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Quantitative determination of zidovudine diaryl phosphate triester pro-drugs in rat plasma by high-performance liquid chromatography–electrospray ionization tandem mass spectrometry

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ABSTRACT

A rapid, simple, and sensitive high-performance liquid chromatography/electrospray ionization tandem mass spectrometry (HPLC/ESI–MS/MS) method was developed and validated for quantitative analysis of 3′-azido-3′-deoxythymidine (zidovudine, AZT) diaryl phosphate triester pro-drugs, in rat plasma using 2′,3′-dideoxy-2′3′-didehydrothymidine (d4T) as internal standard (IS). The analytes were extracted from rat plasma with methanol after protein precipitation. The compounds were separated by HPLC with gradient elution (on a Shim-pack VP-ODS C_{18} analytical column using a mobile phase of methanol/10 mM ammonium acetate). All the analytes were detected in positive ion mode using multiple reaction monitoring (MRM). The method was validated and the specificity, linearity, lower limit of quantitation (LLOQ), precision, accuracy, recoveries and stability were determined. LLOQs were 10 ng mL^{-1} for M1, M2, M3, M4, and M5, respectively. Correlation coefficient (r) values for the linear range of 10–10,000 ng mL^{-1} were greater than 0.999 for all the analytes. The intra-day and inter-day precision and accuracy were higher than 7.13%. The relative and absolute recovery was above 72% and no matrix effects were observed for all the analytes. This validated method provides a modern, rapid, and robust procedure for the pharmacokinetic studies of the pro-drugs after intravenous administration to rats. Some important results of AZT diaryl phosphate triester pro-drugs concerning chemical effect on pharmacokinetic performance are also studied.

1. Introduction

Zidovudine (AZT) is one of the most important antiretroviral agents since it was the first drug approved for the treatment of AIDS [1–5]. However, the clinical application of AZT is limited by its bone marrow toxicity and suppression, low therapeutic index, low localization in brain, and a short half-life in blood [6–8]. Thus, in an attempt to reduce or abolish its side effects and to improve the therapeutic potential [9], research has focused on the discovery of pro-drugs of AZT. Previous studies showed that the diaryl phosphate triester derivatives of AZT had a pronounced and selective anti-HIV-2 effect in CEM/0, MT4 [10,11], the magnitude of which varied considerably with the nature of the aryl substituent [12–14]. The results indicated that the diaryl phosphate triester derivatives of AZT, as pro-drugs of nucleoside analogue, might have therapeutic potential for the treatment of HIV infection.

Furthermore, it showed improved uptake of AZT, a longer residence time and a sustained release of AZT, after administration of some pro-drugs, in our preliminary study on pharmacokinetics (unpublished results). By virtue of the success, the further overall pharmacokinetics research for a series of diaryl phosphate triester compounds will significantly enhance our understanding of the chemical structure effect on therapeutic anti-HIV efficacy and *in vivo* behavior, as well as provide a rich array of opportunities for the design of novel therapeutics [11]. The present study aims at developing a rapid method to satisfy the increasing demand of pharmacokinetics research.

It is now clear that monitoring the levels of active parent drugs and analysis of the major chemical constituents in drug metabolites from complex biological samples, such as plasma and urine, will give overall information about individual drug pharmacokinetics [15]. LC-based methods and extensive techniques have been explored, for quantitative analysis of AZT and its derivatives in biological matrices [16–22]. No study on the quantitative determination of AZT diaryl phosphate triester pro-drugs and their

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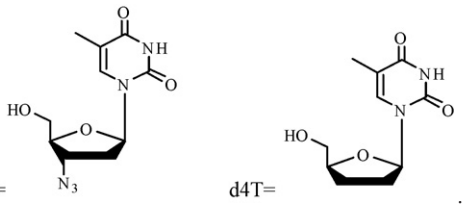
Table 1
Molecular weigh (MW), MRM transitions and structures of five pro-drugs.

Compound	MW	MRM transition	Structure
M1	499	500.2 → 80.9	R=H
M2	751	751.7 → 250.0	R=I
M3	657	658.1 → 250.0	R=Br
M4	567	569.3 → 81.1	R=Cl
M5	559	559.9 → 391.1	R=OCH_3
Internal standard (IS)	224	225.2 → 127.0	d4T



metabolites or LC–MS method for their determination in biological samples has been reported. The objective of the present study was to develop a rapid, efficient, specific, sensitive HPLC/ESI-MS/MS method for the quantitation of the model pronucleosides of AZT. The method was also proved to be a powerful technique for simultaneous quantitative analysis of multiple analytes such as parent pro-drugs and associated AZT metabolites, with minimal sample consumption. Chemical effect on pharmacokinetic performance are given for the first time.

