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Ultrasensitive method to quantify intracellular zidovudine mono-, di- and triphosphate concentrations in peripheral blood mononuclear cells by liquid chromatography–tandem mass spectrometry

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Although zidovudine (AZT) is not the preferred antiretroviral drug for adult HIV-infected patients, it is still widely used in infants for both prevention of mother-to-infant HIV-1 transmission and treatment of HIV-infected children. However, it is difficult to measure intracellular concentrations of AZT metabolites in small blood samples due to their extremely low concentrations in peripheral blood mononuclear cells and interference by endogenous nucleotide triphosphates, residual plasma phosphates and electrolytes. We developed an ultrasensitive assay using liquid chromatography–tandem mass spectrometry (LC–MS/MS) for measurement of intracellular concentrations of zidovudine (AZT)-monophosphate (AZT-MP), -diphosphate (AZT-DP) and -triphosphate (AZT-TP). The high sensitivity was due to the improvement of peripheral blood mononuclear cells extraction for complete removal of plasma and electrolytes, alkalization of LC buffer and use of alkaline-stable high performance liquid chromatography column and tetrabutylammonium hydroxide as the ion pair. Using this method, the lower limits of quantification of AZT, AZT-MP, -DP and -TP were 6, 6, 10 and 10 fmol per sample, respectively. Accuracy ranged 89–115% and precision was lower than 15% in the quantification range of 6–6000 fmol/sample for plasma AZT and intracellular AZT-MP and 10–10 000 fmol/sample for AZT-DP and -TP. The validation parameters met the international requirements. Among nine AZT-treated HIV-infected adult patients, five had low AZT-TP levels (<10 fmol/10^6 cells). Our assay has high sensitivity and is advantageous for evaluation of AZT phosphates in children and infants based on minimum blood sampling requirement. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: LC–MS/MS; ultrasensitive; zidovudine; phosphates; intracellular

Introduction

At present, zidovudine (AZT) is not the preferred antiretroviral drug for adult HIV-infected patients,[1] although it remains one of the main drugs used for prevention of mother-to-infant HIV-1 transmission and also for the treatment of HIV-infected children. Although serious concerns on AZT-associated mitochondrial toxicity have been raised,[2,3] there is little information on the pharmacokinetics of AZT and metabolites in infants,[4] mainly due to limitation of blood sampling volume.

Despite the recent developments in liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for measurement of intracellular concentrations of metabolites of nucleoside reverse transcriptase inhibitors (NRTI) used for the treatment of HIV infection,[5–7] it is still difficult to measure intracellular concentrations of AZT phosphates because of the extremely low concentrations in peripheral blood mononuclear cells (PBMC). Intracellular AZT phosphate levels can be determined by indirect and direct measurements. The indirect measurement involves several complex steps: (1) serial isolation of AZT-monophosphate (MP), -diphosphates (DP) and -triphosphates (TP) using an ion-exchange cartridge; (2) cleavage of the phosphate groups by acid phosphatase; and (3) determination of AZT concentration isolated from each type of AZT phosphate using radioimmunoassay[8] or

LC–MS/MS.[9–13] These assays are quite sensitive and useful for evaluation of intracellular AZT metabolites in AZT-treated patients. However, there is concern about the stability of the different AZT phosphates and reproducibility of the measurement related to the complexity of the aforementioned procedures. Furthermore, because these methods do not directly measure AZT phosphate but rather dephosphorylated products of AZT phosphate, it is difficult to confirm whether this method can precisely discriminate each type of AZT phosphates.

A direct measurement assay of AZT phosphates has been described recently, which is based on the use of the LC–MS/MS system without the dephosphorylation step.[14] However, it was concluded that such LC–MS/MS assay lacks sufficiently high sensitivity for extremely lower concentrations of intracellular AZT

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phosphates, mainly due to interference by endogenous nucleotides triphosphates and other unknown plasma phosphates and electrolytes.[14]

We developed a highly sensitive method for determination of plasma AZT concentration and intracellular AZT-MP, -DP and -TP concentrations using LC–MS/MS. We describe here the validation data of the assay and the clinical data in adult HIV-infected and AZT-treated patients.

Materials and methods

Chemicals and reagents

AZT (>98% purity) and 3′-azido-2′, 3′-dideoxyuridine (AZdU) (>98%), as an internal standard (IS), were obtained from Sigma-Aldrich (St. Quentin-Fallavier, France); AZT-MP (>95%), -DP (>90%) and -TP (>95%), adenosine triphosphate (ATP) (>95%) and 2′-deoxyguanosine-5′-triphosphate (dGTP) (>95%) were obtained from Moravek Biochemicals Inc. (Brea, CA, USA). 5-Methyluridine-5′-triphosphate (5-Methyl-UTP) (>90%), used as an IS, was obtained from TriLink Biotechnologies (San Diego, CA, USA). Tetrabutylammonium hydroxide (TBAH), ammonium formate, ammonium hydroxide, acetonitrle LC gradient grade and methanol LC gradient grade were obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Instrumentation

The LCMS 8030 triple-quadrupole LC/MS/MS system (Shimadzu Corp. Kyoto, Japan) was used in this study. It was monitored using Labsolutions LCMS software (Shimadzu Corp. Kyoto, Japan).

Standard solutions

Standard solutions of AZT, AZT-MP, -DP and -TP were prepared daily from three separate master stock solutions (1 mmol/L of AZT, AZT-MP, -DP and -TP in water) of each compound. Identical stock solutions of AZdU and 5-methyl-UTP were prepared in water from the respective high-concentration master stock solutions (1 mmol/L of AZdU and 5-Methyl-UTP in water). All master solutions were stored at −80 °C.

