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Quantification of zidovudine and individual zidovudine phosphates in peripheral blood mononuclear cells by a combined isocratic high performance liquid chromatography radioimmunoassay method

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Abstract

Zidovudine (ZDV, AZT) is the first clinically effective drug licensed for use in the treatment of human immunodeficiency virus (HIV) infection. Activation of ZDV requires phosphorylation to ZDV triphosphate by cellular kinases. It is important, therefore, to determine the intracellular levels of the active form because measurement of ZDV concentrations in plasma have not reflected any direct relationship with activity or toxicity. In this paper a validated assay for the measurement of both ZDV and its three phosphorylated anabolites, ZDV mono-, di- and triphosphate, in peripheral blood mononuclear cells (PBMCs) is described. The method consisted of a combination of isocratic high performance liquid chromatography (HPLC) separation and radioimmunoassay (RIA). The PBMCs were separated from whole blood and ZDV and ZDV nucleotides were extracted and separated by isocratic elution with an ion-pairing mobile phase on a reversed-phase HPLC column. The collected ZDV and individual ZDV nucleotide fractions were dephosphorylated to ZDV, cleaned by solid phase extraction and assayed by a commercially available RIA kit. The assay developed was successfully used to determine intracellular ZDV and anabolite concentrations of 10 PBMC samples taken from HIV positive patients on ZDV treatment.

Keywords: Zidovudine; Zidovudine phosphates; Intracellular assay; HPLC; RIA; Peripheral blood mononuclear cells

1. Introduction

Zidovudine (ZDV, AZT) is the first drug approved by the Food and Drug Administration (FDA) for use in the treatment of HIV infection. ZDV is effective in increasing peripheral CD4 lymphocyte counts and in decreasing both mortality and the incidence of opportunistic infections in patients with acquired immunodeficiency syndrome (AIDS) and AIDS related complex (ARC) [1]. Although additional nucleoside analogs are now available, ZDV remains one of the most widely used drugs for therapy in HIV infection.

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ZDV is an analog of thymidine but differs from the latter in having an azido (N_3) group in place of the hydroxyl (OH) group at the 3′ position of the deoxyribose ring. ZDV itself is inactive and needs to be converted by cellular kinases to the active ZDV triphosphate (ZDV-TP). ZDV-TP then competitively and potently inhibits the binding of thymidine triphosphate (dTTP) to viral reverse transcriptase. In addition ZDV-TP acts as a DNA chain terminator because the absence of the 3′-OH group prohibits the 5′ to 3′ linkage that is required for chain elongation [1]. In contrast to physiologic deoxynucleosides, ZDV is thought to enter cells by nonfacilitated diffusion [2]. The lipophilic 3′-azido group imparts this membrane permeability. ZDV is converted inside the cell into ZDV monophosphate (ZDV-MP) by thymidine kinase. Thymidilate kinase in turn converts ZDV-MP into the diphosphate (ZDV-DP), which is further phosphorylated to ZDV triphosphate (ZDV-TP), presumably by pyrimidine nucleoside diphosphate kinase. ZDV monophosphate is the predominant compound in human cells exposed to ZDV, suggesting that thymidilate kinase is the rate limiting enzyme in the synthesis of active ZDV-TP [3,4].

