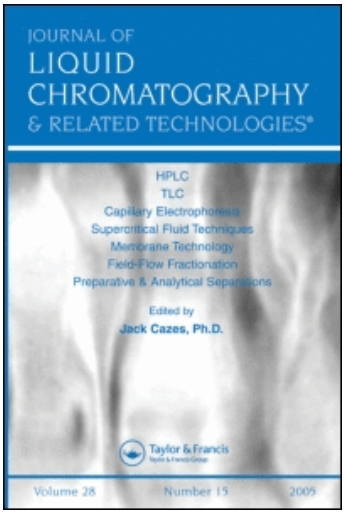

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF INTRACELLULAR dTTP POOLS

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ABSTRACT

The size of intracellular 2'-deoxythymidine-5'-triphosphate (dTTP) pools is an important indicator of cytotoxicity in cells exposed to antiviral drugs and mutagens.

We describe an ion paired reverse-phase high performance liquid chromatographic (HPLC) assay for the quantification of thymidine triphosphate in peripheral blood mononuclear cells (PBMC). Cell extracts are first purified by solid phase extraction using strong anion exchange (SAX) cartridges. The resulting fraction is analyzed by isocratic HPLC using a C_{18} column and a mobile phase consisting of triethylamine phosphate buffer with 2% acetonitrile. UV detection at 267 nm is used. Coefficients of variation for the assay are in the range of 6.0-9.8%. The overall recovery is 86.84%. LOQ for this assay is 48 ng/mL (100 nM), which is equivalent to 5 pmol/10^6 cells and give a signal to noise ratio of 4:1.

The method described is used for the determination of dTTP pools in PHA-stimulated PBMC from healthy volunteers.

INTRODUCTION

The concentration of 2'-deoxynucleoside-5'-triphosphates (dNTPs) regulates the ability of the cell to synthesize DNA and modulates its sensitivity to the genotoxic effects of mutagens and drugs interfering with DNA synthesis.[1]

We have been interested in measuring the 2'-deoxythymidine-5'-triphosphate (dTTP) pool size in phytohemoagglutinin activated peripheral blood mononuclear cells (PHA-PBMC) exposed to 3'-azido-2',3'-dideoxythymidine (zidovudine, ZDV), a nucleoside analog drug used for the treatment of HIV infection. ZDV causes a reduction in the intracellular dTTP pool size in resting cells, although variable results were observed in PHA-PBMC and other cell lines.[2-9] A high intracellular ratio of ZDVTP/dTTP is considered necessary in order to exert antiviral activity, since both ZDVTP and dTTP are in competition for the viral reverse transcriptase.[10-14] It has been observed that upon PHA stimulation of PBMC the ratio of the intracellular ZDVTP/dTTP was more than 10-fold higher than in resting PBMC.[10] This increase correlated with an increased inhibition of HIV replication. However, cytotoxic effects have been linked to dTTP pool depletion. The dTTP levels may interfere with the regulation of ribonucleotide reductase and suppress the synthesis of the other deoxynucleotides. This could lead to DNA fragmentation and apoptosis, [15,16] possibly a cause of the bone marrow suppression frequently associated with ZDV therapy. The measurement of dTTP pools in cells is therefore important for the understanding of nucleotide metabolism in the presence of nucleoside analogs such as ZDV, and for the design of combination chemotherapy.

The most commonly used methods for the determination of the intracellular dNTP pools are HPLC[17-21] and DNA polymerase assays.[22-24] In addition, a specific radioimmunoassay for dTTP has been developed by Aherne et al. [25]