Preparation of spiked plasma and PBMC samples

PBMCs were prepared as described previously.[15] Briefly, anticoagulated whole blood diluted with phosphate-buffered saline was layered gently on 5 mL of a Ficoll-Paque Plus solution (GE Healthcare, Piscataway, NJ, USA) in a centrifuge tube and centrifuged at $600 \times g$ for 25 min. After centrifugation, the plasma portion, which formed above the PBMCs layer, was collected gently and stored at −80 °C. PBMCs, which were sandwiched between the plasma and Ficoll-Paque Plus solution, were collected and counted using a cell counter (Celltac, Nihon Kohden, Tokyo, Japan) after washing twice with phosphate-buffered saline (PBS) containing 2% fetal calf serum. After centrifugation, $1–10 \times 10^6$ cells of the PBMCs were collected and suspended with 100 μL saline. PBMCs were separated from plasma using the following procedure: (i) a mixture of silicone oil (catalog no. SH-550; Nacalai, Kyoto, Japan) and *n*-hexadecane (Nacalai) (800 μl; 90:10 [vol/vol]) was added into the outer tube of a 1.5 ml microcentrifuge tube; (ii) the inner tube (comprising 0.5 ml microcentrifuge tube) with a hole at the bottom, was sunk in the silicone oil/*n*-hexadecane mixture in

the surrounding outer tube; (iii) PBMCs suspended in 100 μl of saline were layered on the silicone oil mixture in the inner tube; (iv) the lid of the inner tube was pressed down; (v) the assembled 'double tube' was centrifuged at $16\,000 \times g$ for 1 min, which resulted in the rapid sedimentation of PBMCs at the bottom of the outer tube; (vi) the inner tube containing plasma and silicone oil was removed, with the lid kept closed to prevent the passage of plasma into the outer tube; and (vii) the silicone was removed from the outer tube while leaving the pelleted PBMCs in place; any remaining silicone oil on the outer tube inner surface was removed after a brief centrifugation. The pelleted PBMCs were suspended in 30 μl of pure water (suspension of cells in PBS is not recommended because the presence of phosphate ions in samples affects ionization of molecules in mass spectrometry and could even sometimes damage the analyzer). Finally, 70 μl of ethanol was added to 30 μl of the cell suspension, and the PBMC solution was stored at −80 °C.

On the day of the analysis, IS (5-Methyl-UTP) and AZT-MP, -DP and -TP standards were added to the PBMC solution for calibration (final spiked amount 6 fmol, 60 fmol, 600 fmol, and 6 pmol of AZT-MP, and 10 fmol, 100 fmol, 1 pmol and 10 pmol of AZT-DP and -TP per each sample). Specifically, AZT and AZdU, and 70 μl of ethanol were added to 30 μl of plasma for calibration (final spiked amount 6 fmol, 60 fmol, 600 fmol and 6 pmol of AZT per each sample, and 20 fmol of AZdU in total volume of 100 μl of solution).

Cellular debris was removed by centrifugation at $18\,000 \times g$ for 2 min at 4 °C, and the supernatant was transferred to a centrifugation tube. Samples were evaporated to dryness using Savant SpeedVac (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The dried samples were re-suspended with 20 μl of Buffer A, and the remaining debris was thoroughly removed by spinning at $18\,000 \times g$ for 2 min followed by the transfer of the supernatant to a fresh tube for LC–MS/MS analysis.

Samples from adult patients

PBMCs collected from the whole blood of adult patients as described earlier were suspended with 70% ethanol solution and stored at −80 °C. On the day of the analysis, the PBMC solution was spiked with 200 fmol of 5-Methyl-UTP per sample. Thirty microliter of plasma collected from whole blood was diluted with 70 μl of ethanol and stored at −80 °C. On the day of the analysis, the plasma solutions were spiked with 10 fmol of AZdU per each run.

Chromatographic and mass spectrometric settings

Assay for plasma AZT concentration

Chromatography was conducted using InnertSil ODS-30, 5 μm, 50 x 1.5 mm (GL Science, Tokyo, Japan). The mobile phase was delivered at a flow rate of 0.3 mL/min.

In the assay for plasma AZT, the mobile phase comprised a mixture of solutions A and B. Solution A was composed of 5 mM of acetate (pH = 5.0) in water, while solution B was composed of the same solute in methanol. A linear gradient was set from 0% to 50% B in 10 min (total run time: 20 min).

Assay for intracellular AZT-MP, -DP and -TP concentrations

Chromatography was conducted using InnertSustain (GL Science), 5 μm, 50 × 1.5 mm, which can be applied for a wide range of pH (pH 2–10). The mobile phase was delivered at a flow rate of 0.3 mL/min. In the assays for intracellular AZT-MP, -DP and -

TP, the mobile phase comprised a mixture of solutions A and B. Solution A was composed of 5 mM of ammonium formate, 2.5 mM ammonium hydroxide and 0.01 mM of TBAH (pH = 8.5) in water, and solution B was composed of the same solute in acetonitrile/methanol (90/10 v/v). A linear gradient was set from 0% to 50% B in 10 min (total run time: 20 min). The instrument was operated in the positive mode under MS/MS conditions using multiple reaction monitoring. Fragmentation was achieved with nitrogen. Ion transitions monitored by m/z were 268.25/127.30 for AZT, 254.10/113.05 for AZdU, 365.10/81.10 for AZT-MP, 445.10/81.15 for AZT-DP, 525.10/81.20 for AZT-TP and 498.85/96.95 for 5-methyl-UTP. Collision energy was set at −15 V for AZT, −10 V for AZdU, −15 V for AZT-MP, −10 V for AZT-DP, −10 V for AZT-TP and −15 V for 5-methyl-UTP.

