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Development of a Direct Assay for Measuring Intracellular AZT Triphosphate in Humans Peripheral Blood Mononuclear Cells

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Direct LC/MS/MS methods have recently been developed for measuring triphosphate anabolites of several nucleosidic reverse transcriptase inhibitor (NRTI) in peripheral blood mononuclear cells (PBMCs) from HIV-positive patients. Whereas AZT is one of the most-used NRTIs, no such method has been developed for AZT-TP, its active anabolite, mainly because of the presence of endogenous nucleotides that interfere with such an assay. In this paper, we first describe the development of two enzyme immunoassays (EIA) of AZT-TP in PBMCs: one directly measuring AZT-TP content; the other, measuring the nucleoside AZT after selective extraction of AZT-TP and dephosphorylation. The precision of these two assays was too low to achieve precise determination of AZT-TP in PBMC samples. Direct LC/MS/MS is not specific enough for AZT-TP, since at least two interfering endogenous nucleotides (same *m*/*z* ratio and fragment as well as retention time close to that of AZT-TP) are found in the intracellular medium of PBMCs. The off-line combination of immunoaffinity extraction (IAE) and LC/MS/MS proved to be a successful strategy allowing without dephosphorylation appropriate specificity and sensitivity (limit of quantification established as 9.3 fmol/10^6 cells) to determine AZT-TP in PBMCs from 7 mL of blood of HIV-infected patients. Validation of this IAE-LC/MS/MS method demonstrated CV percent for repeatability and intermediate precision lower than 15%. More than 150 samples/week can be analyzed by one analyst, making this method suitable for routine analysis during clinical studies.

AZT or zidovudine is the first approved drug against HIV and belongs to the family of nucleosidic reverse transcriptase inhibitors (NRTIs). Like other NRTIs, AZT is a prodrug and is required to be intracellularly phosphorylated to AZT-triphosphate (AZT-TP) for anti-HIV activity. AZT-TP competes with the endogenous nucleotide, deoxythymidine (dT-TP), for binding to HIV reverse transcriptase and stops elongation of viral DNA (because of the lack of the 3′-hydroxyl group on the sugar moiety).[1−3] Measurement of this intracellularly active anabolite is necessary for an accurate understanding of efficacy and toxicity of this drug.

A few methods for quantifying AZT-TP in clinical samples have been published. These include an initial separation of the phosphate anabolites using either ion-exchange cartridges, ion pairing, or ion liquid chromatography, followed by nucleotide dephosphorylation and a further quantification of the nucleoside by radioimmunoassay[4−11] or by liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS).[12,13] All of these methods correspond to indirect measurements, since the quantification is finally carried out on the parent drug (nucleoside) resulting from dephosphorylation of the triphosphorylated anabolite. The multistep process increases the variability of the results

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and makes the method too time-consuming for routine use. A direct method using immunoassay has been described but only applied to the monophosphate anabolite (AZT-MP).[14] Inhibition of HIV reverse transcriptase (RT) activity was also used for quantification of AZT-TP.[15]

Direct LC/MS/MS methods have recently been used to quantify the triphosphate anabolites of several NRTIs, including abacavir (ABC),[16] emtricitabine (FTC),[17] stavudine (d4T),[18] and have also been used with didanosine (ddI) and lamivudine (3TC) in a single run.[19] No equivalent direct method has been developed for AZT-TP measurement, probably because of the presence of high levels of interfering ATP in the intracellular medium.[19]

We describe here several analytical methods for AZT-TP quantification. We first set up different enzyme immunoassays (EIAs) in order to measure the intracellular AZT-TP content either directly or after ion-exchange extraction and dephosphorylation. Parallel attempts were made to quantify AZT-TP using a direct LC/MS/MS assay as for d4T-TP, 3TC-TP, and ddA-TP. Success was achieved using an off-line combination of both techniques, that is, specific extraction of AZT-TP using immunoaffinity and detection of AZT-TP using LC/MS/MS resulting in a highly specific and sensitive method (IAE-LC/MS/MS).

MATERIAL AND METHODS

Chemicals. Phosphorylated anabolite AZT-TP was kindly provided by Drs. C. Guerreiro and R. Sarfati from the Unité de Chimie Organique, Institut Pasteur, Paris. 2-Chloroadenosine 5′-triphosphate (ClA-TP), used as internal standard, was from Sigma (Sigma Chemical Co., St Louis, Mo.). The stable analogue of AZT-TP, 3′-azido-3′deoxy-5′-(α;β:β,γ-bis-methylene)triphosphate-N^3-[1-(5-aminopentyl)]-thymidine (AZX-TP), was synthesized as previously described.[20,21] The 5′-hemisuccinate derivative of AZT (AZT-HS) was also prepared using standard methods.

Materials and Reagents. Bio-Spin columns were from BIO-RAD (California, USA). Human blood from healthy subjects was obtained from EFS (Rungis, France) or Biomédia (Boussens, France). Gradient grade methanol was from Merck (Darmstadt, Germany), HPLC quality acetonitrile from SDS (Peypin, France), and formic acid ammonium salt, analytical formic acid and *N*′, *N*′-dimethylhexylamine (DMH) were from Sigma Aldrich (St Louis, Mo.).

