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Simultaneous analysis of several antiretroviral nucleosides in rat-plasma by high-performance liquid chromatography with UV using acetic acid/hydroxylamine buffer
Test of this new volatile medium-pH for HPLC–ESI-MS/MS

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Abstract

Zalcitabine (ddC), lamivudine (3TC), didanosine (ddI), stavudine (d4T), carbovir (CBV), zidovudine (AZT), tenofovir (PMPA) and its administrated form (tenofovir diisoproxyl fumarate, TDF), are nucleosides currently approved in HIV therapy. To facilitate pharmacokinetics studies, a specific reversed-phase high-performance liquid chromatography (HPLC) method was developed for their analysis in rat plasma. The method involved a quantitative recovery of these drugs from rat plasma by solid-phase extraction on Oasis® HLB Waters cartridges followed by optimised HPLC separation on an Atlantis™ dC_{18} column with acetic acid–hydroxylamine buffer (ionic strength 5 mM, pH 7)-acetonitrile elution gradient. Quantitation was performed by HPLC/UV at 260 nm. Linear calibration curves were obtained within a 30–10,000 ng/mL plasma concentration range. Correlation coefficients (r^2) greater than 0.992 were obtained by least-squares regression and limits of quantification were in 30–90 ng/mL concentration range. Quantitative parameters (accuracy, intra-day repeatability and inter-day reproducibility) yielded satisfactory results. Finally, a new buffer, obtained with acetic acid and hydroxylamine, has been tested in HPLC/ESI-MS/MS and appears to be an efficient volatile buffer in the medium 5–7 pH range. Indeed, at pH 7 and low ionic strength (5 mM), its buffer capacity is one hundred times higher to that obtained for the usual acetic acid/ammonia buffer.

Keywords: HPLC; Hydroxylamine acetate buffer; Nucleoside; Antiretroviral; HIV plasma; Mass spectrometry

1. Introduction

Current therapy of acquired immune deficiency syndrome (AIDS) consists in a combination of several antiretroviral drugs [1] including nucleoside reverse transcriptase inhibitors (NRTIs) [2]. To date eight NRTIs (see Fig. 1 for chemical structures) have been approved in the HIV-1 therapy and are: tenofovir (PMPA, **1**), available as its disoproxil fumarate prodrug (TDF, **8**), zalcitabine (ddC, **2**), lamivudine (3TC, **3**), didanosine (ddI, **4**), which is metabolised to 2′,3′-dideoxyadenosine (ddA), stavudine (d4T, **5**), carbovir (CBV, **6**), zidovudine (AZT, **7**), and the 2004 approved emtricitabine (FTC) [3]. All nucleosides can be released from the cell by passive transport; in the case of the disoproxil fumarate PMPA prodrug (TDF) or didanosine, they are intracellularly and enzymatically (hydrolase, nucleotidase) transformed into PMPA and ddA, respectively [7]. Those forms could thus be also found in more or less slight amounts into the plasma. It

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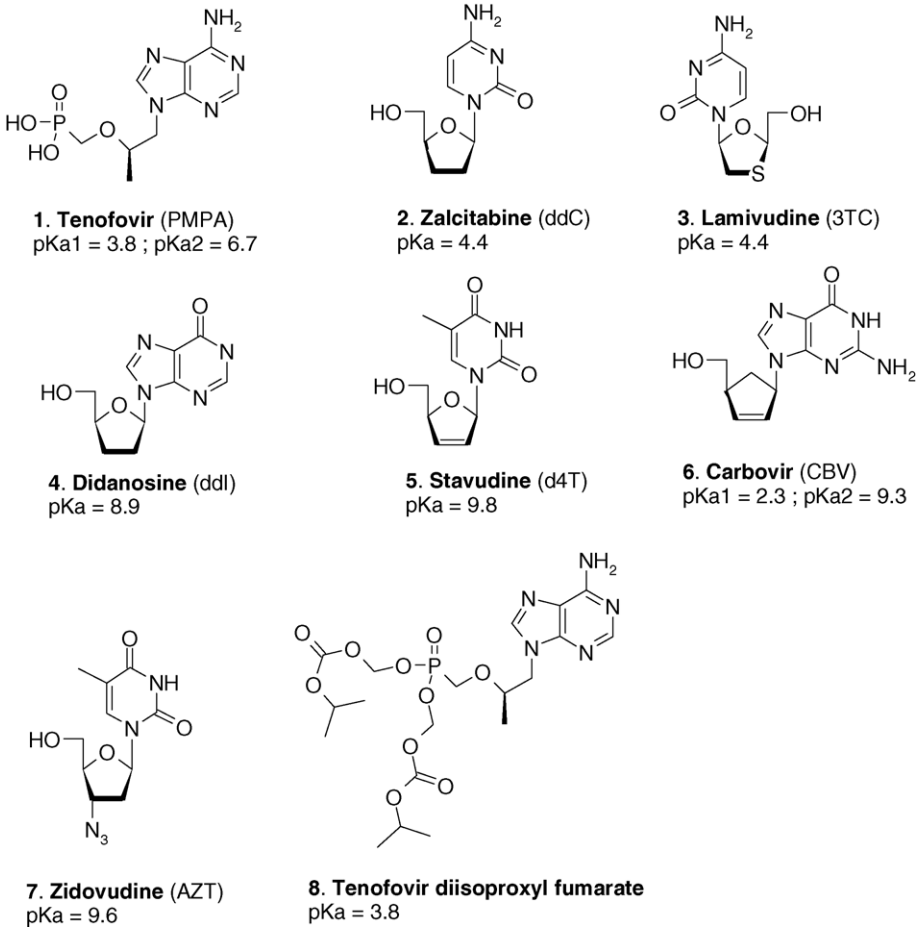

Fig. 1. Chemical structures of studied anti-HIV nucleosides.

is assumed [4,5] that intracellular concentrations of triphosphorylated metabolites of NRTIs may correlate better with HIV viral load than do the plasma concentrations of parent nucleosides. Nevertheless, the measurement of NRTIs in plasma can provide also valuable information about compliance, drug exposure, drug interactions and pharmacokinetics [6].

The measurement of anti-HIV drugs in plasma has been mainly determined by high-performance liquid chromatography (HPLC) methods with UV [8–10], fluorescence [11,12] or tandem mass spectrometry (MS/MS) detection [13–15]. Thus, as part of our on-going research to develop and validate new methodologies for anti-HIV monitoring, we report the development of an assay for the concurrent analysis of various antiviral nucleosides (including the ddI and TDF prodrugs, and excluding the FTC) in rat plasma using a new HPLC/UV method that is an order of magnitude equal or more sensitive than others published procedures. The new hydroxylamine acetate buffer has been found to be a volatile medium-pH buffer able to circumvent the well known acidic instability of nucleosides, and to have a good buffering capacity, which makes it fully suitable for further HPLC–MS analysis.

