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A direct determination of thymidine triphosphate concentrations without dephosphorylation in peripheral blood mononuclear cells by LC/MS/MS

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Abstract

A rapid, sensitive and specific analytical method with minimal sample preparation for the measurement of thymidine triphosphate (TTP) in peripheral blood mononuclear cells (PBMC) by LC/MS/MS has been developed. PBMC were separated from whole blood or buffy coat. The analyte and internal standard were extracted from PBMC with 70% methanol (pH 7.2). These extracts after centrifugation were directly injected onto LC/MS/MS without need for any further sample preparation. The calibration curve was linear over the range 0.8–800 ng/ml. Mean inter- and intra-assay coefficients of variation (CVs) over the range of the standard curve were less than 10%. The overall recovery of TTP was 103.5%. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: TTP; PBMC; LC/MS/MS system

1. Introduction

The concentration of intracellular thymidine triphosphate (TTP) is an important marker of DNA synthesis in cells exposed to nucleoside analog drugs used for human immunodeficiency virus (HIV) [1]. There are several potential ways nucleoside analogs alter intracellular nucleotide pools: (a) inhibition of de-novo biosynthesis of nucleotides; (b) depression of concentrations of substrate essential for DNA polymerase activity; (c) incorporation into cellular nucleic acid following conversion of these drugs to nucleotides. This may perturb intracellular nucleotides acting as regulatory agents of nucleotide synthesis; and (d) antimetabolites can compete for phosphorylation enzymes of endogenous nucleosides. In order to ascertain if one or more of these changes of

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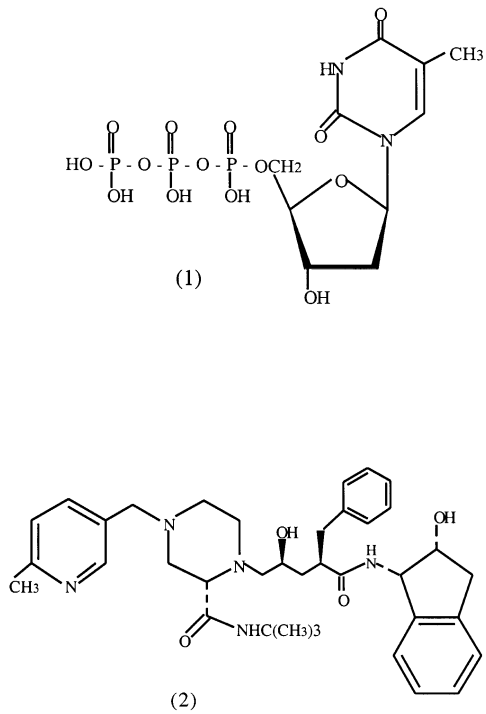

Fig. 1. Chemical structures of TTP (1) and internal standard (2).

cellular functions are taking place during antiviral and anticancer therapy, it is useful to measure the endogenous TTP levels. Further quantitation of TTP in conjunction with quantitation of triphosphate concentrations of drugs such as zidovudine permits a better estimate of antiviral exposure as the zidovudine triphosphate (ZDVTP)/TTP ratio is more representative of antiviral effect.

To accurately determine the low concentration of TTP present in peripheral blood mononuclear cells (PBMC), an analytical method highly sensitive and selective is required. In past years various analytical procedures have been developed for the determination of TTP in cell extracts, including DNA polymerase assay [2–4], radioimmunoassay [5] and HPLC [6–11]. Although the DNA polymerase assay is a sensitive technique, the determination of TTP is hampered by inhibition from other TTP analogs, such as uridine triphosphate. Radioimmunoassay provides an attractive alternative for the measurement of TTP since it is highly sensitive and simple to perform. However, because of cross-reactivity of antibodies with ZDVTP, it cannot be used for samples exposed to zidovudine (ZDV). HPLC methods based on anion exchange or reverse phase columns have been reported, but these methods are characterized by low sensitivity (>48 ng/ml) [6] and require large numbers of PBMC ($1–2 \times 10^7$cells). Other limitations include long run times and time-consuming sample preparation procedures. All HPLC methods require dephosphorylation of TTP to thymidine with phosphatase. To date, no method published has reported use of LC/MS/MS methods for the direct measurement of TTP in human PBMC.