2. Experimental

2.1. Chemicals and reagents

M1 (MW 499), M2 (MW 751), M3 (MW 657), M4 (MW 567), and M5 (MW 559) were synthesized, purified and characterized as described by Mcguigan et al. [9]. The purity of all chemicals was proved above 97% (identified by NMR and HPLC) and their chemical structures are shown in Table 1. d4T (internal standard, IS, MW 224) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade methanol, methylene dichloride was obtained from Tedia Company Inc. (Fairfield, USA). Deionized water was purified using a Classic UVF system (Millipore, Milford, MA, USA). Analytical-grade ammonium acetate was purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Instruments

HPLC was performed using an Agilent 1200 Series (Agilent Corporation, MA, USA) equipped with G1311A quaternary pump, G1322A vacuum degasser unit, G1329A autosampler and G1316A therm. column compartments. The HPLC system was coupled with an Applied Biosystems 3200 Q Trap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ont., Canada) via a TurboIonSpray electrospray ionization (ESI) interface for mass analysis and detection. The system was controlled by Analyst 1.4.1 data acquisition and processing software (Applied Biosystems/MDS Sciex).

2.3. Analytical conditions

The same chromatographic condition was employed for the five pro-drugs, devoted to separate d4T (IS), target analytes, and AZT due to consideration of simultaneous detection of parent drugs and the main metabolites of AZT (data not shown). Samples were analyzed on a Shim-pack VP-ODS C_{18} analytical column (150 mm × 4.6 mm, Shimadzu, Kyoto, Japan) protected by a C_{18}-guard column (10 mm × 4.6 mm, Shimadzu, Kyoto, Japan). The column was set at 35 °C and the injection volume was 10 μL. The flow rate was 0.8 mL min^{-1} and the mobile phase consisted of a mixture of 10 mM ammonium acetate in water (pH 6.5) (A) and methanol (B). The gradient was as follows: a linear gradient from A:B (65:35, v/v) to A:B (10:90, v/v) from 0 to 5 min, followed by an isocratic hold at 90% B for 6 min, then from 11 to 13 min by a linear gradient from A:B (10:90, v/v) to A:B (65:35, v/v). The total run time was 13 min.

The Applied Biosystems 3200Q Trap tandem mass spectrometer, equipped with an electrospray source, was operated in the positive ion mode. The tuning parameters were optimized separately for all five compounds and the internal standard d4T by directly infusing the standard solution. The LC eluate was directed into the electrospray source without splitting. The current of the corona discharge needle was set at 3 mA. Nitrogen was used as the nebulizer, curtain, and collision gas. The ion spray voltage was set at 5300 V. The dwell time of each MRM transition was set at 0.2 s. The collision gas was

Table 2
Optimized mass spectrometric parameters.

Parameters	M1	M2	M3	M4	M5	d4T
Declustering potential (U V^{-1})	50	50	50	50	70	70
Entrance potential (U V^{-1})	5.0	5.0	10	10	5.0	5.0
Collision cell entry potential (U V^{-1})	20	40	20	20	20	20
Collision energy (U V^{-1})	40	30	20	20	50	50
Collision cell exit potential (U V^{-1})	2.3	5.0	5.0	2.0	5.0	5.0
Curtain gas (psi)	25	25	20	20	25	25
Temperature (°C)	400	400	400	400	350	350
Ion source Gas1 (psi)	75	75	70	70	75	75
Ion source Gas2 (psi)	70	70	70	70	70	70

set at medium. Interface-independent instrument parameters were summarized in Table 2.

Quantification was performed using multiple reaction monitoring (MRM) ion mode. The fragment transitions for the five analytes (precursor → metabolite) were monitored (m/z): M1, 500.2 → 80.9; M2, 751.7 → 250.0; M3, 658.1 → 250.0; M4, 569.3 → 81.1; M5, 559.8 → 391.1; and IS, 225.2 → 127.0. The mass resolution was set to a peak width of 0.7 mass units at half-height in the MRM mode. The MRM transitions of precursor ions to product ions optimized for M1, M2, M3, M4, and M5 are also shown in Table 1. The product ion spectra of the protonated molecules are presented in Fig. 1.

2.4. Preparation of standard calibration and quality control (QC) samples

Two primary stock solutions were prepared, based on the expected exposures of five compounds in rat plasma. Stock solutions (1 mg mL^{-1}) of M1, M2, M3, M4, and M5 were prepared separately in methanol. The stock solution of M1, M2, M3, M4, and M5 was further diluted with methanol to achieve standard working solutions at concentrations of 0.1, 0.2, 0.4, 1, 4, 10, 40 and 100 μg mL^{-1}. Internal standard working solution (1 μg mL^{-1}) was prepared by diluting the stock solution (5 mg mL^{-1}) of d4T with methanol. The standard working solutions (20 μL) were used to spike blank plasma samples (180 μL). The final concentrations of M1, M2, M3, M4, and M5 standard calibration samples were 0.01, 0.02, 0.04, 0.1, 0.4, 1, 4 and 10 μg mL^{-1}, respectively.