2. Experimental

2.1. Apparatus

HPLC experiments were carried out on a Thermo Separation Products (Les Ulis, France) model Spectra Series P-4000 quaternary pump, equipped with an on-line filter and an on-line degasser, a Rheodyne (Cotati, CA, USA) Model 7125 injection valve fitted with a 20 μL sample loop. UV detection was performed at 260 nm with a Kratos (Applied Biosystems, Les Ulis, France) Spectroflow 783 UV spectrophotometric detector; data were collected and analyzed using EZ-Chrom Elite software (Version 2.5). Two analytical columns (150 mm × 2.1 mm i.d., particle size 5 μm) Atlantis^{TM} dC_{18} (Waters, Manchester, UK**)** and Zorbax Extend-C_{18} (Agilent, Les Ulis, France) were used for HPLC separations of anti-HIV nucleosides. The mobile phase was delivered at a flow rate of 0.2 mL/min. A guard column (10 mm × 2.1 mm i.d.) with the same composition as the separation column was used to protect the analytical column.

HPLC–ESI-MS/MS experiments were performed on an Agilent (Les Ulis, France) 1100 binary pump, equipped with a μ-Rheodyne (Cotati, CA, USA) injection valve fitted with

40 μL loop, and coupled with a Quattro Ultima (Micromass Ltd, Manchester, UK) mass spectrometer operated simultaneously in negative and positive mode. The electrospray ion source was heated at 120 °C, and the desolvation temperature was set at 360 °C. Nitrogen was used as desolvation (500 L/h) and nebuliser (50 L/h) gas, while the collision gas was argon (0.0033 mbar).

MS/MS parameters and MRM acquisitions were computer controlled using Masslynx 4.0 software. Two simultaneous MRM methods were built for the assay of TDF, 3TC, PMPA, CBV and ddC, and the concurrent analysis of the ddI and AZT (0.20 s dwell time per each) since several acquisition parameters were different.

A Harvard Model 22 syringe pump was used to introduce the standards into the ion source in order to optimise the MS and MS/MS parameters for each compound: individual sample solutions (1 μg/mL in the mobile phase) were directly infused into the ESI source at a flow rate of 3 μL/min. In this optimization step, source and desolvatation temperatures were lowered to 80 and 120 °C, respectively. Sample solutions were diluted in acetonitrile/water (50:50, v/v) buffered with NH_2OH/CH_3COOH (pH 7, ionic strength of 5 mM).

2.2. Chemicals and reagents

Stavudine, didanosine and 2′,3′-dideoxyadenosine were generous gift from Bristol-Myers Squibb (France); carbovir was a gift from Dr. J. Grassi (CEA, Saclay, France); tenofovir DF and PMPA were generously supplied by Gilead Sciences (USA); zalcitabine, lamivudine, and zidovudine were commercially available from Aldrich (St-Quentin-Fallavier, France). HPLC grade-acetonitrile was obtained from J.T. Baker (Noisy-le-Sec, France). Glacial acetic acid, antipyrine (internal standard) and hydroxylamine (NH_2OH) were purchased from Aldrich, respectively. Deionized water (18 MΩ) was obtained from Elgastat UHQ II system (Elga, Antony, France).

Hydroxylamine-acetic acid buffer was selected as a mobile phase for HPLC–UV separations. This buffer was prepared at pH 7 and at fixed ionic strength (5 mM) with the help of Phoebus software (Analis, Namur, Belgium); its composition was 5.03 mM acetic acid and 557.68 mM hydroxylamine. The buffer capacity, calculated with this software, was high enough (10.4 mM/pH unity) to avoid any variation of buffer pH in the column during the experiment. Rat plasma OFA on heparine lithium was purchased from Charles River Laboratoires (L'Arbresle, France). Some pentostatin has been added to the rat-plasma in order to prevent in vitro deamination of 6-NH_2 of adenosine or cytosine heterocycle and to ensure analyte stability [16].

2.3. Preparation of standard solutions

Stock solutions of nucleosides were prepared in water (1 mg/mL) and diluted with rat plasma to give two different mixtures of eight compounds in which each nucleoside was at 20 and 100 μg/mL, respectively. The antipyrine (internal standard) stock solution (1 mg/mL) was prepared in purified water and diluted in plasma (200 μL) to obtain standard working solution (100 μg/mL).

Standard working solutions of eight nucleosides were then obtained by appropriate dilution with plasma to cover the following concentration ranges: 30–10,000 ng/mL for PMPA, ddC, 3TC and d4T, 50–10,000 ng/mL for ddI, 70–10,000 ng/mL for CBV, 80–10,000 ng/mL for AZT and 90–10,000 ng/mL for TDF.

For quality control (QC) samples, 180 μL of a standard solution (as described above) were added to 20 μL of standard working solution of antipyrine to give a 200 μL final volume. Then each of plasma samples was diluted by ×5 with acetonitrile (800 μL), vortex-mixed and centrifuged for 5 min to precipitate the proteins of the samples.

The supernatants were evaporating to dryness under nitrogen stream and reconstitute in 200 μL of water prior to loading on the SPE cartridges.

Stock standard solutions (1 mg/mL) of each nucleoside prepared in water were stable at −40 °C for 6 months without degradation.

2.4. SPE extraction method

The solid-phase extraction cartridges on Oasis® HLB (3 mL, 60 mg) were purchased from Waters (Franklin, MA, USA). SPE columns were placed in a vacuum manifold system from Supelco (Bellefonte, PA, USA) coupled to a vacuum pump. The cartridges were preconditioned with 3 mL methanol and then with 3 mL water. The plasma sample (200 μL) was loaded on each cartridge and a 5 mm Hg-vacuum was applied. The cartridge was then washed with 1 mL water. The anti-HIV nucleosides were retained and eluted with 1.5 mL methanol. Afterwards, the elute was evaporated to dryness under a gentle nitrogen stream at 40 °C. The remaining residue was reconstituted with 100 μL water and a 20 μL volume was injected into the HPLC system.

3. Results and discussion

3.1. Optimisation of reversed-phase HPLC method

The aim of this study was to develop a reversed-phase high-performance liquid chromatography procedure for the simultaneous analysis of some nucleoside HIV reverse transcriptase inhibitors and their prodrugs [zalcitabine, lamivudine, didanosine, stavudine, carbovir, zidovudine, tenofovir and tenofovir diisoproxyl fumarate]. These drugs are relatively polar compounds as expressed by the calculated *n*-octanol-water partition coefficient ($-1.7 < \log P < 1.0$), and are poorly retained on traditional C_{18} bonded phases, which suffer also from poor performances when using mobile phases with more than 90% water content. In this work, the separation of anti-HIV nucleosides was achieved on

reversed-phase Atlantis^{TM} dC_{18} and Zorbax extend-C_{18} columns, which afford much better performance for polar compounds and using conditions that are fully compatible with LC/MS. The first stationary phase is a medium coverage, difunctionally bonded C_{18} that performs well for the retention of both polar and non-polar compounds; this stationary phase is also fully compatible with 100% aqueous-buffered mobile phases, i.e. the pores do not dewet. The second one incorporates a patented bidentate silane, combined with a double-endcapping process that protects the silica from dissolution at high pH. The effect of mobile phase composition (acetonitrile content, pH) on nucleoside retention was investigated to determine the optimum separation conditions.

