, Chiralpak AD-H, AS and IA) in normal phase mode or in reversed phase mode (Chiralcel OJ-R) for compounds **1**–**3** was investigated. In a second step, the global protocol was finally validated for compound **2**, by the study of specificity, linearity, precision, accuracy and recovery, and by determination of the limits of detection and quantification in the optimal analysis conditions. This stereoselective analysis method was applied to the kinetic study of the decomposition of both diastereoisomers in cell extracts.

2. Experimental

2.1. Reagents and chemicals

The mixed phosphotriester derivatives of Ara-C **1–3** (Fig. 1) were obtained as diastereoisomeric mixtures by extension of a published procedure [32]. The diastereoisomeric ratios were determined by ^{31}P NMR; they are (56/44) for **1** and (54/46) for **2** and **3**, respectively.

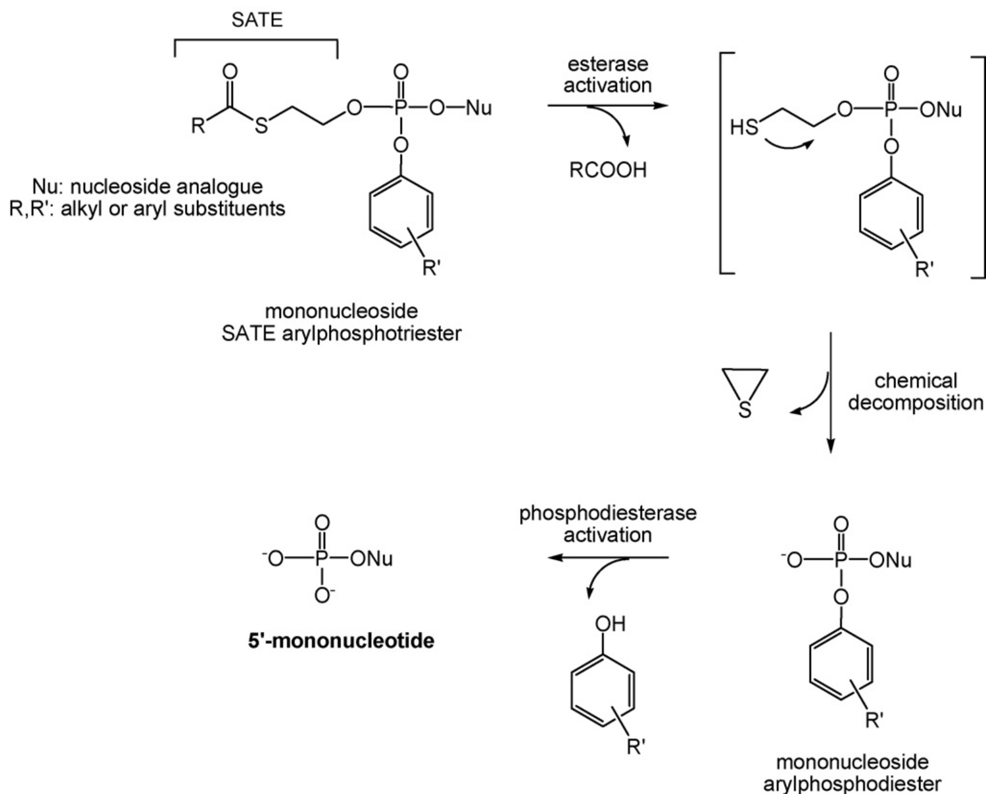

Fig. 2. General decomposition mechanism of phosphotriester derivatives bearing one SATE group and an aryl residue to the corresponding 5′-mononucleotide.

Antipyrin purchased from Sigma–Aldrich (Saint Quentin Fallavier, France) and caffeine from Acros (Halluin, France) were evaluated as internal standards. Methanol, ethanol, 1-propanol, 2-propanol, ethyl acetate, dichloromethane, methyl tertio-butyl ether and *n*-hexane were HPLC grade and obtained from VWR (Strasbourg, France). Acetonitrile, dimethyl sulfoxide (DMSO), glacial acetic acid and triethylamine were purchased from Acros Organics (Halluin, France). Sodium hydroxide 5 M, chlorhydric acid 1N, potassium chloride, TRIS and octane sulfonic acid were obtained from VWR (Strasbourg, France). Deionized (DI) water was obtained from Milli-Q system (Millipore, Saint Quentin en Yvelines). Strata Phenyl, C8 and C18 cartridges (containing 100 mg of stationary phase) and Strata X cartridges (containing 30 mg of stationary phase) were purchased from Phenomenex (Le Pecq, France). The Vac-Elut vacuum manifold was obtained from Varian Sample Preparation Products (Les Ulis, France).

2.2. Preparation of stock and standard solutions

For the optimization of the chromatographic separation, compounds **1**–**3** were dissolved in ethanol to a concentration of about 0.125 mmol L^{-1}. For biological study, stock solutions containing 2 mmol L^{-1} of **2** (mixture of two diastereoisomers) and 2 mmol L^{-1} of antipyrin, used as an internal standard, were prepared by dissolving appropriate amounts in 90/10 (v/v) deionised water/DMSO mixtures. They were stored in the dark at 277 K. Pronucleotide **2** standard solutions, which were used for spiking blank cell extracts, were prepared with 90/10 (v/v) deionised water/DMSO mixtures from stock solution to yield final concentrations of 0.012, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mmol L^{-1}.