All standard solutions were stored at −20 °C, either for calibration curves of five analytes or QCs in the pre-study validation and during the pharmacokinetic study. Stock standard solutions (1 mg mL^{-1}) of each nucleoside pro-drug prepared in methanol were stable at −20 °C for 1 month without degradation.

For quality control (QC) samples, stock solution of M1, M2, M3, M4, and M5 was prepared at 0.1 mg mL^{-1} in methanol. The QC samples were also prepared in the same way by adding 20 μL diluted QC stock solution into 180 μL rat blank plasma. The final concentrations of M1, M2, M3, M4, and M5 in the low, medium and high QC samples were 0.01, 1, and 10 μg mL^{-1}, respectively.

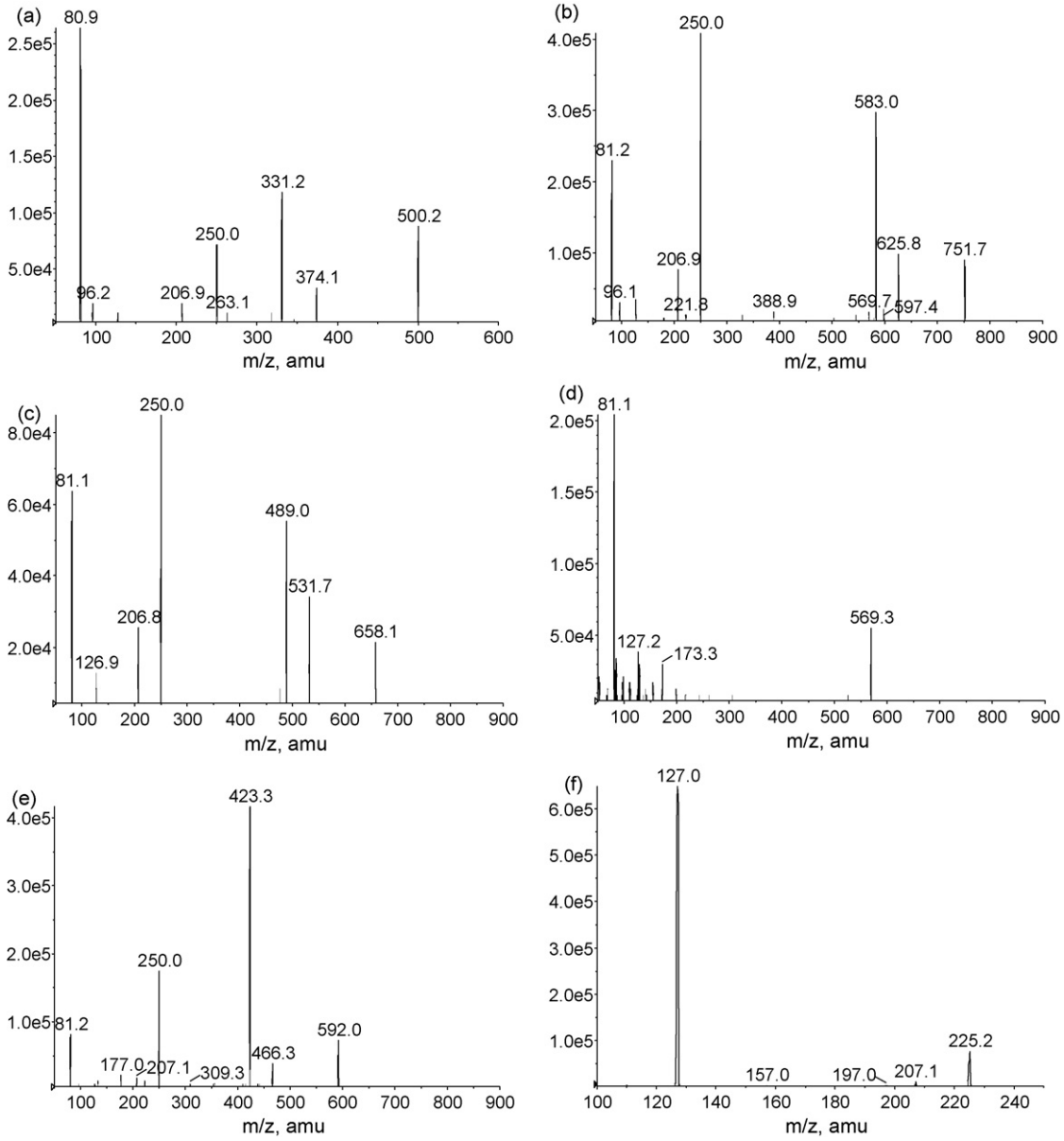


Fig. 1. Full scan ESI (+) product ion spectra of (a) M1: 500.2 → 80.9; (b) M2: 751.7 → 250.0; (c) M3: 658.1 → 250.0; (d) M4: 569.3 → 81.1; (e) M5: 559.9 → 391.1; (f) IS: 225.2 → 127.0.

