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Short communication

Fast separation of antiviral nucleoside phosphoramidate and H-phosphonate diastereoisomers by reversed-phase liquid chromatography

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ABSTRACT

The nucleoside-based antiviral phosphoramidates and H-phosphonates were synthesized and separated using reversed-phase liquid chromatography on bridged ethane hybrid (BEH) C_{18} column packed with 1.7 μm particles of non-chiral stationary phase. The influences of the composition of mobile phase and column temperature have been investigated to optimize the diastereoisomeric separation. Complete separations of the phosphoramidate and H-phosphonate prodrugs with good resolution (R_S = 1.99–2.77) were achieved within a short time (5–9 min). The validation study of the optimized method including linearity, accuracy, repeatability and detection limit has revealed it is better performance versus conventional HPLC method. In addition, HPLC was combined with high resolution electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS), which enabled the exact mass measurement and high sensitivity. Using MS as detection, the limits of detection and limits of quantification of the studied pronucleotide diastereoisomers were determined in the range of several nmol L^{-1} level.
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1. Introduction

Several nucleoside reverse transcriptase inhibitors (NRTIs), such as 3′-azido-2′,3′-dideoxythymidine (AZT) and 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T), are clinically used in the treatment of viral infections [1]. In general, NRTIs require three phosphorylation steps, accomplished by cellular nucleoside and nucleotide kinases, to be converted to their active triphosphorylated forms [2]. In triphosphate forms, NRTIs act as potent competitive inhibitors or alternative substrates and lead to the termination of chain elongation. However, the NRTIs analogues in most situations have a poor affinity for nucleoside kinases and often the efficiency of intracellular phosphorylation limits their therapeutic potential. For example, for d4T the 5′-mono-phosphorylation step is the rate-limiting step, whereas for AZT the conversion of the monophosphate derivatives to nucleoside diphosphate is the slowest step in the activation pathway [3]. Therefore, in order to overcome these limitations, numerous prodrug strategies have been developed and successfully applied to a range of nucleoside analogues of antiviral and anticancer interest [4,5]. According to the previous research, the nucleoside amino acid phosphoramidates [6–9] and nucleoside *O*-alkyl-5′-H-phosphonates [10–12] show promise as potential pronucleotide strategies (Fig. 1). Among them, L-alaninyl aryl phosphoramidates of d4T (**1**) and AZT (**3**), *O*-isopropyl-5′-H-phosphonate of d4T (**4**), and *O*-benzyl-5′-H-phosphonate of d4T (**5**) have got the best active for anti-HIV activity. These ProTide technologies could not only improve inhibitory activity and decrease cytotoxicity compared to their parent nucleotides, but also overcome the poor cell penetration of nucleoside 5′-phosphates by masking the phosphate negative charge with a more lipophilic membrane permeable group and bypass the initial limiting kinase dependence. However, due to the stereochemistry at the phosphorus center, these pronucleotides exist as a mixture of two diastereoisomers. The presence of two diastereoisomers is confirmed by ^{31}P NMR (two signals, Fig. 1). It is often the case that configuration at the phosphorus center may have a significant impact on the in vitro antiviral potency, enzymatic metabolism as well as pharmacokinetic profile [13–17]. From the perspective of new drug research and development, it is preferable to obtain a pure stereoisomer rather than a mixture of diastereoisomers [18]. Therefore, in order to determine diastereoisomeric impurity for pharmaceutical development and their chiral stability, the development of a rapid and reliable analytical method for diastereoisomeric separation is highly desirable.