2.3. Preparation of spiked cell extracts samples

Lymphoblastoid cell line (CEM-SS) cell extracts were kindly prepared by Dr. A. LANSIAUX according to published procedures [31]. Cells were grown at 310 K 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}), sodium bicarbonate (1.5 g L^{-1}), penicillin (100 IU mL^{-1}) and streptomycin (20 μg mL^{-1}) (Gibco BRL, France). After centrifugation (10^4 g, 4 min, 277 K), the cell pellet (about 5×10^7 cells) was resuspended in 2 mL of Tris–HCl buffer, (10 mmol L^{-1}, KCl 140 mmol L^{-1}, pH 7.4) and then sonicated. After lysate centrifugation (10^5 g, 1 h, 277 K), 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 a reference standard. Aliquots of homogeneizated cell extracts were stored at 93 K until use.

Accurately measured standards solutions of **2** (0.012, 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 (0.42 μg μL^{-1}) just before use. Final concentrations in spiked cell extracts were 6, 10, 20, 30, 40, 50, 60 μmol L^{-1} for **2**, respectively and 20 μmol L^{-1} for antipyrin, the final DMSO percentage being 1.5% in all solutions.

2.4. Chromatographic system

Measurements were carried out using a Waters 600E gradient metering pump model equipped with a Waters 996 photodiode array spectrophotometer. Chromatographic data were collected and processed on a computer running Millennium 2010. The column eluate was monitored at 277 nm. The sample loop was 20 μL (Rheodyne 7125 injector).

2.4.1. HPLC optimization

The following columns were tested: (i) a reversed-phase cellulose chiral stationary phases Chiralcel OJ-R (tris-methylbenzoate; 150 mm × 4.6 mm i.d.; 5 μm; Daicel Chemical Industries, Baker, France) was used. Mobile phase elution was made isocratically using reversed phase mode (water and an organic modifier, methanol or acetonitrile). The flow was 0.5 mL min^{-1}. (ii) A normal phase methodology was performed with two silica-based celluloses 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 amyloses Chiralpak AD-H (tris-3,5-dimethylphenylcarbamate; 250 mm × 4.6 mm i.d.; 5 μm), and a Chiralpak AS (tris-(*S*)-1-phenylethylcarbamate; 250 mm × 4.6 mm i.d.; 10 μm) (Daicel Chemical Industries, Baker France). Mobile phase elution was made isocratically using *n*-hexane and a modifier (ethanol, 1-propanol or 2-propanol) at various percentages. The flow was 1 mL min^{-1}. In this separation mode the peak of the solvent front was considered to be equal to the dead time (t_0) and was about 3.20 min for the Chiralcel OD-H and for Chiralpak AD-H and about 3.30 min for the Chiralcel OJ and Chiralpak AS. In all cases retention times were mean values of two replicate determinations. (iii) An isocratic normal phase methodology was performed on an amylose Chiralpak IA (tris-3,5-dimethylphenylcarbamate; 250 mm × 4.6 mm i.d.; 5 μm), using *n*-hexane or methyl tertio-butyl ether and a modifier (ethanol, 2-propanol, ethyl acetate or dichloromethane) at various percentages and a 1 mL min^{-1} flow rate. The separations were carried out at 293 K. Compounds were chromatographed by dissolving them in ethanol to a concentration of about 0.125 mmol L^{-1}.

2.4.2. Cellular extracts analysis

Chiral chromatography was carried out on a Chiralpak AD-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/ethanol – 75/25 (v/v) was filtered through membrane (0.45 μm) and degassed with a Waters in-line degasser apparatus. Use of a LiChrospher® 100 Diol guard column coupled with the Chiralpak AD-H CSP led to an increase in the column back pressure. As a result, to preserve the CSP, a 0.8 mL min^{-1} flow rate was finally selected for the analysis method. The separation was carried out at 293 K.

2.5. Sample preparation

2.5.1. SPE optimization

According to the pK_a values of pronucleotides **2** estimated from the ACD/Labs software (ACD, Toronto, Canada): 3.28 and 13.26 for the aglycon part (N protonation on the cytosine) and the primary hydroxyl function, respectively), the molecular neutral form is predominant for pH between 4.28 and 12.96, whereas the cationic form is predominant under pH 2.28. As a result, in the course of developing a solid phase extraction procedure for clean-up of cell extracts, Strata C8, C18, phenyl and Strata X cartridges, effective for the extraction of neutral compounds, were investigated. Initial clean-up procedures were the following: (i) for Strata C8, C18 and phenyl cartridges: conditioning – 2 mL methanol, 2 mL acetate buffer 20 mM (pH 6.6); sample loading – 100 μL of cellular extract (0.42 μg μL^{-1}) at 60 μmol L^{-1} of **2** and 20 μmol L^{-1} of internal standard; washing – 1.5 mL acetate buffer 20 mM (pH 6.6), 0.5 mL H_2O; elution – 2 mL methanol; (ii) for Strata X: conditioning – 2 mL dichloromethane, 2 mL acetonitrile, 2 mL H_2O; sample loading – 100 μL of cellular extract (0.42 μg μL^{-1}) at 60 μmol L^{-1} of **2** and 20 μmol L^{-1} of internal standard; washing – 2 mL H_2O; elution – 2 mL acetonitrile/dichloromethane (50/50, v/v). After