3.1.1. Effect of acetonitrile content in the mobile phase

The effect of acetonitrile content in the NH_2OH–CH_3COOH buffer (I = 5 mM, pH 7)/acetonitrile mobile phase on nucleoside retention factors was investigated for each C_{18} column (Table 1). Retention factors (k) were calculated for six compositions of the eluent assuming a signal of injection solvent (methanol) as a dead time marker. As expected in reversed-phase HPLC, increasing the water content from 80 to 95% (v/v) resulted in a general increase in the retention factors due to greater hydrophobic interactions between the bonded alkyl stationary phase and the nucleosides. The

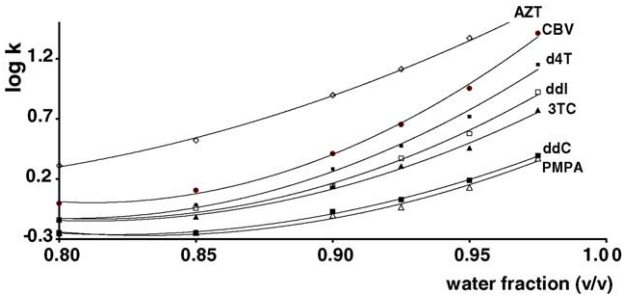

Fig. 2. Variation of log k values of nucleosides vs. water content (% v/v) in the mobile phase on C_{18} silica stationary phase. Column: Atlantis^{TM} dC_{18} (150 mm × 2.1 mm, 5 μm); mobile phase: aqueous NH_2OH–CH_3COOH buffer (ionic strength of 5 mM, pH 7)/NH_2OH–CH_3COOH buffer (ionic strength of 5 mM, pH 7) dissolved in acetonitrile (v/v); detector set at 260 nm; temperature: 25 °C; flow rate: 0.2 mL/min; solutes: see Fig. 1 for peak abbreviation identification.

log k-values of the different nucleobases are plotted against water content (Fig. 2). In the 80–95% water content range the dependence of the logarithmic retention factor on the concentration of acetonitrile in the mobile phase was not linear, but behaves as a quadratic curve. This trend has been previously been observed by several authors [17,18] for a reversed-phase stationary phase and can be expressed by the Eq. (1):

$$\ln k = A\phi^2 + B\phi + C \quad (1)$$

Table 1
Influence of the acetonitrile content (% v/v) of acetic acid–hydroxylamine buffer/acetonitrile mobile phase on HPLC retention factors (k) of anti-HIV nucleosides

Drug name (abbreviation)	Acetonitrile content (%)					
	2.5	5	7.5	10	15	20
Tenofovir (PMPA)						
k_1	2.35	1.35	0.92	0.79	0.57	0.53
k_2	0.76	0.45	0.27	0.19	0.13	0.12
Zalcitabine (ddC)						
k_1	2.48	1.54	1.06	0.85	0.57	0.55
k_2	1.05	0.57	0.36	0.26	0.17	0.15
Lamivudine (3TC)						
k_1	5.91	2.86	2.02	1.373	0.77	0.72
k_2	2.05	0.99	0.59	0.4	0.23	0.15
Didanosine (ddI)						
k_1	8.36	3.78	2.35	1.38	0.90	0.72
k_2	3.28	1.35	0.67	0.40	0.23	0.15
Stavudine (d4T)						
k_1	14.19	5.24	2.99	1.92	0.97	0.72
k_2	3.78	1.71	0.86	0.57	0.23	0.15
Carbovir (CBV)						
k_1	25.85	9.00	4.49	2.57	1.28	0.99
k_2	8.70	6.19	1.30	0.75	0.30	0.15
Zidovudine (AZT)						
k_1	>35	23.51	13.01	7.86	3.32	2.04
k_2	19.68	8.84	5.05	2.52	0.94	0.64
Tenofovir diisoproxyl fumarate (TDF)						
k_1	>35	>35	>35	>35	>35	>35
k_2	>35	>35	>35	>35	>35	>35

Columns: Atlantis^{TM} dC_{18} (150 mm × 2.1 mm, 5 μm) and Zorbax Extend-C_{18} (150 mm × 2.1 mm, 5 μm); mobile phase: NH_2OH/CH_3COOH (ionic strength of 5 mM. (1) pH 7)–acetonitrile (v/v); flow rate: 0.2 mL/min; (2) detector set at 260 nm; temperature: 25° C.

Table 2
Regression analysis of $\ln k = A\phi^2 + B\phi + C$ curves for anti-HIV nucleosides

Drug abbreviation	A	B	C	r^2
PMPA	30.038	−49.942	20.487	0.9902
ddC	27.332	−44.835	18.131	0.998
3TC	33.72	−54.712	22.044	0.9947
ddI	39.476	−64.194	25.964	0.9957
d4T	42.506	−68.369	27.364	0.9946
CBV	52.013	−84.48	34.306	0.9975
AZT	21.50	−30.625	11.04	0.9989

where ϕ is the volume fraction of water in the mobile phase and A, B, C are constants. Retention data have been analyzed by second-order polynomial regression and Table 2 reports experimental values of A, B and C constants and the correlation coefficient. At pH 7, all nucleosides behave as neutral compounds, except for PMPA, which is negatively charged and elutes first. Table 1 compares their retention on Atlantis™ dC_{18} and Zorbax Extend-C_{18} at similar mobile phase compositions. The retention of nucleosides varied with the nature of the bonded phase, with a much more pronounced retention on Atlantis™ dC_{18} (carbon load 12%, surface area 330 m^2/g, pore size 100 Å) than on Zorbax Extend-C_{18} (carbon load 12.5%, surface area 180 m^2/g, pore size 80 Å) column (Table 1). Thus, for instance at 10% acetonitrile content, k-values between two columns varied from 0.79 to 0.19 for polar PMPA ($\log P = -1.7$), and from 7.8 to 2.5 for the less polar AZT ($\log P = -0.05$). Otherwise, first eluting polar nucleosides, PMPA and ddC ($\log P = -1.3$), could be only resolved on Atlantis™ dC_{18} column at the difference of Zorbax Extend-C_{18} column where they co-eluted even at high water content. According to Rezk et al. [19], the sulfur atom in 3TC could be involved in an additional hydrogen-bond interaction with the stationary phase leading to a greater retention (around two times) for 3TC than for ddC. At last, diisoproxylfumarate groups on tenofovir prodrug greatly increase the hydrophobicity of the molecule ($\log P = 0.98$) and induce a stronger retention.

3.1.2. Effect of the pH of the mobile phase

When the pH of the mobile phase increased from pH 4–5.5, nucleosides such as ddC, 3TC, TDF, behave initially as positively charged compounds, then as neutral solutes, and their retention factors slightly increase. As the pH of the mobile phase varied from 7 to 9, acid nucleosides (AZT, d4T, ddI) are initially neutral and then become negatively charged; their retention factors slightly decrease with increasing mobile phase pH (see AZT peak at pH 9 in Fig. 3). In the 4–9 pH range, carbovir (CBV) is neutral and its retention factor is not pH dependent, while PMPA is always negatively charged and elutes firstly. As a consequence of retention behavior, a mobile phase of pH 5–7 seemed suitable for the separation of these nucleosides. Based on the known acidic instability of some anti-HIV nucleosides, we decided to work at pH 7, which is close to the physiological pH.