The DNA polymerase assay allows measurement of intracellular dNTP levels well below 0.5 pmol per 10^6 cells and requires a small number of cells per assay ($\sim 2 \times 10^4$). However, the enzymatic assay is susceptible to inhibition by 2',3'-dideoxynucleoside-5'-triphosphates (ddNTPs), (i.e. ZDVTP)[22] and by certain dTTP analogs, (i.e. dUTP and BrdUTP). The RIA is also very sensitive (0.1 pmols/10^6 cells), but is limited by the cross-reactivity to ZDVTP (unpublished data obtained in our laboratory). HPLC is the most simple and rapid method; however, the interference by ribonucleotides, which are present in much greater amounts than dNTPs, significantly limits the chromatographic resolution and sensitivity. The selective destruction of ribonucleotides in cell extracts can be performed by periodate oxidation, followed by chromatographic analysis of the remaining deoxyribonucleotides.[20] Alternatively, the ribonucleoside triphosphate can be removed from the cell extracts by boronate

chromatography.[19] However, very few assays have been published that can detect concentrations as low as those observed in PBMC starting from a number of cells not superior to 3-4 x 10^7.[5,17] The number of cells used per assay is a critical factor for routine investigation, when the amount of blood available per subject is limited.

We developed an HPLC method for the determination of dTTP in 1 x 10^7 PHA-stimulated PBMC extracts, suitable for concentrations as low as 5 pmols/10^6 cells. This method uses a solid phase extraction SAX column for sample preparation and does not require the removal of the ribonucleotides.

EXPERIMENTAL

Chemicals

During the procedure the following was used: methanol and water HPLC grade (Fisher Scientific, Fair Lawn, New Jersey), RPMI medium 1640 with L-glutamine (Irvine Scientific, Santa Ana, CA), Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden), Dulbecco's phosphate-buffered saline (PBS) and lectin (PHA) (Sigma Chemicals, St. Louis, MO), fetal bovine serum (Gemini-Bio-Products, Calabasas, CA), interleukin-2 (Boheringer Mannheim, Germany), thymidine triphosphate and triethylamine (99%) (Sigma Chemicals, St. Louis, MO.), potassium phosphate monobasic 99%, (Aldrich Chemical Co. Inc., Milwaukee, WI). Human PBMC were obtained from buffy coats of healthy volunteers (Irwin Memorial Blood Center, San Francisco, CA). The cell counting was accomplished using a Brightline Hemacytometer (Hausser Scientific, Horsham, PA) and stained with trypan blue solution (0.4%) (Sigma Chemicals, St. Louis, MO).

Apparatus

The HPLC apparatus consisted of a Waters model 717 plus Autosampler, a Waters 515 HPLC Pump, and a Waters 486 Tuneable Absorbance Detector (Waters, Milford, MA). A model HP 3392A Integrator (Hewlett-Packard, Avondale, PA) was used to collect the chromatographic data.

The separation was achieved through a Waters Nova-Pak C-18 column (3.9 x 300 mm) (Waters, Milford, MA). For sample preparation, we used 1 mL SAX Analytichem Bond Elut SPE cartridges and a Varian Vac-Elut (Varian, Harbor City, CA).

Chromatographic Conditions

The mobile phase was composed of 5 mM triethylamine with pH adjusted to 7.4-7.5 using phosphoric acid (85%) and acetonitrile, 98:2 v/v. The solution was filtered using a 0.22 μm filter and degassed by sonication under vacuum. A flow rate of 1mL/min was used at ambient temperature. Detection of peaks was performed at 267 nm. In order to eliminate building up of interfering substances due to the large injection volume (150 μL), the column was washed once a week with a solution of 60% acetonitrile for two hours and equilibrated with mobile phase before sample injection.

PBMC Isolation and Extraction

Buffy coat was obtained from healthy volunteers and PBMCs were isolated by Ficoll-Paque method. Buffy coat was diluted 1:1 with PBS and 25 mL aliquots were layered onto 15 mL of Ficoll-Paque. White cells were separated by centrifugation for 20 minutes at 37°C at 2500 rpms. Cells were then washed twice with PBS and counted with a hemacytometer. Trypan blue was used to test cell viability. Cells used in these experiments, unless processed immediately after separation (resting PBMC), were stimulated with 10 μg/mL PHA and cultured for 72 h., at 37°C in 5% CO_2, in flasks containing RPMI 1640 medium supplemented with 20% fetal bovine serum, 50 μg/mL IL-2, and 50 μg/mL gentamicin. After counting, aliquots of 10^7 cells were pelleted by centrifugation and extracted with 60% methanol at -20 C° overnight. The extracts were dried under nitrogen and stored at -70°C until analysis.