2.5. Sample processing

100 μL aliquots of plasma sample were mixed with 50 μL of internal standard (IS), and diluted with methanol (350 μL), vortex-mixed and spun in a centrifuge (15,000 rps × 5 min) to precipitate the proteins. The supernatants were evaporated to dryness under nitrogen stream and reconstitute in 100 μL of methanol. Then, a 10 μL aliquot of supernatant was injected into the LC–MS/MS system.

2.6. Assay validation

Standard curves ranging from 0.01 to 10 μg mL^{-1} M1, M2, M3, M4, M5 were run on 3 separate days. Calibration curves were constructed from the peak area ratios of each analyte to IS versus plasma concentrations using a weighted linear least-squares regression analysis according to the equation $y = a + bx$, where y is the peak-area ratio of substance to internal standard, x is the concentration of analyte in the calibration sample, a is the intercept, and b is the slope of the regression line. The concentration of the analytes in the samples was calculated from the regression equation of the calibration curve. A three-point smoothing algorithm was applied to all ion chromatograms.

QC samples at three levels were processed and analyzed five times in the same run (intra-run precision) and one time in five separate runs (inter-run precision). The accuracy was determined as the percentage difference between the mean detected concentrations and the nominal concentrations. The lower limit of quantification (LLOQ) of all of the analytes was verified as the lowest concentration of the calibration curve assessed at which the inter-batch inaccuracy was $< \pm 15\%$ and imprecision $< \pm 15\%$.

The extraction recoveries of the five analytes at three QC levels were determined by comparing peak areas obtained from plasma samples with those found by direct injection of a standard solution of the same concentration.

The stability of five analytes in plasma was assessed by analyzing triplicate QC samples at two different concentration, stored for 2 h at ambient temperatures, three cycles of freezing at −20 °C and thawing, and stored for 1 month at −20 °C, respectively. Each concentration following storage was compared with freshly prepared samples of the same concentration. The results of stability studies indicated that the processed samples were stable at room temperature for at least 2 h, the plasma samples were stable after at least three freeze/thaw cycles, and the plasma samples were stable at −20 °C for at least 4 weeks.

