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Short communication

Simultaneous determination of zidovudine and its monophosphate in mouse plasma and peripheral red blood cells by high-performance liquid chromatography

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Abstract

Novel prodrugs for the intracellular delivery of zidovudine monophosphate (AZTMP) have recently been designed. To investigate the bioconversion and pharmacokinetic profiles of these compounds, an analytical method for the simultaneous determination of zidovudine (AZT) and AZTMP in mouse plasma and peripheral red blood cells was developed. Mouse whole blood samples were treated with TBAHS, EDTA and NaH_2PO_4, and separated into plasma and red blood cell portions. Samples were processed by solid-phase extraction using Bond Elut C_{18} cartridges. Chromatography was performed using an Hypersil ODS column and a mobile phase of 2.9% (v/v) acetonitrile and 97.1% (v/v) phosphate buffer, pH 7.50, with UV detection at 267 nm. The average extraction recoveries of AZTMP and AZT in plasma were approximately 85% and 97% over their linear ranges of 0.05–5 μg/ml and 0.125–25 μg/ml, respectively. Extraction recoveries of AZTMP and AZT from peripheral red blood cells averaged 56 and 69% over their linear ranges of 0.125–5 μg/ml and 0.125–25 μg/ml, respectively. The accuracy of the assay was 90–100%. The intra- and inter-day variations of the assay were less than 14%. The analytical method was found to be applicable, reliable and suitable for pharmacokinetic studies.

Keywords: Zidovudine; Zidovudine monophosphate

1. Introduction

Zidovudine (3′-azido-3′-deoxythymidine; AZT), the first antiviral drug approved for the treatment of human immunodeficiency virus (HIV) infection by the United States Food and Drug Administration (FDA), and one of the first-line therapeutic agents in treating acquired immunodeficiency syndrome (AIDS), is essentially the prodrug of its pharmacologically active 5′-triphosphate (AZTTP) anabolite. AZT is anabolized intracellularly to its 5′-monophosphate (AZTMP) by cellular thymidine kinase, to its 5′-diphosphate (AZTDP) by thymidylate kinase, and subsequently to AZTTP, which acts as an potent inhibitor of HIV reverse transcriptase and terminator of growing proviral DNA [1].

Novel prodrug strategies such as bis(*S*-acyl-2-thioethyl)-AZTMP [bis(SATE)-AZTMP] [2] have been designed to deliver AZTMP intracellularly, and therefore to circumvent the cellular monophos-

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phorylation of AZT, the most restrictive step in the activation of AZT with regard to host species, cell type and cell phase [3–5]. To develop prodrugs of nucleotide analogs as potential antiviral agents for clinical use, preclinical pharmacokinetic and metabolism studies in suitable animal models are necessary. It is of particular interest to investigate the prodrug and drug concentrations in plasma because plasma profiles reflect the overall disposition of the compounds. More importantly, the drug delivery and metabolism in peripheral red blood cells could be used as a cellular model to evaluate the intracellular delivery of AZTMP in vivo.

Due to the ionic nature of the phosphate metabolites of AZT at physiological pH values, simultaneous determination of AZT, AZTMP, AZTDP and AZTTP was achieved by using ion-pair reversed-phase high-performance liquid chromatography (HPLC) [6]. Although this method was successful in measuring AZT and its phosphate anabolites in cell culture, it was not applicable for simultaneous measurement of AZT and AZTMP in mouse plasma and peripheral red blood cells due to the instability of AZTMP. Results of a stability study showed the half-lives of AZTMP in whole blood and mouse serum were approximate 15 min and 28 min, respectively [7]. Nucleotides tend to be dephosphorylated rapidly on the cell surfaces [8].

The present study describes a sensitive and reproducible analytical method for the simultaneous determination of AZT and AZTMP in mouse plasma and peripheral red blood cells.

