Simultaneous Quantitation of Intracellular Zidovudine and Lamivudine Triphosphates in Human Immunodeficiency Virus-Infected Individuals

JOSE F. RODRIGUEZ,1,2* JORGE L. RODRIGUEZ,1 JORGE SANTANA,3 HERMES GARCÍA,4 AND OSVALDO ROSARIO2

Department of Biochemistry1 and Department of Medicine,3 School of Medicine, Medical Sciences Campus, University of Puerto Rico, and Puerto Rico Health Department (CLETS),3 San Juan, and Department of Chemistry, Rio Piedras Campus, University of Puerto Rico, Rio Piedras,2 Puerto Rico

Received 11 February 2000/Returned for modification 28 May 2000/Accepted 21 August 2000

Highly active antiretroviral therapy (HAART) is the standard treatment for infection with human immunodeficiency virus (HIV). The most common HAART regimen consists of the combination of at least one protease inhibitor (PI) with two nucleoside reverse transcriptase inhibitors (NRTIs). Contrary to PIs, NRTIs require intracellular activation from the parent compound of their triphosphate moiety to suppress HIV replication. Simultaneous intracellular determination of two NRTI triphosphates is difficult to accomplish due to their relatively small concentrations in peripheral blood mononuclear cells (PBMCs), requiring large amounts of blood from HIV-positive patients. Recently, we described a method to determine intracellular zidovudine triphosphate (ZDV-TP) concentrations in HIV-infected patients by using solid-phase extraction and tandem mass spectrometry. The limit of quantitation (LOQ) for ZDV-TP was 0.10 pmol, and the method was successfully used for the determination of ZDV-TP in HIV-positive patients. In this study, we enhanced the aforementioned method by the simultaneous quantitation of ZDV-TP and lamivudine triphosphate (3TC-TP) in PBMCs from HIV-infected patients. The LOQ for 3TC-TP was 4.0 pmol, with an interassay coefficient of variation and an accuracy of 7 and 12%, respectively. This method was successfully applied to the simultaneous in vivo determination of the ZDV-TP and 3TC-TP pharmacokinetic profiles from HIV-infected patients receiving HAART.

Highly active antiretroviral therapy (HAART) has been used successfully for treatment of human immunodeficiency virus (HIV) since the discovery of protease inhibitors (PIs) (3, 4, 20). HAART treatment includes a broad category of anti-retroviral drug combinations with the goals of decreasing plasma HIV-1 RNA levels below the limit of detection, limiting disease progression, and delaying the appearance of resistant mutants (12). The most common HAART regimen consists of the combination of one PI with two nucleoside reverse transcriptase inhibitors (NRTIs). This triple drug combination has shown dramatic improvements in viral suppression over the combination of the two nucleosides zidovudine and lamivudine (ZDV and 3TC, respectively) (8–10).

Contrary to PIs, NRTIs require intracellular activation from the parent compound of their triphosphate (TP) moiety to suppress HIV replication. ZDV and 3TC are not active against HIV; they need to be metabolized to 5'-ZDV-TP (ZDV-TP) and 5'-3TC-TP (3TC-TP) to act as competitive inhibitors of HIV reverse transcriptase or be incorporated into the viral genome (2, 7, 11, 23). Studies conducted with HIV-infected populations have not established any relationship between ZDV or 3TC concentrations in plasma and the efficacy of these agents (19). On the other hand, a recent study showed a linear relationship between ZDV-TP intracellular concentrations and an increase in the percent change in CD4+ cells from baseline in HIV-infected adults (5). Furthermore, several studies have shown that intracellular concentrations of NRTI-TPs correlated better with virologic responses than the parent plasma NRTI levels (J. P. Sommadossi, M. A. Valentini, X. J. Zhou, M. Y. Xie, J. Moore, V. Calvez, M. Desa, and C. Kollama, Program Abstr. 5th Conf. Retroviruses Opportunistic Infect., abstr. 262, p. 146; J. P. Sommadossi, X. J. Zhou, J. Moore, D. V. Havlir, G. Friedland, C. Tierney, L. Smeaton, L. Fox, D. Richmann, and R. Pollard, Program Abstr. 5th Conf. Retroviruses Opportunistic Infect., abstr. 3, p. 79).