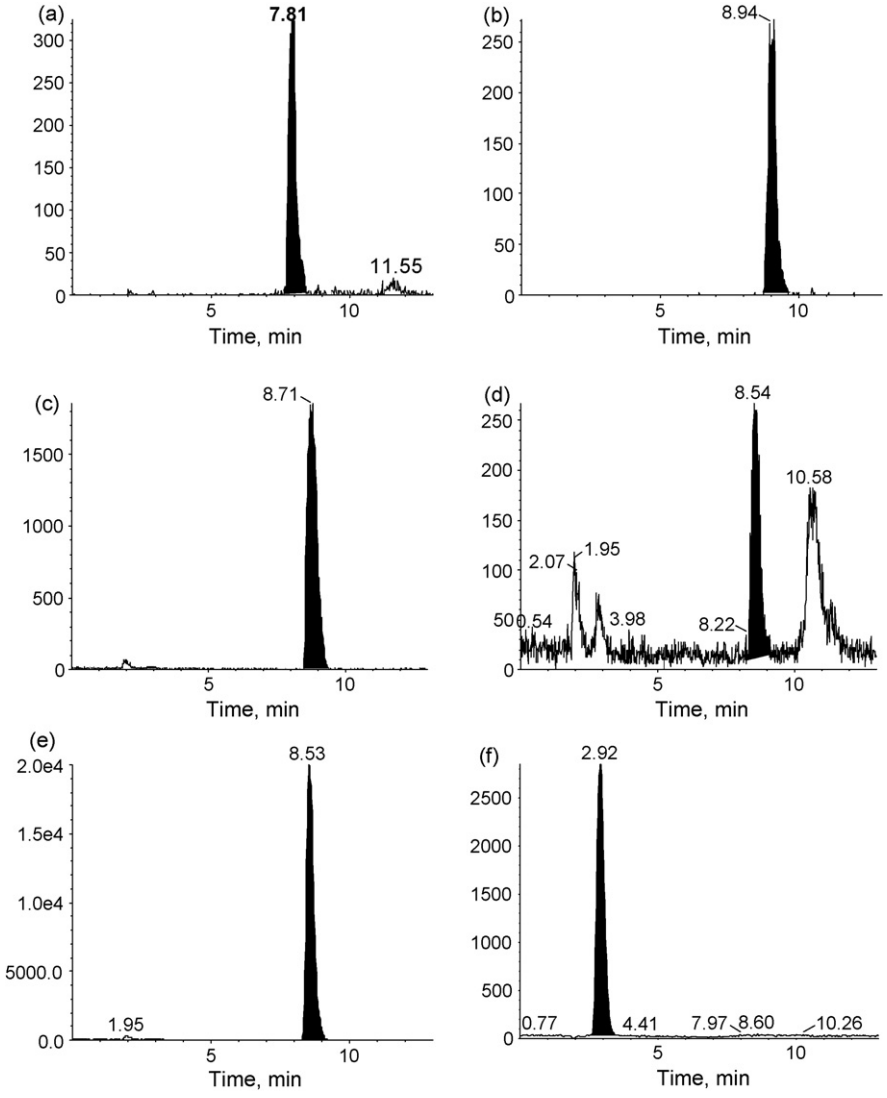


Fig. 2. Representative MRM chromatograms of rat plasmas spiked with six analytes and IS: (a) M1: 500.2 → 80.9; (b) M2: 751.7 → 250.0; (c) M3: 658.1 → 250.0; (d) M4: 569.3 → 81.1; (e) M5: 559.9 → 391.1; (f) IS: 225.2 → 127.0.

2.7. Application of the analytical method

Young adult male and female Sprague–Dawley rats, weighing 250–280 g, were provided by Shanghai SLAC Lab Animal Co. Ltd. (Shanghai, China). Rats ($n = 6$) received an intravenous administration of 5 mg kg^{-1} each pro-drug. Jugular vein and carotid artery cannulation was performed 6 h prior to the administration of the compounds. The compound of interest was dissolved in 5% (v:v) DMSO, then formulated as an emulsion in 45% (v:v) polyethylene glycol 400 and 50% (v:v) sterile normal saline. The rats were placed in metabolism cages. Blood samples (200 μL) were withdrawn from jugular vein using cannulation from each rat at 1, 5, 30, 60, 120, 180, 300, 480, 720, 960, and 1440 min postdosing. The blood samples were placed in heparinized tubes and centrifuged immediately, then the plasma samples were stored at −20 °C until analysis. Drug free rat plasma was collected from 6 vehicle-injected rats.

The pharmacokinetic parameters were calculated by Program Package (DAS 2.0) software. Each value is expressed as mean ± S.D.

3. Results and discussion

3.1. LC MS/MS optimization

Firstly the five pro-drugs and d4T (IS) were characterized by Q1 MS full-scan and Q1/Q3 (MS/MS) product ion scan to ascertain their precursor ions and to select product ions for use in MRM mode, respectively. In order to obtain maximum response and stable product ions, the MS parameters and collision energies were optimized. Interface-independent instrument parameters (Table 2) were optimized during the infusion of a mobile phase composed of 10 mM ammonium acetate in water (pH 6.5): methanol (35:65, v/v), into the Applied Biosystem 3200Q Trap mass spectrometer. Typical MRM chromatograms obtained for rat plasma samples spiked with pro-drugs are shown in Fig. 2. After comparing the ionization methods, positive-ion ESI was chosen for the high ion abundance of precursor protonated molecules $[M+H]^+$ in the positive mode.

In this study, it was found that increase CH_3COONH_4 in mobile phase (CH_3OH/H_2O) resulted in a dramatic increase in signal intensity. For both analytes and IS, there was a uniform reaction of the