2. Experimental

2.1. Chemicals and reagents

AZTMP was purchased from Sigma (St. Louis, MO, USA). AZT and internal standard 3′-azido-2′,3′-dideoxy-5-ethyluridine (CS-85), synthesized as previously described [9], were provided by Dr. Chung K. Chu (University of Georgia, Athens, GA, USA). Bis(*tert*.-butyl-SATE)-AZTMP was provided by Dr. Jean-Louis Imbach (University de Montpellier II, France). The chemical purity of the compounds, as determined by HPLC analysis, was greater than 99%.

HPLC-grade acetonitrile, tetrabutylammonium hydrogensulfate (TBAHS), (ethylenedinitrilo)tetraacetic acid, disodium salt, dihydrate (EDTA) and all other chemicals, analytical grade, were purchased from J.T. Baker (Phillipsburg, NJ, USA). Water used in all sample preparation and analysis was deionized water purified by a Millipore water purification system (Continental Water Systems, El Paso, TX, USA). The Vac-Elut manifold and the C_{18} Bond Elut cartridges (100 mg, 1 ml) were obtained from Varian Analytical Supplies (Harbor City, CA, USA).

2.2. Preparation of standards

Standard stock solutions of AZTMP and AZT were prepared in water. The concentrations of AZTMP, AZT and CS-85 (internal standard) in plasma or peripheral red blood cells were defined as the amount of compounds in the plasma portion or red blood cell portion separated from 1 ml of whole blood. Calibration plots for the compounds in mouse plasma were prepared by adding standard stock solutions to blank plasma yielding concentrations of 0.05–5 μg/ml (0.05, 0.125, 0.25, 0.375, 0.5, 1.25, 2.5, 3.75 and 5 μg/ml) for AZTMP and 0.125–25 μg/ml (0.125, 0.25, 0.5, 1.25, 2.5, 5, 12.5 and 25 μg/ml) for AZT. Standard curves for the analysis of mouse peripheral red blood cells were prepared in blank peripheral red blood cells over the concentration ranges of 0.125–5 μg/ml (0.125, 0.25, 0.375, 0.5, 1.25, 2.5, 3.75 and 5 μg/ml) for AZTMP and 0.125–25 μg/ml (0.125, 0.25, 0.5, 1.25, 2.5, 5, 12.5 and 25 μg/ml) for AZT.

2.3. Extraction procedure

Immediately after AZT, AZTMP and CS-85 were added to 0.1 ml blank plasma or peripheral red blood cells or after samples were collected, 20 μl TBAHS (1 *M*), 10 μl EDTA (0.27 *M*), 100 μl 0.2 *M* NaH_2PO_4 and 50 μl CS-85 (6 μg/ml) as internal standard, were added and mixed thoroughly. Plasma samples were directly loaded on C_{18} solid-phase extraction cartridges, which had been sequentially pre-equilibrated with acetonitrile (2×1 ml), water (2×1 ml) and 0.2 *M* NaH_2PO_4 with 8 m*M* TBAHS (3×1 ml). The cartridges were washed with 2.9%

acetonitrile in buffer containing 0.2 *M* NaH_2PO_4 and 8 m*M* TBAHS, pH 7.5, (5×100 μl), and eluted with 90% acetonitrile in water (3×200 μl). The eluents were collected and evaporated to dryness under a stream of nitrogen gas at 25°C. The residues were reconstituted with 200 μl water and centrifuged at 7500 *g* for 2 min. One hundred fifty microliters (150 μl) of the resulting supernatant was injected onto the HPLC column.

The peripheral red blood cells were hemolyzed in an ultrasonic bath at 0°C for 10 min and centrifuged at 7500 *g* for 2 min. The supernatants were removed and loaded on the pretreated C_{18} solid-phase extraction cartridges. The samples were extracted and injected onto the HPLC column following the procedures described above for plasma samples.

