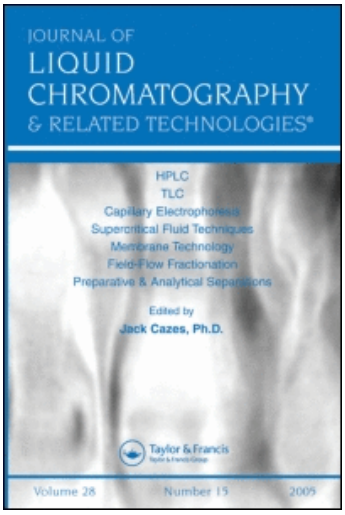

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A Coupled HPLC/Radioimmunoassay for Analysis of Zidovudine Metabolites in Mononuclear Cells

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A COUPLED HPLC/RADIOIMMUNOASSAY FOR ANALYSIS OF ZIDOVUDINE METABOLITES IN MONONUCLEAR CELLS

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ABSTRACT

Reverse-phase HPLC, with the ion-pairing agent tetrabutyl ammonium phosphate, was used to separate zidovudine (ZDV) and its 5'-phosphorylated metabolites in extracts from peripheral blood mononuclear cells incubated with 2 μM ZDV. Because intracellular concentrations were too small to be visualized using UV detection, 1 ml fractions were collected and assayed for ZDV and metabolites using a commercial radioimmunoassay (RIA). Resolution of components was satisfactory, with a total chromatography time of 32 minutes. Interassay variability of peak areas was less than 14%. Comparison of UV detected chromatograms to RIA detected chromatograms from a standard mixture of ZDV and metabolites showed no significant difference between corresponding relative peak areas. The ion-pairing agent elevated baseline concentrations as measured by RIA. Quantitation was therefore performed by concurrent measurement of total phosphorylated ZDV using an established procedure, followed by comparison of relative peak areas. Results indicate that ZDV 5'-triphosphate, the active metabolite, is a major component of total phosphorylated ZDV, with peak heights significantly above baseline in extracts from less than 10^7 mononuclear cells. Therefore, it should be possible to reliably quantitate this metabolite in cells from HIV-infected patients using only 10 to 20 ml of blood.

INTRODUCTION

Zidovudine (ZDV; 3'-azido- 2',3'-dideoxythymidine; AZT) is the only drug approved by the U.S. Food and Drug Administration for the treatment of human immunodeficiency virus (HIV) infection, the etiologic agent of the acquired immunodeficiency syndrome (AIDS). After ZDV enters the cell, it is metabolized through a series of cellular enzymes to ZDV 5'-triphosphate (ZDVTP). ZDVTP is believed to inhibit HIV reverse transcriptase, and block chain elongation as it is incorporated into viral DNA (1). While effective in slowing HIV replication, hence the progression of disease, ZDV can produce serious anemia and neutropenia, requiring reduction or termination of drug therapy (2). Monitoring of plasma or serum ZDV concentrations in an effort to control toxicity while maintaining efficacy has met with little success. This is not surprising, as mechanisms of toxicity and efficacy most likely occur intracellularly, and intracellular concentrations have not correlated with plasma concentrations of ZDV (3). Therefore, it is important to measure the extent of ZDV phosphorylation in patients to determine if toxicity can be managed through dose modifications while maintaining effective intracellular concentrations.

A great deal is known about the intracellular metabolism of ZDV *in vitro*, but relatively little data are available concerning metabolism *in vivo*. The technology commonly used to make intracellular measurements has not been feasible for use in patients, because it requires the exposure of cells to radiolabeled drug and/or excessive numbers of cells ($>10^7$). Therefore, a method designed for patient measurement has been developed and is reported here. The method uses HPLC to separate ZDV and its metabolites in extracts from peripheral blood mononuclear cells (PBMC), and a sensitive radioimmunoassay (RIA) to quantitate ZDV and metabolites in the resultant fractions. The method does not require exposure of cells to radiolabeled drug, and the sensitivity of the RIA has allowed the quantitation of metabolites in as few as 5×10^6 PBMC.