Validation of the LC–MS/MS method

Selectivity and specificity

To investigate whether endogenous compounds ATP and dGTP interfered with the assays, we analyzed 5 pmol of ATP, dGTP and also blank PBMC taken from six healthy subjects and six HIV-infected naïve-to-treatment with AZT or d4T subjects.

Accuracy and precision

The accuracy and precision of the method were assessed by analyzing the intraday and interday accuracies and precisions at concentrations 6–6000 (6, 60, 600 and 6000) fmol per sample of AZT and AZT-MP, and 10–10 000 (10, 100, 1000 and 10 000) fmol per sample of AZT-DP and -TP using spiked plasma (for AZT) and PBMC (for AZT-MP, -DP and -TP) samples together with standard solutions at the same concentrations. For intraday precision and accuracy, five replicates at the four concentrations were assayed in the same run. For both intraday and interday experiments, the mean, accuracy and coefficient of variation were calculated at all tested concentrations. Accuracy was expressed as the absolute percentage of the theoretically determined concentration, and precision was evaluated as the coefficient of variation (CV). Intra-run accuracy was required to be within ±15%, and precision was required not to exceed 15%, except for the lower limit of quantification (LLOQ) in which accuracy was to be within ±20%, and precision was not to exceed 20%. Inter-run accuracy was required to be within ±15%, and precision was required not to exceed 15%, except for the LLOQ in which accuracy was to be within ±20% and precision not to exceed 20%.

Matrix effect

To evaluate the matrix effect, six lots of spiked plasma samples with various dilutions by PBS from 25% to 100% and six lots of spiked PBMC samples with various numbers of cells from 3× to 15 × 10^6 cells at low and high concentrations. Low level was set at three times the LLOQ, and high level was set at the upper limit of quantification (HQ). The mean and the standard deviation of the area ratio between analytes and internal standard for each spiked sample contain 3, 6, 9, 12 and 15 × 10^6 cells. Precision was required to be below 15%.

Pharmacokinetics of intracellular AZT-MP, -DP and -TP concentrations in AZT-exposed PBMC

We studied the association between AZT exposure levels and intracellular AZT metabolites using activated PBMC, according to the standard procedures for phenotypic assay.[16] Briefly, HIV-negative donor PBMC were prepared by Ficoll-Paque plus solution density gradient centrifugation. PBMC were suspended at 1–2 × 10^6/mL in 24-well microtiter culture plates in 2 ml of culture medium (RPMI 1640 medium supplemented with 15% heat-inactivated FCS, 5% purified human interleukin-2, 50U of penicillin per ml, 50 μg of streptomycin and 4 mM L-glutamine) were stimulated with phyto-hemagglutinin (PHA) (3 μg/ml) for 3 days. First, PHA-stimulated PBMC were incubated with different concentrations of AZT (1, 3.2, 10, 100, 1000 and 10 000 nmol/L) for 24 h. Second, PHA-simulated PBMC were incubated with 10 μmol/L of AZT for different time intervals (0.5, 1, 2, 6, 12 and 24 h). These AZT-exposed PBMC were immediately washed twice with PBS, extracted as described earlier, and analyzed to determine the concentrations of AZT metabolites.

Determination of plasma AZT and intracellular AZT-MP,-DP and -TP concentrations in adult AZT-treated patients

We determined the plasma AZT and intracellular AZT-MP, -DP and -TP concentrations in 13 blood samples from nine adult patients. Among 3650 HIV-infected patients who had been treated at our hospital, only nine patients were treated with AZT-containing anti-retroviral therapy. A written informed consent was obtained from all the subjects. Two serial blood samples were obtained from two patients, respectively: the first was obtained immediately before taking AZT and the second was at 4 h after taking AZT. Whole blood was collected into ethylenediaminetetraacetic acid 2 K tube, and plasma and PBMC were extracted immediately and stored. Plasma AZT and intracellular AZT-MP, -DP and -TP concentrations were determined subsequently.