2.4. Chromatography

The HPLC system consisted of a Beckman 110B pump (Fullerton, CA, USA), a Waters 712 WISP autosampler (Waters Associates, Milford, MA, USA), Waters 484 UV detector and Waters 746 data module. The separation of AZTMP and AZT was performed using a Waters Bondpak C_{18} precolumn and an Alltech Hypersil ODS column (150 mm×4.6 mm, particle size 5 μm; Alltech Associates, Deerfield, IL, USA). The mobile phase was comprised of 2.9% acetonitrile in phosphate buffer containing 0.2 *M* NaH_2PO_4 and 8 m*M* TBAHS, pH 7.5, at a flow-rate of 1.5 ml/min. The UV wavelength for the detection of AZTMP and AZT was 267 nm. A detector range setting of 0.005 absorbance units full scale was used.

2.5. Quantitation

Concentrations of AZTMP and AZT in samples were determined from the slopes of standard curves of the peak area ratio of compound to internal standard versus standard compound concentration. Standard curve slopes were generated by weighted $(1/x^2)$ least-squares regression analysis. Use of this weighing factor yielded a normal distribution of weighted residuals over the entire range of compound concentrations.

2.6. Assay specifications

The extraction recoveries of AZTMP, AZT and internal standard were determined by comparing the peak areas for six extracted plasma or peripheral blood cell samples with those of six unextracted samples of the same amount of compound prepared in mobile phase. The extraction recovery was calculated from peak $\text{area}_{\text{extracted}}$/peak $\text{area}_{\text{unextracted}}\times$ 100%.

The intra-day accuracy and precision of the assay methodology in plasma and peripheral red blood cells were determined by assaying six samples per concentration at 0.05 μg/ml, 0.25 μg/ml, 0.5 μg/ml and 5 μg/ml for AZTMP in plasma, 0.125 μg/ml, 0.25 μg/ml, 0.5 μg/ml and 5 μg/ml for AZTMP in peripheral red blood cells, and 0.125 μg/ml, 0.5 μg/ml, 5 μg/ml and 25 μg/ml for AZT in plasma and peripheral red blood cells. For the inter-day accuracy and precision, samples were analyzed on three different days. Accuracy was calculated by comparing measured compound concentrations to the known values. Precision was reported as relative standard deviation (RSD). The limit of quantification (LOQ) was defined as the concentration of the lowest standard in the analytical run which was quantified with an RSD of less than 20% and accuracy greater than 80%.

2.7. In vivo studies in mice

Bis(*tert*.-butyl-SATE)-AZTMP was intravenously administered to female NIH-Swiss mice (Harlan Sprague–Dawley, Indianapolis, IN, USA) at a dose equivalent to 50 mg/kg AZT via a tail vein over 30 s. Whole blood (EDTA as an anticoagulant) was collected and immediately processed for the determination of AZTMP and AZT in plasma and peripheral red blood cells.

3. Results and discussion

Efforts to measure AZT and AZTMP simultaneously in plasma and peripheral red blood cells were first attempted using a previously reported method [6]. However, AZTMP was found to be completely degraded to AZT in blood during sample prepara-