evaporation to dryness under nitrogen at 318 K, the residue was dissolved in 100 μL of ethanol and analysed. On Strata C18 and phenyl cartridges, the nature and the volume of the washing solution (phosphate buffer (pH 6.6), acetate buffer (pH 6.6) or H_2O of 1–2 mL) and the volume of the elution step (1–2.5 mL; methanol) on the cleanup and recovery, were examined.

2.5.2. Optimized cellular extracts protocol

Strata C18 cartridges were attached to the vacuum manifold and conditioned with 2 mL of methanol followed by 2 mL of deionised water. Care was taken to ensure that the cartridges did not run dry. 100 μL of blank or of spiked cell extracts were transferred onto the cartridges and a moderate vacuum was applied. After complete aspiration of the sample through the cartridge it was washed with 1 mL of deionised water and then dried under full vacuum for 15 min. Then, the pronucleotide **2** and antipyrin were eluted with 1.5 mL of methanol. The corresponding eluate was evaporated to dryness under nitrogen at 318 K. The residue was dissolved in 100 μL of ethanol; 20 μL of this solution was injected on 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 the internal standard antipyrin.

2.6.2. Recovery

The recoveries of diastereoisomers of **2** and antipyrin from cell extracts were assessed by using spiked samples at 6, 30 and 60 $\mu mol\,L^{-1}$ of **2** (mixture of both diastereoisomers) and 20 $\mu mol\,L^{-1}$ of antipyrin ($n = 3$). 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 $\mu mol\,L^{-1}$ of antipyrin and the following concentrations of first (P_1) and second (P_2) eluted diastereoisomers of **2** (C_{p1}, C_{p2}) in $\mu mol\,L^{-1}$, respectively, were extracted and analysed in duplicate according to the previously described methods: (2.76, 3.24), (4.60, 5.40), (9.20, 10.80), (13.80, 16.20), (18.40, 21.60), (23.00, 27.00), (27.60, 32.40). Calibration curves were plotted as the ratio of the peak area of the respective compounds to the peak area of 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 20 $\mu mol\,L^{-1}$ of antipyrin and with **2** (diastereoisomers mixture) at the concentration level of 6 $\mu mol\,L^{-1}$ (limit of quantification), 30 and 60 $\mu mol\,L^{-1}$ were extracted and analysed to determine the intra-day precision (repeatability) defined by the relative standard deviation (% RSD) and accuracy defined by the relative error (% error). Inter-day precision (intermediate precision) and accuracy were determined by assaying the same three cell extracts (6, 30 and 60 $\mu mol\,L^{-1}$) in triplicate over a period of 3 days.

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

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

2.7. Kinetic study

The kinetic decomposition of both diastereoisomers of pronucleotide **2** (initial concentration 50 $\mu mol\,L^{-1}$) was studied at 310 K in total cell extracts from lymphocytes (CEM-SS cells) containing 1.5% of DMSO and 20 $\mu mol\,L^{-1}$ of antipyrin as internal standard. A series of 200 μL of identical assay were incubated between 0 and 2947 min, according to the above developed method [34,35]. Hundred microliter of crude aliquots of incubates obtained between 0 and 2947 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 centre by HPLC have been reported, especially on polysaccharide chiral stationary phases used in normal phase [17] or reversed phase mode [18,19]. The separation of the diastereoisomers of **1**–**3** was investigated using coated polysaccharide chiral stationary phases including cellulose (Chiralcel OD-H, OJ, and OJ-R) and amylose (Chiralpak AS and AD-H) in normal phase mode and reversed phase mode. For comparison and to extend the range of mobile phase that can be used, an immobilized amylose chiral stationary phase Chiralpak IA (same chiral selector as Chiralpak AD-H) was also evaluated. In all cases, the influence of the mobile phase composition was investigated. Comparison of the phase nature (coated or immobilized) and operating mode (normal or reversed), indicated that the best baseline separation and the lowest retention times were markedly obtained with coated cellulose and amylose stationary phases used in normal phase mode. Indeed, on Chiralpak IA, with the same mobile phases as those used on AD-H (hexane/ethanol or hexane/2-propanol mixtures) lower resolutions for a similar retention times were observed as peaks are tailing. Use of *n*-hexane or methyl terbutyl ether with 10% to 60% of a modifier (ethanol, 2-propanol, ethyl acetate or dichloromethane) was unsuccessful (lack of resolution even for long retention times). With Chiralcel OJ-R, whatever the proportion of methanol or acetonitrile used in a mixture with deionised water, no separation was obtained in less than 40 min. Among coated cellulose and amylose stationary phases used in normal phase mode, Chiralpak OD-H and Chiralcel AS did not lead to any baseline resolution whatever the mobile phase composition. Table 1 summarizes chromatographic data obtained with Chiralpak AD-H and Chiralcel OJ using different mobile phases. On both columns, the increase in the percentage of organic modifier (ethanol: eluents A and C for example) in the mobile phase, leads to a decrease in the measured parameters *k* and *R*s for all compounds. This expected phenomenon results from the higher polarity of the mobile phase, improving the solubility of the solutes in the mobile phase and decreasing hydrogen bonding between the solute and the stationary phase. Replacement of ethanol (eluent A or C) by 1-propanol (eluent F) or 2-propanol (eluent G or H) does not lead to an increase in the resolution. Thus, for both cellulose and amylose stationary phases ethanol appears to be the best organic modifier. Higher retention and resolution *per* unit time observed on Chiralpak AD-H probably result from the involvement