Instrumentation. An HPLC system 1100 (Agilent Technology, Les Ulis, France) was connected to an API 3000 tandem mass spectrometer equipped with an electrospray source (Sciex, Applied Biosystem, France) and monitored using Analyst v.1.1 software.

Standard Solutions. Stock solutions of AZT-TP and ClA-TP were prepared at 0.1 mg/mL in ultrapure water. Several diluted solutions are prepared by 1/10 dilution of the stock solution in ultrapure water to prepare standards for the calibration curve and quality controls. Stock and diluted solutions were stored at −20 °C. Purity of the stock solution was checked before use using the same chromatographic system coupled with UV detection (λ, 260 nm).

Preparation of Spiked Samples. Peripheral blood mononuclear cells (PBMCs) were prepared using CPT tubes as previously described.[19] Dry pellets of PBMCs contained ∼10 × 10^6 PBMCs. Just before analysis, AZT-TP standards (20 μL of a solution of AZT-TP) were added as calibrators and quality control to the PBMC pellet. Preparation of PBMC extract was performed as previously described.[18]

Samples from Patients. PBMCs collected (in CPT tubes) from 7 mL of blood from patients were submitted to the same treatment as previously described for spiked samples.

For unknown samples taken from subjects, the result obtained by the calibration curve is given in fmol. A single division is then performed according to the number of cells previously counted in the sample.

Production of Polyclonal Antibodies and EIA Development. Different rabbit polyclonal antibodies were produced to develop AZT and AZT-TP EIAs using either an immunogen prepared by covalent coupling of AZT-HS to BSA via an activated ester of the carboxylic function of AZT-HS in 5′ position or keyhole limpet hemocyanin (KLH) as carrier protein and glutaraldehyde to covalently link AZX-TP. To detect antibody production, various enzyme conjugates were prepared using acetylcholinesterase (AchE)[22] by coupling either the activated ester of AZT-HS with the enzyme,[23] a thiol derivative of AZX-TP with AChE previously reacted with succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC)[24] or coupling of AZT-TP by the γ phosphate moiety, as previously described.[25] Competitive enzyme immunoassays were performed in 96-well microtiter plates coated with mouse monoclonal anti-rabbit IgG as previously described,[14] using the enzymatic tracers at a 2 Ellman units/mL concentration (the definition of Ellman unit was previously detailled[23]). After an 18-h incubation at +4 °C, the plates were washed (with 0.01 M phosphate buffer pH 7.4 containing 0.05% Tween 20) before quantitation of the enzyme activity of the bound immunological complex by addition of 200 μL/well of Ellman's reagent (mix of substrate and chromogen).[23] After 1 or 2 h of gentle shaking in the dark at room temperature, the absorbance at 414 nm of each well was measured. Results are expressed in terms of B/B_0% as a function of the dose (logarithmic scale), B and B_0 representing the bound activity in the presence or absence of competitor, respectively. A linear log−logit transformation was used to fit the standard curve. The assay sensitivity was characterized by the dose of standard inducing a 50% lowering of the binding (B/B_0

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50%). The B/B_0 80% corresponds to the minimum detectable concentration.

For the other EIA method, the selective extraction of AZT-TP, the dephosphorylation, and the concentration of the recovered AZT were performed using an ASPEC XL4 apparatus (Gilson, France) that can process 4 samples simultaneously and 24 samples/run. After lysis of the PBMCs, AZT-TP was selectively extracted from samples (at +4 °C) using anion-exchange cartridges (Sep-Pak Vac 3 cm^3 500 mg Accell Plus QMA, Waters). Cartridges were first washed successively with 9 mL of methanol, 8 mL of 2 M KCl, and 9 mL of 100 mM KCl (10 mL/min) before loading the samples (1.5 mL/min). After washing the cartridges with 12 mL of 100 mM KCl (5 mL/min), AZT-TP was eluted with 5 mL of 2 M KCl (3 mL/min). Dephosphorylation was performed by adding four units of acid phosphatase (type XA, Sigma) and 12.5 μL of 1 M sodium acetate pH 4 for a 1-h incubation at 37 °C. The resulting AZT was concentrated using Oasis HLB Vac 3 cm^3 cartridges (Waters). Cartridges were successively washed with 3 mL of methanol, 5 mL of acetonitrile, 5 mL of H_2O, and 3 mL of 2 M KCl (6 mL/min) before loading the samples (1.5 mL/min). After washing with 10 mL of H_2O (15 mL/min), AZT was eluted with 2 mL of acetonitrile (3 mL/min). After drying under nitrogen, AZT was resuspended in 500 μL of EIA buffer (100 mM phosphate buffer pH 7.4, 1 g/L BSA, 0.15 M NaCl, 0.01% NaN_3) before assaying.

Immunoaffinity Extraction. The total IgG fraction from anti-AZX-TP polyclonal antiserum was purified by precipitation using caprylic acid.[26] The purity of the recovered IgG was assessed by SDS−PAGE experiment. A 13.5 mg portion of purified IgG was further covalently linked to 2 g of CN−Br-activated Sepharose 4B (Pharmacia, Sweden) following the manufacturer's instructions.