Results and discussion

Validation of the LC–MS/MS method

The method used in the present analysis markedly improved the sensitivity of measurement of intracellular AZT-MP, -DP and -TP through several technical improvements. First, there is a need to overcome interference by endogenous nucleotide phosphates by detecting specific precursor-product ions and positive ionization mode.[7] We succeeded in discriminating AZT phosphates from ATP and dGTP by adopting a positive ionization mode. The fragmentation patterns of the precursor ions of AZT, AZT-MP, -DP, -TP, AZdU, 5-Methyl-UTP, ATP, and dGTP obtained in the positive ionization mode are shown in Figure 1. Relatively little attention has been paid to the problem of unknown phosphate compounds and electrolytes in residual plasma or PBS used for washing PBMC, which also can inhibit ionization of AZT phosphates.[7] Even double washing with saline does not completely remove residual plasma phosphates or electrolytes. To remove residual plasma phosphates and sodium ions, we used a double tube filled with silicon oil for PBMC extraction.[15] Furthermore, for complete elimination of sodium ions, the extracted PBMC aliquot was suspended not in saline but pure water. These improvements of PBMC extraction procedures markedly eliminated various factors known to interfere with ionization. Second, our mobile phase was set at a high pH (around 8.5), and it contained low concentrations of ion-pairing agent. Because NRTI phosphates are highly hydrophilic negative ions, a high pH buffer (pH 8.5) and a positive ion pair are necessary for separation in LC. Because a conventional HPLC column is vulnerable to exposure to a high pH buffer, we used a highly stable HPLC column with a high pH buffer. Several studies employing NRTI phosphates assay
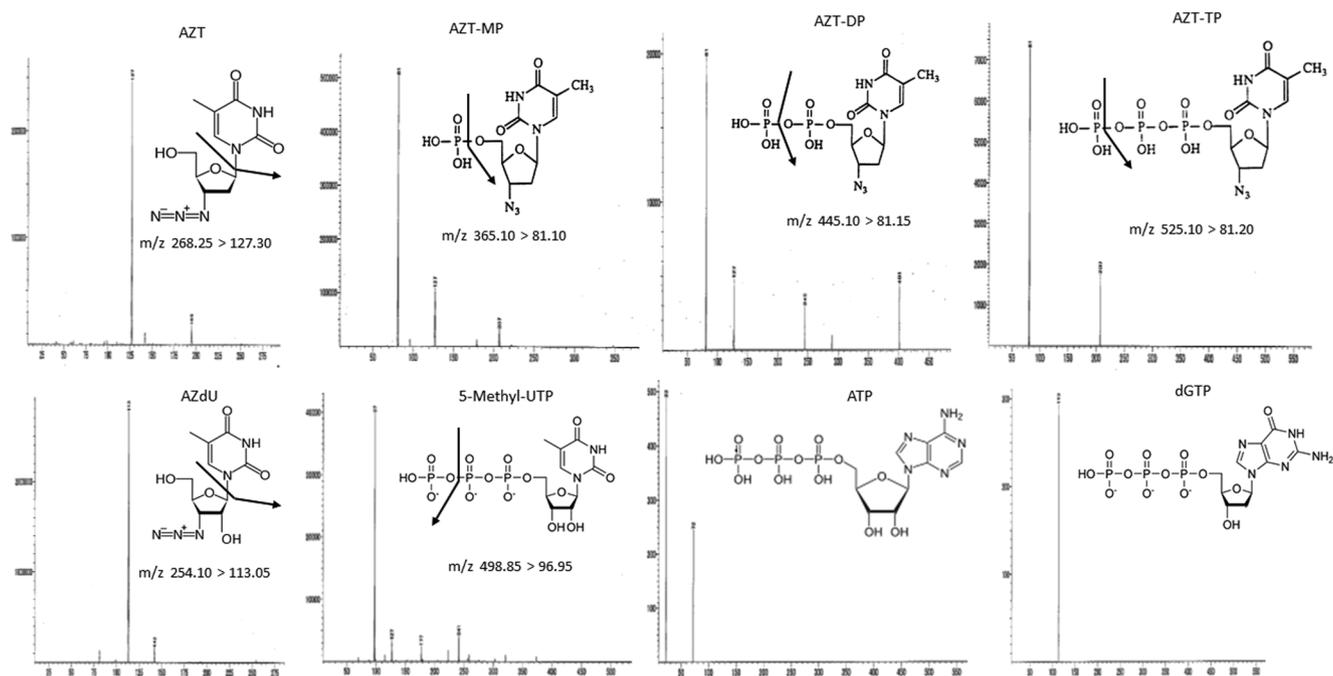
wileyonlinelibrary.com/journal/jms

Figure 1. Chemical structures, precursor/product ions used in MRM and MS/MS spectra of fragmentation patterns of AZT, AZT-MP, AZT-DP, AZT-TP, AZdU, 5-Methyl-UTP and chemical structures and MS/MS spectra of fragmentation patterns of ATP and dGTP.

used 1,5-dimethylhexylamine as an ion pair.[5–7,14] However, while high concentrations of 1,5-dimethylhexylamine can increase the pH of buffers, they can inhibit the ionization of NRTI-TPs. To improve this procedure, we used the combination of ammonium hydrate, as an alkalization agent, and low concentrations of TBAH, as an ion pair, which allowed clear separation and increased the sensitivity. Furthermore, the low concentration of an ion pair interferes less with the ionization of AZT metabolites and can be easily washed from LC lines, which contributed to the stability of this assay (Fig. 1).

The chromatogram of each of the compounds analyzed in this study is shown in Fig. 2. The total runtime of the two assays were 20 min, respectively. The retention times were about 10, 11 for AZT, AZdU (IS), and 5, 6, 6.5 and 6 min for AZT-MP, AZT-DP, AZT-TP and 5-Methyl-UTP (IS), respectively.