Table 1
Chromatographic parameters: retention time (*t*), retention factors (*k*) and resolution (*Rs*) of compounds **1–3** on Chiralpak AD-H and Chiralcel OJ.

Compound	CSP	Eluent	k_2	*Rs*
1	AD-H	A	11.89	1.20
		C	2.20	<0.5
		F	8.68	0.60
		G	12.56	<0.5
		H	1.77	<0.5
	OJ	A	4.70	<0.5
		C	0.93	0.00
		F	6.80	0.00
		G	5.73	<0.5
		H	0.86	0.00
2	AD-H	A	44.37	5.16
		C	6.87	3.99
		D	4.31	3.25
		F	7.31	0.00
		H	4.31	0.00
	OJ	A	17.87	2.73
		B	5.20	1.71
		C	2.54	1.39
		E	0.88	0.75
3	AD-H	A	76.24	5.12
		C	9.41	3.93
		D	4.21	2.75
		H	5.97	0.00
	OJ	A	25.50	1.40
		C	3.11	0.71
		G	2.49	0.00

A: hex/EtOH – 90/10; B: hex/EtOH – 85/15; C: hex/EtOH – 80/20; D: hex/EtOH – 75/25; E: hex/EtOH – 70/30; F: hex/1-prop – 90/10; G: hex/2-prop – 90/10; H: hex/2-prop – 80/20.

of a greater number of chiral interactions. So, the amylose stationary phase was selected for the further separation of compounds **1**–**3** diastereoisomers. In conclusion, despite the number of experimental conditions evaluated, baseline separation of compound **1** was not achieved (maximal resolution: 1.20 in about 42 min on Chiralpak AD-H; Hexane/ethanol – 90/10). For compounds **2** and **3**, the best results in terms of resolution *per* unit time were obtained on a Chiralpak AD-H using hexane/ethanol – 75/25 at 1 mL min^{-1} and 293 K: retention time of the second eluted diastereoisomer was 17.51 and 17.19 min and *Rs* was 3.25 and 2.75 for **2** and **3**, respectively. Chromatograms obtained in these optimal conditions are shown in Fig. 3.

Exact composition of the diastereoisomers mixture was determined by internal normalization. It contains 46.4% of first eluted diastereoisomer and 53.6% of second eluted diastereoisomer for **2** and **3**, which is in accordance with ^{31}P NMR data (unpublished data).

The development of a separation method allowing the analysis of diastereoisomeric pronucleotides in biological media, especially in cell extracts, was then performed with pronucleotide **2**. In order to improve the precision of the method, use of an internal standard with similar chromatographic behaviour (on Strata Phenyl, C8, C18, X and on Chiralpak AD-H) and UV characteristics to **2** was necessary. Antipyrin and caffeine were evaluated according to their hydrophobic character and ionisation capacity estimated from the ACD Labs software (ln *P* equal to −0.5 and 1.2 for antipyrin and caffeine, respectively; ionisation capacity: $pK_a < 1$, neutral compounds in the experimental conditions) and UV properties (maximum absorption wavelength equal to 272 and 275 nm for antipyrin and caffeine, respectively). Chromatographic parameters of pronucleotide **2**, antipyrin and caffeine, using hexane/ethanol – 75/25 at 0.8 mL min^{-1} at 298 K on Chiralpak AD-H coupled to a guard column were determined. Whereas caffeine is eluted after the second eluting diastereoisomer of **2** (P_2) ($t_{caffeine}$ = 51.35 min; $Rs_{caffeine-P_2}$ = 12.40), antipyrin is eluted before the first eluting diastereoisomer

