

Enzymatic Assay for Measurement of Zidovudine Triphosphate in Peripheral Blood Mononuclear Cells

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In this report, we describe a new method to measure intracellular zidovudine triphosphate (ZDV-TP) levels in peripheral blood mononuclear cells (PBMCs) from patients treated with ZDV by utilizing inhibition of human immunodeficiency virus type 1 reverse transcriptase activity by ZDV-TP. Intracellular levels of ZDV-TP were determined with our enzymatic assay in PBMCs isolated from the blood of healthy individuals incubated with different concentrations of labeled ZDV and were validated by high-performance liquid chromatography separation and liquid scintillation counting of the radioactive ZDV-TP. These methods gave virtually identical results over a range of ZDV-TP concentrations from 150 to 900 fmol. ZDV-TP recoveries were over 90%, and the limit of quantitation of ZDV-TP by this method was 20 to 50 fmol. To demonstrate the utility of the method, plasma ZDV and intracellular ZDV-TP concentrations were measured at serial time points over 6 h in 12 human immunodeficiency virus-infected volunteers following a single 100- or 500-mg oral dose of ZDV. Systemic oral clearance rates were similar to those in previous studies with adults but were highly variable (range, 0.86 to 2.75 liters/h/kg of body weight). The area under the plasma concentration versus time curve increased significantly ($P < 0.0005$) with the dose from a median value of 1.2 mg · h/liter at the lower dose to 4.2 mg · h/liter at the higher dose. Median intracellular ZDV-TP levels ranged from 5 to 57 and 42 to 92 fmol/10⁶ cells in volunteers administered 100 and 500 mg of ZDV, respectively. Intracellular ZDV-TP levels rose to a plateau value by 2 h and remained consistent to 6 h. Although the higher dose and higher areas under the curve yielded consistently higher intracellular ZDV-TP levels, systemic pharmacokinetics explains only a modest proportion of the variability in cellular pharmacokinetics. The ZDV-TP bioassay should prove useful in further studies of ZDV metabolism in patient-derived PBMCs at the doses of ZDV currently administered.

Zidovudine (3'-azido-3'-deoxythymidine [ZDV]) is one of three nucleoside analogs approved for the treatment of AIDS and AIDS-related complex (3, 5, 6, 10, 11, 13, 16). Investigation of the mode of action of ZDV has shown that the drug is phosphorylated to its 5'-monophosphate (MP), diphosphate, and triphosphate (TP) derivatives by cellular kinases. ZDV-5'-TP (ZDV-TP hereafter) is a potent inhibitor of human immunodeficiency virus reverse transcriptase (HIV-RT) and thus of HIV replication (8, 12). Much of what is presently known about the intracellular metabolism of ZDV has been elucidated with radiolabeled drugs in cultured human lymphoid cells. For example, marked differences in the activation and accumulation of ZDV nucleotides have been noted among different cells upon incubation with ZDV which correlate with differences in the in vitro effectiveness of the drug (1, 2).

Because in vitro results from human cell lines in culture cannot necessarily be extrapolated to the in vivo situation and because differences in drug disposition and clinical effects exist in patients, it is of interest to measure the intracellular level of ZDV-TP, the major active metabolite of the drug. More importantly, the evaluation of the intracellular metabolism and pharmacology of the proximate inhibitor of ZDV would lead to a better understanding of the pharmacological properties of ZDV in vivo than can be obtained

from measurement of the plasma pharmacokinetics of ZDV alone. Two methods have recently been described for quantitative determination of intracellular ZDV metabolites in HIV-infected patients. One method involves multidimensional high-performance liquid chromatography (HPLC), but its application to measuring cellular ZDV nucleotides in patients undergoing therapy has not been evaluated (15). The other method is an indirect assay which utilizes HPLC and radioimmunoassay (RIA). However, this method is quite cumbersome and requires prior purification of ZDV metabolites from cell extracts, treatment of extracts with phosphatases, and quantitation of the resultant ZDV with an RIA (9, 14).

Herein, we describe a bioassay based on the inhibition of reverse transcriptase (RT) activity to measure intracellular ZDV-TP in cell extracts. Two types of cells were analyzed: CEM human T-lymphoid cells and human peripheral blood mononuclear cells (PBMCs) both in vitro and in vivo (after the administration of ZDV to patients). Significant differences in the ability of CEM cells and PBMCs to anabolize ZDV could be detected. The reproducibility and reliability of this method have also been examined and compared with those of previously studied procedures.