PBMC extract (~150 μL) was mixed with 500 μL of the immunoaffinity matrix disposed in a biospin column. After 20 min of rotation at 20 °C, the column was washed with 5 mL of Tris HCl (0.1 M, pH = 7.4) before elution of AZT-TP with 5 mL of formate buffer (0.2 M pH 2.5)/acetonitrile (80/20,v/v). The eluted extract was dried under vacuum overnight using a SpeedVac Savant apparatus before reconstitution with 150 μL of Tris-HCl (0.05 M pH 7.4). Internal standard (ClA-TP, 20 μL, 102.5 ng/mL) was then added. A 40-μL portion of the reconstituted extract was injected into the LC/MS/MS.

The column was regenerated by successive washes with 5 mL of 0.1 M acetate buffer pH 4 containing 0.5 M NaCl and 5 mL of 0.1 M carbonate buffer pH 8.5 containing 0.5 M NaCl, and stored in 0.1 M Tris−HCl buffer pH 7.4 containing 0.02% sodium azide at 4 °C.

Chromatographic and Mass Spectrometric Conditions. Chromatography was achieved on Supelcogel ODP-50 5 μm, 150 × 2.1 mm (Supelco, St Quentin-Fallavier, France). The mobile phase was delivered at a flow rate of 0.3 mL/min. The mobile phase and gradient were as previously described.[18]

The instrument was operated in the electrospray negative mode under MS/MS conditions (MRM). Fragmentation was achieved with nitrogen. The ion transitions monitored were 506/159 for AZT-TP and 540/159 for ClA-TP. The dwell time for each transition was 0.5 s. For maximum sensitivity of AZT-TP detection, the mass spectrometer parameters were optimized as follows: capillary voltage, −4 KV; source temperature, 450 °C. Cone voltage (CV) and collision energy (CE) were optimized for AZT-TP and internal standard (I.S.), that is, −70 and −75 V (CV), −34 and −45 V (CE).

Validation of the IAE-LC/MS/MS Method. Calibration Curves. Calibrators were prepared by spiking PBMC samples at six concentrations (final amount of AZT-TP: 93.0, 186, 839, 1680, 2610, and 3360 fmol)

Selectivity and Specificity. To investigate whether endogenous compounds interfered with the assays, PBMC extracts taken from 4 different healthy subjects and from 10 HIV-infected subjects not treated with AZT were analyzed.

Accuracy and Precision. QC samples at three levels (261, 1120, and 2240 fmol) were processed and analyzed three times in the same run (intrarun precision or repeatability) and one time in five separate runs (inter-run precision or intermediate precision). The accuracy was calculated at each concentration as the ratio of the measured concentration to the nominal concentration multiplied by 100%, both using the intra- and inter-run precision experiment.

Limits of Detection and Quantification. Limit of detection and limit of quantification were considered as the lowest intracellular concentration that the software could differentiate from background noise (ratio 3/1 and ratio 9/1, respectively). The QC samples were processed and analyzed six times in the same run.

Recovery. Recovery experiments were performed in triplicate at the three QC concentrations. Ratios of the peak area of AZT-TP to that of the internal standard were compared for QC samples spiked before and after immunoaffinity extraction and evaporation.

Stability. Stability of AZT-TP was tested in the injection solvent at 4 °C and light exposure (on the HPLC autosampler).

RESULTS

Enzyme Immunoassays for Measuring Intracellular AZT-TP Levels. Because of in vivo degradation of AZT-TP, polyclonal anti-AZT-TP antibodies were obtained using a synthetic analogue of AZT-TP, that is, AZX-TP, including a spacer arm on the base and stable methylene bisphosphonate groups mimicking the pyrophosphate moieties of the 5′-triphosphate (see Materials and Methods). This compound showed good in vivo stability, since good immunological responses were recorded using all the AZT-TP enzyme conjugates prepared in our laboratory (see Materials and Methods). The best competitive immunoassay was obtained using as tracer AZT-TP coupled by the γ phosphate through carbodiimide. The sensitivity at B/B_0 50% was ~420 pg/mL (828 pM), with a limit of detection close to 91 pg/mL (8.95 fmol/well). AZT-diphosphate (DP) and AZT-monophosphate (MP) metabolites were partly recognized by the antisera, exhibiting cross-reactivity of 12.5 and 8.3%, respectively. Conversely, neither the parent drug AZT nor the different nucleotides tested (ATP, TTP, dATP and dCTP) presented any significant cross-reactivity (CR inferior to 0.01%).

Unfortunately, using this competitive immunoassay, we have never been able to achieve reliable determination of intracellular AZT-TP concentrations as found in cellular extracts spiked with known amount of AZT-TP or in cells cultured in the presence of AZT. Our interpretation is that the assay was impaired because