Solid Phase Extraction

The dried PBMC extracts were reconstituted with water and applied to the SAX column previously equilibrated by washing with 5 mL of 0.7 KH_2PO_4 followed by 5 mL of 5 mM KH_2PO_4. After loading the sample, the column was washed with 1mL of 5 mM KH_2PO_4 and 0.5 mL of 0.1 M KH_2PO_4. dTTP was eluted with 0.5 mL of 0.7 M KH_2PO_4.

RESULTS

HPLC Analysis of Cell Extracts

150 μL aliquots of each purified cell extract were injected onto the HPLC column. dTTP was identified by its retention time and by evaluating the

absorption spectra on a diode array detector set up for data acquisition at an absorbance between 200 and 300 nm. Figure 1.a shows the chromatogram of an extract of 10^7 resting PBMC and Figure 1.b shows the same extract spiked with 75 pmols of dTTP. An example of the chromatographic data obtained for PHA-PBMC is shown in Figure 1.c. No coeluting peak was detected in the control samples at the retention time of dTTP. ZDVTP eluted at about 40 minutes. The lowest limit of quantification (LOQ) was 5 pmols/10^6 cells or 100 nM standard solution for 150 μL injection.

Calibration and Linearity

Calibration curve samples were made using resting cell extracts in order to obtain points on the curve below the concentration of dTTP expected in PHA-stimulated PBMC. Thirty spiked resting PBMC samples were measured on six days to produce six different calibration graphs, each with five different concentrations (50, 75, 100, 150, 400 pmols/10^7 cells). The mean slope for these samples was 2786.32 with an r^2 of 0.996 and C.V. of 5.42%. The regression equation was y= 2786.32x+106196.

Recovery, Inter-Assay, and Intra-Assay Coefficient of Variation

The overall recovery was calculated by measuring the peak area of spiked dTTP in PBMC after the whole process of extraction and purification and comparing it with the dTTP peak areas in PBMC extracts spiked only after purification. dTTP was spiked at three different concentrations (60, 125, and 250 pmols/10^7 cells). The mean recovery was 86.8% for the three concentrations (C.V. 8.1%). Inter-assay and intra-assay variability was evaluated using frozen controls at three different concentrations, low, medium, and high (60, 125, and 250 pmols/10^7 cells).

Inter-assay C.V. for PBMC samples was calculated on the basis of five measurements each performed for the three concentrations on five different days. One calibration curve was run each day. The coefficients of variation for dTTP concentrations of 60, 125, and 250 pmols/10^7 cells were 9.8% (5.9, standard deviation), 9.3% (10.7), and 6.9% (16.9) respectively.

The intra-assay C.V. was determined by measuring five different samples for each concentration on four different days. One calibration curve was run each day. Coefficients of variation for dTTP concentrations of 60, 125 and 250 pmols/10^7 cells were 6.4% (3.9, standard deviation), 5.6% (6.7), 7.4% (18.3), respectively.