Several approaches have been reported for the individual determination of ZDV-TP and 3TC-TP (6, 13, 15–18, 21, 22, 24). A recent approach was developed in which strong anion-exchange–solid-phase extraction separated ZDV anabolites (ZDV-MP, ZDV-DP, and ZDV-TP), followed by enzyme digestion and quantification by radioimmunoassay (18). A similar approach was employed by the same group to determine intracellular levels of 3TC-TP (17). The combination of both methods was used to individually measure ZDV-TP and 3TC-TP concentrations in HIV-infected subjects. Limitations of the aforementioned method include the lack of an internal standard in the quantitation process and the use of parent compounds (ZDV and 3TC) to produce the calibration curve instead of ZDV-TP and 3TC-TP.

Another approach has been proposed to measure intracellular 3TC metabolites by a combination of solid-phase extraction and high-performance liquid chromatography (HPLC) with UV detection (22). The use of UV detection is possible with 3TC metabolites (3TC-MP, 3TC-DP, and 3TC-TP) because of the large amounts (picomoles per 10⁶ cells instead of femtomoles per 10⁶ cells) formed in vivo. However, as well as in the aforementioned methods, no internal standard was used with this methodology. In addition, this method can only be

* Corresponding author. Mailing address: Department of Biochemistry, P.O. Box 365067, School of Medicine, Medical Sciences Campus, University of Puerto Rico, San Juan Puerto Rico 00936-5067. Phone and Fax: (787) 754-4929. E-mail: j_rodriguez@rcmaxp.upr.clu.edu.
used for 3TC, since ZDV does not produce the large amounts of intracellular metabolites made by 3TC.

In this study, we report the simultaneous determination of intracellular ZDV-TP and 3TC-TP concentrations in human peripheral blood mononuclear cells (PBMCs) with azidothymidine (AZdU) as the internal standard. With this methodology, the limits of quantitation (LOQ) for 3TC-TP and ZDV-TP are 4.0 and 0.10 pmol, respectively. This method was successfully used to determine the in vivo pharmacokinetic profile of ZDV-TP and 3TC-TP from HIV-infected patients receiving HAART.

**MATERIALS AND METHODS**

Chemicals. ZDV, AZdU, sodium acetate, and acid phosphatase (type XA) were obtained from Sigma Chemical Co. (St. Louis, Mo.). ZDV-TP, 3TC, and 3TC-TP were purchased from Moravek Biochemicals (Brea, Calif.). Potassium chloride, acetone, triethylamine, and glacial acetic acid (American Chemical Society certified) were obtained from Fisher Scientific (Fairlawn, N.J.). Strong anion-exchange Sep-Pak plus (SAX-QMA) cartridges were purchased from Waters Co. (Milford, Mass.). HPLC-grade water was obtained from Serva (Heidelberg, N.Y.). RPMI 1640, glutamine, nonessential amino acids, penicillin-streptomycin, and fetal calf serum were obtained from BioWhittaker (Baltimore, Md.).

**Preparation of standard solutions.** ZDV-TP and 3TC-TP standard solutions were prepared by serial dilution starting with a stock concentration of 500 μM for ZDV-TP and 1,000 μM for 3TC-TP spiked with PBMCs from HIV-negative individuals.

**Sample collection from HIV-infected patients.** Patients signed an informed consent form approved by the Medical Sciences Campus Institutional Review Board at the University of Puerto Rico. Blood samples (16 ml) were drawn at predose (time zero) and 1, 2, 4, 8, 12, 16, and 24 h postdose from five HIV-infected patients treated with the standard ZDV (300 mg twice a day [BID]) and 3TC therapy (150 mg BID). ZDV-TP and 3TC therapy concentrations were measured at steady state, since patients had been receiving ZDV or 3TC therapy for at least 24 weeks. PBMCs (usually 16 million cells) were separated from erythrocytes by centrifugation at 1,500 × g for 20 min at room temperature. PBMCs were recovered and counted in a Coulter Z2 series system (Hialeah, Fla.), followed by an extraction with 70% methanol, and stored at −80°C until analysis.

**Intracellular ZDV-TP and 3TC-TP isolation.** SAX-QMA was used for the separation of ZDV and AZdU nucleotides as previously described (22). Briefly, the cartridges were preconditioned with 500 μl of deionized water. The cell extract sample was loaded onto the cartridge and eluted under reduced pressure. The parent drugs were eluted with a serial wash of 150 and 500 μl of water. ZDV-MP and 3TC-SP were obtained in the fraction rinsed with 400 μl of 100 mM KCl, and ZDV-DP and 3TC-DP were eluted with 400 μl of 120 mM KCl. The fraction containing ZDV-TP and 3TC-TP was eluted with 500 μl of 400 mM KCl. Recovery experiments were performed comparing SAX-QMA cartridgates with SAX-QMA cartridges. With SAX-QMA cartridgates, we observed that at least 95% of AZdU, ZDV, and 3TC were recovered and counted in a Coulter Z2 series system (Hialeah, Fla.), followed by an extraction with 70% methanol, and stored at −80°C until analysis.