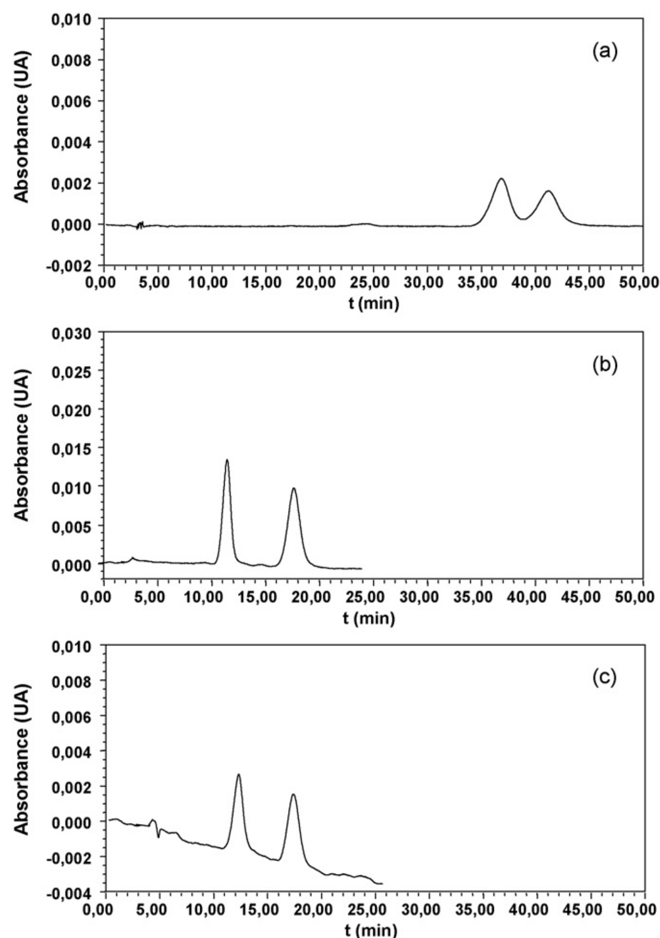

Fig. 3. Chromatograms obtained for the separations on Chiralcel AD-H for (a) compound **1**; Hexane/ethanol – 90/10, (b) compound **2**; hexane/ethanol – 75/25, (c) compound **3**; hexane/ethanol – 75/25, at 0.125 mmol L^{-1} in ethanol; flow-rate 1 mL min^{-1}, 293 K and λ = 277 nm.

of **2** (P_1) ($t_{antipyrin}$ = 9.52 min; $Rs_{antipyrin-P_1}$ = 4.85). Antipyrin was then chosen as an internal standard.

3.1.2. Solid phase extraction method

In a first step, the effectiveness of Strata C8, C18, phenyl and Strata X cartridges for the clean-up of cell extracts was studied using the general procedure described in the Section 2.4. Better recoveries obtained with Strata C18 and phenyl (71% and 61%, respectively) compared to Strata C8 and Strata X cartridges (32% and 30%, respectively) led us to follow the optimisation using these first two. In a second step, the influence of the washing and elution solutions on the cleanup and recovery was examined. Previous reported SPE procedure [15] using acetate buffer leads unfortunately to impurity peaks between 3.3 and 10 min interfering with antipyrin. The same result was obtained with phosphate buffer, whereas water allows total sample cleanup. Influence of the water volume to be used during the washing step was then evaluated on C18 cartridges in the range 1–2 mL (0.5 mL steps). Increase in the water volume from 1 to 2 mL leads to a decrease in the recovery from 80% to 72%. A 1 mL volume of water was then chosen, as sample cleanup was efficient. The volume of methanol to be used during elution step was then varied in the range 1–2.5 mL (0.5 mL steps). An optimal recovery of 86% was obtained with a 1.5 mL of methanol. Finally, the optimal procedure, leading to the best extraction recoveries of both diastereoisomers and the internal standard (minimum recoveries obtained at the three concentration levels studied for the first and second eluted diastereoisomers: 84.67% and 82.05%;

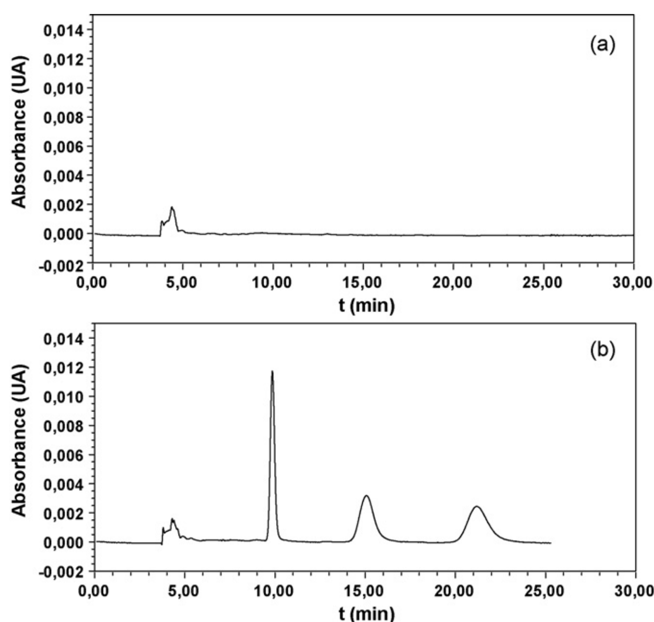

Fig. 4. Chromatogram obtained for the separation of (a) blank cell extracts and (b) spiked cell extracts containing antipyrin (20 μmol L^{-1}, used as an internal standard) and both diastereoisomers of **2** (60 μmol L^{-1}). Chromatographic conditions were: Chiralpak AD-H with a LiChrospher® 100 Diol guard column at λ = 277 nm, eluant hexane/ethanol – 75/25, 293 K, flow rate 0.8 mL min^{-1}.

for the internal standard: 85.55%), includes a 1 mL water washing step followed by a 1.5 mL elution step with methanol using C18 cartridges. Fig. 4 shows the chromatograms of blank cell extracts and spiked cell extracts containing antipyrin and both diastereoisomers of **2** obtained in optimal conditions.