In some cases, non-chiral chromatographic methods, such as the repeating silica gel column chromatography or reverse-phase HPLC on C_8 or C_{18} columns, can be used for the resolution of diastereoisomers resulting from the chirality at the phosphorus atom [19–21]. Nevertheless, the separation of nucleoside phos-

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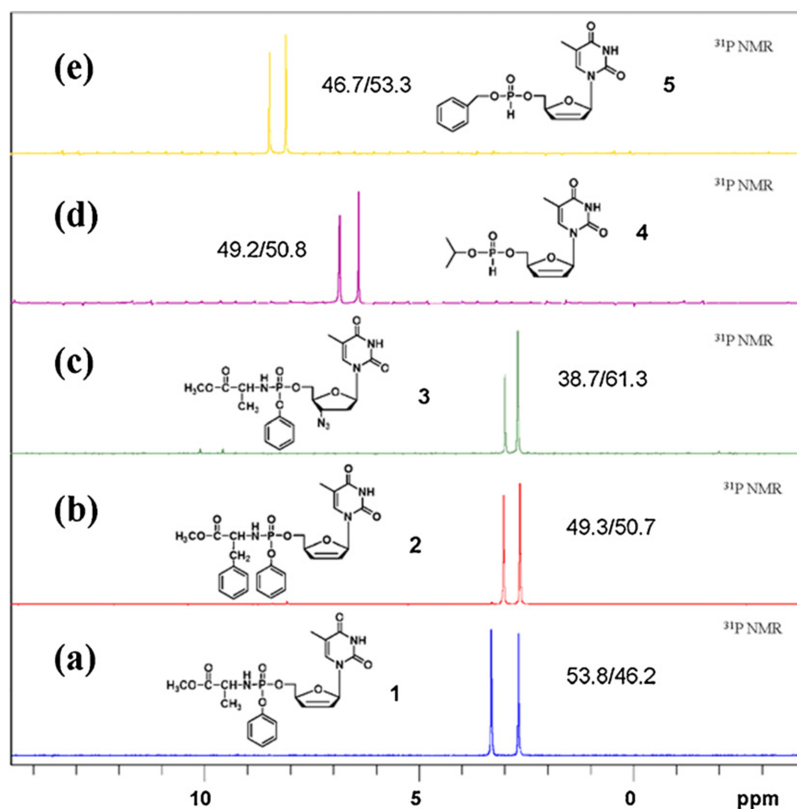

Fig. 1. Chemical structures and ^{31}P NMR spectra of the studied pronucleotides **1**–**5**.

phates and phosphoramidates remains a hard task to achieve by these non-chiral liquid chromatographic methods [22–24]. Thus, several chiral separation approaches using polysaccharide stationary phases have been investigated in order to achieve sufficient separations [24–32]. Furthermore, as an alternative technique to HPLC, separations of pronucleotide diastereoisomers, such as the phosphoramidates and phosphotriesters of AZT, have been efficiently resolved by capillary electrophoresis (CE) using anionic cyclodextrin as chiral selector added in the background electrolyte [26,33]. Recently, the development of HPLC as a powerful tool has made it possible to achieve higher resolutions, better sensitivities, and more rapid separations for pharmaceutical analysis [34–36]. Thus, we expected that these important antiviral pronucleotides containing an asymmetric phosphorus atom might be whether separated by reversed-phase liquid chromatography on non-chiral C_8 or C_{18} columns. The aim of present work was to develop a HPLC method using C_{18} stationary phase for direct separation of the diastereoisomers of nucleoside phosphoramidates and H-phosphonates (**1**–**5**). After optimization of various analytical parameters (variation of eluent and temperature), all of the studied pronucleotides were sufficiently resolved in short time. The validation of the method including linearity, repeatability, accuracy, and limits of detection (LODs) and quantification (LOQs) was performed. Finally, HPLC was combined with electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS), which enabled the exact mass measurement and high sensitivity.

2. Experimental

2.1. Reagents and chemicals

Phosphoramidate and H-phosphonate derivatives of antiviral nucleosides **1**–**5** (Fig. 1) were obtained as diastereoisomeric mixtures according to the published procedures [12,37]. All compounds synthesized were identified by ^{31}P NMR, ^{1}H NMR, ^{13}C NMR and ESI-MS. The diastereoisomeric ratio of products was calculated from the integral area of ^{31}P NMR signals: **1** (53.8:46.2), **2** (49.3:50.7), **3** (38.7:61.3), **4** (49.2:50.8), and **5** (46.7:53.3), respectively.