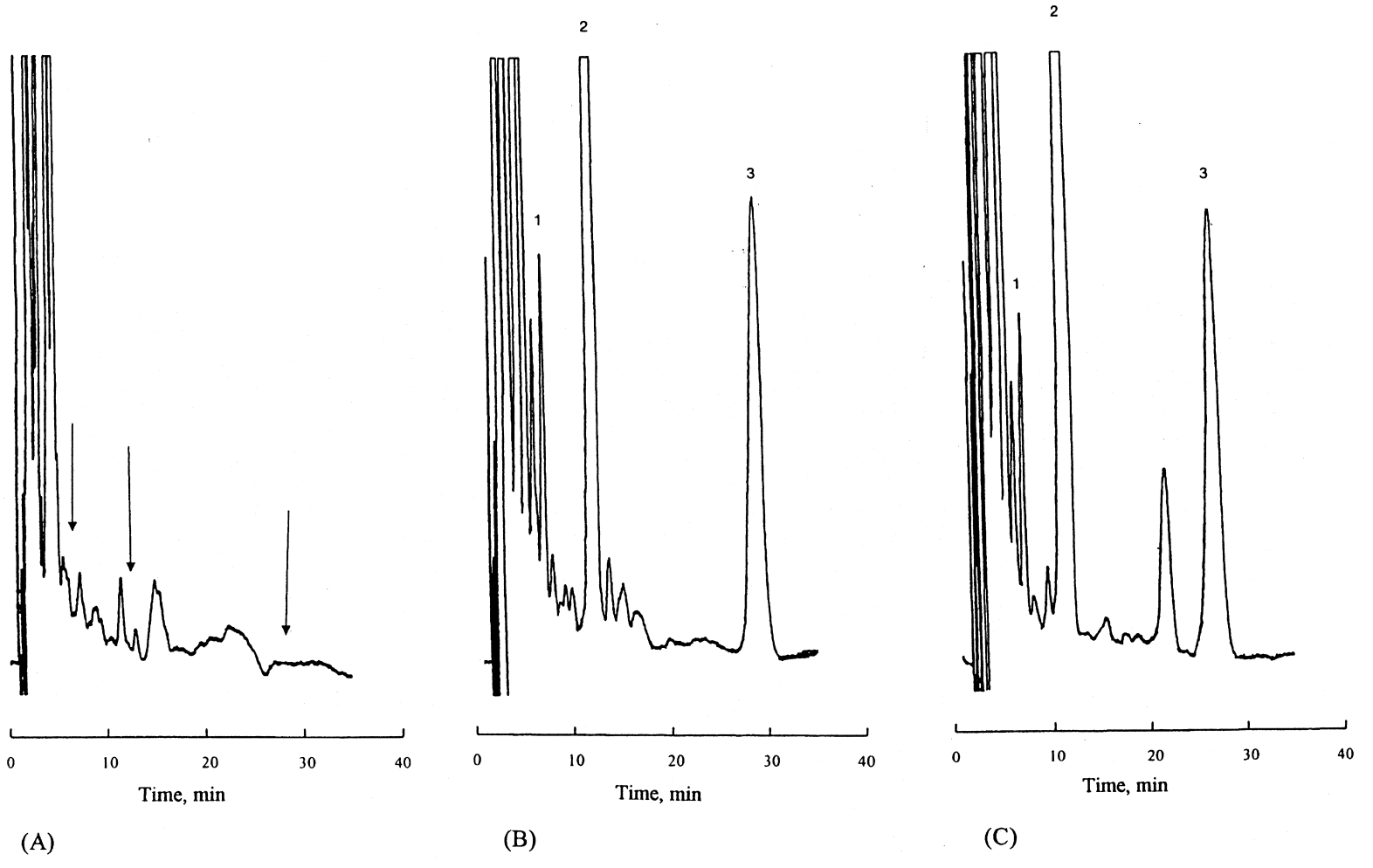

Fig. 1. Chromatograms of (A) blank mouse plasma, (B) mouse plasma spiked with (1) 0.5 μg/ml AZTMP, (2) 5 μg/ml AZT and (3) 1.5 μg/ml CS-85, and (C) a mouse plasma sample (0.5 μg/ml AZTMP and 9.5 μg/ml AZT) collected 30 min after intravenous administration of bis(*tert*.-butyl-SATE)-AZTMP at a dose equivalent to 50 mg/kg AZT.