We have developed a rapid, sensitive and selective LC/MS/MS method for the direct determination of TTP in human PBMC without prior dephosphorylation.

2. Experimental

2.1. Materials

Acetonitrile, methanol and water (Fisher Scientific, Fair Lawn, NJ) were HPLC grade. Ammonium formate and ammonium hydroxide solution were from Fisher Scientific (Fair Lawn, NJ). Thymidine triphosphate and methylindinavir sulfate (internal standard, IS), (Fig. 1) were supplied by Sigma (St. Louis, MO) and Merck Research Laboratory (Rahway, NJ), respectively. Phosphate-buffered saline (PBS) was obtained from Sigma. Ficoll Paque was purchased from Pharmacia Biotechnology (Piscataway, NJ). 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).

Stock standard solutions of TTP and Me-IDV were prepared at concentrations of 10 μg/ml in 50% methanol and when stored at -70 °C were stable for at least 4 months. The calibration standards were prepared each day from these stock solutions.

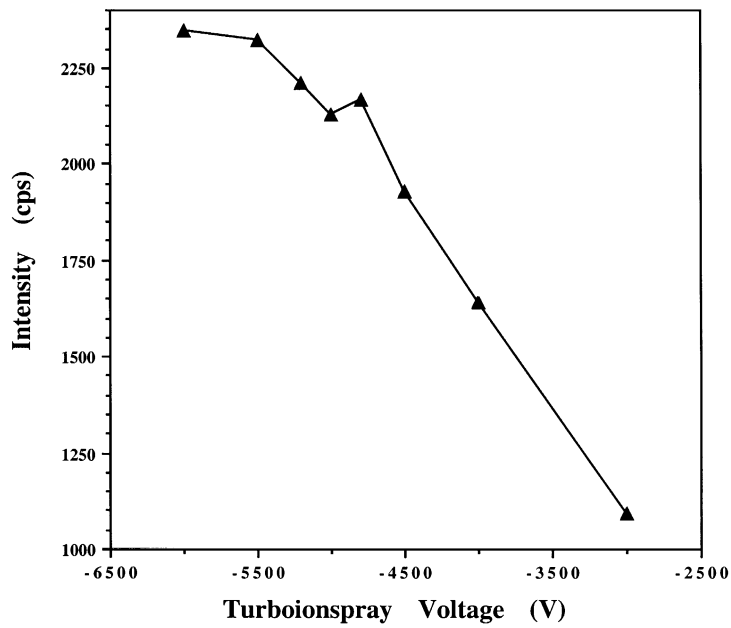

Fig. 2. Optimization of turboionspray voltage.

2.2. Apparatus

Perkin Elmer, Norwalk, CT supplied the PE Biosystems 200 series Autosampler and twin PE Biosystems series 200 micro HPLC pumps. The PE Sciex API 2000 triple quadrupole mass spectrometer with turboion spray sample inlet was purchased from. Perkin Elmer-Sciex (Concord, Ontario, Canada).