MATERIALS AND METHODS

Reagents

ZDV, ZDV 5'-monophosphate (ZDVMP), and ZDVTP were donated by Burroughs-Wellcome Co. (Research Triangle Park, NC, USA). Leucoprep 8 ml

separation tubes, used for the isolation of PBMC, were purchased from Becton-Dickinson Labware (Lincoln Park, NJ, USA). All cell culture reagents were purchased from GIBCO Laboratories (Grand Island, NY, USA). HPLC grade methanol was purchased from EM Science (Gibbstown, NJ, USA), as was ammonium dihydrogen phosphate. The ion- pairing agent, tetrabutyl ammonium phosphate (TBAP) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Tris (Ultrapur grade) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA). Magnesium chloride hexahydrate was purchased from Aldrich Chemical Co. Bovine alkaline phosphatase (10 units activity/mg) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). ZDV-Trac RIA kits were donated by INCStar Corp. (Stillwater, MN, USA).

Uptake and Phosphorylation of ZDV

Blood was collected from healthy volunteers into sterile 8 ml Leucoprep separation tubes, and PBMC were isolated by centrifuging according to package instructions. Cells were washed twice with RPMI 1640 medium, and viable cells were quantitated by Trypan Blue dye exclusion. Cells were then resuspended (cell density approximately 2 x 10^6 cells/ml) in incubation medium consisting of RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. ZDV was added to yield a final incubation concentration of 2 µM. The suspension was mixed thoroughly and placed in a 5% CO_2 humidified incubator at 37° C for 72 to 96 hours. Cells were again quantitated, harvested by centrifugation in 4 ml aliquots and washed twice with Dulbecco's 1x phosphate buffered saline. The cells were extracted overnight with 5ml of 60% methanol at -20° C and centrifuged to remove cellular debris. The supernatant was decanted and evaporated to dryness under a stream of nitrogen. The resulting residue was reconstituted in 250 µl water prior to separation by HPLC.

HPLC Separation of Intracellular ZDV and its Phosphorylated Metabolites

Separations of 100 µl of reconstituted residues or standard mixtures were performed on a Waters Associates (Milford, MA, USA) HPLC system consisting of a U6K Injector, a 510/501 dual pump system controlled by a 680 Automated

Gradient Controller and a Model 441 Absorbance Detector. The column was an Alltech (Deerfield, IL, USA) Adsorbosphere nucleotide/nucleoside 7μ C18 reverse-phase column (4.6 x 250 mm) preceded by an Alltech direct connect cartridge 7μ guard column (4.6 x 10 mm). Two mobile phases were used: mobile phase A was 20 mM ammonium phosphate with 2 mM TBAP, pH 5.0; mobile phase B was 2 mM TBAP in methanol. The gradient consisted of 25% B increased linearly over 15 minutes to 35% B. Flow rate was 1 ml/min, and column effluent was monitored at 254 nm. One ml fractions were collected throughout each chromatographic run.

Measurement of ZDV and Metabolites in HPLC Fractions

HPLC fractions were dried under reduced pressure at 60° C, then reconstituted in 190 μl of 0.2 M tris/10 mM $MgCl_2$ buffer, pH 9.5. Ten μl of a 20 mg/ml solution of alkaline phosphatase in tris/$MgCl_2$ was added to each fraction, to hydrolyze all 5'-phosphate groups. Each fraction was vortexed, then placed in a 37° C water bath for at least 2 hours. After incubation, each fraction was assayed in duplicate for ZDV content using the ZDV-Trac RIA (range 0.2 to 220 ng/ml) modified as previously described (4). Peaks were identified as ZDV or its metabolites by comparison of retention time with known standards. For calculation of the concentration of individual metabolites, relative peak ratios were compared with separately measured total phosphorylated ZDV (4) in simultaneously aliquoted cells.