MATERIALS AND METHODS

Materials. [³H]ZDV (~20 μCi/mmol), [³H]TTP, and ZDV-TP were purchased from Moravек Biochemical, Brea, Calif. Recombinant HIV-RT was purchased from American BioTechnologies Inc., Cambridge, Mass. ZDV and ZDV-MP

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were a gift from David Johns at the National Cancer Institute, Washington, D.C. Human recombinant interleukin 2 was obtained from Boehringer Mannheim, Indianapolis, Ind., and lymphocyte separation medium (Ficoll-Hypaque) was purchased from the Organon Teknika Corp., Durham, N.C. The Sephadex G-50 (fine), poly(rA), and oligo(dT)₁₂₋₁₈ were obtained from Pharmacia-LKB Biotechnology, Piscataway, N.J. The RIA kits for ZDV analysis were purchased from Incstar, Stillwater, Minn. Phytohemagglutinin (PHA-P) was purchased from Sigma Chemical Co., St. Louis, Mo. Other fine chemicals were purchased from Sigma Chemical Co.; Fisher Scientific, Fair Lawn, N.J.; or Calbiochem, La Jolla, Calif. Tissue culture media RPMI-1640, Hank's balanced salt solution, glutamine, and fetal calf serum were purchased from BioWhittaker, Baltimore, Md.

Cells. CCRF/CEM cells were obtained from the American Type Culture Collection (Rockville, Md.) and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated newborn calf serum. PBMCs were isolated as described below.

PBMC isolation. Heparinized blood from healthy human volunteers or asymptomatic HIV-seropositive individuals was diluted 1:1 with Hank's balanced salt solution, layered onto lymphocyte separation medium (Ficoll-Hypaque), and separated by centrifugation for 30 min at 400 × *g*. The mononuclear cell layer was removed, washed once with growth medium, counted, and pelleted by centrifugation. The cells were then resuspended in growth medium (RPMI containing 10% fetal calf serum), and the residual erythrocytes were lysed by the addition of 3 volumes of cold water for 30 s. Erythrocyte lysis was stopped by the addition of 1 volume of 0.6 M sodium chloride, and the mononuclear cells were pelleted by centrifugation.

Culture of human PBMCs. The PBMCs were suspended in growth media and transferred to plastic tissue culture flasks. The monocyte and macrophage cells were allowed to adhere to the flask for at least 60 min, after which the nonadherent cells (primarily lymphocytes) were transferred to a new flask. The cells were stimulated by addition of 5 μg of PHA-P per ml and 10 U of interleukin 2 per ml and were incubated for 72 h at 37°C prior to use. Fresh medium was added each 24 h to maintain the interleukin 2 concentration. If quiescent cells were required, the PHA-P and interleukin 2 were omitted and the cells were used the following day.

Enumeration of cell numbers. All cell numbers were determined with a Coulter Counter. When cell clumping prevented normal determination of cell numbers, especially in stimulated PBMC cultures, cell counting was accomplished by counting nuclei as described by Butler (4).

Preparation of cell extracts. Extracts suitable for the analysis of intracellular ZDV metabolites were prepared either from cells (CEM and quiescent or activated PBMCs) incubated unlabelled or with [³H]ZDV or from fresh PBMCs from ZDV-treated HIV-seropositive individuals. Cultured cells incubated with [³H]ZDV were first harvested by centrifugation at 1,000 × *g* for 5 min. The cell pellets were resuspended in 1 ml of residual medium, layered onto 150 μl of Nyosil oil, and spun through the oil. The medium above the oil layer was removed, and any residual medium was removed by rinsing the area above the oil layer with two gentle water washes. Finally, the oil was removed and the cells were extracted with 70% methanol–15 mM Tris-HCl buffer (pH 7.4) for at least 15 min on ice. The debris was removed by centrifugation, and the samples were stored at –20°C until required.

Uncultured PBMCs from ZDV-treated patients were re-

suspended in 1 ml of medium and transferred to a 1.5-ml centrifuge tube and spun for 1 min at 16,000 × *g*. The medium was then removed and pulsed again to allow the removal of any residual medium. The cells were extracted for at least 15 min with 200 μl of ice-cold 70% methanol per 10⁷ cells. The cell debris was removed by centrifugation for 15 min at 16,000 × *g*, and the supernatant was stored at –20°C until required for analysis. Prior to use, the extracts were dried under a vacuum and resuspended in 50 μl of 5 mM Tris-HCl buffer (pH 8.5); extracts from 0.5 × 10⁷ CEM cells or 10⁷ cultured PBMCs were reconstituted in 200 μl of buffer.