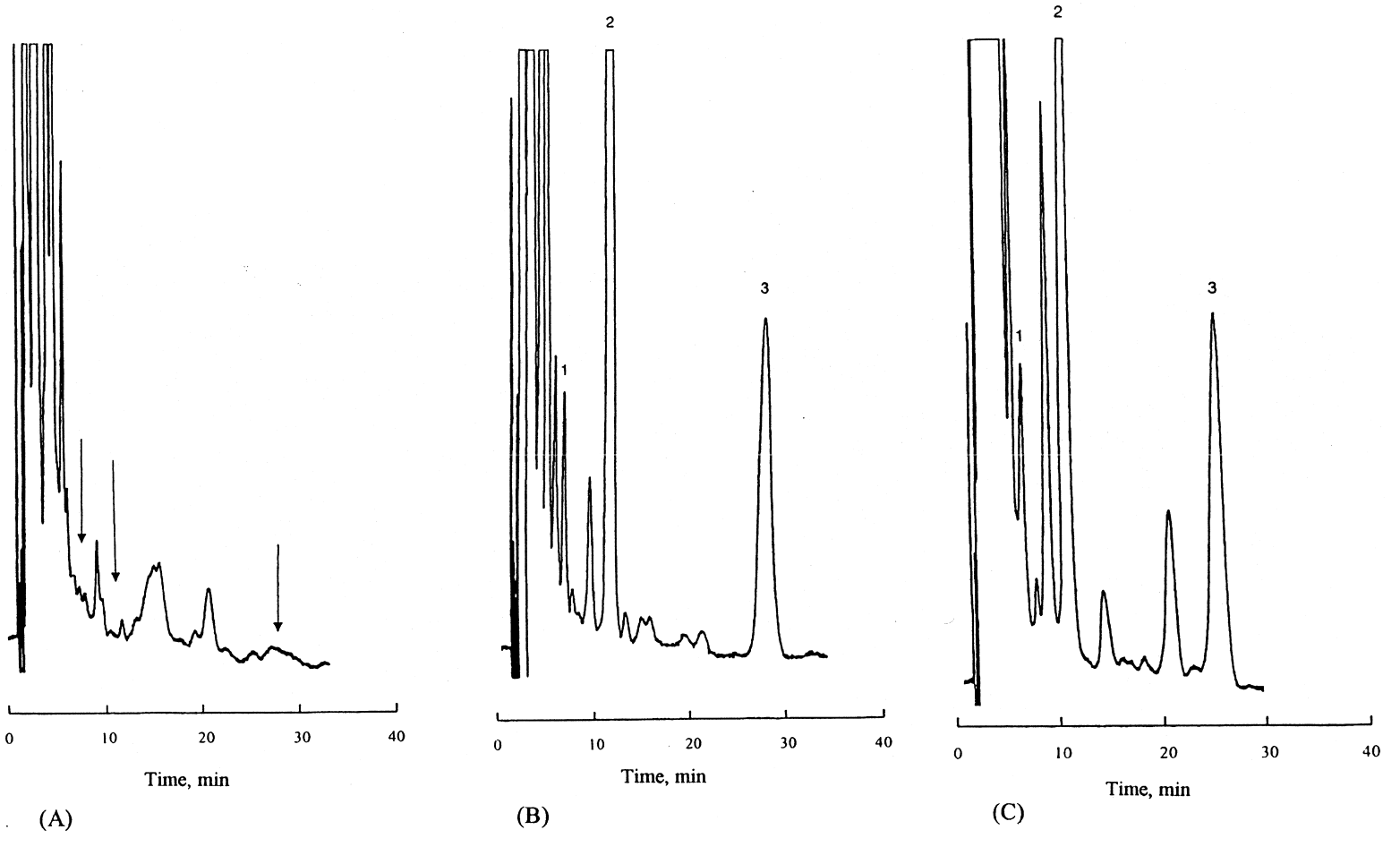

Fig. 2. Chromatograms of (A) blank mouse peripheral red blood cells, (B) mouse peripheral red blood cells spiked with (1) 0.5 μg/ml AZTMP, (2) 5 μg/ml AZT and (3) 1.5 μg/ml CS-85, and (C) a mouse peripheral red blood cell sample (0.6 μg/ml AZTMP and 6.6 μg/ml AZT) collected 30 min after intravenous administration of bis(*tert*.-butyl-SATE)-AZTMP at a dose equivalent to 50 mg/kg AZT.