Most measurements of ZDV are performed in plasma or serum, but because ZDV is phosphorylated to its active form inside the cells, measurements of plasma levels of ZDV may not directly correlate with antiviral activity or toxicity. Therefore, it is important to determine the intracellular levels of ZDV and its anabolites. These measurements will allow one to address questions concerning intracellular pharmacokinetics, metabolism, activity, and toxicity and such results may help to optimize treatment with ZDV or combination therapy. Several attempts have been made to measure the intracellular concentrations of ZDV and ZDV anabolites. Concentrations of ZDV nucleotides are very low and impossible to detect with HPLC or any other separation method directly. More sensitive approaches, such as bioassays, are therefore needed to detect these low levels. Robbins et al. [5] use a sensitive reverse transcriptase enzyme assay to determine triphosphate levels. This assay has the advantage of being able to measure the triphosphate directly without elaborate separation or purification steps prior to analysis. Unfortunately the monophosphate, which may be responsible in part for some of the toxicity of ZDV, cannot be measured by this method [6,7]. Toyoshima et al. [8] developed a high performance liquid chromatography (HPLC) method with a column switching technique while Stretcher et al. [9], Kuster et al. [10] and Slusher et al. [11] used a coupled HPLC–radioimmunoassay (RIA) assay. These assays are either not validated or only partially validated. A fully validated assay with a similar HPLC–RIA approach is described below. The RIA was commercially available and therefore the method of choice. Major differences from the other methods were in the HPLC separation and sample cleanup steps. Although this approach is still cumbersome, the assay was simplified by using an isocratic elution on a reversed-phase column similar to that used by Molema et al. [12], where the separation time is reduced markedly without the need for a more complicated gradient system.

2. Experimental

2.1. Apparatus

Whole blood was drawn into green top Vacutainers containing sodium heparin (Becton Dickinson, Rutherford, NJ) and for the separation of PBMCs a Marathon centrifuge model 21 K/R (Fisher Scientific Co., Fair Lawn, NJ) was used. Cell counts were performed with a Neubauer Hemacytometer (Fisher Scientific). The HPLC system used consisted of a Waters M-510 HPLC pump, a model 717 Plus WISP autoinjector, a model 441 UV detector (Waters Assoc., Milford, MA) with a fixed wavelength of 254 nm and an HP integrator model 3396A (Hewlett-Packard, Avondale, PA). Separation was achieved with a C18 reversed-phase column (Novapak, 3.9 mm $\times$ 300 mm, Waters), guarded with an Adsorbosphere C18, 5μ cartridge (Alltech Inc., Deerfield, IL). Solid phase extraction was performed using C18 catridges (3 cm^3, Bond Elut, Varian, Harbor City, CA). For evaporation of solvents a Meyer N-EVAP model 112 (Organomation Associate

Inc., South Berlin, MA) and a Speed Vac concentrator Savant model SVC 100H (Savant Instruments Inc., Farmingdale, NY) were used.

2.2. Reagents

All solvents were of HPLC grade (Fisher Scientific Co., Fair Lawn, NJ). Tetrabutylammonium dihydrogenphosphate [(ion pair agent, 1 M solution), Aldrich Chemical Company Inc., Milwaukee, WI], sodium dihydrogenphosphate, disodium hydrogenphosphate (Fisher Scientific Co.), Ficoll-Paque (Pharmacia Biotechnology Inc., Piscataway, NJ), PBS (phosphate buffered saline), fetal calf serum (Cell Culture Facility, University of California, San Francisco, CA), alkaline phosphatase from calf intestine [(1U μl^{-1}), Boehringer Mannheim GmbH, Germany] were used as received. ZDV, ZDV mono-, di- and triphosphate standards, and the internal standard A22U were obtained from Burroughs Wellcome (Research Triangle Park, NC). ^{14}C-Labeled ZDV and ZDV-monophosphate and *p*-nitrophenyl phosphate were received from Sigma Chemicals (St. Louis, MO). For the RIA the commercially available ZDV RIA kit (Incstar, Stillwater, MN) was used. Drug free human PBMCs and plasma were obtained from healthy volunteers.

2.3. Mobile phase

The mobile phase consisted of a sodium dihydrogenphosphate–disodium hydrogenphosphate buffer (140 mM), tetrabutylammonium dihydrogenphosphate (1 M) (pH 7.5), and acetonitrile (957.3:7.7:35, v/v/v) at an isocratic flow rate of 1.0 ml min^{-1}. The mixture was filtered through a 0.22 μm filter and degassed under vacuum.