RESULTS

Figure 1a shows an HPLC chromatogram of a standard mixture of ZDV (14.8 min), ZDVMP (18.6 min), and ZDVTP (29.2 min), as monitored at 254 nm. ZDV 5'-diphosphate (ZDVDP), formed from the breakdown of ZDVTP, was identified at 24.1 min. Figure 1b shows the same separation as visualized by RIA of the resultant fractions. While accurate only to the nearest fraction, retention times are essentially identical to those in the UV tracing. Similarly, comparison of calculated peak area ratios for the two chromatograms shows that there is little difference between the detection methods (Table 1). Table 2 illustrates chromatographic reproducibility using data from several chromatograms of ZDV

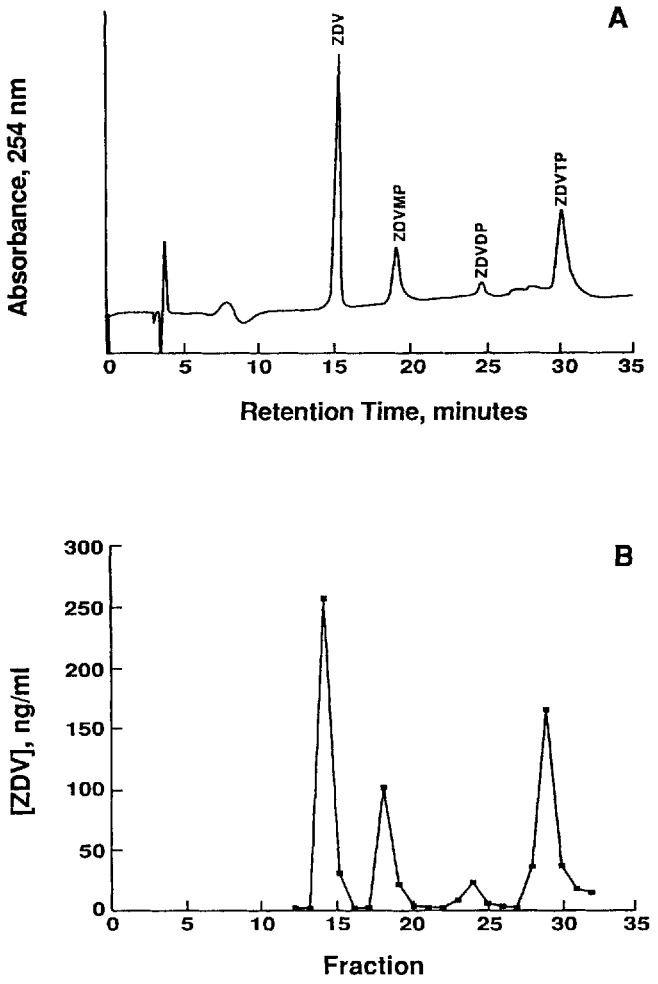

Figure 1. Chromatograms of a standard mixture of ZDV (14.8 min.), ZDVMP (18.6 min), ZDVDP (24.1 min.) and ZDVTP (29.2 min.). A) Chromatogram generated by UV detection at 254 nm. B) Chromatogram generated by RIA of 1 ml fractions collected during the same run as A. See text for chromatographic conditions.

TABLE 1

Relative Peak Area Ratios By Detection Method

Method	Ratio ZDV:ZDVMP:ZDVTP
UV	2.6 : 1 : 1.9
RIA	2.4 : 1 : 2.0

TABLE 2

Chromatographic Reproducibility with Standards (n=4)

Metabolite	Relative Area +/- S.D. (%CV)
ZDV	13.8 ± 1.4 (10.3)
ZDVMP	5.0 ± 0.5 (10.0)
ZDVDP	1.0 ± 0.14 (14.0)
ZDVTP	10.0 ± 0.9 (9.0)

S.D., standard deviation. C.V., coefficient of variation.

standards, showing coefficients of variation of 14% or less, even for the small diphosphate peak. Figure 2a shows a UV detected chromatogram from a cell extract blank (no ZDV added). This chromatogram shows the background signal from 13.7 x 10^6 PBMC. Some of the endogenous nucleosides and nucleotides were identified by comparison to retention times of known nucleoside and nucleotide standards run on the same gradient.