HPLC separation of ZDV metabolites. Radioactive cell extracts were analyzed for ZDV metabolite concentration by separating the radioactive ZDV metabolites on a 250-mm-diameter Whatman Partisil-10 SAX anion-exchange column and collecting the fractions for scintillation counting. Buffers used in the separation procedure were 0.005 M ammonium phosphate (pH 4.0)–10% methanol (buffer A) and 0.7 M ammonium phosphate (pH 4.6)–10% methanol (buffer B). The gradients used for the separation of ZDV metabolites were 20 min with 0% buffer B, a 16-min concave gradient with from 0 to 100% buffer B, and finally an 8-min gradient with 100% buffer B. A flow rate of 1.5 ml/min was used, and fractions were collected every 40 s and analyzed by liquid scintillation counting.

RT assay. RT assays were performed with purified HIV-1-RT and poly(rA)-oligo(dT)₁₂₋₁₈ as primer template. The reactions were carried out in a total volume of 10 μl; a 4.5-μl volume of the sample was mixed with an equal volume of a reaction mixture to provide a final concentration of 50 mM Tris (pH 8.5), 6 mM MgCl₂, 10 mM dithiothreitol, 2.0 μg of bovine serum albumin, 0.5 μCi of TTP, 80 mM KCl, and 0.05% Triton X-100. The reaction was initiated by the addition of 1.0 μl of HIV-RT (containing ~1 ng of protein [American BioTechnologies Inc.; catalog no. 26001]). After 1 h of incubation at 37°C, the reaction was stopped by the addition of 5.0 μl of stop buffer (50 mM EDTA, 3% sodium dodecyl sulfate). The RT products were separated from the unincorporated label by chromatography on Sephadex G-25 (superfine) columns. The fractions corresponding to the void volume were collected and counted by scintillation counting. The inhibition of HIV-RT by ZDV-TP was determined with known amounts of ZDV-TP, both in buffer and in the presence of cell extracts from normal PBMCs, to obtain standard curves which were used to estimate the amounts of ZDV-TP in assay samples.

HPLC-RIA determination of ZDV-TP. CEM cells were extracted in the same manner as cultured PBMC samples. The ZDV metabolites were separated by anion-exchange HPLC with the same buffers as described previously but with a gradient of 10 min with 0% buffer B, a 26-min concave gradient, and a gradient of 10 min with 100% buffer B to further separate the diphosphates and TPs from the ZDV-MP. The fractions containing ZDV-TP were pooled, vacuum desiccated to ~2 ml, and treated with acid phosphatase at 37°C overnight to remove the phosphate groups. The samples were cleaned up with Waters Sep-Pak C-18 cartridges. The cartridges were conditioned with 10% methanol and then rinsed with water. The sample was applied and eluted with 50% methanol. The samples were vacuum desiccated to a volume of less than 500 μl and were further purified with a 250-mm-diameter Whatman ODS-3 column. Buffer A was 35% methanol, and buffer B was 50% methanol. The gradient was 15 min with 0% B followed by 20 min with 100% B, with a flow rate of 0.5 ml/min and fractions were collected every

min. The fractions containing ZDV were pooled and vacuum desiccated to dryness. The samples were resuspended in the sample dilution solution provided in the kit and were analyzed for ZDV as described previously with modifications described by Slusher et al. (14). Briefly, the antibody was diluted 1:3 and preincubated with the sample for 2 h. A 1:6 dilution of ^{125}I -ZDV was added and incubated at room temperature for another 2 h, and finally the volume of antibody-precipitating complex was reduced to 300 μl .

RIA for plasma ZDV. Plasma samples were heat inactivated at 56°C for 4 h prior to assaying. The samples were diluted to obtain values within a standard curve range of 10 to 200 ng/ml with 200 μl of sample. One hundred microliters of ^{125}I -ZDV and 100 μl of antibody directed against ZDV were added to the 200- μl sample, vortexed, and incubated at room temperature for 2 h. Five hundred microliters of a secondary antibody-precipitating complex was added and incubated for 20 min at room temperature. The resultant mixtures were centrifuged, the supernatant was decanted, and the precipitate was counted with an LKB gamma counter connected to an IBM PC computer with an RIA CALC software program.