**Chemical and enzymatic hydrolysis.** The eluted sample was acidified with 200 μl of 400 mM KCl. The solution was processed throughout the complete procedure, and AZdU was used as the internal standard. Retention times for ZDV, 3TC, and AZdU were 3.08, 3.41, and 2.72 min, respectively. Chromatographic conditions are described in Materials and Methods. The y axis is the ion intensity obtained for each compound.

**RESULTS AND DISCUSSION**

**Ion chromatograms for ZDV-TP and 3TC-TP.** Figure 1 shows the ion chromatograms obtained from an HIV-infected patient treated with ZDV or 3TC. The upper panel shows the ion chromatogram for ZDV (after removal of the phosphate groups from ZDV-TP) with a retention time of 3.08 min. The middle panel shows the ion chromatogram of 3TC (after removal of the phosphate groups from 3TC-TP) with a retention time of 3.41 min, which provides a higher signal than ZDV. This result is not unexpected, since in vitro and in vivo studies have demonstrated that the intracellular production of 3TC-TP is higher than that of ZDV-TP (1, 2, 17, 22). In the lower panel, the chromatogram of AZdU (internal standard) shows a strong signal with a retention time of 2.74 min. We determined that AZdU did not interfere with the signal of ZDV or 3TC, despite the proximity in their retention times (data not shown). Similarly, 3TC did not interfere with the signal of ZDV or AZdU. In addition, endogenous nucleotides or other compounds from cell samples do not interfere with the quantitation process of ZDV-TP or 3TC-TP. The short retention times for the three compounds increase the throughput of samples (20 samples/h), providing the opportunity to quantify ZDV-TP or 3TC-TP concentrations in clinical trials.

**Standard curve and statistics.** Figure 2 shows a typical calibration curve constructed from the ratio of the areas from 3TC and AZdU chromatograms plotted against 3TC-TP concentration. This calibration curve was obtained by using different 3TC-TP standard concentrations spiked with PBMCs and passed through the entire methodology (SAX-QMA, enzyme cleavage of phosphate groups was accomplished by the addition of 2 U of acid phosphatase per ml for 30 min at 37°C, adjusting the fraction to pH 4.0 with sodium acetate. After enzyme digestion, AZdU (internal standard, 100 ng/ml) was added to the extract and recovered simultaneously with ZDV and 3TC by using an XAD column. The XAD column was preconditioned with water before sample loading. The sample was desalted with 5 ml of water. ZDV, 3TC, and AZdU were eluted with 2.0 ml of methanol. Desalting and recovery of ZDV, 3TC, and AZdU by using XAD columns were >98%. Samples were dried in a Labconco CentriVap console (Kansas City, Miss.) and reconstituted with 100 μl of mobile phase, prior to HPLC analysis.

**Data analysis.** Concentrations of analytes were determined by using ZDV/AZdU and 3TC/AZdU peak area ratios. Calibration curves from ZDV-TP and 3TC-TP standard solutions were prepared every time a series of samples were analyzed. Linear regression analyses were performed with five ZDV-TP and 3TC-TP standard concentrations. Regression coefficients (r) were better than 0.998 for all calibration curves.
digestion, and XAD column). The LOQ obtained with this methodology is 4.0 pmol (coefficient of variation [CV], <8%, n = 5; accuracy, <15%, n = 5). The regression coefficients for similar calibration curves were better than 0.998. We have previously reported calibration curves for ZDV-TP with regression coefficients better than 0.999 and an LOQ of 0.10 pmol (6). The quantitation process for ZDV-TP was not affected by the simultaneous quantitation of 3TC-TP, as shown in Table 1.