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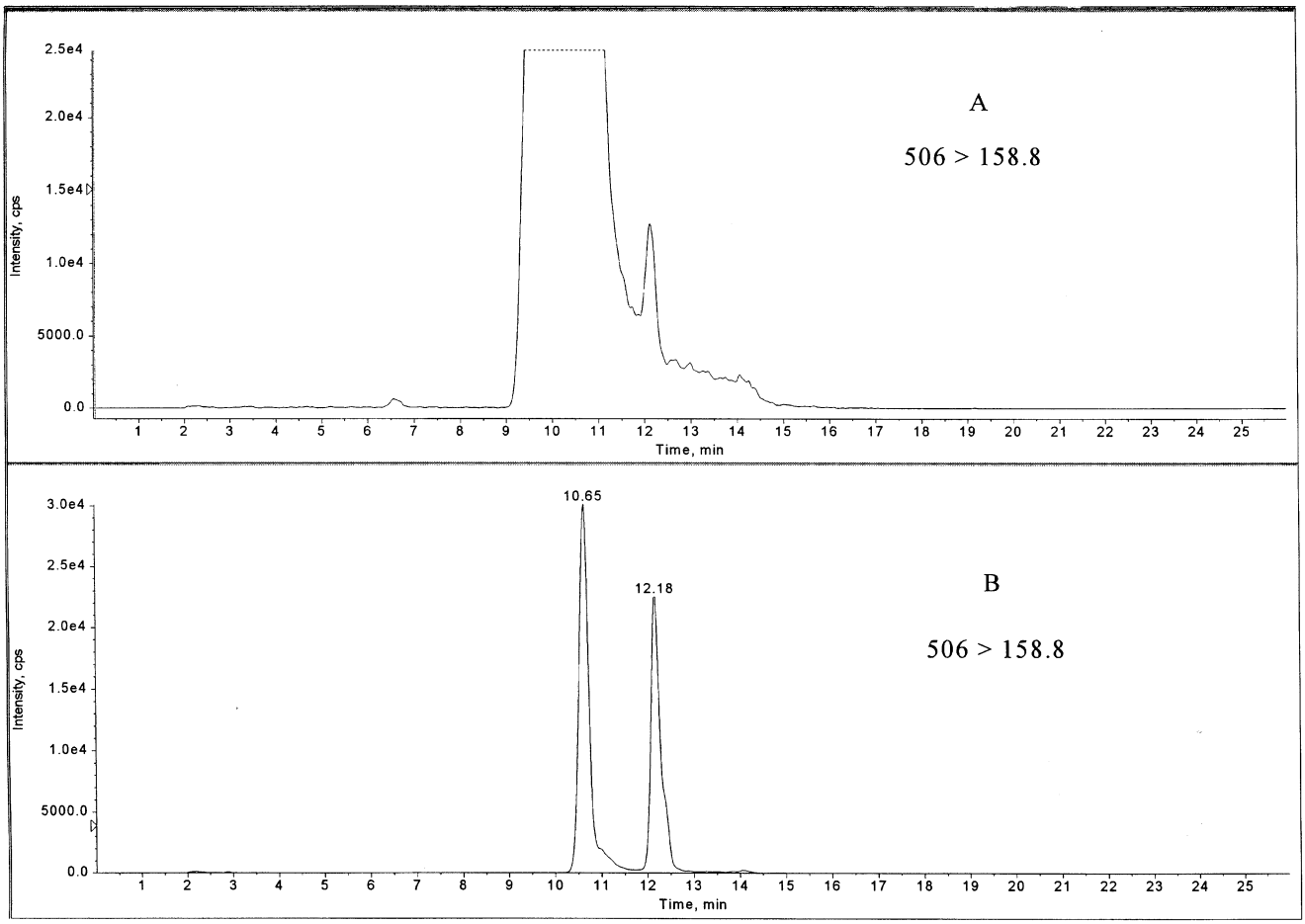

Figure 1. MRM ion chromatograms for the LC/MS/MS determination of AZT-TP in human PBMCs before (A) and after (B) periodate-methylamine degradation of ribonucleotides. (TR 10.65 min, ATP; TR 12.18 min, AZT-TP + dG-TP)

of an important matrix effect, the presence of large amounts of AZT-MP (the major phosphorylated anabolite of AZT), or both.

To overcome these problems, we moved to an indirect measurement involving, as previously described,[27] a selective extraction of AZT-TP, a dephosphorylation step, and the concentration of the recovered AZT before assaying. Using antibodies directed against AZT-HS analogue and the corresponding enzyme conjugate (see method), we have developed a specific AZT EIA whose sensitivity was characterized by a B/B_0 50% close to 110 pg/mL (411 pM) and a limit of detection ~18 pg/mL (3.36 fmol/well). The enzymatic dephosphorylation and the concentration step proved to be quantitative with a yield superior to 95%. Conversely, in our hands, in opposition with previously published results,[27] the selective separation of AZT-TP from AZT-DP using anion-exchange cartridges appears to be very difficult to achieve. Different ionic strength buffers, volumes, and flow rates were compared to optimize this extraction, leading either to a very low recovery of AZT-TP (≈40%) or, when better yields were obtained (>90%), to an important AZT-DP contamination (≈20%). Moreover, despite the complete automation of the overall sample preparation, this method exhibited substantial variability when applied to spiked extracts (CV > 25%).

Both EIA methods were applied to a limited number PBMC samples from HIV-infected patients. Most of the samples were quantified (data not shown), but the low AZT-TP content (close to the limit of detection of the methods) prevented dilution of the samples to limit the matrix effect. This problem associated with the low precision previously observed led us to give up the quantification of AZT-TP in PBMC extracts using EIA and to focus on a physicochemical method, that is, LC/MS/MS, which could provide better results.