Table 3
Mobile phase gradient program of the chromatographic method used

Retention time (min)	% solvent A (aqueous buffer)	% solvent B (buffered acetonitrile)
0	93	7
4	90	10
5	50	50
9	30	70
15	30	70

3.1.3. Selected elution gradient

Finally, the separation of eight antiviral nucleosides was compared on these two C_{18} columns by using the same elution gradient described in Table 3, where solvents A and B have a constant ionic strength (5 mM). The Atlantis™ dC_{18} column was selected due to its ability to resolve PMPA and ddC (Fig. 4). Peak efficiencies of different nucleosides were high enough (around 40,000 theoretical plates for CBV, AZT, TDF) and resolutions between two consecutive solutes ranged between 2.0 and 6.9 (Table 4). Peak shapes are satisfactorily with an asymmetry factor around 1.3, except for PMPA (1.7); indeed, non-endcapped silanols on Atlantis™ dC_{18} interact with free phosphate groups of PMPA, which induces peak tailing.

3.2. Quantitative analysis in HPLC/UV

This section does not intend to fully validate the method but presents the preliminary assessments of several main

Table 4
Separation parameters obtained under optimized elution gradient

Drug abbreviation	Retention time (min)	Efficiency (theoretical plates)	Asymmetry factor (10%)	Resolution
PMPA	4.84	2200	1.7	–
ddC	5.78	6670	1.3	2.6
3TC	7.56	13020	1.2	5.0
ddI	8.37	18450	1.2	2.4
d4T	8.82	26730	1.2	2.5
CBV	9.32	37910	1.3	2.0
AZT	10.01	43100	1.3	4.2
TDF	12.13	47540	1.3	6.9

Column: Atlantis™ dC_{18}(150 mm × 2.1 mm, 5 μm): mobile phase: solvent A: aqueous NH_2OH–CH_3COOH buffer (ionic strength of 5 mM, pH 7); solvent B: NH_2OH–CH_3COOH buffer (ionic strength of 5 mM, pH 7) dissolved in acetonitrile; gradient profile: 0–4 min from 7 to 10% B; 4–5 min from 10 to 50% B; 5–9 min from 50 to 70% B; 9–15 min 70% B; detector set at 260 nm; temperature: 25 °C; flow rate: 0.2 mL/min.

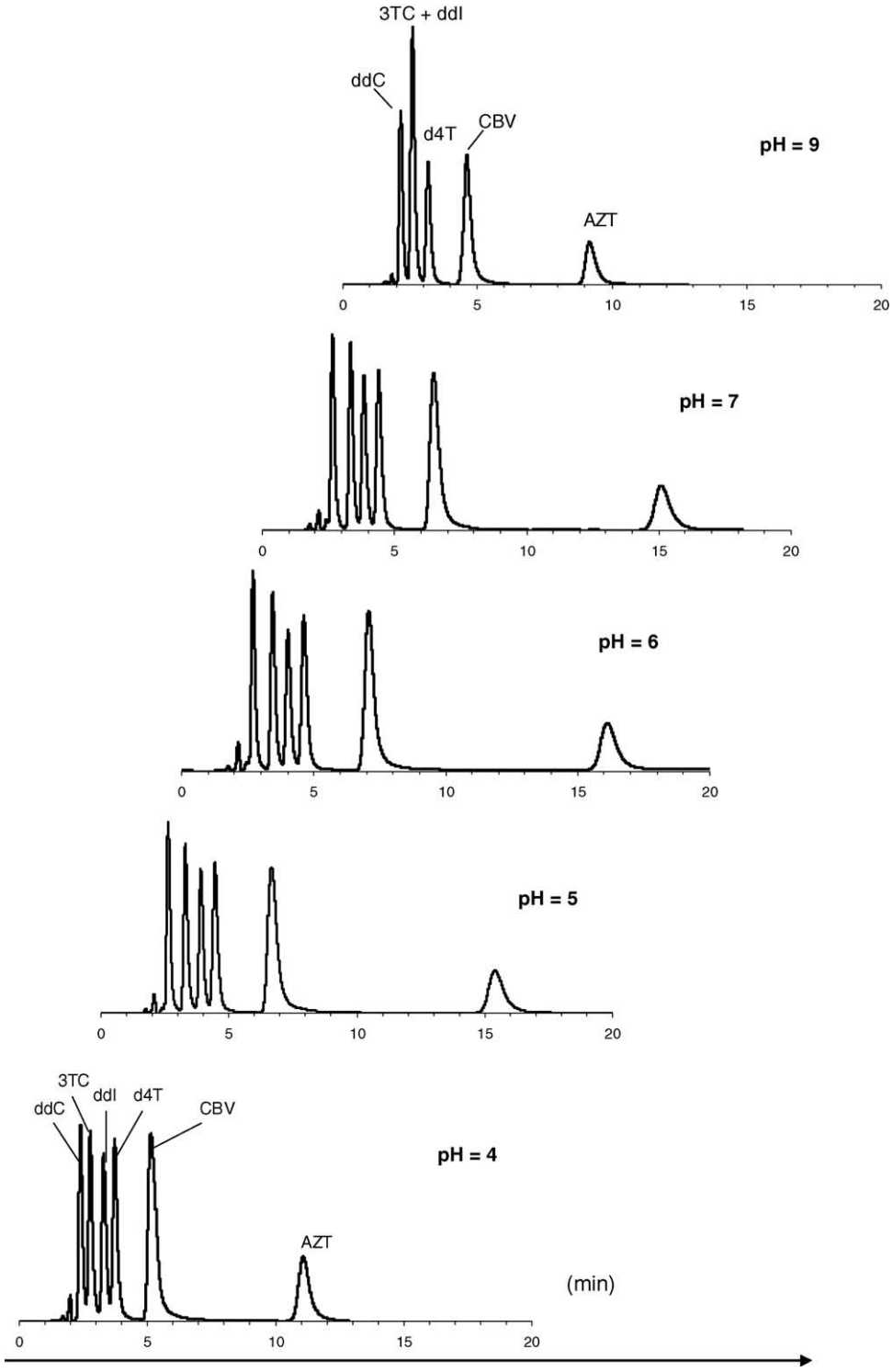

Fig. 3. Influence of mobile phase pH on HPLC separation of anti-HIV nucleosides. Column: Zorbax Extend-C_{18} (150 mm × 2.1 mm, 5 μm); mobile phase: solvent A: NH_2OH–CH_3COOH buffer (ionic strength of 5 mM), solvent B: NH_2OH–CH_3COOH buffer (ionic strength of 5 mM) dissolved in acetonitrile; gradient profile: 0–4 min from 7 to 10% B; 4–5 min from 10 to 50% B; 5–9 min from 50 to 70% B; 9–15 min 70% B; UV detection at 260 nm; temperature: 25 °C; flow rate: 0.2 mL/min; solutes: see Fig. 1 for peak abbreviation identification.