2.4. Sample preparation

2.4.1. PBMC isolation

Whole blood (30 ml) was diluted 1:1 with PBS and 30 ml aliquots were layered onto 15 ml of cold Ficoll-Paque and centrifuged at 500 *g* for 30 min. Cells were washed twice with PBS and counted. Viability of cells was tested with Trypan Blue.

2.4.2. Extraction, separation and collection of ZDV anabolites

ZDV and ZDV nucleotides were extracted from PBMCs with 3 ml of 60% methanol at −20°C overnight [9,11]. The extracts were concentrated under nitrogen, transferred into a 1.5 ml Eppendorf tube, and dried in a Speed Vac centrifuge. The dried extracts were stored at −80°C until analysis. The cell extract residues were reconstituted in 150 μl of HPLC eluent and 130 μl was injected onto the HPLC column. After each sample injection, fractions were collected 1.25 min before and after the respective retention time of each compound.

2.4.3. Dephosphorylation of nucleotides and sample cleanup

The fractions collected from the HPLC separation were dried under nitrogen and reconstituted in 0.5 ml of phosphatase buffer. After adding 30 μl of alkaline phosphatase, the samples were incubated for 20 h at 37°C. The products of phosphatase digestion were then cleaned by solid phase extraction. The cartridges were activated with 1 volume of methanol and washed with 2 volumes of water (pH 2.5). After the samples had been applied, the columns were washed with 2 volumes of 15% methanol in water (pH 2.5), followed by elution with 3 ml of methanol.

2.4.4. RIA

The samples from solid phase elution were dried under nitrogen and the residues reconstituted in 450 μl of assay diluent buffer. The RIA kit was used with modifications similar to those of Slusher et al. [11]: the primary antibody was diluted 1:3 with assay diluent supplemented with 1% fetal calf serum and the samples were preincubated with diluted antibody for 2 h before a 1:6 dilution of the ^{125}I-labeled ZDV was added (0.005 μCi per assay tube). The volume of the secondary antibody was reduced to 300 μl. For preparation of the standard curve, 2.5 ml mobile phase samples were dried under nitrogen and reconstituted in phosphatase buffer. After adding alkaline phosphatase, the samples were spiked with ZDV before passing them through solid phase extraction.

2.5. Calculations of intracellular concentrations

The concentrations of ZDV in nanograms per milliliter obtained using the RIA calibration curve were converted to the equivalent concentrations in the number of PBMCs in each sample. Finally the concentrations were expressed in picomoles per million cells. This step makes it easier to compare the concentrations of the different nucleotides.

2.6. Measurements of plasma concentrations

For the determination of plasma concentrations of ZDV, the commercially available RIA kit was used following the instructions of the manufacturer. For the standard curve, pooled plasma from three different persons was spiked with the same ZDV solutions as used for the intracellular method.

3. Results and discussion

3.1. Assay and validation

The assay for the measurement of intracellular ZDV and ZDV nucleotides involved several steps as outlined in Fig. 1. A complete validation of the assay, including stability and recovery of the compounds during the process and determination of the variability and accuracy of the obtained results is discussed below.

3.1.1. Stability of compounds during methanol extraction

All four substances were added to 60% methanol and kept at −20°C overnight. Samples were then analyzed by HPLC using the internal standard A22U, a stereoisomer of ZDV, and the peak height ratios compared to a solution of standards in water. The results demonstrated that ZDV and ZDV mono-, di-, and triphosphate were stable.

3.1.2. Separation of ZDV and its nucleotides by HPLC

Fig. 2 represents a chromatogram of the separation of ZDV and ZDV mono-, di-, and triphosphate standards in the mobile phase. Typical