2.3. MS–MS Parameters

Mass spectral analyses were accomplished on a Perkin Elmer Sciex Model API 2000 triple quadrupole mass spectrometer, fitted with Turboion spray source and operated in the negative ionization mode. Nebulizer, turbo and certain gases were ultra high purity nitrogen (99.999%) delivered at settings of 42, 55 and 20 psi, respectively. Nitrogen was also used as the collision gas at a setting of 4, which produced an overall analyzer pressure of approximately 3.0×10^{-5} torr. The Turboion spray temperature was maintained at 495 °C. The orifice and focusing ring voltages were -56 and -350 V. The collision energy was 42 V. All the tuning parameters were optimized in order to get the best signal to noise (S/N) ratio for TTP. For the optimization of needle potential, the sensitivity increased with increasing negative voltage (Fig. 2). However a corona discharge appeared at a voltage of -4725 V, therefor a setting at -4500 was used as the optimum needle voltage. The mass spectrometer was operated in MS/MS mode using multiple reaction monitoring (MRM) to detect specific precursor ion to product ion transitions for each analyte. Following chromatographic separation, the pseudomolecular ion ($M-H^-$) for each analyte was selected by the first quadrupole (Q1) and focused into the collision cell (Q2) where it fragmented into product ions. For each $M-H^-$ precursor ion, a distinct product ion was selected by the third quadrupole (Q3). The precursor ion intensity was monitored and subsequently stored by the computer system of mass spectrometer. These mass spectral Q1/Q3 transitions, monitored for TTP and IS, were 481.1/158.8 and 625.6/288.8 (m/z), respectively. A 5 ms delay between scans was found to be adequate for eliminating potential cross talk. The control software, including LC Tune, Multiview, Turboquan, Method Editor and

Experiment Editor was installed on a Macintosh OS 8.5 platform with 64 MB of RAM and a 16 GB hard disk.

Instrument tuning parameters were optimized using a 500 ng/ml solution of TTP prepared in acetonitrile–ammonium formate buffer (pH 7.5, 2 mM) (50:50, v/v). This solution was infused at 10 μl/min into the LC/MS/MS interface using a built in syringe pump.

2.4. Liquid chromatography parameters

The specific liquid chromatographic parameters for the assay were as follows: The column was C8 Waters Sentry reverse phase guard column (3.9 mm × 20 mm) with 5 μm particle size packing. The mobile phase consisted of acetonitrile–2 mM ammonium buffer (pH 7.5) (55:45). The mobile phase was filtered and degassed prior to use. The flow rate was initially kept at 200 μl/min for 1 min, then increased to 400 μl/min for 1.5 min and then returned to 200 μl/min for 1.5 min prior to the injection of another sample. The volume of injection was 30 μl through a 100 μl loop.

2.5. PBMC isolation, extraction and processing of samples

Buffy coat was obtained from healthy volunteers and PBMC were isolated by the Ficoll Paque method. Buffy coat was diluted 1:1 with PBS and 30 ml aliquots were layered onto 15 ml of Ficoll Paque. White cells were separated by centrifugation for 30 min at 37 °C at 2000 rpms. Cells were then washed twice with PBS and counted with a hemacytometer. Trypan blue was used to test cell viability. After counting, aliquots of 10^6 cells were pelleted by centrifugation and extracted with 1 ml of 70% methanol at −20 °C overnight. The extracted PBMC sample was then centrifuged at 12 000 × *g* for 5 min to get rid of the cell residue and give a clear supernatant. A total of 100 μl of the resultant clear extract were transferred to a 1.5 ml eppendorf tube and mixed with 100 μl mobile phase and 50 μl of internal standard. This solution was transferred to an autosampler vial and placed in the autosampler tray for injection onto the LC/MS/MS system.

2.6. Data analysis

Calibration standards in cell extract containing 0.8, 2.0, 4.0, 8.0, 20.0, 40.0, 80.0, 200.0, 400.0 and 800.0 ng/ml were used to establish a single calibration curve with non-weighted linear regression. TTP concentrations versus peak area ratios were plotted in Turboquan, a separate quantitation software program supplied with the mass spectrometer.

2.7. Inter-assay and intra-assay precision

Inter-assay and intra-assay precision was evaluated at 4 concentrations of 1.4, 14, 140 and 600 ng/ml designated as low, medium, high and extra high. For inter-assay precision, six samples of each concentration were assayed on six different days using six sets of standard curves. Means and S.D. were obtained for the calculated analyte concentrations over all six days and coefficients of variation (CV%) for the four different levels (n = 6 for each) were determined. For intra-assay precision, ten control samples from each of four concentrations were assayed with a single calibration curve and coefficients of variation for the calculated analyte concentrations were determined. The accuracy was calculated using the equation.