HPLC-grade acetonitrile was obtained from Tedia (Fairfield, OH, USA). Deionized water (18 MΩ) was produced by Milli-Q system (Millipore, Bedford, MA, USA). All the mobile phases were filtered through membrane (0.2 μm) and degassed with a Waters in-line degasser apparatus. The mobile phase used were: (A) CH_3CN/H_2O, 13:87 (v/v); (B) CH_3CN/H_2O, 15:85 (v/v); (C) CH_3CN/H_2O, 20:80 (v/v); (D) CH_3CN/H_2O, 25:75 (v/v); (E) CH_3CN/H_2O, 30:70 (v/v); (F) CH_3CN/H_2O, 40:60 (v/v). Stock solutions containing about 2.0 mmol L^{-1} of studied pronucleotides **1**–**5** were prepared by dissolving appropriate amounts in CH_3CN/H_2O (10/90, v/v) and passed through a 0.2 μm membrane filter. The stock solutions are stored in the dark at 4 °C.

2.2. Chromatographic system

Chromatographic analyses were carried out using an Acquity UPLC system (Waters, Milford, MA, USA) fitted with a binary pump, a plate autosampler, a thermostated column compartment, sample organizer and a Waters 996 photodiode array spectrophotometer. A Waters Acquity BEH C_{18} column (1.7 μm particles, 2.1 mm × 50 mm) with an in-line vanguard column was used for chromatographic separation. CH_3CN/H_2O (10:90, v/v) and CH_3CN/H_2O (50:50, v/v) were used as weak and strong wash solutions for cleaning the injection needle respectively. The flow rate was set at 0.20 mL/min and the injection volume was 5 μL. The peak of the solvent front was considered to be equal to the dead time (t_0). The column eluate was monitored at 254 nm for all compounds.

Table 1
Influence of the acetonitrile content (%) of the mobile phase (CH_3CN/H_2O) on the retention factors (k), separation factors (α) and resolution (R_S) of pronucleotides **1**–**5**.

Entry	Compound	Eluent	T (°C)	k_1	k_2	α	R_S
1	d4T-P-*N*-AlaOMe	C	25	4.78	5.39	1.13	2.60
		C	30	4.46	5.04	1.13	2.53
		C	35	4.34	4.89	1.12	2.49
		C	40	4.33	4.88	1.12	2.47
		D	25	2.28	2.49	1.09	2.04
		E	25	1.44	1.54	1.06	1.40
2	d4T-P-*N*-PheOMe	C	25	7.07	11.05	1.56	2.58
		E	25	5.14	5.64	1.10	2.04
		F	25	1.75	1.83	1.04	1.11
3	AZT-P-*N*-AlaOMe	C	25	19.85	23.50	1.18	3.25
		D	25	6.69	7.64	1.14	2.77
		E	25	3.20	3.49	1.09	2.11
4	d4T-P-Isopropyl-H	A	25	3.56	4.12	1.16	2.55
		C	25	1.41	1.51	1.07	1.43
		D	25	n.r.	n.r.	n.r.	n.r.
5	d4T-P-Benzyl-H	B	25	9.87	11.37	1.15	2.95
		C	25	3.84	4.21	1.10	1.99
		D	25	2.04	2.15	1.05	1.19

Conditions: Waters Acquity BEH C_{18} column (1.7 μm particles, 2.1 mm × 50 mm), 25 °C, and λ = 254 nm. Eluent: A: CH_3CN/H_2O 13:87 (v/v); B: CH_3CN/H_2O 15:85 (v/v); C: CH_3CN/H_2O 20:80 (v/v); D: CH_3CN/H_2O 25:75 (v/v); E: CH_3CN/H_2O 30:70 (v/v); F: CH_3CN/H_2O 40:60 (v/v); n.r., non resolved.