Pharmacokinetic studies in patients. The clinical study was approved by the Institutional Review Board of St. Jude Children's Research Hospital, and informed consent was obtained from each subject according to institutional procedures. Subjects in this study were HIV-infected individuals recruited from an area clinic. All patients had received no prior ZDV therapy and were in good health. After informed consent, a single dose of 100 or 500 mg of ZDV was administered orally. Samples of peripheral blood (20 ml) were obtained at baseline and at 1, 2, 4, and 6 h after the dose of ZDV. CD4 and CD8 cell counts were determined from baseline samples. Plasma ZDV concentrations were determined by RIA, and the intracellular ZDV-TP concentration was determined after ZDV administration.

Pharmacokinetic parameters for a one-compartment model (oral clearance $[\text{CL}_o]$, half-life $[t_{1/2}]$, apparent volume of distribution, and absorption rate) were estimated from plasma ZDV concentrations for each subject with Bayesian estimation as implemented in the ADAPTI modeling software (Biomedical Simulation Resource, University of Southern California, Los Angeles). Prior distributions for the parameters were assumed to be log-normally distributed with known but independent variances (i.e., no parameter covariance) and were taken from previous studies in similar patients (7). For absorption, both zero and first-order inputs were examined and similar results were obtained for the remaining parameters (CL_o , apparent volume of distribution) with no difference in the criterion values for convergence. First-order absorption was selected as being most consistent with previous pharmacokinetic reports.

RESULTS

Standard curve. Figure 1 shows typical inhibition kinetics by ZDV-TP of HIV-RT-catalyzed incorporation of TTP into products with poly(rA)-oligo(dT)₁₂₋₁₈ as template primer. These results (expressed as the reciprocal of the incorporation of radioactivity) show linear inhibition kinetics (coefficient of regression, 0.99) over concentrations of ZDV-TP ranging from 10 to 500 fmol. One potential problem with using an enzymatic assay of this type for measuring ZDV-TP in biological material is the dilution of ^{3}H TTP by endogenous deoxynucleotides, particularly TTP. Figure 1 shows the effect of spiking the standard curve with extract of

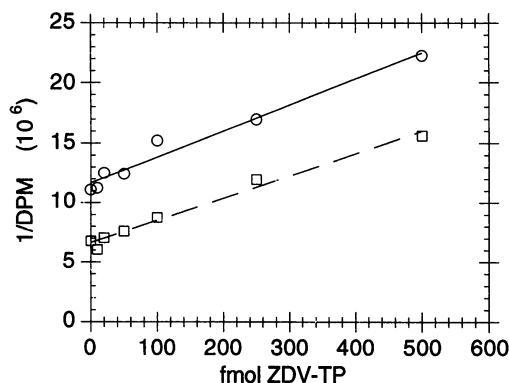


FIG. 1. The effect of ZDV-TP and PBMC extract addition on HIV-RT activity. RT assay mixtures were incubated at 37°C with 0, 10, 20, 50, 100, 250, and 500 fmol of ZDV-TP in the absence (□) or presence (○) of 4.5 μl of untreated PBMC extract.

PBMCs from about 10^6 cells (which was the amount routinely assayed in patient samples). There was some decrease of the radioactivity incorporated from ^{3}H TTP (as indicated by an increase in the y-intercept), but the standard curve still gave good linear kinetics over the concentration of ZDV-TP being evaluated ($r > 0.98$, where r is the coefficient of regression). Other metabolites, including ZDV-MP and TMP, were also tested for inhibition of RT activity, but they did not affect the reaction kinetics at concentrations of up to 10,000 fmol (data not shown).

ZDV-TP levels in CEM cell extracts. The next step was to determine whether it was possible to quantitate the level of ZDV-TP in extracts from cells exposed to ZDV with this RT assay. T-lymphoid CEM cells were incubated with 1 μM ^{3}H ZDV for 4 h and split into three fractions. One fraction was analyzed by HPLC, and ZDV metabolites were quantitated by the usual radioisotopic methods. The other two cell fractions were used to quantitate ZDV-TP by the RIA and RT assay. Cells were extracted with 70% methanol, dried under a vacuum, and resuspended in 5 mM Tris (for the RT assay) or the diluent solution provided with the RIA kit. A ZDV-free extract was used to construct the standard curve for the RT assay. As shown in Table 1, the ZDV-TP levels in CEM cells determined after incubation with 1 μM ZDV from the three methods were in good agreement.