Table 1
Assay specifications for the determination of AZTMP and AZT in mouse plasma

	Concentration	Recovery[a]	Precision (%)		Accuracy (%)	
	(μg/ml)	(%)	Intra-day	Inter-day	Intra-day	Inter-day
AZTMP	0.05	81.9±9.1	7.3	11.9	92.9	96.7
	0.25	78.6±8.8	6.1	6.7	97.8	95.4
	0.5	92.5±8.1	5.7	3.2	97.7	96.3
	5	89.7±7.4	3.4	2.1	98.4	97.2
AZT	0.125	94.7±10.0	9.5	12.4	93.8	98.7
	0.5	99.5±3.6	6.6	3.5	98.6	98.3
	5	96.6±2.0	3.1	4.5	99.5	98.0
	25	98.1±2.7	7.0	5.1	99.1	99.6
CS-85	1.5	94.9±2.8				

[a] Mean±SD (n=6).

tion. The current assay method was developed based on the previously reported method [6]. However, in the present assay AZTMP is stabilized by the addition of EDTA and TBAHS. Further, additional modifications such as the detection wavelength (254 nm vs. 267 nm), the composition of washing buffer and eluting solution during solid-phase extraction, were made in order to obtain cleaner samples. This was especially important for peripheral blood cell samples.

Chromatograms of (A) blank mouse plasma, (B) mouse plasma spiked with AZTMP, AZT and CS-85, and (C) a mouse plasma sample collected following intravenous administration of bis(*tert*.-butyl-SATE)-AZTMP are shown in Fig. 1. The corresponding chromatograms for mouse peripheral red blood cells are illustrated in Fig. 2. The retention times of AZTMP, AZT and CS-85 were 7.2, 12.6 and 28.8 min, respectively. There were no interferences by endogenous compounds.

In Figs. 1C and 2C, peaks which appeared at around 20 min were possibly an intermediate or other metabolite of bis(*tert*.-butyl-SATE)-AZTMP biotransformation, however no further analysis was performed to identify the structure of this peak. In vitro bioconversion of bis(*tert*.-butyl-SATE)-AZTMP in various media such as whole blood, serum and tissue homogenates was studied. Evidence showed that the degradation of the prodrug was very rapid and there were possible intermediates existing

Table 2
Assay specifications for the determination of AZTMP and AZT in mouse peripheral red blood cells

	Concentration	Recovery[a]	Precision (%)		Accuracy (%)	
	(μg/ml)	(%)	Intra-day	Inter-day	Intra-day	Inter-day
AZTMP	0.125	51.6±4.8	12.6	12.1	93.4	90.8
	0.25	51.8±6.3	11.9	8.5	90.3	94.7
	0.5	59.9±5.8	6.7	2.2	95.8	96.3
	5	58.7±3.5	3.6	2.6	94.1	98.2
AZT	0.125	60.7±2.7	13.9	13.1	90.2	97.9
	0.5	65.4±4.1	4.8	5.2	95.6	97.0
	5	77.9±6.2	3.8	7.7	97.9	96.6
	25	74.4±5.6	6.5	2.5	93.7	97.5
CS-85	1.5	77.8±4.3				

[a] Mean±SD (n=6).

prior to the formation of AZTMP [7]. The detailed pharmacokinetics of bis(*tert*.-butyl-SATE)-AZTMP are reported elsewhere [7].