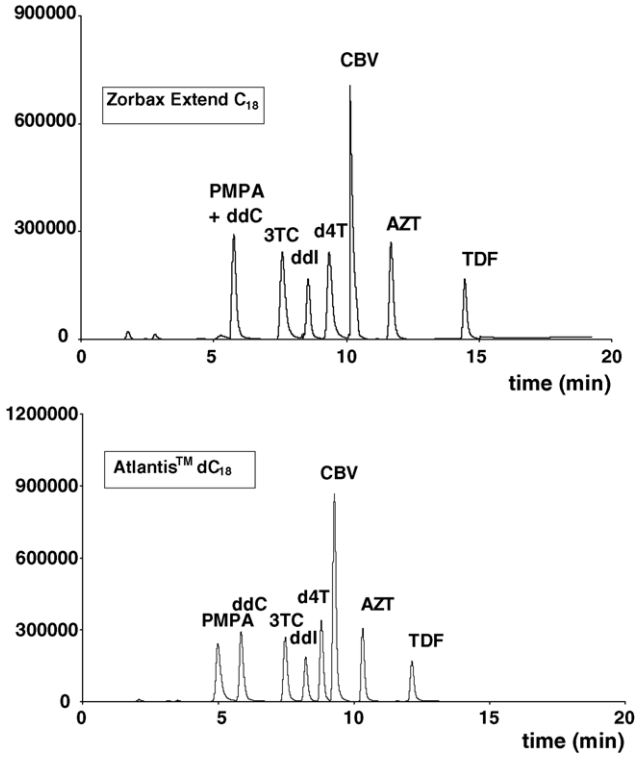

Fig. 4. Chromatograms of studied anti-HIV nucleosides on two both C18 silica columns. Column: (1) Zorbax Extend-C_{18} (150 mm × 2.1 mm, 5 μm); (2) Atlantis™ dC_{18} (150 mm × 2.1 mm, 5 μm); mobile phase: solvent A: aqueous NH_2OH–CH_3COOH buffer (ionic strength of 5 mM, pH 7), solvent B: NH_2OH–CH_3COOH buffer (ionic strength of 5 mM, pH 7) dissolved in acetonitrile; gradient profile: 0–4 min from 7 to 10% B; 4–5 min from 10 to 50% B; 5–9 min from 50 to 70% B; 9–15 min 70% of B; UV detection at 260 nm; temperature: 25 °C; flow rate: 0.2 mL/min; solutes: see Fig. 1 for peak abbreviation identification.

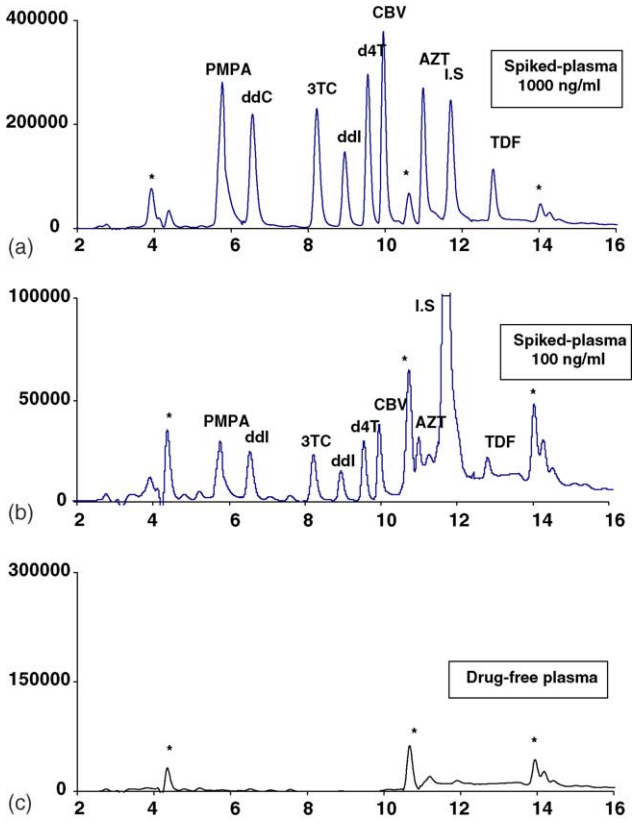

Fig. 5. Chromatograms of (a) spiked plasma sample at high level concentration (1000 ng/mL); (b) spiked plasma sample at low level concentration (100 ng/mL); (c) drug-free rat plasma extract. Experimental conditions as in Fig. 4; (*) endogenous plasma component.

criteria, e.g. linearity, accuracy and intra-day repeatability and inter-day reproducibility.

First, a SPE procedure of plasma samples was performed in order to avoid interferences from endogenous components in HPLC–UV chromatograms. The SPE step often requires the use of an internal standard to access reproducible linearity and for the validation process. We have tested several nucleosides and found that the non-nucleoside antipyrine (2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) afforded the best results with an extraction recovery closely related to the NRTIs one. The SPE pre-treatment on Oasis® HLB cartridges yielded to acceptable recoveries for these antiretroviral nucleosides (1 μg/mL concentration): 71% for PMPA, 92% for ddC, 88% for 3TC, 91% for ddI, 86% for d4T, 92% for CBV, 93% for AZT and 86% for TDF.

3.2.1. Linearity, limit of quantification (LOQs)

For each nucleoside, linearity was assessed using five standard solutions (each injected in triplicate). Linear relationships were obtained for all tested drugs in the following concentration range investigated: (30–10,000 ng/mL for PMPA, ddC, 3TC and d4T, 50–10,000 ng/mL for ddI, 70–10,000 ng/mL for CBV, 80–10,000 ng/mL for AZT and 90–10,000 ng/mL for TDF); least squares regression yielded

Table 5
Results of linearity studies and LOD values determination for each anti-HIV nucleoside

Drug	Slope	RSD (%)	Intercept	RSD (%)	r^2	LOQ (ng/mL)
PMPA	0.2117	2.4	−0.0171	1.8	0.9962	30
ddC	0.2012	1.8	−0.0342	1.5	0.9960	30
3TC	0.1686	2.1	−0.0421	1.2	0.9976	30
ddI	0.276	2.6	−0.0296	2.4	0.9924	50
d4T	0.1847	1.4	0.0038	1.7	0.9981	30
CBV	0.0921	1.6	0.0101	1.5	0.9996	70
AZT	0.1521	1.8	0.0462	2.1	0.9928	80
TDF	0.0899	2.1	0.0094	1.8	0.9952	90

Same conditions as in Table 4.

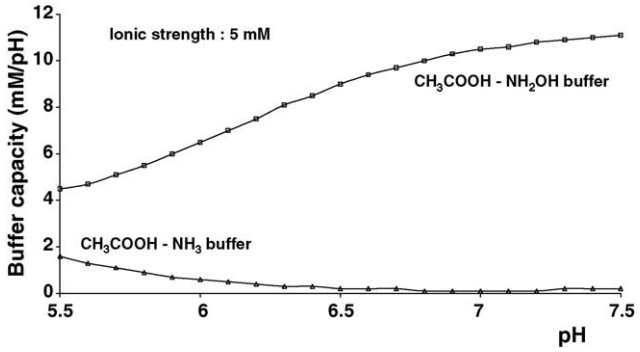

Fig. 6. Variation of buffer capacity of volatile acetic acid/hydroxylamine buffer at medium-pH range.

good correlation coefficients ($r^2 > 0.992$) and relative standard deviations (RSD) on the slope ranged from 1.4 to 2.6%, and on the intercept from 1.2 to 2.4% (Table 5).

In this report, the limits of quantitation were calculated by linear extrapolation of signal-to-noise ratios as a function of concentration. For each nucleoside, a S/N value of 10 was used to determine the LOQ, ranging from 30 to 90 ng/mL, respectively (Table 5). HPLC–UV chromatograms obtained after SPE step of drug-free rat plasma and of spiked rat plasma at low (100 ng/mL) and high (1000 ng/mL) concentration levels are shown in Fig. 5. No significant interference of endogenous components in rat plasma occurred with any of the anti-HIV nucleoside.