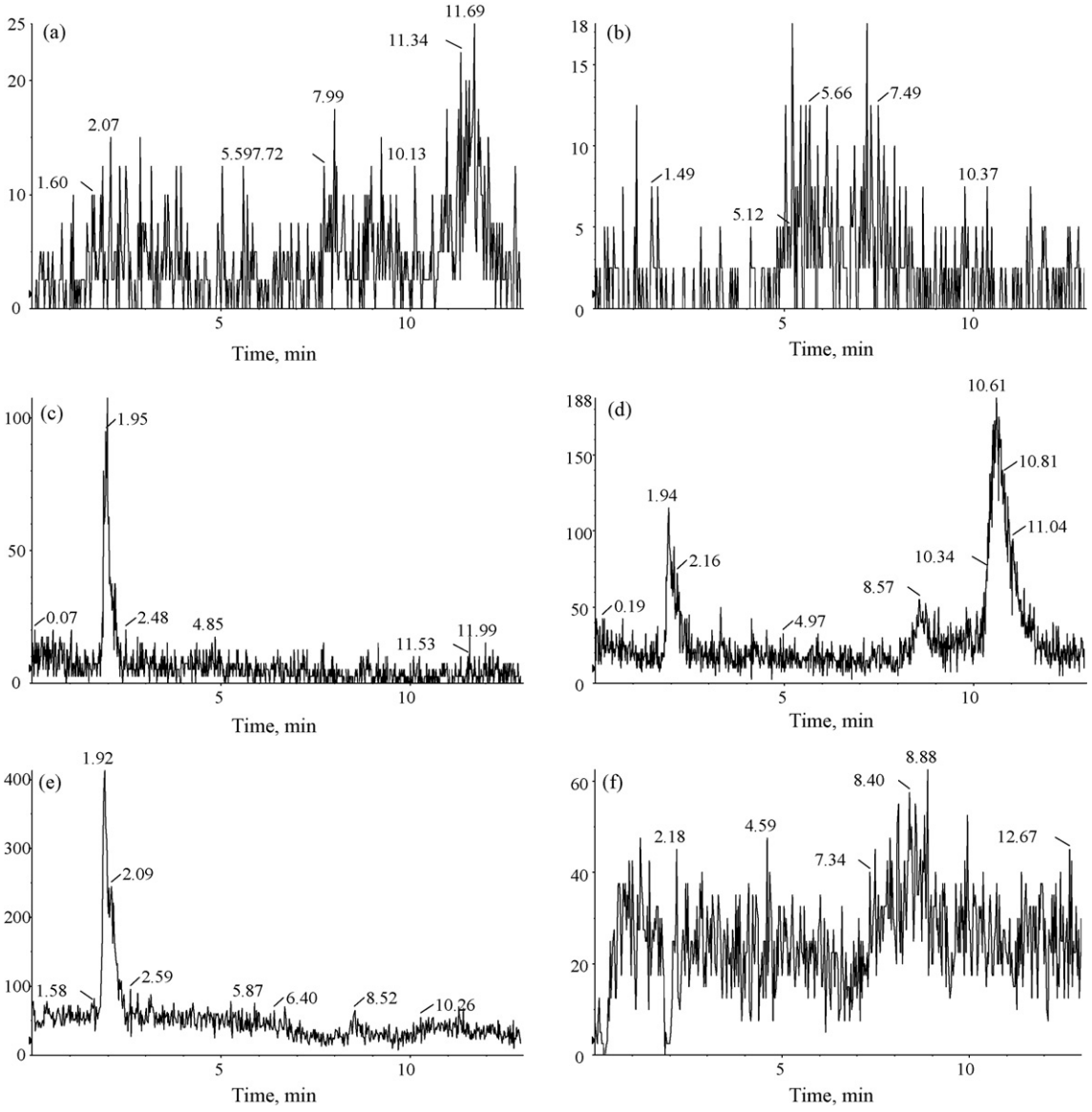


Fig. 3. Representative MRM chromatograms of a drug-free rat plasma sample: (a) M1; (b) M2; (c) M3; (d) M4; (e) M5; (f) IS.

signal intensity to the change of CH_3COONH_4 concentration 10 mM (>10-fold higher than that in electrolyte-free mobile phase, data not shown).

Secondly, investigation on whether the benefit of the low concentration of mobile phase additive might be offset by a decreased chromatographic performance and/or undesirable matrix effects was carried out. Instead, it appeared that the use of CH_3COONH_4 had beneficial effects on the chromatographic peak widths, peak asymmetry, and peak retention times. A mobile phase containing 10 mM CH_3COONH_4 resulted in only negligible matrix effects for all of the tested compounds. The compounds showed good chromatographic behavior and sufficient sensitivity under the given LC/MS^n conditions. These results indicated that positive ion ESI with a low concentration of mobile phase additive (10 mM) was an effective protocol for this study.

3.2. Method validation

3.2.1. Selectivity

Under the analytical conditions described above, retention times were approximately 7.8 min for M1, 8.9 min for M2, 8.7 min for M3, 8.5 min for M4, 7.9 min for M5, and 2.9 min for the IS. The five pro-drugs and the internal standard d4T were all rapidly eluted within 10 min. The drug-free rat plasma samples were allowed to run a relatively long time up to 30 min to observe any possible late-eluting interfering peaks. No interferences were observed up to 30 min due to the high selectivity of multiple reaction mode. Therefore, prolongation of the analytical time was not necessary. Fig. 2 shows the representative chromatograms of samples spiked with five compounds and internal standard, suggesting the high selectivity of this method.

During method development, two mobile phase compositions were compared, i.e. methanol/water and methanol/10 mM ammonium acetate in water. The latter gave a better peak shape and dramatically high sensitivity. Pooled compound-free, control rat blood samples were investigated in order to assess interferences from endogenous compounds. Representative MRM chromatograms of pro-drugs and IS in drug-free rat plasma (Fig. 3) showed no matrix-specific interfering peaks at the retention times of all pro-drugs and the IS.