2.3. *Validation of the method*

2.3.1. *Linearity*

For each pronucleotides, seven standard solutions were analyzed in triplicate according to the previously described methods. Total concentrations of two diastereoisomers were (μmol L^{-1}): d4T-P-*N*-AlaOMe **1**: 0.33, 2.15, 10.75, 21.51, 107.53, 215.05, 1075.27; d4T-P-*N*-PheOMe **2**: 0.74, 1.85, 9.24, 18.48, 92.42, 184.84, 924.21; AZT-P-*N*-AlaOMe **3**: 0.79, 1.97, 9.84, 19.69, 98.43, 196.85, 984.25; d4T-P-Isopropyl-H **4**: 2.21, 3.03, 15.15, 30.30, 151.52, 303.03, 1515.15; d4T-P-Benzyl-H **5**: 1.59, 2.65, 13.23, 26.46, 132.28, 264.55, 1322.75. The exact concentrations for each diastereoisomers could be calculated through the ratio of ^{31}P NMR integrations. The seven-point calibration curves were plotted as the peak area versus the concentration. A linear regression using analysis of variance was used to assess linearity [27].

2.3.2. *Limit of detection (LOD) and limit of quantification (LOQ)*

Limits of detection and limits of quantification achieved by UV and ESI-TOF MS were defined as signal to noise ratio equal to 3 and 10, respectively, from the analysis of the mixtures of two diastereoisomers in the optimal conditions previously determined.

2.3.3. *Accuracy*

For each pronucleotides (**1**–**5**), three standard solutions (total concentrations of two diastereoisomers in μmol L^{-1}: d4T-P-*N*-AlaOMe **1**: 2.15, 107.53, 215.05; d4T-P-*N*-PheOMe **2**: 1.85, 92.42, 184.84; AZT-P-*N*-AlaOMe **3**: 1.97, 98.43, 196.85; d4T-P-isopropyl-H **4**: 3.03, 151.52, 303.03; d4T-P-benzyl-H **5**: 2.65, 132.28, 264.55) were injected in triplicate to assess the intra-day accuracy by relative error (error, %).

2.3.4. *Repeatability*

Standard solutions of each pronucleotides (total concentrations of two diastereoisomers in μmol L^{-1}: d4T-P-*N*-AlaOMe **1**: 21.51; d4T-P-*N*-PheOMe **2**: 18.48; AZT-P-*N*-AlaOMe **3**: 19.69; d4T-P-isopropyl-H **4**: 30.30; d4T-P-benzyl-H **5**: 26.46) were injected six times to determine the intra-day precision defined by the relative standard deviation (RSD, %) of peak area and retention time.

2.4. *HPLC–ESI-TOF MS analysis*

HPLC–MS analysis was performed on a Waters Acquity UPLC chromatograph interfaced to a Waters QTOF-Micro mass spectrometer. Analysis was carried out using positive ion mode, with the TOF data being collected between m/z 100 and 800. Nitrogen was used as nebulizer and curtain gas. The ionization source conditions were as follows: source temperature of 120 °C, desolvation temperature 300 °C, capillary voltage 2500 V and sample cone voltage 50 V. The cone and desolvation gas flows were 10 and 800 L/h, respectively. With these HPLC/MS conditions, the compounds were analyzed by selected ion monitoring (SIM) with a dwell time of 0.10 s.

3. Results and discussion

The tetrahedral phosphorus atoms of amino acid phosphoramidates and H-phosphonates are chiral center because of the three groups appended to phosphorus atom are different. Actually, the chirality of the adjacent nucleosides or amino acids lead these prodrugs to exist as two diastereoisomers. Generally, the enantiomers (a racemate) of organophosphorus compounds are successfully separated by chiral HPLC on polysaccharide-based stationary phase [31,32] or CE with addition of chiral selectors [38]. It goes without saying that the non-chiral HPLC methods on the reversed-mode C_{18} stationary phase or normal-mode silica columns do not have the power of molecule recognition and thereby cannot be used to resolve enantiomers. On the contrary, the separations of the diastereoisomeric phosphoramidates and H-phosphonates on a non-chiral C_{18}-bonded stationary phase are possible because the two organophosphorus diastereoisomers are actually two different compounds with distinct physical properties. However, methods described in the literatures for the resolution of nucleoside phosphoramidate derivatives on conventional C_{18}-bonded phases lead to poor separation even though using a small particle size column (3 μm) [24]. According to the van Deemter equation, an empirical formula that describes the relation between linear velocity and plate height, reducing the particle diameter from 5 or 3 μm (conventional HPLC columns) to sub-2 μm results in better chromatographic resolution and increased peak capacity and analysis speed [36,39,40]. The objective of the following work was to explore the possibilities of the separation of antiviral pronucleotides using HPLC on bridged ethane hybrid (BEH) C_{18} column packed with 1.7 μm particles.