Development of an Off-Line Combination of IAE and LC/MS/MS. To detect AZT-TP, we initially used the chromatographic conditions previously developed to monitor the PBMC's intracellular content of ddA-TP, d4T-TP, and 3TC-TP. As previously described,[19] when applied to PBMC extracts spiked with AZT-TP concentrations ranging between 90 and 3000 fmol (thus supposed to correspond to clinical samples), no detectable signal was found. This was mainly due to the interference of endogenous nucleotides possessing the same retention time, m/z ratio and fragment (i.e., pyrophosphate ion) as AZT-TP, probably including ATP that has an m/z ratio very close (differing only at the second decimal) to that of AZT-TP: 506.2. Modification of the LC gradient was unsuccessful, since the ATP peak remained too broad to be accurately separated from AZT-TP, and a second interfering compound was also present (previously masked by ATP), assumed to correspond to deoxyguanosine triphosphate or dG-TP (m/z = 506.2).

Periodate-methylamine degradation of ribonucleotides such as ATP, previously described by Garrett and Santi,[28] was tested.

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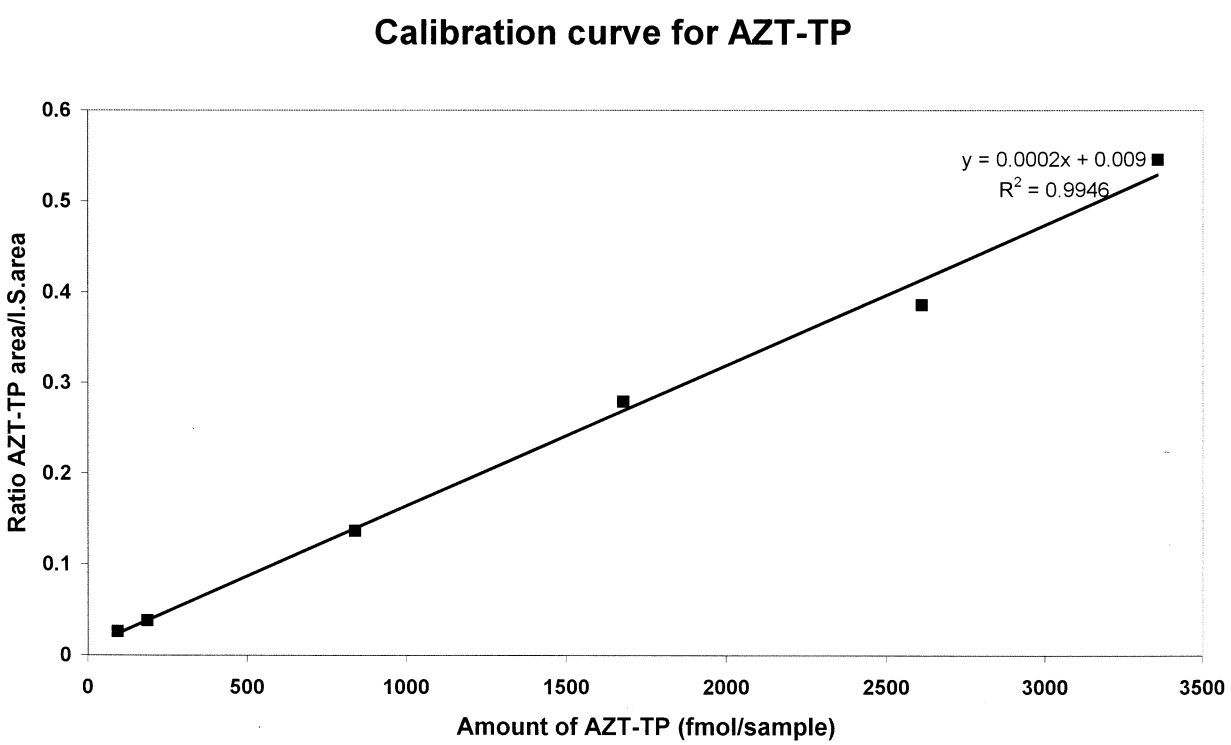

Figure 2. Representative calibration curves for AZT-TP in human PBMCs using IAE-LC/MS/MS.

Although this method was able to reduce the ATP signal, no effect on the second interfering nucleotide was observed, strengthening the previous dG-TP hypothesis, since this degradation method works for ribonucleotide but not for deoxynucleotide (see chromatograms in Figure 1). This nucleotide exhibits the same retention time as AZT-TP (~12 min) and provides a very intense signal in clinical samples, thus preventing a reliable quantification of AZT-TP with this method.

To circumvent this problem, we decided to apply immunoaffinity extraction using anti-AZT-TP polyclonal antibodies obtained using AZX-TP (see above) that selectively recognize the AZT core and could allow a highly specific extraction of this analyte from complex biological matrixes. Methanol is usually used for eluting haptens from immunosorbent and was therefore tested first, but it provided only a 5% recovery of the AZT-TP loaded onto the column. To enhance this yield, different more drastic solvents or combinations were used. The best results (recovery >90%) were obtained using a mix of 0.2 M formate buffer pH 2.5/acetonitrile, 50/50. The requirement of these drastic conditions follows from the use of polyclonal antibodies that involve different types of interaction. Unfortunately, these conditions also totally denature the antibodies, leading to a single use of the immunochromatographic matrix. After optimization, intermediary conditions (0.2 M formate buffer pH 2.5/actetonitrile; 80/20, v/v) were adopted, allowing both a good extraction (close to 75% recovery over a large range of AZT-TP concentrations) and the preserving of the chromatographic column, which can be reused more than 30 times after regeneration without modification of its capacity. About 25% of AZT-TP was lost during evaporation as a plausible result of thermal degradation, and the final recovery was therefore close to 50%. After immunoaffinity extraction, <1% of the initial ATP content remained, having a poor influence on the quantification of AZT-TP (TR: 10.65 min, see Figure 1).