3.2. Assessment of performance characteristics

3.2.1. Linearity

The linearity of the analyte response was assessed in the range 2.76–27.6 and 3.24–32.4 μmol L^{-1} for the first and second eluting diastereoisomers, respectively. An analysis of variance (ANOVA) showed a linear relationship between the peak area ratio of each diastereoisomer to the internal standard (int.st.) ($A/A_{\text{int.st.}}$) and the analyte nominal concentration in the standards (spiked cell extracts) (C, μmol L^{-1}). The regression equation was:

$$\frac{A_{P_1}}{A_{\text{int.st.}}} = -0.0010 \pm 0.0009 + (0.0357 \pm 0.0008)C_{P_1}$$

$$\frac{A_{P_2}}{A_{\text{int.st.}}} = -0.0718 \pm 0.0173 + (0.0357 \pm 0.0008)C_{P_2}$$

With confidence interval calculated at $\alpha = 0.05$, $r^2 = 0.9927$ and 0.9920 for first and second eluting diastereoisomers, respectively; the subscripts P_1 and P_2 denote the first and second eluting diastereoisomers, respectively. The y-intercepts were not significantly different from zero and the regression lines had an identical slope at the level $\alpha = 0.05$.

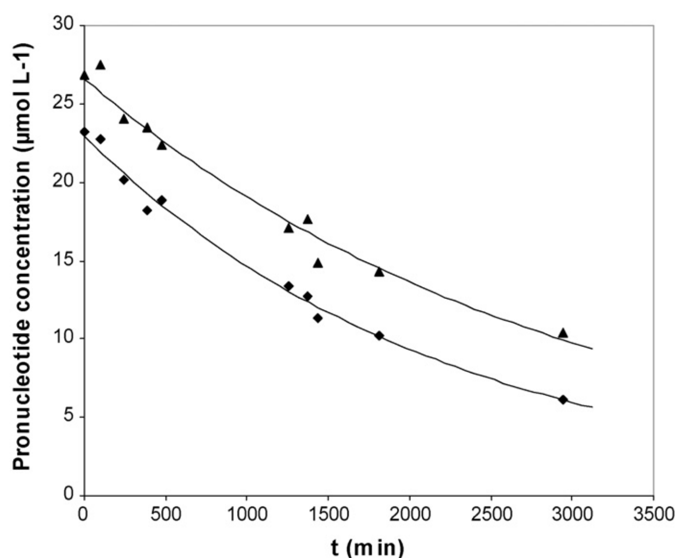

Fig. 5. Kinetic data of each diastereoisomer decomposition in cell extracts: first eluting diastereoisomer (♦) and second eluting diastereoisomer (▲).

3.2.2. Precision and accuracy

A summary of the precision and accuracy results is given in Table 2. In the range 6–60 μmol L^{-1} (diastereoisomers mixture) of pronucleotide **2**, the data indicate that intra-day precision and accuracy ($n = 7$) as expressed by % RSD and % error were in the range 0.84–2.34% and 2.0–6.5%, respectively for both diastereoisomers. At these concentrations, the inter-day precision and accuracy ($n = 9$) were in the range 1.47–5.98% and 3.1–6.8%, respectively. These values fulfil the recommendations for bioanalytical method validation [34].

3.2.3. Limit of detection and quantification

The limits of detection for the first and second eluting diastereoisomers of **2** were 0.83 and 0.97 μmol L^{-1}, respectively. The limits of quantification, which corresponds to the first point in the calibration graph, were 2.76 and 3.24 μmol L^{-1} for the first and second eluting diastereoisomers, respectively.

3.3. Degradation kinetics 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 behaviour of the pronucleotide inside the cells. According to previously reported studies in the mononucleotide SATE arylphos-

Table 2
The precision (%RSD) 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		
		RSD (%)	Found concentration (μmol L^{-1})	Error (%)	RSD (%)	Found concentration (μmol L^{-1})	Error (%)
P_1	2.76	2.34	2.94	+6.5	5.98	2.94	+6.8
	13.8	1.21	13.22	−4.2	3.84	14.41	+4.4
	27.6	1.05	27.04	−2.0	1.95	26.74	−3.1
P_2	3.24	2.17	3.07	−5.3	4.32	3.44	+6.2
	16.2	1.19	16.83	+3.9	3.26	15.31	−5.5
	32.4	0.84	30.97	−4.4	1.47	31.14	−3.9