Of particular interest was the large ATP peak, which eluted at about 21 minutes, between the elution times of ZDVMP and ZDVDP. There were no significant endogenous peaks in the ZDVTP region. Figure 2b shows the RIA detected chromatogram from the same cell extract. No prominent peaks were observed although the baseline was not entirely stable. This instability was probably due to non-specific interactions between the RIA antibody, endogenous nucleosides and nucleotides, cellular proteins, and other cellular components not removed in the extraction process. There is also a general elevation in baseline of approximately 5 ng/ml which was traced to the ion-pairing agent, TBAP. TBAP has a concentration dependent effect on RIA response (data not shown).

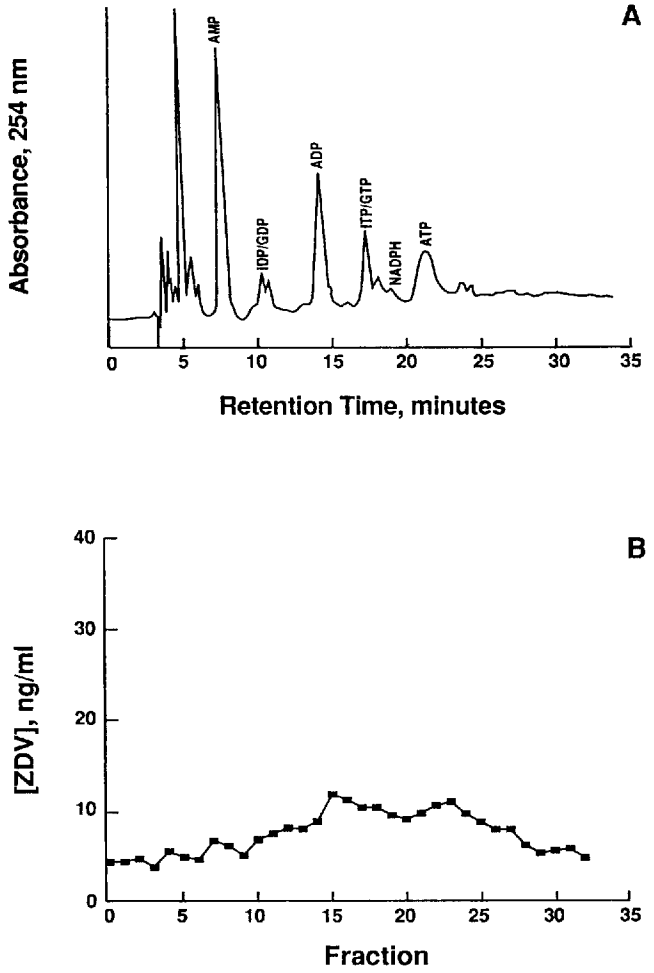

Figure 2. Chromatograms of an extract from 13.7 x 10^6 PBMC without added ZDV. The chromatograms portray background due to interferences. A) Chromatogram generated by UV detection at 254 nm. Endogenous nucleosides/nucleotides were identified as shown. B) RIA of resultant fractions from the same analysis.