ZDV-TP levels in PBMCs in vitro. In order to assess the usefulness of the RT assay for measuring intracellular concentrations in PBMCs, ZDV-TP concentrations were determined in quiescent or PHA-P-stimulated PBMCs incubated with 1 μM ^{3}H ZDV. Proliferating PBMCs had a high rate of phosphorylation of ZDV compared with that of resting

TABLE 1. Comparison of ZDV-TP levels in CEM cells by various analytical methods^a

Method of determination	ZDV-TP level (pmol/ 10^6 cells)	n
HPLC radioisotopic	1.42	1
HPLC-RIA	1.50 \pm 0.65	3
RT bioassay	1.39 \pm 0.17	3

^a Intracellular ZDV-TP concentrations in CEM were determined cells after a 4-h incubation with 1 μM ZDV in the presence of 1 μCi of ^{3}H ZDV per ml. Cell extracts were analyzed and quantified by using the different assays as described in Materials and Methods. Statistical parameters represent the standard deviation of the mean of three determinations of parallel samples.

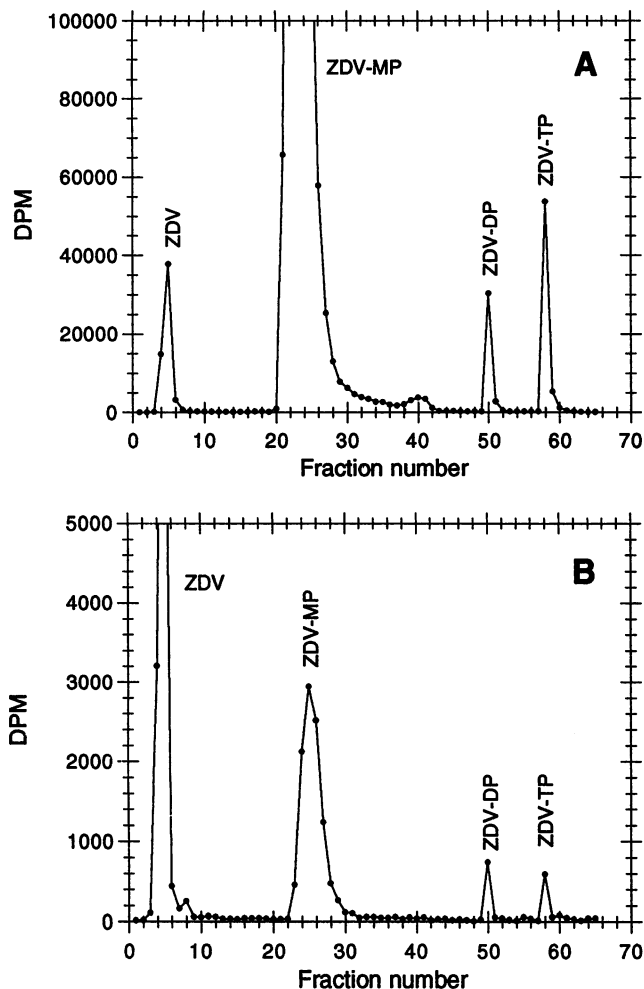


FIG. 2. HPLC separation of ZDV metabolites in PHA-P-stimulated and resting PBMCs. ZDV metabolites from stimulated (A) or resting (B) PBMCs incubated with $1 \mu\text{M}$ [^3H]ZDV were separated by anion-exchange HPLC, and metabolite concentrations were determined by scintillation counting of the radioactive fractions. DP, diphosphate.

PBMCs (Fig. 2). To determine the stability of ZDV during isolation of PBMCs by Ficoll-Hypaque, PHA-P-stimulated cells were incubated with $1 \mu\text{M}$ [^3H]ZDV for 4 h and divided into two fractions. One fraction was extracted with 70% methanol, and the second was layered on Ficoll-Hypaque and purified as whole blood. Recovery of [^3H]ZDV metabolites after the separation procedure was greater than 90%, indicating that ZDV metabolites are stable during Ficoll-Hypaque isolation.