Selectivity and specificity

For the blank PBMCs prepared from six different patients, there was no interference at the retention time of the nucleotides and IS. Two endogenous nucleotides triphosphates, ATP and dGTP, can
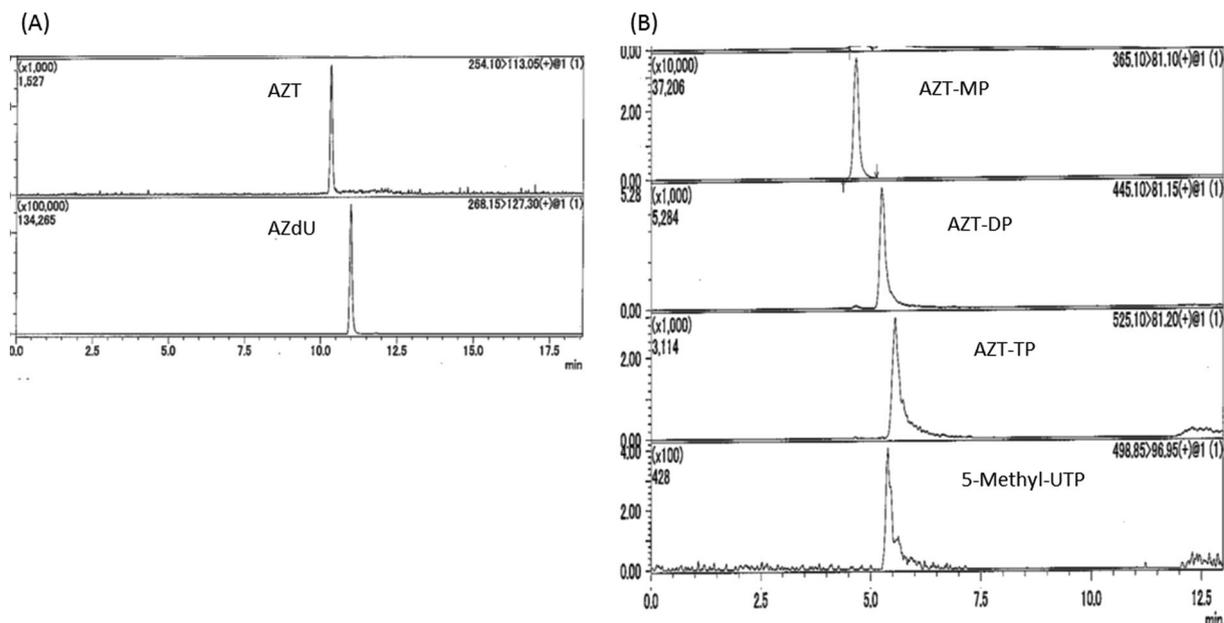

Figure 2. Multiple reaction monitoring ion chromatograms for determination of (A) AZT and AZdU (IS) in spiked plasma and (B) AZT-MP, AZT-DP, AZT-TP and 5-Methyl-UTP (IS) in spiked human peripheral blood mononuclear cells.

interfere with the detection of AZT-TP due to the similarity in molecular weights (ATP: 507, dGTP: 506, AZT-TP: 507) and almost identical fragmentation patterns, especially in the negative electrospray ionization mode. In our method, although both ATP and dGTP can be detected in the same precursor-product ion pattern as AZT-TP, with comparable retention time (4.7 min for AZT-TP, 4.5 min for ATP and 4.0 min for dGTP), they were discriminated by the large differences in the peaks of the large amounts of these compounds (areas under curve for 5 pmol of ATP and dGTP versus AZT-TP were 60 and 400 versus 1,00 000. Fig. 3).

Quantification range and lower limit of quantification

Intraday precision (CV) was less than 15%, and intraday accuracy ranged from 89% to 109% for AZT-MP, -DP and -TP at quantification range (Table 1). Interday precision was less than 15%, and interday accuracy was between 89% and 115% for all analytes. Figure 4 shows typical chromatograms of each analyte in standard solution and spiked samples at LLOQ and HQ.

Despite recent advances in LC–MS/MS technology for measurement of NRTI-TP,[1–3] it is difficult to determine intracellular AZT metabolites (especially AZT-DP and -TP) due to their low concentrations in AZT-treated patients (reported to be around 10–40 fmol/10^6 cell).[4,12] In the present assay, the LLOQ for each run was 6 fmol per sample for plasma AZT, and 6, 10 and 10 fmol per sample for intracellular AZT-MP, -DP and -TP, respectively.

These values can be translated into 0.4 nmol/L of plasma and 0.6, 1.0 and 1.0 fmol/10^6 cells containing 10×10^6 cells, for intracellular AZT-MP, -DP and -TP, respectively. In comparison with previously reported LLOQ of AZT-MP (300 fmol per sample) and -TP (150 fmol per sample),[14] the LLOQ of our assay (6 and 10 fmol per sample for AZT-MP and -TP) improved the sensitivity by 50 and 15 times, respectively. These LLOQ were low for the measurement of intracellular AZT metabolites concentrations in AZT-treated adult patients.

Matrix effect

Data of the matrix effect are summarized in Table 2. The precision of area ratios between AZT and AZdU (IS) in spiked plasma with 25%, 50% and 100% of plasma were 7.9% at three times of LLOQ and 2.5% at HQ. The precision of area ratios between AZT phosphates and 5-Methyl-UTP (IS) in spiked PBMC with 3×, 6×, 9×, 12× and 15×10^6 cells ranged from 6.9% to 11.2% at LQ and 3.8% to 6.9% at HQ, respectively.

In vitro pharmacokinetics of intracellular AZT-MP, -DP and -TP concentrations in PBMC from AZT-treated patients

Figure 5(A) shows the dose-dependent relationship between the AZT exposure level and intracellular AZT metabolites in activated PBMC. Interestingly, AZT-MP level increased proportionally with the increases in AZT level, whereas the increase in AZT-DP and -TP levels blunted at high AZT levels. AZT-TP increased only 94-fold
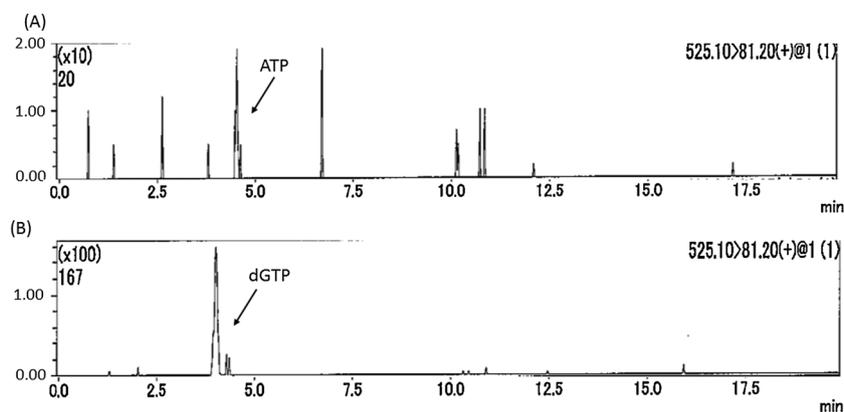

Figure 3. Multiple reaction monitoring (MRM) ion chromatograms of 5 pmol of ATP and dGTP using MRM setting for determination of AZT-TP.