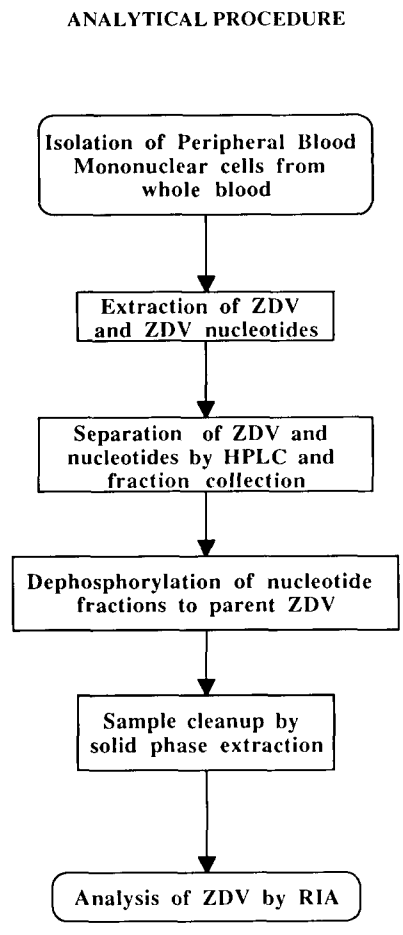

Fig. 1. Flow diagram of the complete analytical procedure.

retention times were 14.64 min for the monophosphate, 20.40 min for ZDV, 25.83 min for the diphosphate and 43.76 min for the triphosphate. These retention times varied slightly, depending on room temperature. The HPLC system was therefore kept in a temperature controlled room and retention times were determined each time before patient sample separations were performed. With steady room temperatures the retention times were stable and the RSDs for three consecutive runs were 0.49% for the monophosphate, 0.84% for ZDV, 0.67% for the diphosphate and 0.68% for the triphosphate.

To exclude the possibility that peaks shift when injected in a mixture with endogenous compounds from PBMCs, the collection intervals of ZDV and

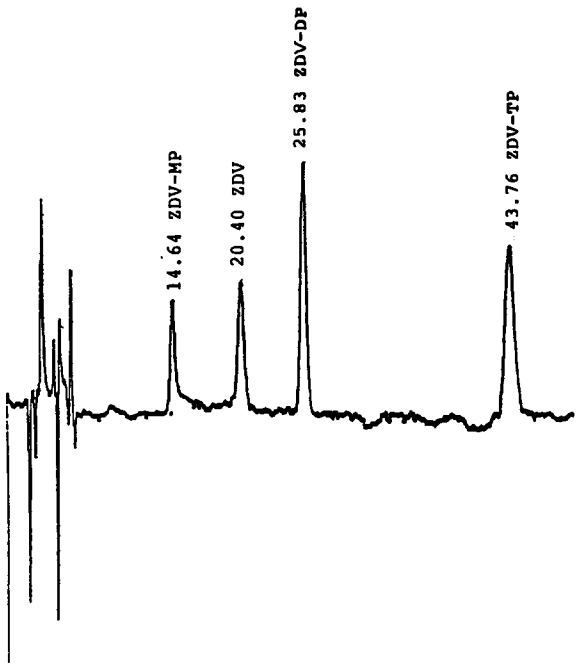

Fig. 2. HPLC separation of ZDV and ZDV mono-, di- and triphosphate standards dissolved in the mobile phase. The injection volume was 130 μl.

ZDV-MP were tested by adding 10 ng (0.002 μCi) of ^{14}C-labeled ZDV and 13.7 ng (0.002 μCi) of ^{14}C-labeled ZDV-MP to extracts of about 20×10^6 cells (average number of cells analyzed with the assay). The fractions were collected at 0.5 min intervals 3 min before and after the determined retention times and quantitated by scintillation counting. The compounds eluted at the expected retention times and the recovery was greater than 99%.

For testing the stability of the compounds in the mobile phase, the substances were added to the eluent and kept for at least 1 h at room temperature. The samples were then analysed by HPLC in the same way as described for stability in methanol (Fig. 3). Comparing the peak height ratios revealed that the compounds were stable in the mobile phase.