3.2.2. Recovery and matrix effect

Sample preparation of rat plasma involved a simple single-step liquid–liquid protein precipitation procedure for which the extraction efficiency and matrix effects of two protein precipitants (acetonitrile and methanol) were compared. Both solvents gave >70% extraction efficiency for five analytes but methanol produced a better peak shape with lower matrix effects. Recoveries of analytes at concentrations of 0.01, 1, and 10 μg mL^{-1} were in the range of 72–94%, indicating low matrix effects for the analytes as well. Since plasma samples were precipitated with methanol and centrifugated, the background was very low and reconstituted samples did not cause any type of tubing or needle sprayer clog caused by original proteins.

The carryover effect was tested by injecting the high concentration standard (1000 ng mL^{-1} for pro-drugs and IS) into the system followed by methanol injection. Autosampler carry-over was minimized (≤0.5%) using organic wash solvent (HPLC grade methylene dichloride) for the G1329A autosampler.

3.2.3. Linearity of calibration curves and lower limit of quantification (LLOQ)

The standard calibration curves for spiked rat plasma containing M1, M2, M3, M4, and M5 were linear over the range 0.01–10 μg mL^{-1} with correlation coefficient (r) > 0.999. Typical equations for the calibration curve were as follows:

$$M1: Y = 0.00136 + 0.1209X, \quad r = 0.99995$$

$$M2: Y = -0.03485 + 6.40872X, \quad r = 0.99996$$

$$M3: Y = -0.02149 + 0.66086X, \quad r = 0.99929$$

$$M4: Y = 0.00428 + 0.10818X, \quad r = 0.99949$$

$$M5: Y = -0.01934 + 1.93393X, \quad r = 0.99989$$

where X is the plasma concentration of each analyte (ng mL^{-1}) and Y is the peak-area ratios of each analyte to IS.

The LLOQs were calculated on the basis of a signal-to-noise ratio of 10:1, for determination of M1, M2, M3, M4, and M5 in plasma, resulting in 10 ng mL^{-1} for M1, M2, M3, M4, and M5, respectively. These limits are sufficient for the pharmacokinetics studies of pro-drugs following an intravenous administration.

3.2.4. Assay precision and accuracy

Intra- and inter-day precision was assessed from the results of QCs. The mean values and R.S.D. for QC samples at three concentration levels were calculated over five validation runs. Five replicates of each QC level were determined in each run. The intra-day and inter-day precision were determined by calculating the relative standard deviation values. The accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QCs and expressed as the relative error (R.E.). Table 3 summarizes the intra- and inter-day precision and accuracy for M1, M2, M3, M4, and M5 from the QC samples.

Table 3
Accuracy and precision for the analysis of five compounds (n = 5).

Analytes	Statistical variables	Intra-day			Inter-day		
		10 ng mL^{-1}	1000 ng mL^{-1}	10,000 ng mL^{-1}	10 ng mL^{-1}	1000 ng mL^{-1}	10,000 ng mL^{-1}
M1	R.S.D. (%)	0.673	2.040	1.158	1.663	1.950	1.966
	R.E. (%)	−0.366	−1.563	−1.135	1.682	−3.077	4.411
M2	R.S.D. (%)	2.840	5.547	1.748	2.615	2.466	5.442
	R.E. (%)	3.592	−0.475	−3.852	−1.168	−4.275	−2.643
M3	R.S.D. (%)	0.605	1.682	0.661	4.079	3.359	2.556
	R.E. (%)	−1.592	0.429	−4.849	−3.432	−1.046	−5.879
M4	R.S.D. (%)	1.733	2.173	1.585	4.146	3.361	4.058
	R.E. (%)	0.522	2.741	−3.280	−3.118	−2.766	−1.376
M5	R.S.D. (%)	1.335	2.588	1.185	2.228	2.326	2.000
	R.E. (%)	−3.406	2.610	−4.217	−0.648	1.085	−0.174

Table 4
Stability of five pro-drugs in plasma during sample storage, preparation and analysis ($n = 3$).

Analyte	Nominal concentration (ng mL^{-1})	−20 °C/4 weeks R.S.D. (%)	−20 °C/3 freeze/thaw cycles R.S.D. (%)	Plasma samples at RT for 2 h R.S.D. (%)
M1	10	0.7	0.2	1.0
	1000	5.0	4.2	4.2
M2	10	0.5	3.1	0.8
	1000	2.4	0.9	5.0
M3	10	0.6	1.5	2.7
	1000	1.8	2.1	2.5
M4	10	0.4	2.3	2.5
	1000	7.6	5.9	3.5
M5	10	1.7	3.4	1.3
	1000	6.1	4.1	3.9

RT: Room temperature.

Table 5
The main pharmacokinetic parameters of five pro-drugs after i.v. administration to six rats at a dose of 5 mg kg^{-1} (mean).