photriester series of AZT [35–38], pronucleotide **2** should be bioconverted into its corresponding phenylphosphodiester derivative through an esterase-mediated activation mechanism (Fig. 2). The kinetic profile of both diastereoisomers of **2** was studied (Fig. 5) using the method developed above. Kinetic data were treated according the Michaëlis–Menten model [39]. 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 second first-order kinetic model [32]. The convenience of the model used is attested by the linearity of ln C *versus* t ($r^2 > 0.977$). Significant difference in the decomposition kinetics of each diastereoisomer was observed. The first eluting diastereoisomer (P_1) is hydrolysed faster than the second (P_2) ($k_{P_1} = 4.49 \times 10^{-4}\,\text{min}^{-1}$ and $t_{1/2\,P_1} = 26\,\text{h}$; $k_{P_2} = 3.34 \times 10^{-4}\,\text{min}^{-1}$ and $t_{1/2\,P_2} = 34\,\text{h}$). 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 for other pronucleotides series [11–15]. In order to verify the enzymatic nature involved in the decomposition mechanism, similar incubation experiment were performed after a 333 K heating pre-treatment of the total CEM-SS. The results show a greater stability of **2** with 99.7% recovery after 10 days.

4. Conclusion

In a first step, separation of diastereoisomers of three mixed phosphotriester derivatives was investigated in HPLC using cellulose and amylose chiral stationary phases. Use of Chiralpak AD-H CSP and mixtures of *n*-hexane/ethanol led to the best results, allowing baseline separation of both pronucleotides ((**2**) and (**3**)) in less than 20 min with resolution up to 2.75. In a second step, a rapid and specific isocratic chiral HPLC method including a solid phase extraction clean-up step was developed for the study of the behaviour of pronucleotide **2** in biological media. This method is significantly different from the previously developed method for the study of the same analogue of AZT (unsuccessful separation of the diastereoisomers using the same HPLC methodology, *i.e.* Cellulose OD-H PSC and hexane/2-propanol – 70/30 (v/v) mobile phase; insufficient sample cleanup using the same SPE protocol, *i.e.* acetate buffer as washing solution). Moreover, recoveries obtained for this Ara-C pronucleotide are greater than for its AZT analogue (about 85% *versus* 75%). The method shows good specificity, linearity, precision, accuracy and limit of quantification. This method has been applied to the degradation kinetic study of a diastereoisomeric mixture of a SATE phenylphosphotriester prodrug of Ara-C incubated in cellular extracts preparation. Differences between the decomposition kinetics of each diastereoisomer illustrate the stereoselectivity of the decomposition process mediated by an esterase activity. Moreover, a major difference between the kinetics of decomposition of this Ara-C pronucleotide and its AZT analogue (for the most rapidly hydrolyzed isomer kinetic constants were 4.49×10^{-4} and $3.27 \times 10^{-3}\,\text{min}^{-1}$, respectively) illustrates the influence of the pyrimidic base. As a result, although numerous data are available in the literature concerning the hydrolysis of AZT pronucleotides, no direct parallel with the comportment of Ara-C pronucleotides can be performed, making compound specific studies necessary.