3.1.3. Dephosphorylation of phosphates

The commercially available RIA kit only detects ZDV. The individual nucleotides were therefore dephosphorylated to ZDV with alkaline phosphatase. Dephosphorylation was first tested on ZDV and ZDV nucleotide standards in the HPLC mobile phase. Aliquots of 2.5 ml of mobile phase (the volume collected during fraction collection) were dried, reconstituted in 0.5 ml of phosphatase buffer and spiked with constant amounts of standards (approximately 4 μg of each nucleotide). Increasing amounts of alkaline phosphatase were then added and tested during an incubation time course. The optimal conditions for complete dephosphorylation were found to be 25 U of alkaline phosphatase for 20 h at 37°C. Both the incubation time and phosphatase concentration were much higher than required for a standard solution in water, which is probably due to either the high buffer salt concentration or the tetrabutylammonium phosphate present in the mobile phase.

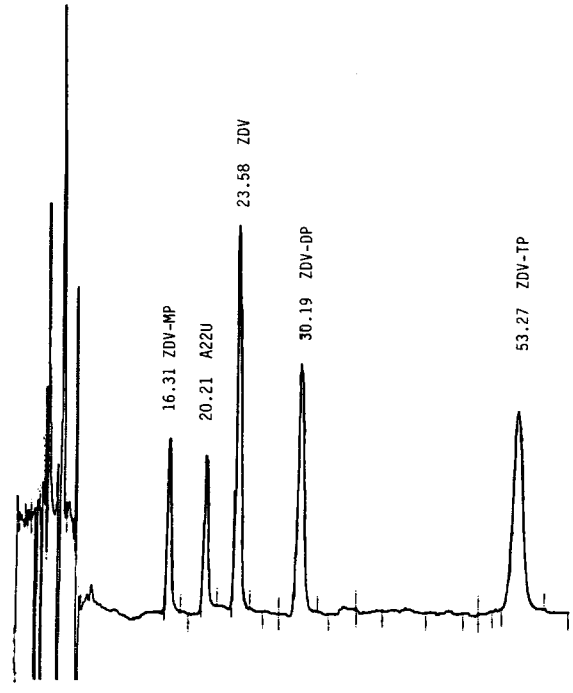

Fig. 3. HPLC separation of a mixture of ZDV and ZDV nucleotides containing the internal standard A22U to show noninterference with other compounds. The injection volume was 20 μl. Since a different lot number of column and a different batch of mobile phase were used, retention times were different compared to the previous chromatogram (see Fig. 2).

To determine if any inhibition of alkaline phosphatase by cellular extracts occurs a test with *p*-nitrophenyl phosphate was performed. A solution of *p*-nitrophenyl phosphate turns yellow after dephosphorylation, which can be easily quantified with a spectrophotometer.

Four fractions corresponding to the respective retention times of ZDV and ZDV phosphates were collected after injecting an extract of a blank PBMC sample. The fractions were dried under nitrogen and reconstituted with a 1 mM solution of *p*-nitrophenyl phosphate in phosphatase buffer. After adding the phosphatase, the absorption was measured at different times and compared to the values of a sample containing no cell extracts. In all the fractions the phosphatase was inhibited by 20%. Therefore, the amount of phosphatase was increased to 30 μl per sample. This amount of phosphatase was considered sufficient, since the concentrations of nucleotides used to test dephosphorylation were much higher than concentrations in patient samples.

The stability of ZDV during the dephosphorylation process was determined in the same way as described above and was found to be stable under these conditions.

3.1.4. Recovery of ZDV during solid phase extraction using Bond Elut columns

The fractions obtained after HPLC separation and dephosphorylation contain salts in high concentrations and tetrabutylammonium phosphate, both of which interfere with the antibodies used in the RIA. These compounds could be eliminated in part by using solid phase extraction. The recovery of ZDV during this step was determined by using three different concentrations of ^{14}C-labeled ZDV (1 ng, 0.0002 μCi, 5 ng, 0.001 μCi, 10 ng 0.002 μCi) and five samples for each concentration. The samples were analysed by scintillation counting and mean recoveries were 93.9% (RSD 6.6%) for 1 ng, 93.3% (RSD 2.5%) for 5 ng and 91.7% (RSD 3.6%) for 10 ng. The overall mean recovery was 93.0%.