3.1. *Optimization of the chromatographic conditions*

The influence of the mobile phase composition and temperature for the separation of diastereoisomeric phosphoramidates

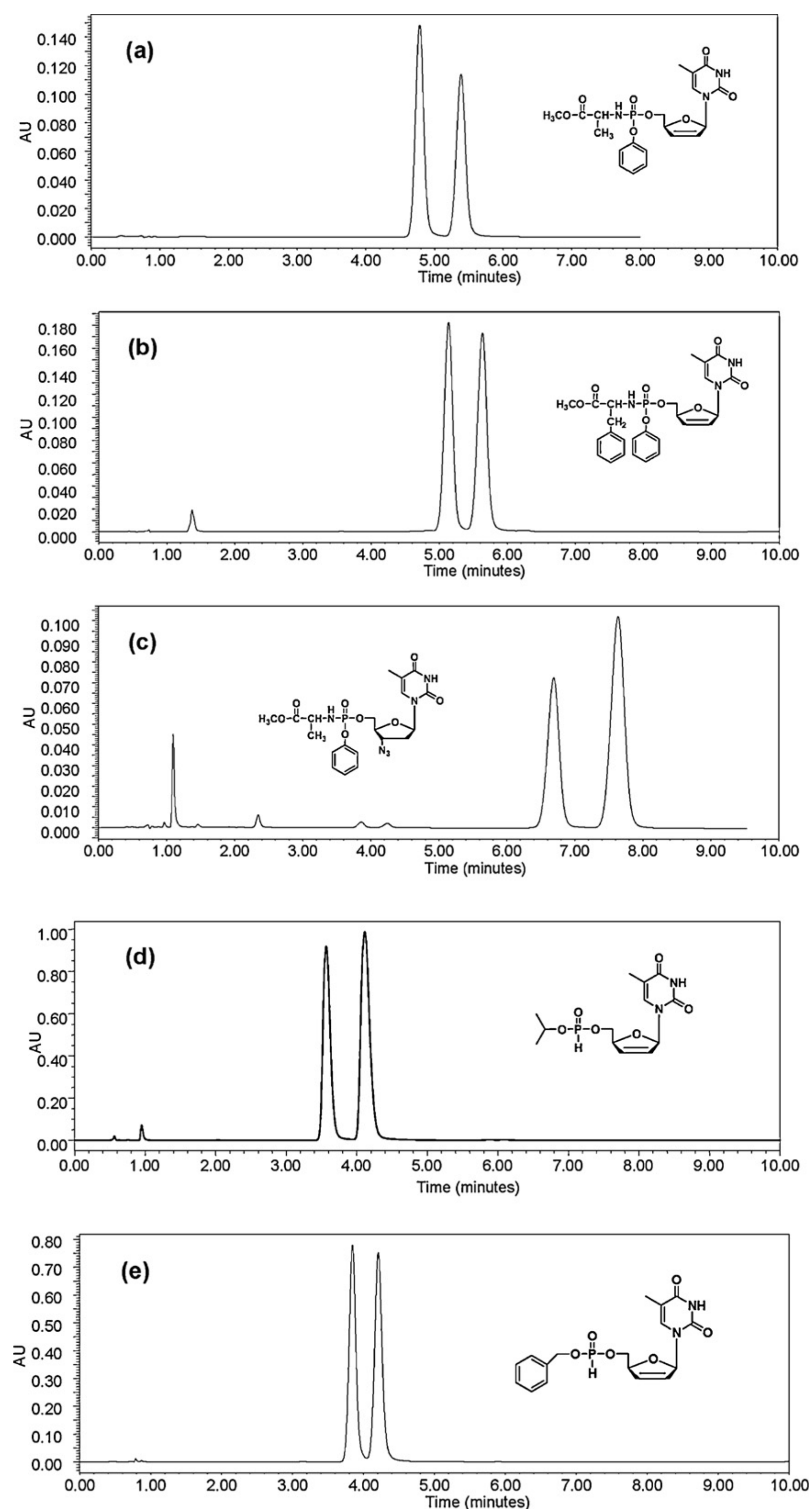

Fig. 2. Chromatograms obtained for the separations of phosphoramidates (**1**–**3**) and H-phosphonates (**4**, **5**) using HPLC on Waters Acquity BEH C_{18} column (1.7 μm particles, 2.1 mm × 50 mm): (a) d4T-P-*N*-AlaOMe (**1**), CH_3CN/H_2O-20:80 (v/v); (b) d4T-P-*N*-PheOMe (**2**), CH_3CN/H_2O-30:70 (v/v); (c) AZT-P-*N*-AlaOMe (**3**), CH_3CN/H_2O-25:75 (v/v); (d) d4T-P-Isopropyl-H (**4**), CH_3CN/H_2O-13:87 (v/v); (e) d4T-P-benzyl-H (**5**), CH_3CN/H_2O-20:80 (v/v); flow rate: 0.2 mL/min, 25 °C and λ = 254 nm.

and H-phosphonates was investigated and the chromatographic data obtained were summarized in Table 1. As shown in Table 1, the increase in the percentage of organic modifier in the mobile phase leads to a decrease in retention factors (k), separation factors (α), and resolution (R_S) for all compounds (**1**–**5**). This is the typical behaviour of the reversed-phase chromatography, which can be explained that the higher polarity of mobile phase improves the solubility of the solutes and decreases the hydrophobic interactions between the solutes and the C_{18} stationary phase. However, it was found that the separation performance, such as the k and R_S,

Table 2
Limits of detection (LODs) and quantification (LOQs) of the studied pronucleotides using optimal HPLC methods.

Entry	Compound	Eluent	Diastereoisomer	LOD (μmol L^{-1})	LOQ (μmol L^{-1})
1	d4T-P-*N*-AlaOMe	C	P_1	0.043 (0.006)[a]	0.140 (0.021)[a]