Table 1. Intraday and interday assay precision and accuracy for AZT and AZT-MP, -DP and -TP in human plasma and human PBMC

	Theoretical Concentration (fmol/sample)															
	LLOQ				Low				Middle				High			
	6	6	10	10	60	60	100	100	600	600	1000	1000	6000	6000	10 000	10 000
	AZT	AZT-MP	AZT-DP	AZT-TP	AZT	AZT-MP	AZT-DP	AZT-TP	AZT	AZT-MP	AZT-DP	AZT-TP	AZT	AZT-MP	AZT-DP	AZT-TP
Intraday (n = 5)																
Precision	8.9	10.2	8.0	8.0	7.4	2.2	5.8	7.5	0.5	2.5	3.9	5.6	2.2	1.1	2.1	1.9
Accuracy	93.8	108.8	92.8	91.6	102.9	98.4	102.2	110.3	91	100.7	96.4	97.5	89	101.2	100.1	101.7
Interday (n = 5)																
Precision	8.9	10.9	8.3	9.7	2.6	6.2	9.9	9.1	0.7	2.4	4.9	4.5	1.5	1.3	1.8	1.7
Accuracy	93.8	103.6	104.6	114.9	107.7	97.3	96.7	91.1	91.0	103.1	102.3	96.0	89.3	102.7	102.3	100.3

AZT, zidovudine; MP, monophosphate; DP, diphosphate; TP, triphosphate; PBMC, peripheral blood mononuclear cells; LLOQ. lower limit of quantification.
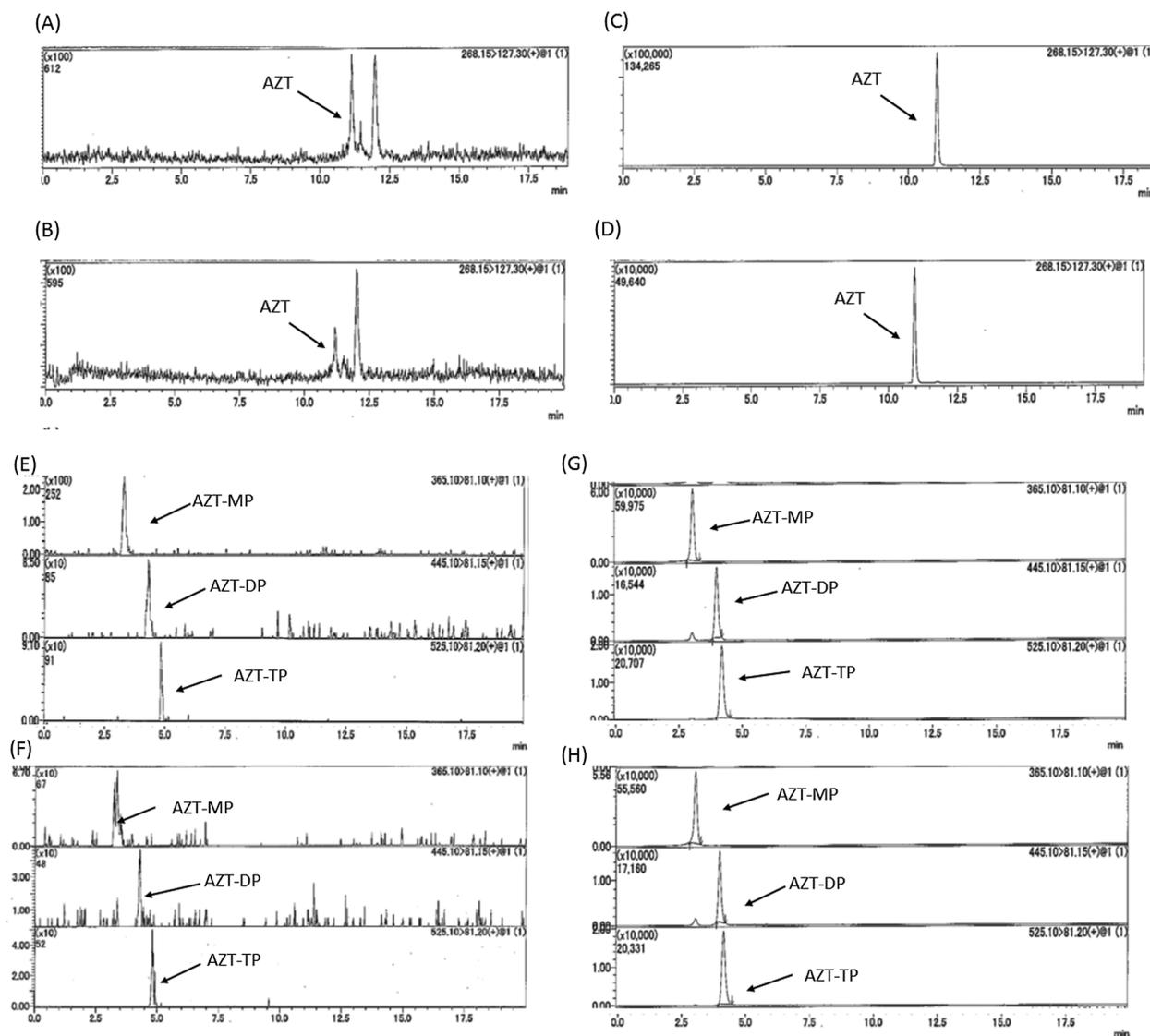
Figure 4. Multiple reaction monitoring ion chromatograms of (A) lower limit of quantification (LLOQ:6 fmol per sample) of AZT in standard solution; (B) LLOQ of AZT spiked in plasma; (C) high calibration point (6000 fmol per sample) of AZT in standard solution; (D) high calibration point of AZT in spiked plasma; (E) LLOQ (6, 10 and 10 fmol per sample) of AZT-MP, -DP and -TP in standard solution; (F) LLOQ of AZT-MP, -DP and -TP in spiked peripheral blood mononuclear cells (PBMCs); (G) high calibration point of AZT-MP (6000 fmol per sample), -DP (10 000 fmol per sample) and -TP (10 000 fmol per sample) in standard solution; (H) high calibration point of AZT-MP, -DP and -TP in spiked PBMCs.