Table 1. Precision of the Analytical Method

Intra-Assay Precision			
theor concn (fmol/10^6 cells)	26.1	111.8	224
CV%	5.1	2.5	3.7
accuracy	94.3	87.0	99.3
Interassay Precision			
theor concn (fmol/10^6 cells)	26.1	111.8	224
CV%	10.5	4.9	8.0
accuracy	100.1	90.3	95.2
Limit of Quantification (Intra-Assay)			
theor concn (fmol/10^6 cells)	9.3		
CV%	9.9		
accuracy	94.1		

Validation of the IAE-LC/MS/MS Method. Standard curves were further established for AZT-TP using PBMCs from healthy subjects (Figure 2). Linear regression ($Y = Ax + B$) was performed between the ratio of the peak area of AZT-TP to that of the internal standard (ClA-TP) versus the corresponding theoretical spiked concentration (X). A $1/X$ weighted regression was used. The average calibration slope was 1.7×10^{-4} ($n = 5$), and the correlation coefficient was 0.998 ($n = 5$).

Precision of the assay was checked with PBMCs samples spiked with AZT-TP at concentrations within the calibration curve range (see Materials and Methods). Average CV% was found to be 3.8% for repeatability and at 7.8% for intermediate precision. Accuracy was between 87 and 112%. All precision and accuracy results are summarized in Table 1.

The limit of quantification (LOQ) was established at 93 fmol (or 9.3 fmol/10^6 cells; 10×10^6 cells/sample). Intra-assay CV%

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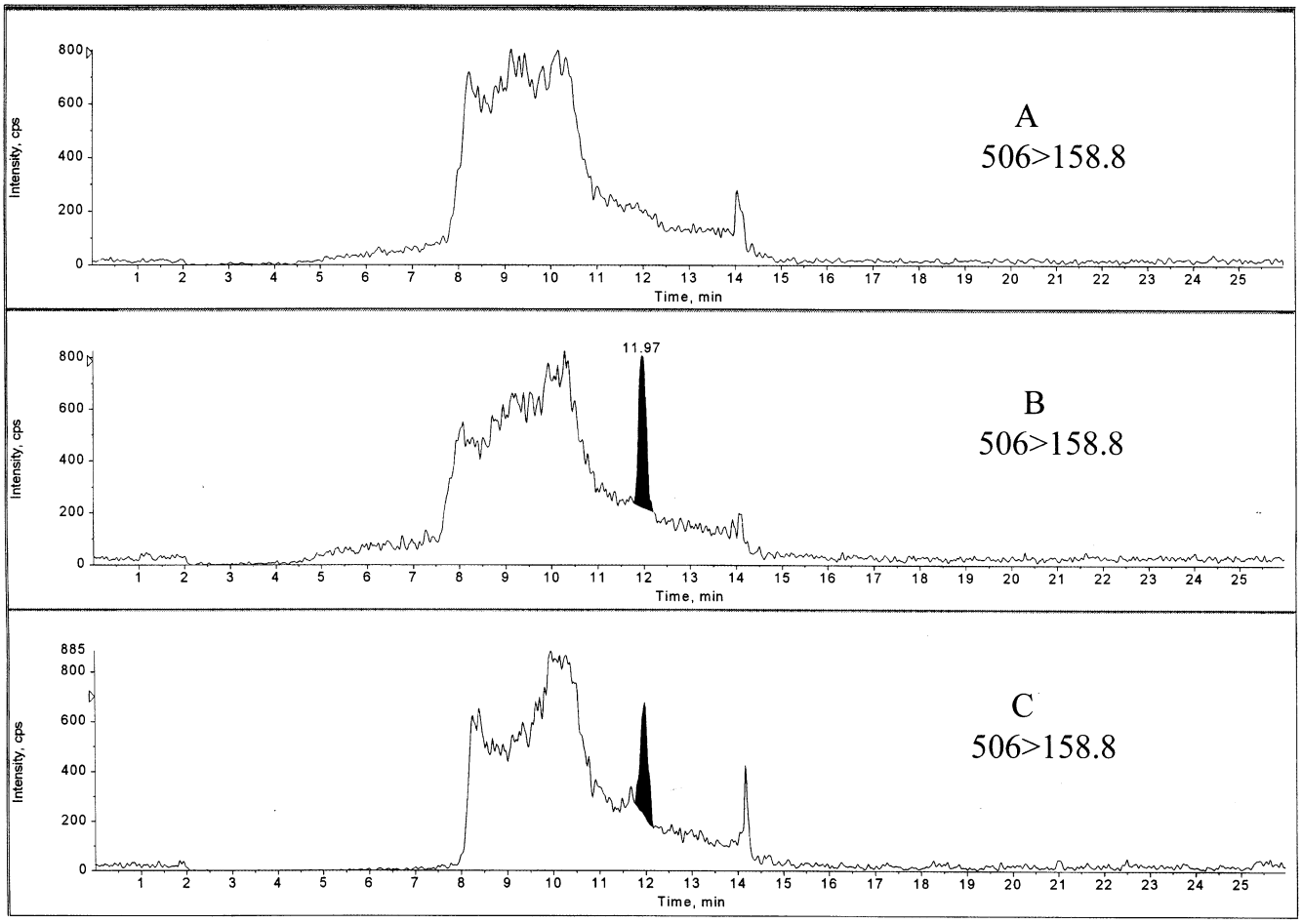

Figure 3. MRM ion chromatograms for the IAE-LC/MS/MS determination of AZT-TP in drug-free human PBMCs (A) and in PBMCs collected after dosing of AZT (B and C).