3.1.5. Overall recovery of ZDV during the assay

Three PBMC samples (about 20×10^6 cells) were spiked with 20 ng (0.004 μCi) of ^{14}C-ZDV.

Table 1
Accuracy of standard curves. Back calculated concentrations from standard curves are compared with the theoretical spiked concentrations. The values represent the means from the six validation days.

Theoretical ZDV concentration (ng ml^{-1})	Calculated ZDV concentration (mean ng ml^{-1})	% Difference
0.21	0.26	+23.8
0.52	0.49	−5.8
1.05	0.91	−13.4
2.10	1.93	−8.1
5.24	5.44	+3.8
10.49	11.47	+9.3

The samples were carried through the complete assay procedure except that instead of RIA measurement the ZDV was determined by scintillation counting. The mean recovery was 83.94% (SD 0.57, RSD 0.68%).

3.1.6. RIA precision and accuracy

Mobile phase samples of 2.5 ml (amount obtained during HPLC fraction collection) were carried through the whole process. Known amounts of ZDV were added before solid phase extraction. Two different ZDV solutions were used for the standard curve and the controls. The standard curve was linear over a range of 0.21 ng ml^{-1} to 10.5 ng ml^{-1} ($r^2 \geq 0.98$, Table 1).

For the interday and intraday precision and accuracy, five duplicates of each of three control concentrations were run during 6 days. Tables 2 and 3 show the results of the interday and intraday variability respectively. The lower limit of quantitation (LOQ) was 0.21 ng ml^{-1} with an RSD of 20.2%.

3.1.7. Interferences during RIA

Thymidine is reported to have the highest cross reactivity during the RIA for ZDV. However, with the authors' HPLC separation method, thymidine and thymidine phosphates eluted within the first 10 min and did not interfere with ZDV and ZDV mono-, di- and triphosphate peaks, which permitted the collection of fractions free of these compounds.

Table 2
Interday variability. Five samples for three different concentrations were spiked each day (during 6 days). The Table shows the mean values for each concentration for the individual days. In addition the overall means from the 6 days are calculated together with the SD and %RSD.

Concn.	Day 1 $n=5$	Day 2 $n=5$	Day 3 $n=5$	Day 4 $n=5$	Day 5 $n=5$	Day 6 $n=5$	Mean Day 1–6	SD Day 1–6	%RSD Day 1–6
Low (0.306 ng ml^{-1} mean)	0.326	0.322	0.358	0.374	0.307	0.310	0.333	0.027	8.15
Med. (2.039 ng ml^{-1}mean)	1.855	1.705	2.087	1.875	1.898	1.956	1.896	0.125	6.62
High (8.156 ng ml^{-1} mean)	8.438	8.644	8.354	7.551	7.304	8.673	8.161	0.586	7.18

Tetrabutylammonium phosphate, used as the ion-pairing reagent in the HPLC separation, elevated the background markedly. Most of this tetrabutylammonium phosphate could be eliminated by solid phase extraction. However, since the background persisted, the standard curve was prepared in HPLC eluent rather than in serum as described.

The background caused by PBMCs was tested with cells (approximately 20×10^6 cells) from four different subjects. The blank samples were processed the same way as patient samples and assayed by RIA. This background was below the LOQ and was therefore considered negligible.

Table 3
The individual concentrations for low, medium and high levels of one day are shown for the intraday variability.