Table 6
Results of accuracy studies for each anti-HIV nucleoside

Drug	Concentration (μg/mL)	Accuracy (%)
PMPA	0.03	104.2
	1.0	101.3
	10	101.1
ddC	0.03	97.7
	1.0	100.5
	10	102.6
3TC	0.03	96.9
	1.0	101.4
	10	102.9
ddI	0.05	104.8
	1.0	101.1
	10	102.7
d4T	0.03	97.8
	1.0	101.0
	10	100.3
CBV	0.07	103.2
	1	101.4
	10	100.8
AZT	0.08	94.9
	1.0	103.4
	10	99.9
TDF	0.09	95.6
	1.0	101.2
	10	102.4

Same conditions as in Table 4.

Table 7
Results of (a) intra-day repeatability and (b) inter-day reproductibility studies for each anti-HIV nucleoside

Drug	Concentration (μg/mL)	RSD on retention time (%)	RSD on peak area (%)
(a) Repeatability			
PMPA	0.03	1.1	6.9
	1	1.3	3.2
	10	0.8	4.8
ddC	0.03	1.2	5.6
	1	1.1	3.8
	10	1.3	3.2
3TC	0.03	0.9	4.2
	1	0.9	2.6
	10	0.7	2.8
ddI	0.05	1.4	3.1
	1	1.1	2.4
	10	0.9	2.6
d4T	0.03	1.1	3.5
	1	1.3	2.4
	10	1.2	2.4
CBV	0.07	1.3	4.2
	1	1.1	2.6
	10	1.2	2.8
AZT	0.08	0.9	5.9
	1	0.8	2.6
	10	1.0	3.2
TDF	0.09	1.1	3.6
	1	1.2	2.4
	10	0.9	2.7
(b) Reproducibility			
PMPA	0.03	1.5	7.8
	1	1.8	4.5
	10	1.2	5.6
ddC	0.03	1.4	6.5
	1	1.3	4.2
	10	1.7	4.5
3TC	0.03	1.2	5.1
	1	1.4	3.5
	10	1.6	3.8
ddI	0.05	1.7	4.1
	1	1.4	3.2
	10	1.5	2.9
d4T	0.03	1.3	4.2
	1	1.4	2.9
	10	1.5	3.0
CBV	0.07	1.8	4.8
	1	1.4	3.2
	10	1.5	3.5
AZT	0.08	1.3	6.7
	1	1.4	3.9
	10	1.6	4.6
TDF	0.09	1.7	5.0
	1	1.4	3.3
	10	1.6	3.9

Same conditions as in Table 4.

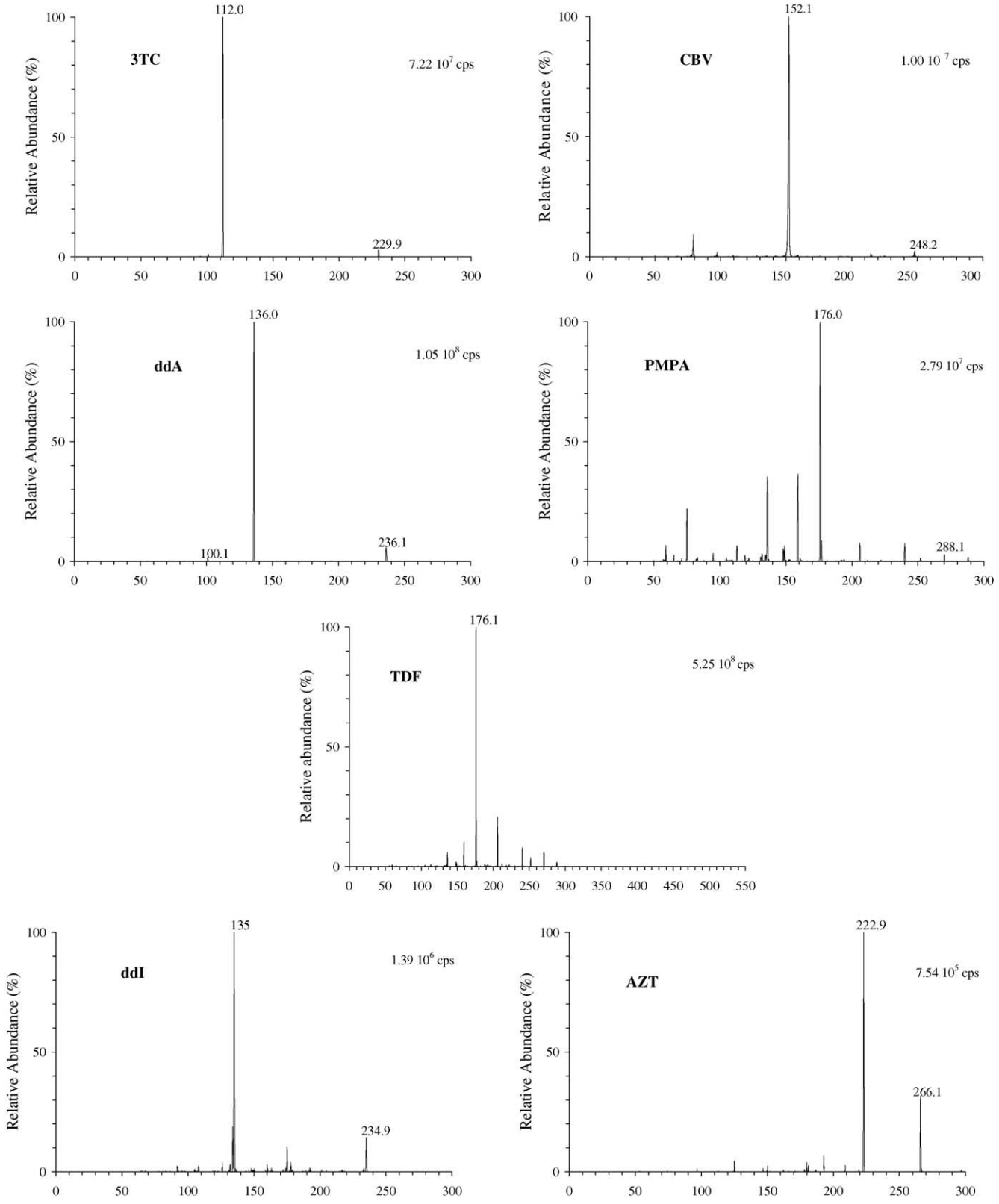

Fig. 7. Full-scan product ion spectra of the protonated and deprotonated molecules of NRTIs. Acquisition in MCA mode (10 scans); infusion of each solute (1000 ng/mL in the mobile phase) at a flow rate of 3 μL/min.