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References

[1] R.R. Ellison, J.F. Holland, M. Weill, C. Jacquillat, M. Boiron, I. Bernard, A. Sawitsky, F. Rosner, B. Gussoff, R.T. Silver, A. Karanas, J. Cutner, C.L. Spurr, D.M. Hayes, J. Blom, L.A. Leone, F. Haurani, R. Kyle, J.L. Hutchinson, R.L. Forcier, J.H. Moon, Blood 32 (1968) 507.
[2] B. Colin, N.M. Jones, C. Mcguigan, P. Riley, Nucl. Acids Res. 17 (1989) 7195.
[3] B. Gröschel, J. Cinalt, R. Kotchetkov, U. Kôhl, E. Rohrbach, B. Kornhuber, R. Bazzarini, C. Périgaud, G. Gosselin, J.L. Imbach, H.W. Doeer, J. Cinalt, Experimental Approaches and Novel Therapies, Springer-Verlag, Heidelberg, Berlin, 1998, p. 628.
[4] R. Bazzanini, S. Manfredini, E. Durini, B. Gröschel, J. Cinatl, J. Balzarini, E. DeClercq, J.L. Imbach, C. Périgaud, G. Gosselin, Nucleos. Nucleot. 18 (1999) 971.
[5] L. Jordheim, E. Cros, M.H. Gouy, C.M. Galmarini, S. Peyrottes, J. Mackey, C. Périgaud, C. Dumontet, Clin. Cancer Res. 10 (2004) 5614.
[6] R. Bazzanini, M.H. Gouy, S. Peyrottes, G. Gosselin, C. Périgaud, S. Manfredini, Nucleos, Nucleot. Nucl. Acid 24 (2005) 1635.
[7] M.H. Gouy, H. Fabre, M.-D. Blanchin, S. Peyrottes, C. Périgaud, I. Lefebvre, Anal. Chim. Acta 566 (2006) 178.
[8] C. Meier, T. Knispel, E. De Clercq, J. Balzarini, J. Med. Chem. 42 (1999) 1604.
[9] J. Zemlicka, Biochim. Biophys. Acta 1587 (2002) 276.
[10] C. Meier, C. Ducho, U. Görbig, R. Esnouif, J. Balzarini, J. Med. Chem. 47 (2004) 2839.
[11] N. Diaz-Alejo, A. Monroy, E. Vilanova, J.L. Vicedo, M.A. Sogorb, Chem. Biol. Interact. 108 (1998) 187.
[12] A. Monroy-Noyola, M.A. Sogorb, E. Vilanova, Toxicol. In vitro 13 (1999) 689.
[13] T.K. Venkatachalam, S. Qazi, F.M. Uckun, Bioorg. Med. Chem. 14 (2006) 5161.
[14] D. Siccardi, M. Gumbleton, Y. Omidi, C. McGuigan, Eur. J. Pharm. Sci. 22 (2004) 25.
[15] C. Foulon, C. Vaccher, A.L. Villard, J.Y. Puy, I. Lefebvre, C. Périgaud, J.P. Bonte, J.F. Goossens, J. Pharm. Biomed. Anal. 42 (2006) 245.
[16] M. Shaffiee, S. Deferme, A.L. Villard, D. Egron, G. Gosselin, J.L. Imbach, T. Lioux, A. Pompon, S. Varray, A.M. Aubertin, G. van Den Mooter, R. Kinget, C. Périgaud, P. Augustijns, J. Pharm. Sci. 90 (2001) 448.
[17] A.M. Sørensen, K.E. Nielson, B. Vogg, J.P. Jacobsen, P. Nielsen, Tetrahedron 57 (2001) 10191.
[18] N. Mesplet, Y. Saito, P. Morin, L.A. Agrofoglio, J. Chromatogr. A 983 (2003) 115.
[19] H. Chapman, M. Kernan, J. Rohloff, M. Sparacino, T. Terhorst, Nucleos. Nucleot. Nucl. Acid 20 (2001) 1085.
[20] J.J. Ellington, J.J. Evans, K.B. Prickett Jr., W.L. Champion, J. Chromatogr. A 928 (2001) 145.
[21] J.F. Goossens, C. Foulon, A.L. Villard, J.Y. Puy, I. Lefebvre, C. Périgaud, C. Vaccher, J.P. Bonte, Biomed. Chromatogr. 19 (2005) 415.
[22] C.J. Allender, K.R. Brain, C. Ballatore, D. Cahard, A. Siddiqui, C. McGuigan, Anal. Chim. Acta 435 (2001) 107.
[23] R.Y. Gao, G.S. Yang, H.Z. Yang, Z.Y. Chen, Q.S. Wang, J. Chromatogr. A 763 (1997) 125.
[24] Y.S. Wang, K.T. Tai, J.H. Hen, Ecotoxicol. Environ. Safe. 57 (2004) 346.
[25] J.F. Goossens, S. Roux, D. Egron, C. Périgaud, J.P. Bonte, C. Vaccher, C. Foulon, J. Chromatogr. B 875 (2008) 288.
[26] X. Cahours, T.T. Tran, N. Mesplet, C. Kieda, P. Morin, L.A. Agrofoglio, J. Pharm. Biomed. Anal. 26 (2001) 819.
[27] L.D. Williams, L.S. Von Tungeln, F.A. Beland, D.R. Doerge, J. Chromatogr. B 798 (2003) 55.
[28] C. Perrin, L. Meyer, C. Meijahid, C.J. Blaka, Food Chem. 74 (2001) 245.
[29] I. Lefebvre, J.Y. Puy, C. Perrin, C. Périgaud, J. Chromatogr. B 858 (2007) 2.
[30] N. Amini, C. Crescenzi, J. Chromatogr. B 795 (2003) 245.
[31] R.K. Juhler, J. Chromatogr. A 786 (1997) 145.
[32] I. Lefebvre, C. Périgaud, A. pompon, A.M. Aubertin, J.L. Girardet, A. Kirn, G. Gosselin, J.L. Imbach, J. Med. Chem. 38 (1995) 3941.
[33] A.L. Villard, G. Coussot, I. Lefebvre, P. Augustijns, A.M. Aubertin, G. Gosselin, S. Peyrottes, C. Périgaud, Bioorg. Med. Chem. 16 (2008) 7321.
[34] V.P. Shah, K.K. Midha, J.W.A. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551.
[35] N. Schlienger, T. Beltran, C. Périgaud, I. Lefebvre, A. Pompon, A.M. Aubertin, G. Gosselin, J.L. Imbach, Bioorg. Med. Chem. Lett. 21 (1998) 3003.
[36] S. Peyrottes, G. Coussot, I. Lefebvre, J.L. Imbach, G. Gosselin, A.M. Aubertin, C. Périgaud, J. Med. Chem. 46 (2003) 782.
[37] A. Jochum, N. Schlienger, D. Egron, S. Peyrottes, C. Périgaud, J. Organomet. Chem. 690 (2005) 2614.
[38] S. Peyrottes, N. Schlienger, T. Beltran, I. Lefebvre, A. Pompon, G. Gosselin, A.M. Aubertin, J.L. Imbach, C. Périgaud, Nucleos. Nucleot. Nucl. Acid 20 (2001) 315.
[39] P.C. Engel, Enzyme Kinetics: The steady State Approach. Outline Studies in Biology, 2nd ed., Chapman & Hall, London, 1981.