	$t_{1/2}$ (min)	Ke (L min^{-1})	CL (L min^{-1} kg^{-1})	Vss (L kg^{-1})	MRT$_{(0-t)}$ (min)	AUC$_{(0-t)}$ (ng min mL^{-1})
M1	79.4	0.009	0.007	0.760	133.5	877365.3
M2	378.1	0.002	0.014	7.439	271.0	565543.5
M3	196.1	0.004	0.074	21.028	181.8	146579.4
M4	109.4	0.006	0.004	0.613	109.8	1936381.5
M5	87.2	0.009	0.021	3.016	111.1	418756.7

3.2.5. Analyte stability

The stability of five analytes and the IS in rat plasma and mobile phase were investigated. The results of stability studies are shown in Table 4. After storage at −20 °C for 1 month, no obvious reduction (<5% reduction) was found in the standard working solutions. The analytes were found to be stable after three freeze–thaw cycles with a reduction of less than 8%. The analytes were also shown to be stable in rat plasma with a reduction of less than 5%, at room temperature for 2 h, at which time the control samples were processed and extracted.

Samples of M1, M2, M3, M4 and M5 were found to be stable at three different storage conditions, as seen the deviation from nominal concentrations, which were within the acceptable limits of ±15% at all concentration levels.

3.3. Application of the analytical method

The present method proved to be reproducible and sensitive, and was successfully applied to rat plasma samples for the pharmacokinetic study of five compounds. Both pro-drugs M1, M5 and the parent drug AZT were found in plasma samples. The mean ± S.D. ($n = 6$) plasma concentration versus time profiles for M1, M5 are depicted in Fig. 4, after administration to six rats at a dose of 5 mg kg^{-1}. The main pharmacokinetic parameters for M1, M5 are summarized in Table 5. Nevertheless, the complete conversion of M2, M3, M4 to AZT were observed in rat plasma without detection of pro-drugs (data not shown). The pharmacokinetics of M2, M3, and M4 are shown in Table 5 and depicted in Fig. 5, in terms of the pattern of the plasma concentration of metabolite AZT. The results implied that the overall pharmacokinetics were associated with structure of pro-drugs. In particular, strongly electron-withdrawing aryl substituents correlate with their complete conversion to AZT in rat plasma. Further research is necessary to clarify the mechanism of this interesting phenomenon.

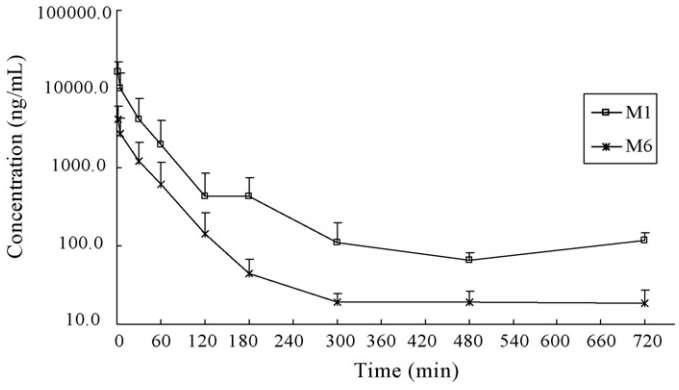


Fig. 4. Mean drug plasma concentration time curve (mean ± S.D., $n = 6$) of pro-drugs in rats after i.v. administration of M1, and M5.

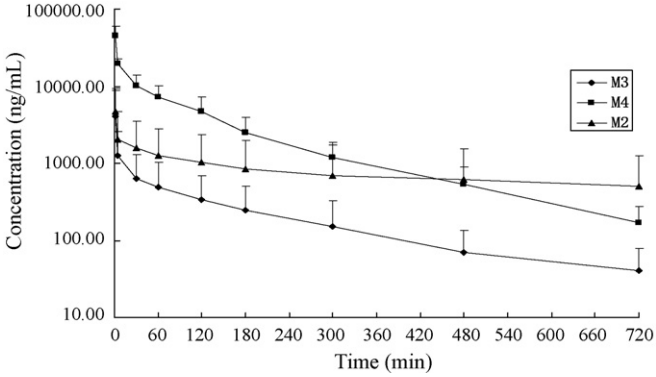


Fig. 5. Mean drug plasma concentration time curve (mean ± S.D., $n = 6$) of metabolite AZT in rats after i.v. administration of M2, M3, and M4.

4. Conclusions

A LC/MS/MS method for the quantification of five pronucleosides in rat plasma was developed and fully validated. The diaryl phosphate triester derivatives of AZT were well chromatographed by a HPLC system coupled with ESI$^+$–MS/MS. The method showed high precision (≤7.13% R.S.D.) and accuracy (≤6.85% R.E.), and could be modified for microsample analysis or lower concentration level analysis. The total analysis time for the five analytes and the IS was only 13.0 min. The method was proved to be sensitive, specific, accurate and reproducible and has been successfully applied to

determine the levels of M1, M2, M3, M4, and M5. This approach has also been utilized in the characterization of the pharmacokinetic profile of the five pro-drugs in rats after intravenous administration to rats.

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