Table 2. Intra-Assay Recovery of Immunoaffinity Extraction Process

theor concn (fmol/10^6 cells)	recovery	CV%
26.1	56.1	
26.1	55.3	
26.1	55.4	0.8
111.8	51.8	
111.8	49.9	
111.8	49.9	2.2
224	44.4	
224	42.2	
224	45.3	3.6
mean	50.0	10.3

was 9.9%, and accuracy, 94%. The limit of detection (LOD) was evaluated as 31 fmol or 3.1 fmol/10^6 cells. In view of the previously published in vivo concentration,[29] this method is applicable to clinical samples.

Recovery of the full analytical procedure was determined on spiked PBMC extract. Within the spiking range 261−2238 fmol, the average recovery was 50% (intra-assay CV% between 0.8 and 3.6% (Table 2).

Stability of AZT-TP was checked in the autosampler in the injection solvent (i.e., Tris buffer). The results showed that the analyte was stable for over 24 h.

(29) Stein, D. S.; Moore, K. H. P. *Pharmacotherapy* **2001**, *21*, 11−34.

Clinical Samples. A total of 33 cellular samples from HIV-infected patients, AZT-treated or AZT-free, were analyzed with this method. HPLC/MS/MS chromatograms recorded for the PBMCs from one AZT-free and two AZT-treated patients are shown in Figure 3. It can be seen that significant peaks corresponding to AZT-TP were observed in the cell extracts obtained from AZT-treated patients, whereas no peak corresponding to AZT-TP was evidenced in the cell extract from patients not treated with AZT. Of 23 of the AZT-treated patients, 22 exhibited concentrations above the limit of quantification. One sample was below the limit of detection. Table 3 shows the intracellular concentration of AZT-TP determined for these HIV-infected patients, who were receiving 300 mg of AZT bid. No signal was observed for AZT-TP in the 10 samples from patients not under AZT therapy, nor for d4T-TP in the two d4T-treated patients, showing that, despite similarity in chemical structure, antibodies did not bind d4T-TP. This result was confirmed with d4T-TP-spiked PBMCs.

DISCUSSION

The present paper describes different attempts to develop an analytical method allowing accurate and reliable quantification of PBMC's intracellular content of AZT-TP, the active anabolite of the widely used anti-HIV drug AZT. The ultimate goal should be to develop a direct determination of AZT-TP, rather than an indirect measurement, since this approach should lead to im-

Table 3. AZT-TP Determination in Samples from HIV-infected Patients

patient	time between AZT dosing and blood sampling (h)	other NRTIs	AZT-TP (fmol/10^6 cells)
		Patients on AZT Therapy	
1	12.5	3TC	81.1
2	1.5	ddI	316
3	13.75	3TC	54.1
4	3	ddI	60.7
5		ddI	241
6	11.5	3TC	386
7	2.5	3TC	137
8	2.5	3TC, ABC	97.0
9	2.5	3TC	128
10	2.5	3TC	190
11	0.5	3TC	240
12	2	3TC-ABC	31.3
13	5	ddC	97.9
14	6.25	3TC, ddI	ILD
15	0.75	3TC	72.6
16	12.5	3TC	97.7
17	12	3TC	152
18	12.25	3TC	55.6
19	11.75	3TC	212
20	0.5	3TC	64.5
21	13.5	3TC	105
22	3.25	ddC	376
23	1	3TC	188
		Patients not on AZT therapy	
24		3TC-d4T	ILD
25		d4T	ILD
26			ILD
27		3TC-ABC	ILD
28		3TC	ILD
29		3TC-ddI	ILD
30		ddI-ABC	ILD
31		ddI−Tenofovir	ILD
32		3TC-ddI-ABC	ILD
33			ILD

ILD: inferior to the limit of detection (3.1 fmol/10^6 cells).

proved precision and a decrease in the analysis time, as compared to the multistep indirect measurement.

Since we have previously successfully set up a specific EIA to measure the AZT-MP metabolite,[14] we initially adopted the same strategy with AZT-TP. To overcome the limited AZT-TP stability in biological media, a synthetic compound comprising a methylene bisphosphonate group mimicking the natural pyrophosphate was used to raise polyclonal antibodies in rabbit and to set up a sensitive EIA for AZT-TP. Unfortunately, when applied to PBMC samples, this EIA appears sensitive to matrix interference, failing to preserve the original characteristics determined in buffer and preventing correct quantification of the AZT-TP content. Moreover, the observed cross-reactivity with AZT-MP was leading to an overvaluation of AZT-TP, since AZT-MP accumulates in PBMCs from HIV-infected patients and represents ~95% of all phosphorylated forms.[29]

The alternative strategy involving successively the selective extraction of AZT-TP, an enzymatic dephosphorylation, and a final concentration of the recovered AZT before assaying AZT with a specific sensitive EIA (instead of RIA in a previous published method[27]) proved to be unsuccessful in our hands, unlike in previous works. This was mainly due to the poor yield of the specific extraction of AZT-TP from AZT-DP combined with poor precision, despite the complete automation of the overall sample handling process.