Correlation of the RT assay for detection of ZDV-TP with the radioisotopic method. PHA-P-stimulated PBMCs were incubated with a range of labeled ZDV concentrations for 4 h, and samples of these extracts were diluted to provide a wide range of concentrations. These samples were divided into two fractions; one fraction was analyzed by the HPLC-radioisotopic method, and the other was analyzed by the RT assay. A ZDV-free extract served as the control to eliminate cell background for the bioassay. Figure 3 shows a scatter plot of radioisotopic measurements versus concurrent measurement of ZDV-TP by the RT assay. The data distribution around the line of unity indicated good agreement between

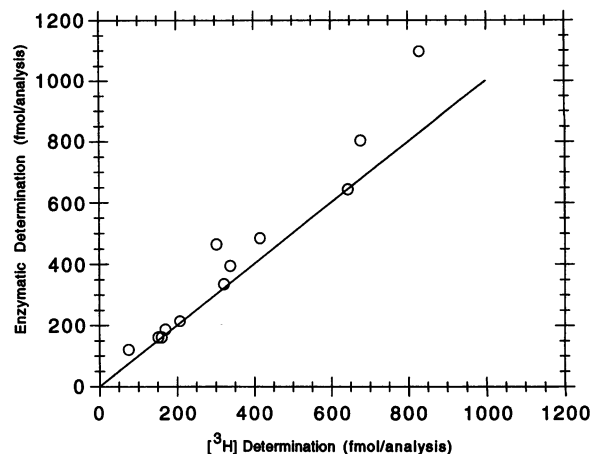


FIG. 3. Correlation between ZDV-TP levels determined with the RT assay and measured radioisotopically. PBMCs were incubated with labeled ZDV at final concentrations of 0.5, 5, 10, and $50 \mu\text{M}$. These samples were diluted to provide a wide range of ZDV-TP concentrations. The concentrations of ZDV-TP were determined by both enzymatic and isotopic methods. The results of the two determinations were plotted with the line representing unity.

the two methods, with a slight bias towards higher estimates with larger concentrations of ZDV-TP.

Intracellular ZDV-TP and plasma ZDV levels in HIV-infected volunteers. ZDV-TP in PBMCs and plasma ZDV concentrations were measured in 12 HIV-infected adult volunteers receiving ZDV at St. Jude Children's Research Hospital. All 12 volunteers studied were administered a single 100- or 500-mg oral dose of ZDV. Plasma ZDV concentrations and intracellular ZDV-TP levels were determined at 1, 2, 4, and 6 h after administration of the drug. The ZDV-free PBMCs from each individual at baseline served as the cell background to construct the standard curve for determination of ZDV-TP levels. ZDV-TP could be measured in all 12 volunteers after ZDV administration.

ZDV was determined in the plasma of HIV-infected volunteers by RIA to ascertain the relationship between ZDV

TABLE 2. Summary of ZDV concentrations in plasma in patients administered 500 or 100 mg of ZDV^a

Time (h)	Concn of ZDV in plasma in patients given:					
	100 mg of ZDV			500 mg of ZDV		
	Median (μM)	Range (μM)	%CV	Median (μM)	Range (μM)	%CV
1	1.79	1.07-2.7	37	6.3	5.7-14.0	50
2	0.76	0.48-1.1	34	3.0	0.8-3.54	38
4	0.26	0.16-0.9	77	0.9	0.21-1.08	39
6	ND ^b			0.36	0.36-0.45	18

^a Results are from five patients administered 100 mg of ZDV and six patients administered 500 mg of ZDV and monitored for the indicated time periods. %CV = (standard deviation/mean) \times 100. At the 100-mg dose, the values for $t_{1/2}$, CL_{50} , and AUC were as follows: $t_{1/2}$, median, 1.29 h; range, 0.8 to 1.6 h; %CV, 26; CL_{50} , median, 1.28 liters/h/kg of body weight; range, 0.86 to 2.1 liters/h/kg; %CV, 36; and AUC, median, 1.2 mg \cdot h/liter; range, 0.8 to 1.8 mg \cdot h/liter; %CV, 31. At the 500-mg dose, the values for $t_{1/2}$, CL_{50} , and AUC were as follows: $t_{1/2}$, median, 1.07 h; range, 0.7 to 1.2 h; %CV, 21; CL_{50} , median, 1.56 liters/h/kg; range, 1.2 to 2.75 liters/h/kg; %CV, 35; and AUC, median, 4.2 mg \cdot h/liter; range, 2.1 to 6.2 mg \cdot h/liter; %CV, 33.

^b ND, concentrations of ZDV in plasma below the standard curve in four of five patients.