The ranges of linearity for AZTMP were 0.05–5 μg/ml in mouse plasma [$y=0.3259(0.0152)x-0.0048(0.0017)$, $r=0.999$] and 0.125–5 μg/ml in mouse peripheral red blood cells [$y=0.3657(0.0329)x+0.0003(0.0095)$, $r=0.993$]. The range of linearity for AZT was 0.125–25 μg/ml in both mouse plasma [$y=0.5574(0.0217)x+0.0116(0.0074)$, $r=0.999$]and peripheral red blood cells [$y=0.5964(0.0162)x+0.0363(0.0063)$, $r=0.999$]. The assay specifications including extraction recovery, assay precision and accuracy are presented in Tables 1 and 2. The recoveries of AZTMP and AZT in peripheral red blood cells were somewhat lower than recoveries in plasma. This may due to the greater tendency of adsorption/binding of these compounds by the insoluble proteins in the cell debris.

The low recovery of AZTMP in peripheral blood cells did not result from incomplete stabilization of AZTMP since there was no AZT detected if only AZTMP was spiked in the sample. Although the extraction recovery of AZTMP was low in peripheral red blood cells the limit of quantification of AZTMP in red blood cells was 0.125 μg/ml. Based on the in vivo study in mice, there was no AZTMP detectable following intravenous administration of AZT. However, following intravenous administration of bis(*tert*.-butyl-SATE)-AZTMP, AZTMP concentrations in blood cells were above 1 μ*M* (0.36 μg/ml) until 2 h. Furthermore, the accuracy and precision of the method were acceptable. Therefore the present assay method is indeed useful for pharmacokinetic study of AZTMP prodrugs.

At low, medium and high concentrations for AZTMP and AZT, the intra- and inter-day RSDs were less than 14% in both plasma and peripheral red blood cells. The intra- and inter-day accuracy values were greater than 90%. Because of the satisfactory precision and accuracy, lower ends of the linear ranges were determined as LOQ for AZTMP and AZT in mouse plasma and peripheral red blood cells.

4. Conclusion

The assay methodology described here provides sensitive, accurate and reproducible determination of AZTMP and AZT in mouse blood samples. AZTMP was completely stabilized in mouse plasma and peripheral red blood cells by the addition of EDTA and TBAHS. This made it possible to process samples by solid-phase extraction yielding significant cleaner chromatograms and higher recoveries than those by protein precipitation methods. This method was found to be applicable for pharmacokinetic studies of AZTMP prodrugs in mice. The results of this study are reported elsewhere [7].

References

[1] P.A. Furman, J.A. Fyfe, M.H. St. Clair, K. Weinhold, J.L. Rideout, G.A. Freeman, S. Nusinoff Lehrman, D.P. Bolognesi, S. Broder, H. Mitsuya, D.W. Barry, Proc. Natl. Acad. Sci. USA 83 (1986) 8333.
[2] I. Lefebvre, C. Perigaud, A. Pompon, A.-M. Aubertin, J.-L. Girardet, A. Kirn, G. Gosselin, J.-L. Imbach, J. Med. Chem. 38 (1995) 3941.
[3] W.-Y. Gao, Y. Shirasaka, D.G. Johns, S. Broder, H. Mitsuya, J. Clin. Invest. 91 (1993) 2326.
[4] W.-Y. Gao, R. Agbaria, J.S. Driscoll, H. Mitsuya, J. Biol. Chem. 269 (1994) 12633.
[5] J. Balzarini, P. Herdewijn, E. De Clercq, J. Biol. Chem. 264 (1988) 6127.
[6] G. Molema, R.W. Jansen, J. Visser, D.K.F. Meijer, J. Chromatogr. B 579 (1992) 107.
[7] X. Tan, C.K. Chu, D. Egron, C. Perigaud, G. Gosselin, J.-L. Imbach, F.D. Boudinot, Pharm. Res. Suppl. 1 (1998) S681.
[8] K.C. Leibman, C. Heideberg, J. Biol. Chem. 216 (1955) 823.
[9] C.K. Chu, R.F. Schinazi, M.K. Ahn, G.V. Ullas, Z.P. Gu, J. Med. Chem. 32 (1989) 612.