			P_2	0.039 (0.005)[a]	0.123 (0.018)[a]
2	d4T-P-*N*-PheOMe	E	P_1	0.110	0.323
			P_2	0.115	0.333
3	AZT-P-*N*-AlaOMe	D	P_1	0.112	0.347
			P_2	0.079	0.286
4	d4T-P-Isopropyl-H	A	P_1	0.315 (0.008)[a]	0.933 (0.025)[a]
			P_2	0.324 (0.009)[a]	0.955 (0.029)[a]
5	d4T-P-Benzyl-H	C	P_1	0.218	0.669
			P_2	0.244	0.698

[a] Limits of detection and quantification determined by HPLC–ESI-TOF MS were shown in parentheses.

were more sensitive to the changes of the organic modifier than conventional HPLC. For example, for AZT-P-*N*-AlaOMe (**3**), when the proportion of CH_3CN in the eluent was increased from 20 to 25% (v/v), k_1 and k_2 for each diastereoisomers noticeably decreased from 19.85 and 23.50 to 6.69 and 7.64, respectively. Meanwhile, the resolution factor decreased form 3.25 to 2.77. Indeed, if the working solutions of prodrugs were prepared in CH_3CN without the addition of water, all of the mixtures of two diastereoisomers could not be resolved and very broad peaks were observed. Generally, for many chiral compounds, the separation factor increases when the column temperature is lowered [30]. In order to clarify the effect of temperature on diastereoisomeric separations, d4T-P-*N*-AlaOMe (**1**) was investigated with stepwise increasing the column temperature from 25 °C to 40 °C using CH_3CN/H_2O 20:80 (v/v). As shown in Table 1, the increasement of the separation temperature leads to slight decrease in the retention factors (k) and resolution (R_S). However, the separation factors were almost unchanged from 1.13 at 25 °C to 1.12 at 40 °C. Therefore, 25 °C temperature was selected for the further separation of two diastereoisomers of compounds **1**–**5**.