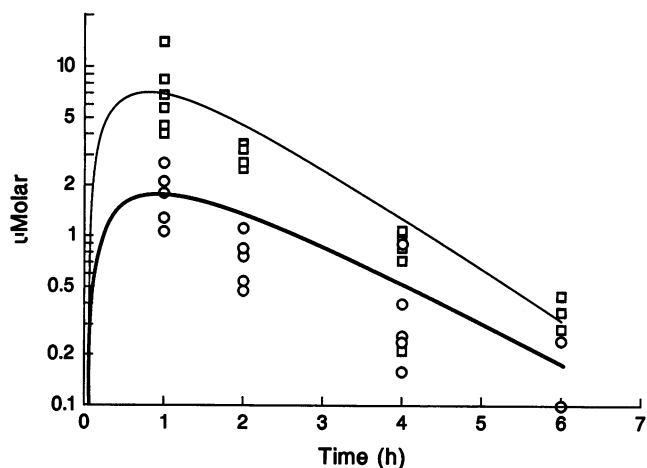


FIG. 4. Plasma ZDV concentrations in HIV-infected individuals. Volunteers were administered a single 100-mg (○) or 500-mg (□) dose of ZDV. Symbols represent data for each subject, and the solid line is a simulation of medium parameters determined for each of the two dose levels.

concentrations and intracellular ZDV-TP. Table 2 shows that median plasma ZDV concentrations at 1 h were 1.79 μM for the 100-mg dose and 6.3 μM for the 500-mg dose. There was substantial variability in plasma ZDV concentrations at any single time point, even for patients receiving the same dose, with coefficients of variation ranging from 18 to 77%. Figure 4 shows the plasma ZDV concentrations from volunteers given 100 or 500 mg of ZDV and the predicted concentrations from median parameters estimated for each group. The median CL_0 (1.28 liters/h/kg of body weight) in the 100-mg group of subjects was approximately 80% of the median CL_0 (1.56 liters/h/kg of body weight) in the subjects given 500 mg, but the two groups were not significantly different ($P > 0.2$ by the Mann-Whitney U test). This substantially lower CL_0 in the low-dose group resulted in systemic exposures between the two groups, as reflected by the area under the curve (AUC), that differed by a ratio of approximately 4 rather than 5, as would be predicted only from the dose ratio.

Intracellular ZDV-TP levels ranged from 3 to 326 fmol/ 10^6 cells, with coefficients of variation ranging from 52 to 157%, indicating a higher variation in ZDV-TP levels than in plasma ZDV concentrations (Table 3). In parallel incubations, the extracts of ZDV-treated patients were spiked with a known concentration of ZDV-TP, as shown in Table 4. The measured concentrations of spiked samples by the RT assay had an average variation of about 20% from the expected

TABLE 4. Variations from expected concentrations of samples spiked with ZDV-TP^a

Time (h)	% of variation from expected concn in samples spiked with:			
	100 mg of ZDV		500 mg of ZDV	
	Mean	Range	Mean	Range
1	33.3	12.8–58.3	16.6	11.1–21.0
2	15.2	0.5–36.3	17.5	7.6–28.0
4	21.6	2.5–33.6	14.2	3.2–28.6
6	11.1	3.0–19.9	21.7	9.4–30.3

^a Aliquots of patient extract were spiked with 0.1 pmol of ZDV-TP, and the concentration was determined with the enzymatic assay. The data are from five patients administered 100 mg of ZDV or four patients administered 500 mg of ZDV.

ZDV-TP levels. ZDV-TP values increased from 1 to 2 h (Table 3) and then remained at a plateau value through 6 h that was consistent within patients but quite variable between subjects. The median plateau value (average of the 2-, 4-, and 6-h measurements) at the 500-mg dose was 68 fmol/ 10^6 cells, with 41 fmol/ 10^6 cells for the 100-mg dose. This proportion of 1.7 is in contrast to the systemic AUC ratio of 4 noted above.