(from 103 to 9640 fmol/10^6 cells) in accordance with the 10 000-fold increase in exposure to AZT (from 1 to 10 000 nmol/L). Especially, AZT-TP increased only 2.6-fold (from 3703 to 9640 fmol/10^6 cells) when exposure to AZT increased by 100-fold from 100 to 10 000 nmol/L. Consequently, AZT-MP levels were lower than those of AZT-DP and -TP at low AZT exposure (less than 100 nmol/L), which are comparable to the range in AZT-treated HIV-infected adult patients. Although AZT-MP can be underestimated because of the wash-out of AZT-MP through the extraction procedures, it is suggested that AZT-MP can rapidly be metabolized into AZT-DP and -TP upon exposure to low levels of AZT. Our highly sensitive method first highlighted the pharmacokinetics of AZT metabolites at extremely low concentrations of AZT (1 and 10 nmol/L), which has not been determined even by conventional methods.

Figure 5(B) shows intracellular concentrations of AZT metabolites using different incubation times with 10 μmol/L of AZT. The levels of AZT metabolites increased rapidly after the start of incubation,

then reached plateaus within 2 h of incubation. Interestingly, AZT metabolites did not increase after longer exposure to AZT or high levels of AZT.

Quantification of AZT metabolites in AZT-treated adult patients

The demographic characteristics of the patients, sampling time, AZT plasma concentrations and intracellular concentrations of AZT-MP, -DP and -TP are listed in Table 3. Low levels of intracellular AZT-TP (<10 fmol/10^6 cells) were noted in eight samples from five patients. These results were consistent with those determined by the conventional but highly sensitive method of radioimmunoassay (10–40 fmol/10^6 cell).[8,17] In contrast, these values were lower than those reported previously in a study using LC–MS/MS assay (50–200 fmol/10^6 cells).[9,10,14]

Table 2. Matrix effect of human PBMC number on quantitation of AZT, AZT-MP, -DP and -TP

	Theoretical concentration (fmol/sample)			Theoretical concentration (fmol/sample)					
	Low	High		Low			High		
	18	6000		18	30	30	6000	10 000	10 000
	AZT	AZT		AZT-MP	AZT-DP	AZT-TP	AZT-MP	AZT-DP	AZT-TP
Plasma %			Cells ($\times 10^6$)						
25	0.085	17.12	3	0.0069	0.0027	0.0014	14.69	7.055	4.582
50	0.099	16.76	6	0.0077	0.0025	0.0016	15.30	6.793	4.484
100	0.092	17.60	9	0.0079	0.0026	0.0015	16.10	7.179	4.711
			12	0.0071	0.0026	0.0013	14.92	6.525	4.367
			15	0.0075	0.0023	0.0014	15.80	6.806	4.446
Mean	0.092	17.16		0.0074	0.0026	0.0014	15.36	6.872	4.518
SD	0.007	0.422		0.0005	0.0001	0.0001	1.058	0.272	0.171
CV%	7.9	2.5		6.9	9.8	11.2	6.9	4.0	3.8

Figures represent mean ratio (*n* = 6) of chromatographic peak area of analytes to internal standard.
AZT, zidovudine; MP, monophosphate; DP, diphosphate; TP, triphosphate; PBMC, peripheral blood mononuclear cells; LLOQ. lower limit of quantification; CV, intraday precision; SD, standard deviation.
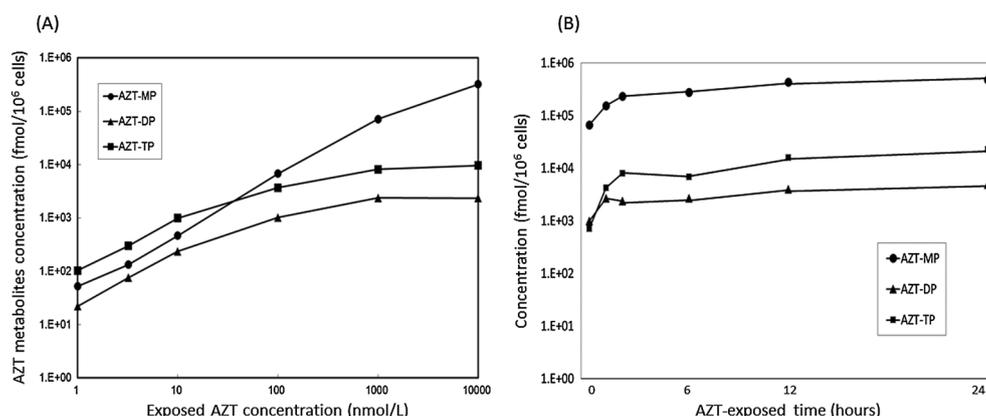

Figure 5. *In vitro* assay using phytohemagglutinin-activated peripheral blood mononuclear cells. Intracellular AZT-MP, -DP and -TP concentrations by (A) 24 h-incubation of different concentrations of AZT and by (B) different incubation time period using 10 µmol/L of AZT.