In conclusion, after optimization of the composition of mobile phase and column temperature, baseline separation is achieved for all studied pronucleotides (**1**–**5**) on BEH C_{18} column, using CH_3CN/H_2O 13:87 (v/v) for **4** (R_S = 2.55), 20:80 (v/v) for **1** (R_S = 2.60) and **5** (R_S = 1.99), 25:75 (v/v) for **3** (R_S = 2.77), and 30:70 (v/v) for **2** (R_S = 2.04), respectively (Fig. 2). In addition, it was worth noted that diastereoisomeric d4T-P-isopropyl-H (**4**) and d4T-P-benzyl-H (**5**) could be completely resolved within 5 min (Table 1). Furthermore, it was found that the diastereoisomers of valine methyl ester phosphoramidate of d4T (d4t-P-*N*-ValOMe) and *O*-cyclohexyl-H-phosphonate of AZT (AZT-P-cyclohexyl-H) could also be successfully separated using HPLC on C_{18} column (data not shown). However, the retention time for diastereoisomers of AZT-P-cyclohexyl-H increased to about 20 min in order to achieve baseline separation, which might be attributed to the more lipophilic structures.

3.2. Validation study

3.2.1. Linearity

For the studied pronucleotides, the linearity was assessed using seven standard solutions in the range 0.33–1075.27 (**1**), 0.74–924.21 (**2**), 0.79–984.52 (**3**), 2.21–1515.15 (**4**), and 1.59–1322.75 (**5**) μmol L^{-1}, respectively. The linear regression analysis for each diastereoisomer was constructed by plotting the peak area against the added concentration in the standards. As shown in Table S1 (Supporting information), good correlation coefficients were observed for each diastereoisomers ($r^2 > 0.9998$) with confidence interval calculated at $\alpha = 0.05$. The y-intercepts were not significantly different from the origin (RSD%: 0.9–4.8%) and both regression lines of the diastereoisomers have identical value on the slope (RSD%: 0.1–1.2%).

3.2.2. Accuracy and repeatability

Table S2 (Supporting information) summarized the results of intra-day precision and accuracy experiments. As shown in Table S1, the intra-day repeatability as expressed by %RSD were in the range 0.1–0.6% on peak area and 0.03–0.07% on retention times, respectively. The intra-day accuracy as expressed by % error were in the range −1.0 to 1.7% for **1**, −0.3 to 4.3% for **2**, −2.6 to 3.3% for **3**, −0.3 to 2.6% for **4**, and −1.4 to 2.4% for **5**, respectively. These values fulfill the recommendations for the validation of bioanalytical methods [41].

3.2.3. Limit of detection and quantification

The limits of detection for the first and second eluting diastereoisomers of compounds **1**–**5** were in the range 0.043–0.315 and 0.039–0.324 μmol L^{-1}, respectively (Table 2). The limits of quantification for the first and second eluting diastereoisomers were in the range 0.140–0.933 and 0.123–0.955 μmol L^{-1}, respectively. It was found that the LOD and LOQ of phosphoramidate derivatives were smaller than those of H-phosphonates of d4T. For all the diastereoisomers of the studied prodrugs, the LOD and LOQ were smaller than the results obtained using HPLC on chiral stationary phase and chiral capillary electrophoresis [24,33].