DISCUSSION

Quantitation of ZDV metabolites to date has been performed mainly with in vitro cell systems using radiolabeled ZDV (8, 12). These studies on the mechanism of action of ZDV have shown that ZDV is phosphorylated to its MP, diphosphate, and TP forms via thymidine kinase and other cellular kinases. ZDV-TP, as the active form of the drug, is directly responsible for inhibition of RT, which ultimately results in inhibition of viral replication by ZDV (10). However, determination of ZDV-TP levels in human clinical studies has proven difficult because the use of radiolabeled ZDV is not feasible in patients. In the present study, we describe the application of the RT assay to quantify unlabeled intracellular ZDV-TP. On the basis of a close correlation between the cellular levels of ZDV-TP obtained by the bioassay and by the HPLC-radioisotopic assay and RIA, the precision and specificity of the bioassay seem sufficiently accurate to quantify the ZDV-TP levels in extracts of PBMCs from patients treated with therapeutic doses of ZDV. A drawback associated with the bioassay when cell extracts are used is the inability to differentiate ZDV-TP from interfering endogenous nucleotides. However, as described in this paper, we addressed this problem by including in each determination the patient's own ZDV-free peripheral blood

TABLE 3. ZDV-TP concentration in PBMCs of patients administered 100 or 500 mg of ZDV^a

Time (h)	Concn of ZDV-TP in PBMCs of patients given:						
	100 mg of ZDV			500 mg of ZDV			Median ratio (500/100)
	Median (fmol/ 10^6 cells)	Range (fmol/ 10^6 cells)	%CV	Median (fmol/ 10^6 cells)	Range (fmol/ 10^6 cells)	%CV	
1	5	1–49	157	42	28–98	52	8.4
2	57	11–207	95	92	1–182	80	1.6
4	41	11–89	62	63	19–76	35	1.5
6	37	24–326	118	78	1–207	79	2.1

^a Results were from six patients given the indicated doses of ZDV. %CV = (standard deviation/mean) \times 100.

lymphocyte extract to construct the standard curve. In addition, the accuracy of ZDV-TP levels was determined by obtaining the results of the RT assay spiked with a known concentration of ZDV-TP. The average variation from expected values (20%) was less than sample variation (30%) seen with RIA measurements by Slusher et al. (14).

The results of our enzymatic assay for ZDV-TP levels in PBMC extracts were compared with results obtained from the HPLC-RIA (9). Although different procedures were used, the data from the two methods were in good agreement. Moreover, our results with the bioassay of the patient samples are similar to those reported recently by Slusher et al. (14). Slusher et al., using the HPLC-RIA, reported levels of 50 to 140 fmol of ZDV-TP per 10^6 cells in PBMC extracts from seven patients 2 h after receiving a 300-mg dose of oral ZDV. We observed average peak ZDV-TP levels of 107 and 119 fmol/ 10^6 cells after 100- and 500-mg doses, respectively. However, our results were markedly different from those of Toyoshima et al. (15), who reported 10-fold higher levels of ZDV-TP in PBMCs from AIDS patients receiving a 200-mg oral dose of ZDV with an elaborate HPLC column-switching and UV-spectrophotometric method.

These initial data describing the systemic and cellular pharmacokinetics of ZDV suggest a relationship between systemic exposure and cellular metabolism to the active form. Increased doses yielded increased plasma AUCs and increased levels of ZDV-TP in cells. However, the limited duration over which samples were obtained and the relatively small number of subjects with substantial intersubject variability preclude complex or extensive modeling of these data.

Relatively small numbers of ZDV-treated PBMCs were needed for this bioassay (10×10^6 to 15×10^6 cells), numbers which could be obtained from 10 to 20 ml of blood. Several important observations resulted from the present measurements. First, we were able to measure ZDV-TP levels in blood samples from patients receiving ZDV without the use of radiolabeled drug and the elaborate separation by HPLC required by the RIA. With appropriate modification, this assay should be adaptable to analysis of other thymidine analogs, such as stavudine and other dideoxynucleotide analogs in clinical use. Second, a relationship between the systemic pharmacokinetics of ZDV and intracellular ZDV-TP is suggested by the increase in the median ZDV-TP values as dose and systemic AUC are increased. However, the time frame for these measurements will have to be extended to allow a quantitative assessment of the importance of systemic exposure (e.g., plasma AUC) to intracellular ZDV-TP formation. Third, there appears to be a greater variation in the intracellular ZDV-TP concentrations than in the plasma ZDV concentrations. The greater variability may be caused by differences in metabolism introduced by phosphorylation of ZDV. This supports the notion of continued measurement of ZDV-TP levels directly and raises questions as to the actual time frame of intracellular ZDV-TP degradation. Thus, the enzymatic assay may be of significant value in the more extensive studies of the intracellular pharmacology of ZDV and other such antiviral agents required to correlate such observations with therapeutic activity and toxicity.

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