Accuracy %

= [Calculated Concentration/ Nominal Concentration] × 100

2.8. Limit of quantitation and recovery of TTP

The intra-assay lower limit of quantitation was determined by adding TTP to six aliquots of processed blank cell extract at concentrations of 0.8 ng/ml and assaying them with a set of calibration standards. The mean value of the lower limit of quantitation was determined with the S.D. and the CV%.

Recovery of TTP from cell extracts (5×10^5cells/ml) was assessed by comparing the concentration of TTP from cell extract samples to the concentration of TTP spiked into the mobile phase at the same concentration as in the cell

extract samples. In order to avoid the loss of internal standard (IS) during sample preparation, the IS was added after cell extract samples were centrifuged. Mean recovery was calculated as:

$$\text{Mean Recovery} = \frac{\text{Mean calculated cell extract TTP concentration}}{\text{Mean calculated buffer TTP concentration}} \times 100$$

2.9. Freeze/thaw stability

Three aliquots of 5 ml PBMC extracts (5×10^5 cells/ml) were spiked with TTP at concentrations of 14, 140 and 600 ng/ml. These samples were vortexed and stored at -70 °C. For each freeze/thaw cycle, the containers of spiked PBMC extracts were thawed and six aliquots were removed from each concentration. These samples were processed along with a set of TTP calibration standards. The containers with the remainder of the spiked PBMC extracts were re-frozen at -70 °C. The same process was repeated three times during the period of assay validation. The data for the freeze/thaw stability studies was obtained by calculating the change of TTP concentrations over three cycles of freeze/thaw.

2.10. Storage stability of TTP at -70 °C freezer

A set of six each of frozen PBMC controls at concentrations of 14, 140 and 600 ng/ml and a set of calibration standards were analyzed while these samples were fresh and also after storage at -70 °C freezer for 1 and 2 weeks, and 1 and 4 months. TTP concentrations were compared to determine the stability of the TTP during storage at -70 °C temperature.

3. Results

Resolution and sensitivity was determined by injection of an extracted PBMC standard (Fig. 3). The retention times of TTP and internal standard were 0.8 and 1.5 min, respectively. The linearity was verified over the assay range (0.8–800 ng/ml). This was done by assaying pooled drug-free PBMC extract solution (5×10^5 cells/ml) (which had been previously processed and screened for extraneous peaks) spiked with known amounts of each analyte. A calibration curve was calculated for the analyte using its concentration and the area ratio of the analyte to internal standard over the standard range.

The mean equation for the TTP calibration curve using linear regression analysis, was $y = 0.0087x + 0.0056$ ($R^2 = 0.9995$, $n = 6$). The inter and intra-assay CVs are given in Table 1 with all values reported as less than 10%. The mean extraction recovery for TTP QCs 1.4, 14, 140 and 600 ng/ml was 103.4 (CVs% = 1.9, $n = 12$). The lower limit of quantitation for TTP by this method was 0.8 ng/ml with a CV of 3.5% (six replicates) and a mean accuracy of 102.3%. For the inter-assay LOQ over 6 days the CV was 3.3% and accuracy was 103.8%. For freeze/thaw stability, the mean change was 17% decrease in TTP concentration over three cycles of freeze/thaw (Table 2), and for storage stability, TTP was stable up to 4 months at temperature of -70 °C.

4. Discussion

Quantitative analysis of compounds with high selectivity and sensitivity by LC/MS/MS uses the technique of selected multiple reaction monitoring (MRM). In this mode only a selected MS/MS or collision induced dissociation (CID) transition needs to be monitored. This is now considered the best analytical approach for accurate and highly selective quantitative measurement of drugs and metabolites in complex matrices such as biological samples. In this method the unique pair of ions with m/z 481.1 and m/z 158.8 for TTP and m/z 626.1 and m/z 288.9 for the internal standard, respectively constitute the parent/daughter ion pairs which are scanned alternatively every 200 ms with 5 ms delay between scans. It is the capability of the MRM mode of LC/MS/MS that allows a significant gain in signal strength over noise, and permits the tandem mass spectrometer to detect