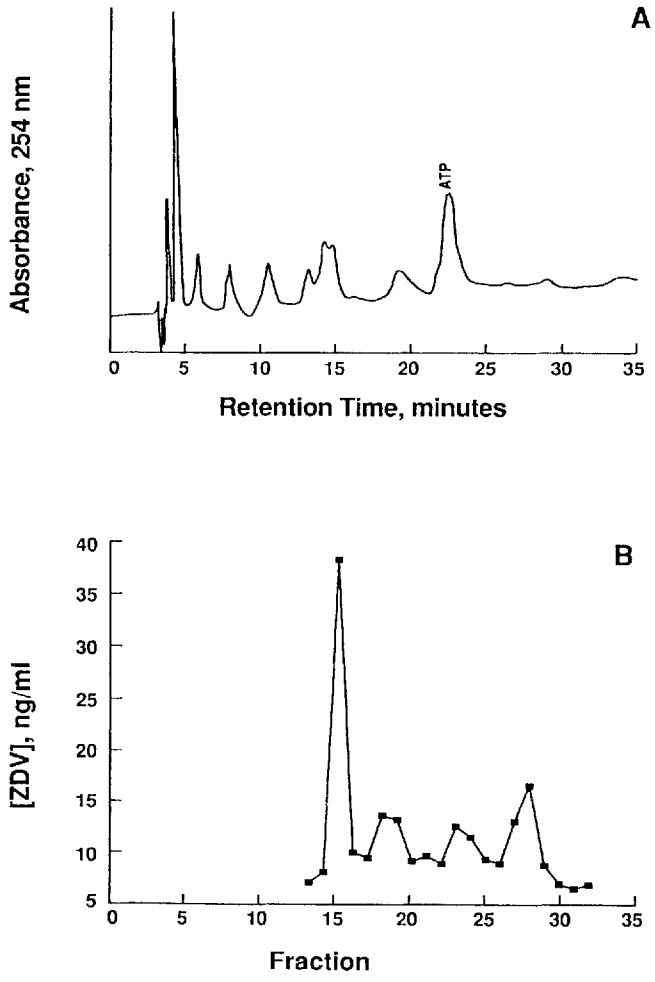

Figure 3. Chromatograms of an extract from 8.4 x 10^6 PBMC incubated for 90 hours with 2 μm ZDV. A) Chromatogram generated by UV detection at 254 nm. The ATP peak is identified. B) RIA of resultant fractions from the same analysis, showing the characteristic pattern of ZDV plus metabolites. Peak assignments were ZDV (fraction 15), ZDVMP (fractions 17 and 18), ZDVDP (fractions 23 and 24) and ZDVTP (fractions 27 and 28).

A chromatogram obtained at 254 nm from extracts of 8.4 x 10^6 cells incubated with 2 μM ZDV is shown in figure 3a. ZDV and its metabolites could not be conclusively identified as peaks in this or other chromatograms obtained by UV monitoring. However, as seen in figure 3b, ZDV and its metabolites were readily apparent in corresponding chromatograms obtained by RIA detection. No interference associated with identified nucleosides/nucleotides was observed.

Because baseline elevation logarithmically increases RIA measured concentrations, quantitation of components directly by fraction content was not appropriate. Instead, relative metabolite peak areas were compared to total phosphorylated ZDV, measured in a separate aliquot from the same sample. For the example shown in fig. 3b, total phosphate was found to be 0.16 pmole/10^6 cells. Peak area ratios (ZDVMP:ZDVDP:ZDVTP) were 1.4 : 1.0 : 2.3. The respective metabolite concentrations were 0.048, 0.034, and 0.078 pmole/10^6 cells.

DISCUSSION

Virtually all studies investigating metabolism of ZDV in human white cells have been performed in activated cells from immortalized lines (1, 5-7). However, because phosphorylation varies in different types and states of white cells (5), extrapolation of data from highly active and dividing cells to relatively quiescent, normal human mononuclear cells must be made with caution. Furthermore, these and other studies have been performed by following the *in vitro* uptake and phosphorylation of tritiated ZDV by quantitating radioactivity in resultant fractions after HPLC separation of the intracellular components in cell extracts. Use of radiolabeled ZDV for studies of *in vivo* metabolism in HIV-infected patients is clearly not feasible. In addition, use of tritiated ZDV necessitates the use of up to 30 million cells for a single determination (7). The relatively high specific activity of the ^{125}I-based RIA allowed determinations in PBMC isolated from a single 10 ml blood sample (2 to 10 million cells). Therefore, this report describes a procedure for study of ZDV metabolism potentially applicable to patient samples.

