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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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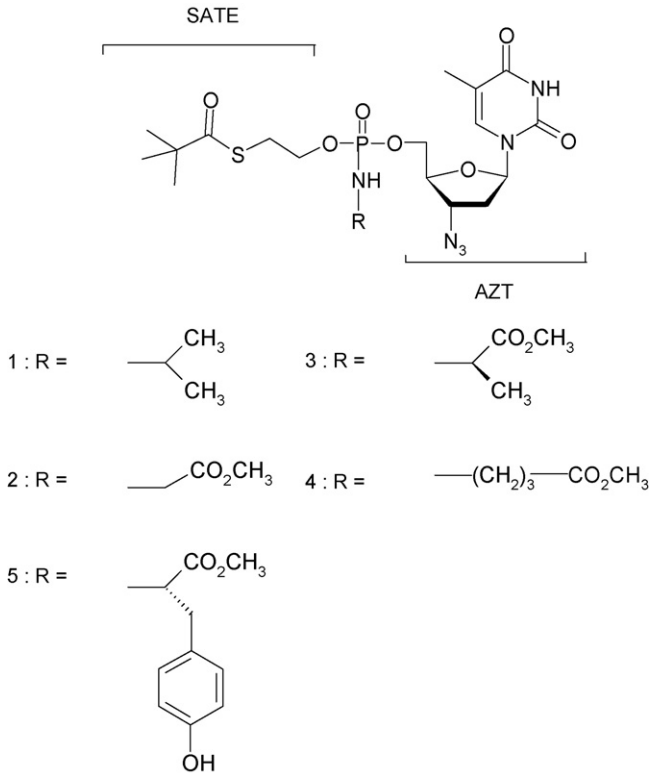

Fig. 1. Structure of the studied pronucleotides of AZT **1**–**5**.

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.