In the validation process for ZDV-TP, we pointed out the necessity of the internal standard to obtain precise and accurate results (6). For 3TC-TP, we found a similar situation. With AZdU (internal standard), we improved the 3TC-TP regression coefficient for the calibration curve from 0.937 to 0.998. Furthermore, the intra-assay variability improved with AZdU for a 3TC-TP concentration of 43.0 pmol from a CV of 6% to 1%. The accuracy (percent error) of the method with AZdU improved from 42% to 3% for 4.0 pmol (n = 5) and from 37% to 8% for 43.0 pmol (n = 5). Thus, the use of AZdU as the internal standard improved dramatically the accuracy of the assay and provided a precise method to measure low concentrations of 3TC-TP.

Table 1 shows the results for the interassay variability (10.0, 50.0, and 150.0 pmol) used in the validation process. These results confirm the excellent accuracy and precision of this new methodology. For all concentrations studied, recoveries were ≥93% and the CV was <10%. Similar results were obtained for the ZDV-TP quality control standards (0.10, 0.50, and 5.00 pmol). Thus, the methodology is suitable to measure intracellular 3TC-TP and ZDV-TP from HIV-infected patients treated with ZDV or 3TC.

Patient samples. Figure 3 shows the concentration-time curves for 3TC-TP and ZDV-TP from five HIV-infected patients treated with ZDV or 3TC. Patients were taking the standard dose for these antiretroviral agents: ZDV, 300 mg BID; and 3TC, 150 mg BID. For the pharmacokinetic study, measurements were performed at steady state, and patients did not take the dose at 12 h in order to obtain the complete profile for 24 h. Despite the large variability in intracellular 3TC-TP and ZDV-TP concentrations between patients, differences between the pharmacokinetic profiles of the two metabolites are evident. Intracellular 3TC-TP concentrations are 20-fold higher than those of ZDV-TP, due to the greater efficiency of the 3TC phosphorylation process (2). The elimination process appears to be different for both nucleotides, since the estimated median 3TC-TP half-life for these five patients is approximately 32 h (range, 23 to 49 h), while for ZDV-TP, it is approximately 11 h (range, 5 to 13 h). In all five patients, a robust signal was observed for 3TC-TP at 24 h, while only one out of five had measurable concentrations of ZDV-TP. In a recent study, Moore et al. measured the intracellular concentration of 3TC-TP in 10 HIV-infected patients (14). The median intracellular half-life for 3TC-TP was reported to be 15 h (range, 6 to 32 h). The discrepancy between both studies can be attributed to the large variability in the measurements made by Moore et al. and to the small number of patients used in both studies. They used HPLC to separate their metabolites, and quantitation was performed by radioimmunoassay. They did not use an internal standard in their methodology, which may account for their large variability (CV, >50%). The lack of an internal standard also diminishes the certainty of the measure-

![Figure 2: Calibration standard curve for 3TC-TP constructed from standard solutions (4, 21, 43, 85, and 128 pmol) and passed through the complete methodology. The data plotted represent the average of two determinations for each concentration. Error bars reside within the points if not shown. The equation describing the complete range is y = 0.121x – 0.018, with a regression coefficient (r) of 0.998.](http://aac.asm.org/)

![Figure 3: Intracellular pharmacokinetic profile for 3TC-TP (top) and ZDV-TP (bottom) from five HIV-infected patients. Patients stayed overnight at the University of Puerto Rico-Clinical Research Center facilities, and samples were collected at predose (time zero) and 1, 2, 4, 8, 12, 16, and 24 h postdose. The line represents the median value for each time point.](http://aac.asm.org/)
ments, since the method cannot account for losses during the experimental procedure for each sample. This could explain the higher 3TC-TP intracellular concentrations observed in our study.

The values observed for 3TC-TP and ZDV-TP are similar to those reported previously by other methodologies. Interestingly, the ZDV-TP concentrations between 2 and 4 h are similar to those obtained by Fletcher et al. for patients that had an increase in the percent change of CD4+ cells from baseline (5). 3TC-TP concentrations are in the same range observed by other laboratories (17, 24). To the best of our knowledge, this is the first time that intensive intracellular pharmacokinetic profiles have been obtained for ZDV-TP and 3TC-TP, providing the opportunity to understand better the in vivo intracellular interactions between these antiretroviral agents. These interactions will provide information that could be essential for the clinical management of HIV-infected patients. We now have the capacity to establish a correlation between intracellular pharmacological parameters and drug efficacy or toxicity. These studies are currently in progress at our institution.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grants 2U01AI32906, 1P20RR11126, G12-RR03051, AI34858, and R01AI39191 (J.F.R.).

We acknowledge the technical assistance of Marianela Pérez and Raul Blanco.

REFERENCES