The chromatography system performed well in the separation of ZDV and its metabolites. Resolution was satisfactory without significant peak overlap in either the UV or RIA chromatograms. In the RIA chromatograms some peak trailing was apparent, especially in the ZDVDP and ZDVTP peaks, but not enough to affect

peak areas in comparison to UV chromatograms. Although ZDVDP was not available as a reference compound, its peak was identified by location and by its increase in area with time as the standard mixture aged. It was also reproducibly observed by RIA analysis. Reference retention times were confirmed by chromatography of standard solutions with UV detection between all experimental runs. Since 1 ml HPLC fractions were collected, retention times in the RIA chromatograms were accurate only to the nearest fraction. RIA detected peaks were generally within one fraction of the retention time determined by standard UV detection.

Examination of the RIA detected chromatogram from unexposed cells (fig. 2b) shows that interference from cellular material was not a significant concern. Analyses were generally performed on less than 10^7 cells, therefore background was less than that shown. Since the peaks of interest were considerably above background (50 to 150%), error introduced from cellular interference was minimal.

The use of 2 mM TBAP optimizes HPLC separation by maintaining resolution and minimum peak broadening while minimizing baseline elevation in the RIA detected chromatograms. Residual contribution to baseline by TBAP was equivalent to 5 ng/ml of ZDV. However, background cannot simply be subtracted in order to calculate a per fraction concentration because TBAP logarithmically shifts the concentration range of assayed fractions and cellular interference is somewhat variable between samples. This would result in extremely high calculations of intracellular concentrations. Hence, comparison of relative peak areas to total phosphorylated ZDV is used to calculate concentrations of individual metabolites. Coefficients of variation in measurement of total phosphate are less than 10% (3). Since recovery of total phosphate from cell extract is 88% or greater (4), relative peak areas are an accurate representation of the composition of phosphorylated ZDV at the time of measurement. The alternative, a calibration curve from known ZDV and metabolite mixtures, would not be practical because of the required manipulations of numerous HPLC fractions and the small concentration range (0 to 10 ng/ml) that would be necessary to measure intracellular concentrations in a minimum number of cells.

Because the method is a multi-procedure process, the assay required three to four days and the multiple manipulations were tedious. Despite these limitations, the procedures were basically simple and designed to be performed in a clinical laboratory with HPLC and RIA capability. Streamlining the procedure by deleting

the first 12 HPLC fractions from RIA analysis helped considerably by permitting entire chromatograms to be comfortably assayed in one RIA (see figures).

This method has primarily been used to investigate ZDV metabolism *in vitro* and serves as a model for future determinations in patients. The results suggest that under simulated steady-state conditions, ZDVTP is a major component of total phosphate. This observation is contrary to results from earlier studies in immortalized cells, which found ZDVTP levels only 2% or less of total phosphate (1). That the triphosphate is a major component in patients has recently been reported by Kuster et al. (8), who found ZDVTP levels comprising as much as 30% of total phosphate with a method very similar to the one reported here.

CONCLUSIONS

An HPLC/RIA method has successfully been used for analysis of ZDV and its 5'-phosphorylated metabolites in drug-exposed PBMC. Although time consuming, the method is straightforward and could be used by clinical laboratories for monitoring of ZDV metabolites in cells from HIV-infected patients. The data suggest that ZDV 5'-triphosphate is a major intracellular metabolite in PBMC in the steady-state. Current efforts are focused on using this technique with patient samples so that the intracellular metabolism of ZDV *in vivo* can be thoroughly assessed.

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