	Low (0.306 ng ml^{-1})	Medium (2.039 ng ml^{-1})	High (8.516 ng ml^{-1})
Control 1	0.294	2.059	8.153
Control 2	0.316	2.181	8.506
Control 3	0.332	1.194	8.624
Control 4	0.285	1.735	9.348
Control 5	0.322	1.892	8.733
Mean	0.310	1.956	8.673
SD	0.020	0.170	0.436
RSD %	6.34	8.70	5.03

3.2. Measurement of intracellular concentrations of ZDV and ZDV mono-, di- and triphosphate from patient samples

The assay described above was used to measure the intracellular concentrations of ZDV and ZDV anabolites of ten samples from HIV positive patients treated with ZDV. The blood samples were drawn approximately 1 h after a 200 mg oral ZDV dose. This time was chosen because peak plasma levels of ZDV are normally reached in 1 h. Table 4 summarizes the intracellular concentrations, exact times of sampling after the ZDV dose, duration of ZDV treatment prior to measurement, and patient CD4 counts. In addition, corresponding ZDV plasma concentrations are shown in the same Table. Two samples each were drawn from patients 1 and 4 at different times of treatment. Consistent with earlier reports [11], the data show that the monophosphate is the predominant nucleotide, suggesting that phosphorylation to the diphosphate is the rate limiting step in vivo as has been demonstrated in vitro. The triphosphate contributed on average only 8.5% (4.0%–11.8%) to the total phosphorylated compounds, except for patient 5, where it was 21.8%. Furthermore, no correlation was detected between plasma ZDV concentrations and intracellular mono-, di- or triphosphate levels. The measurement of individual intracellular nucleotide concentrations in addition to plasma concentrations may therefore increase the capability of predicting activity or

Table 4
The intracellular concentrations of the individual nucleotides from 10 patient samples are summarized. The concentrations are expressed in pmol per 10^6 cells. Also included in the Table are the duration of ZDV treatment, CD4 count, the exact time of the blood draw, plasma concentrations and the % triphosphate of total phosphorylated compounds

Subject	Months of ZDV therapy prior to measurement	CD4 count (cells mm^{-3})	Time sample drawn (min)	ZDV plasma conc. (ng ml^{-1})	ZDV plasma conc. (μM)	ZDV intracellular (pmol per 10^6 cells)	ZDV-MP intracellular (pmol per 10^6 cells)	ZDV-DP intracellular (pmol per 10^6 cells)	ZDV-TP intracellular (pmol per 10^6 cells)	% Triphosphate of total phosphate conc.
1	40	126	60	566.9	2.13	0.086	0.545	[a]	0.061	11.19
1	44	165	71	689.9	2.59	0.112	0.617	0.041	0.041	11.09
2	60	375	60	387.0	1.45	0.073	0.464	0.044	0.041	8.07
3	24	18	64	61.2	0.23	[a]	0.126	0.065	[a]	[b]
4	38	161	60	382.3	1.43	0.042	0.363	0.027	0.041	10.51
4	40	[b]	60	439.2	1.65	0.057	0.729	0.022	0.033	3.99
5	55	377	60	418.3	1.57	0.057	0.267	0.050	0.069	21.77
6	11	99	60	987.6	3.67	0.041	0.337	0.043	0.045	11.84
7	27	56	60	1020.4	3.83	[a]	0.802	0.039	0.055	6.54
8	39	351	60	722.6	2.71	0.093	0.820	0.037	0.043	5.02

[a] Not detected.
[b] Not determined.

toxicity of ZDV. In this small sample size, there was no apparent relationship between duration of ZDV treatment prior to measurement, CD4 count, and intracellular concentrations. One reason for the variability of intracellular levels among different patients could be due to varying degrees of enzyme activities, depending on the activation of the PBMCs [13]. Furthermore, the intracellular pharmacokinetics of ZDV mono-, di- and triphosphate in vivo have not been described.

Toyoshima et al. [8] and Kuster et al. [10] reported much higher intracellular concentrations, which may be due to the differences in their methodology. The levels measured with this assay were in general lower than those from Slusher et al. [11]. This may be due to the lower dosage of ZDV or the different sampling time.

In conclusion, a fully validated assay for the measurement of intracellular ZDV and ZDV mono-, di- and triphosphate in PBMCs was developed. This assay will allow the determination of intracellular pharmacokinetics of ZDV anabolites and help to establish possible correlations between nucleotide concentrations and efficacy and toxicity.

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