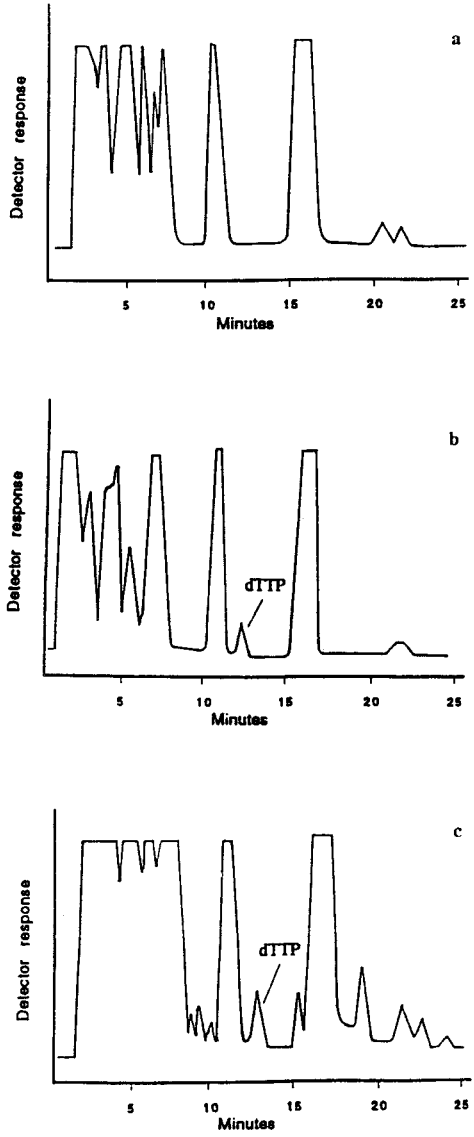

Figure 1. Chromatograms of (a) blank of 10^7 resting PBMC extract, (b) 10^7 resting PBMC extract spiked with 75 pmols of dTTP, (c) an extract of 10^7 PHA activated PBMC. Experimental details are described in the text.

Table 1

dTTP Concentration in PHA-Stimulated PBMC

Subject	pmoL/10^6 Cells (Mean)	S.D.	Experiment
1	23.2	3.5	n=3
2	6.7	0.2	n=2
3	<5	--	n=5

Application

This HPLC method has been used to measure the concentration of dTTP in drug-free PHA-PBMC. We found a marked inter-subject variability: three experiments in three different subjects using the same amount of cells and the same analytical procedure gave very different results, one of the pools being below the LOQ (Table 1).

DISCUSSION

The measurement of the intracellular dTTP pool is useful for the study of drugs interfering with DNA synthesis. Various analytical procedures have been developed for the determination of dNTPs in cell extracts including DNA polymerase assay, radioimmunoassay, and HPLC. The DNA polymerase assay is perhaps the most sensitive technique, but the determination of dTTP is hampered by the inhibition of certain dTTP analogs. The radioimmunoassay provides an attractive alternative for the measurement of dTTP because it is highly sensitive and quite simple to perform. However, because of the cross-reactivity of the antibodies with ZDVTP, it cannot be used when cells are exposed to ZDV. HPLC methods based on anion exchange or reversed-phase columns have been described, but the sensitivity of these methods is low (generally >10 pmol/10^6 cells) and a high number of cells is required when lymphocytes or PBMC are analyzed (> 3-4 x 10^7). Other limitations include long run times using gradient elution and low resolution when isocratic elution is used.

The HPLC assay described here is rapid, simple, sensitive, and relatively inexpensive and is ideally suited for routine investigations. The number of cells required per experiment is relatively low (10^7) and the simple manipulation required provides improved reproducibility as well as a short analysis time. This method could be a valid tool for the analysis of dTTP in tumor cells or

other cell lines containing high dNTP concentrations. In the lymphoblastic CCRF-CEM cell line, for example, the dTTP pool is estimated to be around 80 pmol/10^6 cells.[3] However, the experiments we performed on PBMC showed that the dTTP pool size can vary considerably between subjects. If only a small number of cells is available for analysis, it may not be possible to determine by UV the concentration of dTTP in every donor. In cells exposed to ZDV, the concentration of dTTP is expected to be even lower and a more sensitive method will be required for these measurements.

The variety of assay conditions employed by several groups has resulted in reports of divergent amounts of intracellular deoxynucleotides. The concentration of dTTP in stimulated human lymphocytes have been reported to range from 1.1 to 21.7 pmol/10^6 cells.[17,23,24] In stimulated PBMC preparations from three different donors the mean pool size was 6.18 pmols/10^6 (S.D. 1.24).[10] We believe that the discrepancy of data reported in the literature about the intracellular concentration of dNTPs is not only the result of the different analytical procedures, but also of the natural differences of availability among subjects, (and maybe within the same subject at different time points). Large intersubject variability in dNTP pools could explain the differences in antiviral activity of ZDV and other nucleoside analogs and the differences in side effects observed in patients.

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