Interestingly, no marked increases in intracellular AZT phosphate levels were noted from 0 to 4 h in the two patients, despite the rapid increases in plasma AZT levels. Several studies have also reported that AZT phosphorylation is quite poor,[18–20] and recent clinical data also confirmed that the efficiency of AZT phosphorylation is far lower than tenofovir DF.[17] AZT is phosphorylated sequentially by thymidine kinase, thymidylate kinase and nucleoside diphosphate kinase, with thymidylate kinase acting as a rate-limiting enzyme in this process.[21] Our study also showed that AZT-DP levels are comparable to those of AZT-TP, which added support to the aforementioned hypothesis.

Our study identified relatively low levels of intracellular AZT-MP concentrations compared with those reported in previous studies.[9,10,14] The different findings can be explained as follows: first, low plasma AZT concentrations (<100 nmol/L), which are observed in the majority of AZT-treated adult patients, are associated with low intracellular AZT-MP levels. Second, AZT-MP concentration decreases rapidly due to the short half-life.[10] Third,

low levels of AZT-MP can be easily washed out during the PBMC extraction procedures, because AZT-MP rapidly diffuses out of the cell membrane. Furthermore, in Japan, AZT is commonly administered in a small dose of 200 mg twice daily based on the high incidence of severe nausea in adult patients treated with the standard dose. Thus, a smaller dose of AZT is associated with low plasma AZT concentrations and consequently results in low intracellular AZT-MP levels.

AZT is exclusively and widely used in infants to prevent mother-to-infant HIV-1 transmission. The pharmacokinetics of AZT phosphates in infants have not been fully evaluated except for one study that employed the LC–MS/MS assay.[4] However, 4 mL of blood sample was required for analysis in the earlier study, which can be a heavy burden in most infants. In contrast, our assay determined AZT phosphates level using smaller volume of blood sample due to the improved sensitivity. Therefore, our sensitive assay is potentially useful for the evaluation of the pharmacokinetics of intracellular phosphates in infants and children using only a small amount of blood samples.

Table 3. Characteristics, plasma AZT and intracellular AZT-MP, -DP and -TP concentrations in adult HIV-infected patients

Patients	Age (yrs)/sex	Weight (kg)	AZT dose (mg)	concurrent ARV	Latency (hrs)*	Cell number (x10^6)	plasma AZT (nM)	AZT-MP (fmol/10^6 cells)	AZT-DP (fmol/10^6 cells)	AZT-TP (fmol/10^6 cells)	CD4 cell count	HIV RNA (copies/ml)	Hb	MCV	Remarks
1	49/M	61	400	3TC, NVP	1	5.3	2240	5.88	23.2	46.8	1099	UD	16.1	113	
2	40/F	64	400	3TC, LPV/r	12	5.3	21.6	<LLOQ	2.87	3.41	784	UD	13.1	114	
3	34/F	60	400	3TC, LPV/r	2	1.7	219	10.7	16.5	28.5	243	2700	10.2	90.4	Pregnant woman
4	40/M	74	400	3TC, NVP	12	5.5	18.3	2.02	21.7	15.9	635	UD	16	107	
5	38/M	75	400	3TC, LPV/r	4	2	158	4.07	20.0	14.7	971	UD	15.2	105	
6	56/M	64	400	3TC, LPV/r	4	7.2	64.7	1.71	30.5	22.3	811	UD	13.8	111	Samples collected at different days
					6	2.1	16.8	<LLOQ	<LLOQ	5.29	723	UD	13.6	111	Samples collected at different days
7	36/F	47	400	3TC, LPV/r	13	4.6	2.12	<LLOQ	<LLOQ	<LLOQ	371	UD	12.4	103	Samples collected at different days
					10	3.4	34.1	4.21	3.78	9.56	296	UD	12.8	105	
8	38/F	59	400	3TC, LPV/r	12	5.4	12.3	<LLOQ	<LLOQ	<LLOQ	371	UD	11.1	116	Pregnant woman. Samples collected on same day
					4	5.8	22.4	1.06	1.89	1.98					Samples collected on same day
9	42/M	78	600	3TC, EFV	12	10	5.62	1.47	2.22	4.21	612	UD	15.3	112	
					4	10	163	<LLOQ	2.19	3.28					

* Latency, time between previous AZT dosing and blood sampling; ARV, antiretrovirals; 3TC, lamivudine; NVP, nevirapine; LPV/r, lopinavir/ritonavir; EFV, efavirenz; UD, Undetectable; Hb, hemoglobin; MCV, mean corpuscular volume; AZT, zidovudine; MP, monophosphate; DP, diphosphate; TP, triphosphate; LLOQ, lower limit of quantification

Conclusion

We described here a highly sensitive method for analysis of intracellular AZT phosphates by improving PBMC extraction procedures, alkalization of LC buffer, alkaline-stable HPLC column and the use of low concentration of tetrabutylammonium hydroxide (TBAH) as ion pair. We were able to determine the presence of extremely low concentrations of intracellular AZT metabolites in AZT-treated adult patients. This improved method can be applied to measure other NRTI metabolites because their chemical characteristics are closely similar to those of AZT phosphates. Our method requires small volume of blood samples for determination of intracellular concentrations of AZT metabolites, which can be highly advantageous in children and infants.

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