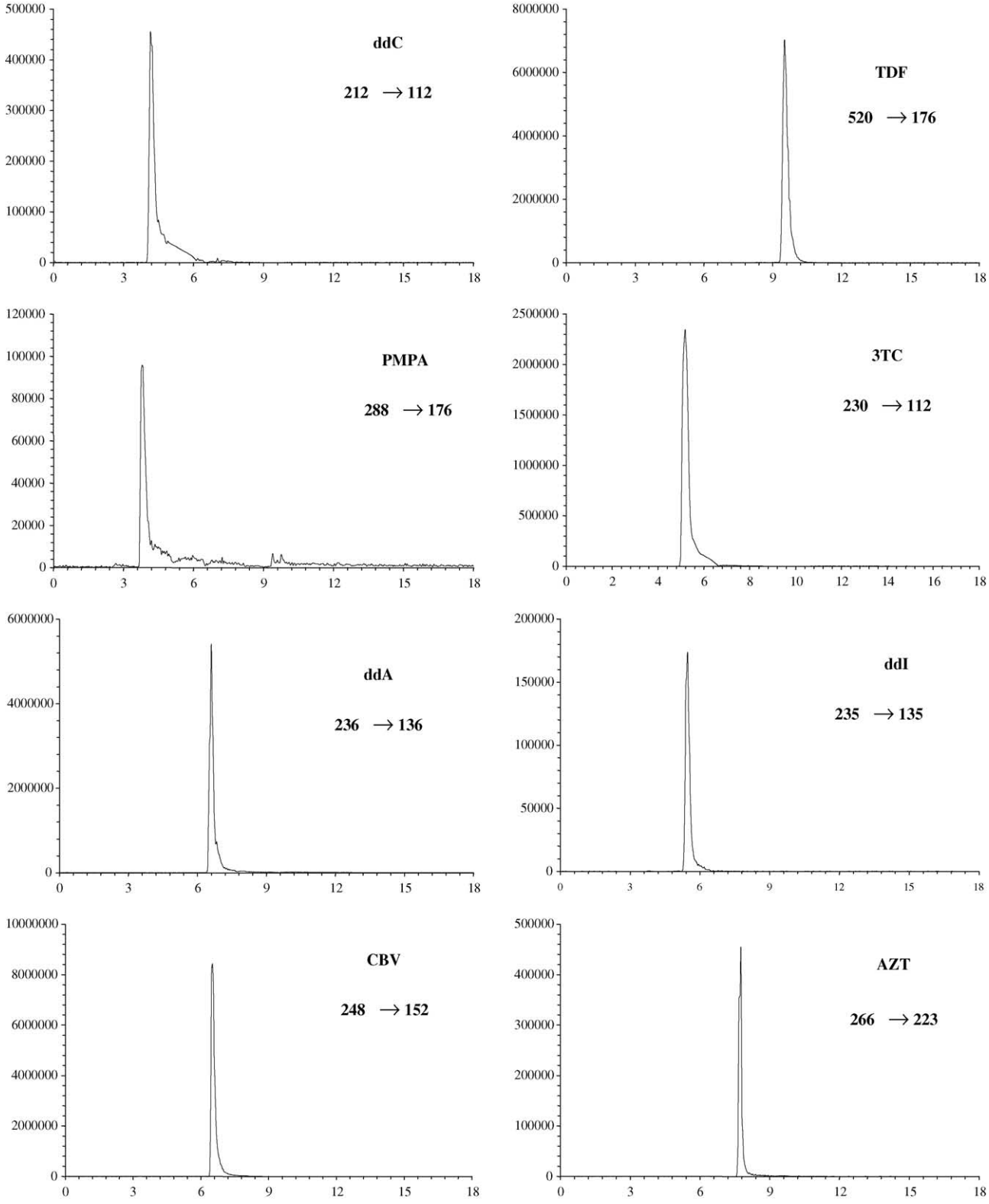

Fig. 8. HPLC–ESI-MS/MS analysis of the nucleosides pool: extracted ion currents of ddC, CBV, 3TC, ddA, PMPA, TDF, AZT and ddI. Column: Atlantis^{TM} dC_{18} (150 mm × 2.1 mm i.d., 5 μm); mobile phase: solvent A: aqueous NH_2OH–CH_3COOH buffer (ionic strength of 5 mM, pH 7), solvent B: NH_2OH–CH_3COOH buffer (ionic strength of 5 mM, pH 7) dissolved in acetonitrile; linear gradient profile: 5–70% of B in 10 min then 70% of B during 5 min; temperature: 25 °C; flow rate: 0.2 mL/min. MS/MS parameters: capillary voltage: ±3 kV; cone voltage: ±50 V; source temperature: 120 °C; desolvatation gas temperature: 360 °C; MRM transitions and collision energies: CBV: 248 → 152 (−14 eV); 3TC: 230 → 112 (−12 eV); ddA: 236 → 136 (−15 eV); ddC: 212 → 112 (−15 eV); PMPA: 288 → 176 (−25 eV); TDF: 520 → 176 (−30 eV); AZT: 266 → 223 (+ 11 eV); ddI: 235 → 135 (+ 24 eV).

3.2.2. Accuracy

The quality control samples were injected – at three levels of concentration low (LOQ), medium (1 μg/mL) and high (10 μg/mL) – in triplicate to evaluate the intra-day accuracy. For each nucleoside and concentrations tested, the accuracies were calculated and expressed as a percentage of the theoretical concentration (Table 6). The intra-day accuracy values were found to range from 94.9 to 104.8%. These acceptable results confirm that an internal standard was required.

3.2.3. Repeatability/reproducibility

The intra-day repeatability of the method was assessed by injecting six times quality control plasma samples at three different concentrations (LOQ, 1.0 and 10 μg/mL) (Table 7(a)). Repeatabilities, expressed as RSD of retention times were found to be generally less than 1.4% while RSD of peak areas ranged from 2.4 to 4.8% at the highest concentration (10 μg/mL), from 2.4 to 3.8% at the medium concentration (1 μg/mL), and from 3.1 to 6.9% at the lowest concentration (LOQ).

The inter-dayreproducibility of the procedure was determined by injecting in triplicate quality control plasma samples at three different concentrations (LOQ, 1.0 and 10 μg/mL), repeated on three days (Table 7(b)). RSD of retention times were found to be less than 1.8% while RSD of peak areas ranged from 2.9 to 5.6% at 10 μg/mL concentration, from 2.9 to 4.5% at 1 μg/mL concentration, and from 4.1 to 7.8% at LOQ concentration. Results of PMPA are the worst due to the asymmetric shape of its peak.

3.3. Test of hydroxylamine/acetic acid buffer in ESI-MS

Until now, the use of a volatile medium-pH buffered eluent compatible with MS appears rather limited. Indeed, the commonly used HPLC–UV phosphate buffer is non-volatile and has also the drawback to fasten the degradation of C_{18} columns. Otherwise, formic acid–ammonia and acetic acid–ammonia buffers are generally used in 2–4 and 3–5 pH ranges, respectively, and their volatility make them compatible with mass spectrometry.

So, a volatile buffer, obtained with acetic acid and hydroxylamine ($pK_a = 5.94$), has been tested in the medium-pH range with an electrospray-MS detection. Table 8 compares the buffer capacities at pH 5, 6 and 7 of several buffers—non volatile phosphoric acid/sodium hydroxide, semi-volatile phosphoric acid/ammonia, volatile acetic acid/ammonia and volatile acetic acid/hydroxylamine—at low ionic strength (5 mM). The acetic acid/hydroxylamine buffer appears to be an efficient volatile buffer in the medium pH range between 5 and 7. Thus, for an ionic strength of 5 mM, acetic acid/ hydroxylamine buffer has a 10, 100 and 19 times higher buffer capacities compared to those of acetic acid/ammonia buffer at pH 6, 7, 8, respectively (Fig. 6). Besides this volatile buffer works well down to pH 5, where its buffer capacity is quite the same to that of acetic acid/ammonia buffer.