Since we have in parallel developed sensitive LC/MS/MS methods to measure intracellular triphosphate metabolites for other anti-HIV drugs, such as stavudine (d4T), didanosine (ddI), and lamivudine (3TC),[18,19] the EIA approach was abandoned in favor of this detection method. However, despite the common perception that LC/MS/MS comes close to the highest degree of selectivity, it was impossible to separate AZT-TP from two other interfering endogenous nucleotide triphosphates (very likely ATP and dGTP). This is mainly due to the ion pairing chromatography technique that was used, which led to chromatographic separation based on the analyte's charge, thus resulting in poor separation of all triphosphorylated nucleotides. Clearly, a more specific sample preparation was necessary.

Among the series of available efficient extraction processes, that is, solid phase, liquid/liquid or immunoaffinity (IA), IAE was chosen because of its specificity characteristics.

The developed off-line combination of immunoaffinity extraction and LC/MS/MS offers the best selectivity toward interfering nucleotides and also to compounds structurally related to AZT-TP, like d4T-TP. The linearity of the calibration curves shows that the column capacity is sufficient for AZT-TP amounts contained in samples from treated patients. Moreover, as usually performed for assay in biological fluids, calibration standards and quality controls were processed in the same way as patient samples. The overall recovery (extraction and evaporation) reached 50%, since 25% of the AZT-TP loaded on the column was not eluted, and 25% of the eluted AZT-TP was lost during evaporation. This yield could be increased by using monoclonal antibodies, which by presenting a similar restricted specificity and a lower affinity, would not require a drastic buffer, unlike the polyclonal antibodies, to achieve a higher recovery. Internal standard was added after the immunoaffinity process, because no stable-isotope-labeled AZT-TP was available. CV percent of the recovery measured at the three quality control values was low (between 0.8 and 3.6%). CV percent of the nine measurements rose by 10%, since a slight decrease of the recovery was observed at the highest amount of AZT-TP. Despite this, standard curves were linear from 93 to 3360 fmol of AZT-TP. The accuracy measured during intra- and interassay was close to 100%, and precision was in the recommended ranges.[30] Stability of AZT-TP between blood collection and sample treatment was not evaluated during this preliminary validation, but this parameter has been tested for the structurally related d4T-TP.[18] Previously published results indicated that a storage temperature of around −70 °C was necessary for AZT-TP in cell extracts.[31] The method's characteristics were suitable for determination of AZT-TP in PBMCs from HIV-infected and AZT-treated patients using 7 mL of blood. Only one of the 23 samples from the AZT-treated patients was below the limit of detection. Mean [range] AZT-TP concentration for the 22 patients was calculated as 154 [31.3−386] fmol/10^6 cells. These results were in agreement with those previously reported, describing mean AZT-TP concentrations in PBMCs ranging between 5 and 720 fmol/10^6 cells.[29]

(30) Shah, V. P.; Midha, K. K.; Findlay, J. W.; Hill, H. M.; Hulse, J. D.; McGilveray, I. J.; McKay, G.; Miller, K. J.; Patnaik, R. N.; Powell, M. L.; Tonelli, A.; Viswanathan, C. T.; Yacobi, A. *Pharm Res.* **2000**, *17*, 1551−1557.

(31) Thevanayagam, L. N.; Jayewardene, A. L.; Gambertoglio, J. G. *J. Pharm. Biomed. Anal.* **2000**, *22*, 597−603.

The proposed elution scheme allows easy reuse of the same affinity column, since more than 30 PBMC extracts can be cleaned-up with one immunoaffinity column. One operator can perform 150 extractions and LC/MS/MS analysis of clinical samples per week, making this method suitable for routine analysis in the framework of clinical studies. Interestingly, other triphosphorylated NRTIs and their ratios with their natural competitors can be determined in the same blood sample using an aliquot of the PBMC extract directly analyzed with the previously described LC/MS/MS method,[19] which should give interesting information on the metabolism and efficacy of associated NRTIs.

In conclusion, the quantification of the intracellular AZT-TP content of PBMCs appears to pose a difficult challenge. Direct or indirect measurement using EIA failed as a result of different problems encountered, such as a lack of precision and the poor selectivity of AZT-TP extraction, even using an automated device. Some major interference due to natural compounds was also observed after injection of the PBMC extracts without extraction in LC/MS/MS, thus preventing the use of LC/MS/MS analysis previously successfully developed for other triphosphate metabolites of ddI, 3TC, and d4T. This led us to take advantage of the selectivity of the antibody developed for EIA to clean up the samples before using LC/MS/MS. The present IAE-LC/MS/MS method has the required analytical characteristics for routine analysis and could provide more precise and accurate information on the efficiency of AZT phosphorylation in PBMCs from HIV-infected patients, thereby enabling future individual drug monitoring and enhancing understanding of drug efficacy and toxicity.

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