3.3. HPLC–ESI-TOF MS coupling

The combination of an HPLC system with high resolution mass spectrometers, such as the TOF MS with a high capture rate, present the most advanced LC–MS systems [42–44]. The feasibility of such HPLC–TOF MS coupling is shown in Fig. 3. After optimization of the ESI-MS conditions in positive ion mode, d4T-P-*N*-AlaOMe (**1**) and d4T-P-isopropyl-H (**4**) as the typical presentatives for two kinds of pronucleotides with different chemical structures were separated by HPLC and directly introduced into TOF MS. As shown in Fig. 3(a), two peaks of the selected ion chromatogram corresponded to diastereoisomers of d4T-P-*N*-AlaOMe (**1**) were observed at 4.57 and 5.17 min with identical mass spectra, in which two intense mass signals corresponded to $[M+H]^+$ and $[M+NH_4]^+$ of compound **1** were detected at m/z 466.1427 and 483.1667, respectively. It should be pointed out that the LOD and LOQ for d4T-P-isopropyl-H (**4**) were observed at least 30 fold lower than LOD and LOQ obtained by UV detection. Indeed, the LOD and LOQ for each diastereoisomers of compounds **1** and **4** were in concentration of several nmol L^{-1} (Table 2). This HPLC–ESI-TOF MS analysis, which not only provides the improved sensitivity but also enables the exact mass measurement, is undoubtedly a suitable method for the meticulous determination of diastereoisomeric impurity and the chiral stability of the studied prodrugs.

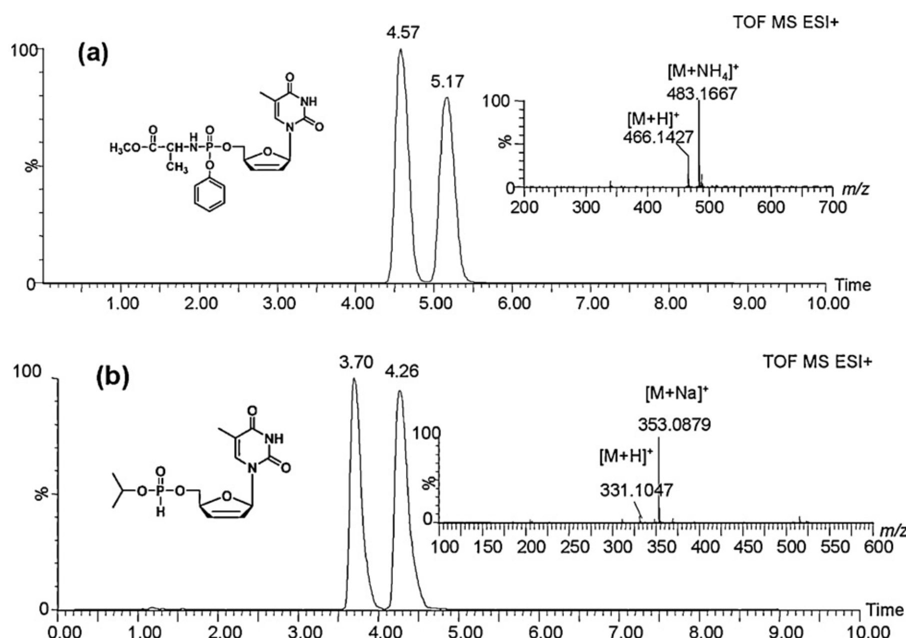

Fig. 3. HPLC–ESI-TOF MS chromatograms of the studied pronucleotides. (a) Extracted ion chromatogram of $[M+H]^+$ at *m/z* 466 for d4T-P-*N*-AlaOMe (**1**), CH_3CN/H_2O 20:80 (v/v); (b) extracted ion chromatogram of $[M+H]^+$ at *m/z* 331 for d4T-P-isopropyl-H (**4**), CH_3CN/H_2O 13:87 (v/v).

4. Conclusion

In summary, a new fast and sensitive reversed-phase liquid chromatography method for separation of phosphoramidates and H-phosphonates diastereoisomers was developed using non-chiral column packed with 1.7 μm particles of C_{18} stationary phase. After optimization of the composition of mobile phase and column temperature, baseline separation of all of the studied pronucleotides was obtained in less than 10 min with high resolution. In addition, this method shows good results in terms of linearity, accuracy, repeatability, and limits of detection and quantification. Furthermore, the coupling of high resolution TOF MS with HPLC enables to obtain improved sensitivity and exact mass measurement. The method can be applied not only to determine diastereoisomeric impurity of antiviral phosphoramidates and H-phosphonates and related nucleoside-based phosphorus prodrugs, but also to study their chiral stability.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.01.046.

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