Preliminary experiments were carried out, in positive and negative MS scan mode, by direct infusion into the ESI source of individual standard solutions of each nucleoside dissolved in acetic acid–hydroxylamine buffer (ionic strength 5 mM, pH 7)-acetonitrile mobile phase. The ESI-MS analysis of nucleosides revealed intense protonated (PMPA, ddC, ddA, 3TC, CBV, TDF) or deprotonated (ddI and AZT) molecule signals. PMPA is negatively charged in aqueous HPLC mobile phase, but gives positively charged ions during the ESI process. The ESI parameters (capillary and cone voltages) were optimized for each compound to get the highest signal intensity. The mass spectrometer was then switched to the daughter scan mode, and the precursor ions were fragmented by collision-induced dissociation (CID) with argon. Collision energies were optimized to obtain the best intensity of the most abundant product ions (see Table 9).

Fig. 7 illustrates the typical full-scan ESI-MS/MS spectra of studied nucleosides, acquired in multi-channel analyser (MCA) mode, under optimized conditions. The MS/MS detection of these compounds, performed in the MRM mode, was demonstrated to be analyte-specific since selected transitions are different from one to another (Table 9). No full chromatographic separation is needed, thus an eluting mobile phase allowed to shorten the analysis time. Besides, the use of a high organic content in the mobile phase improves the MS signal stability. HPLC separation was carried out on an Atlantis^{TM} dC_{18} column (150 mm × 2.1 mm i.d., 5 μm) with an elution gradient. The eluent was driven at a 200 μL/min flow-rate and no split was required. Fig. 8 reported MRM chromatograms of several antiretroviral nucleosides, which have been resolved in HPLC with an aqueous hydroxylamine acetate volatile buffer (pH 7)-acetonitrile elution gradient. Thus, hydroxylammonium acetate appears to be an interesting volatile buffer in the medium pH range and its volatility makes it an useful tool for LC–MS.

Table 8
Comparison of buffer capacities (mM/pH) of common non-volatile, semi-volatile buffers vs. volatile acetic acid–hydroxylamine buffer in medium-pH range

Buffer pH	Phosphoric acid–sodium hydroxide[a]	Phosphoric acid–ammonia[b]	Acetic acid–ammonia[c]	Acetic acid–hydroxylamine[c]
5.0	0.1	0.1	4.0	5.1
6.0	0.7	0.7	0.6	6.4
7.0	1.6	1.6	0.1	10.4

[a] Non volatile.
[b] Semi-volatile.
[c] Volatile.

Table 9
Selected ion transitions (*m*/*z* values) and optimised ESI-MS/MS parameters for HPLC–ESI-MS/MS analysis of NRTIs, in multiple reaction monitoring (MRM) mode

	Precursor ion $[M+H]^+$ (*m*/*z*)	Product ion (*m*/*z*)	ESI capillary voltage (kV)	Cone voltage (V)	Collision energy (Ev)
$[M+H]^+$					
PMPA	288.3	176.0	+3.4	−50	−25
ddC	212.0	112.1	+2.0	−31	−15
3TC	230.2	112.1	+3.4	−40	−12
ddA	236	135.9	+3.4	−31	−15
CBV	248.2	152.1	+3.4	−31	−14
TDF	520.2	176.0	+3.4	−40	−50
$[M-H]^-$					
ddI	235.1	135.1	−3.4	+50	+24
AZT	265.9	223.0	−3.4	+31	+11

4. Conclusion

A specific reversed-phase high-performance liquid chromatography has been developed for the simultaneous analysis of eight antiretroviral nucleosides. These drugs were quantitatively recovered from rat-plasma by solid-phase extraction on Oasis® HLB cartridges. Their separations were achieved on the Atlantis™ dC_{18} column with an hydroxylamine acetate buffer (pH 7)-acetonitrile elution gradient. With an UV detection at 260 nm, LOQs ranged from 30 ng/mL (PMPA, ddC, 3TC, d4T), 50 for ddI, to 70–90 ng/mL (CBV, AZT, TDF). This HPLC/UV method is simple, selective and sensitive and can be used to evaluate pharmacokinetics in mouse model.

Finally, the volatile acetic acid/hydroxylamine buffer appears to be very useful in the medium 5–7 pH range for HPLC–ESI-MS/MS. Indeed, at pH 7 and low ionic strength (5 mM), its buffer capacity is one hundred times higher to that obtained for the usual acetic acid/ammonia buffer.

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References

[1] M. Sension, HIV Clin. Trials 5 (2004) 99.
[2] L.A. Agrofoglio, S.R. Challand, Acyclic, carbocyclic and L-nucleosides, Kluwer Academic Publishers, Dordrecht, 1998.
[3] Due to its recent FDA approval, the authors were not able to include it within the frame of this study.
[4] C. Zanone, L.R. Chiarelli, G. Valentini, E. Perani, L. Annovazzi, S. Viglio, P. Iadarola, Electrophoresis 25 (2004) 3270.
[5] P.A. Furman, J.A. Fyfe, M.H. St. Clair, K. Weinhold, J.L. Rideout, G.A. Freeman, S.N. Lehrman, D.P. Bolognesi, S. Broder, H. Mitsuya, et al., Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 8333.
[6] C.V. Fletcher, E.P. Acosta, K. Henry, L.M. Page, C.R. Gross, S.P. Kawle, R.P. Remmel, A. Erice, H.H. Balfour, Clin. Pharm. Ther. 64 (1998) 331.
[7] M. Barry, F. Mulcahy, C. Merry, S. Gibbons, D. Back, Clin. Pharmacokinet. 36 (1999) 289.
[8] D.L. Walters, D.L. Jacobs, J.E. Tomaszewski, S. Graves, J. Pharm. Biomed. Anal. 19 (1999) 955.
[9] A.R. Swagler, M. Qian, J.M. Gallo, J. Pharm. Pharmacol. 43 (1991) 823.
[10] K.B. Kenney, S.A. Wring, R.M. Carr, G.N. Wells, J.A. Dunn, J. Pharm. Biomed. Anal. 22 (2000) 967.
[11] C.A. Knupp, F.A. Stancato, E.A. Papp, R.H. Barbhaiya, J. Chromatogr. B 533 (1990) 282.
[12] R.W. Sparidans, K.M.L. Crommentuyn, J.H.M. Schellens, J.H. Beijnen, J. Chromatogr. B 791 (2003) 227.
[13] A. Volosov, C. Alexander, L. Ting, S. Soldin, Clin. Biochem. 35 (2002) 99.
[14] A.S. Pereira, K.B. Kenney, M.S. Cohen, J.E. Hall, J.J. Eron, R.R. Tidwell, J.A. Dunn, J. Chromatogr. B 742 (2000) 173.
[15] G. Aymard, M. Legrand, N. Trichereau, B. Diquet, J. Chromatogr. B 744 (2000) 227.
[16] D.L. Walters, D.L. Jacobs, J.E. Tomaszewski, S. Graves, J. Pharm. Biomed. Anal. 19 (1999) 955.
[17] P.J. Schoenmakers, H.A. Billiet, L. de Galan, J. Chromatogr. 185 (1979) 179.
[18] M.C. Henion, C. Picard, C. Combellas, M. Caude, R. Rosset, J. Chromatogr. 210 (1981) 211.
[19] N.L. Rezk, R.R. Tidwell, A.D.M. Kashuba, J. Chromatogr. B 791 (2003) 137.