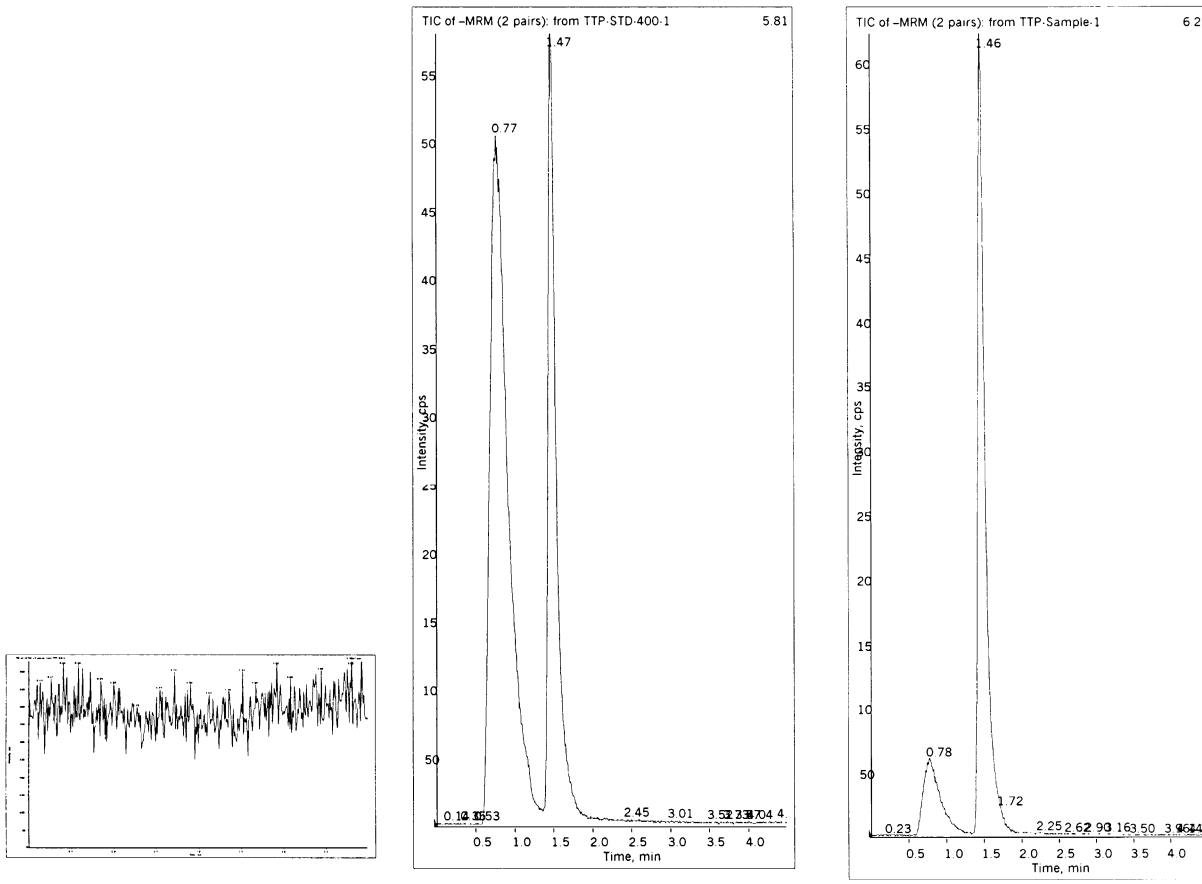

Fig. 3. Chromatogram of (A) blank TTP-free PBMC; (B) TTP-free PBMC (5×10^5 cells/ml) spiked with 400 ng/ml TTP and 400 ng/ml of the internal standard; (C) chromatogram of a sample from a patient following administration of an anti-HIV drug, AZT. The first and second peaks represent TTP and the internal standard, respectively. The injection volume was 30 μl on column.

Table 1
Intra- and inter-assay precision (RSDs) and accuracy data for the determination of TTP in PBMC

Actual value (ng/ml)	Observed value (ng/ml)		CV (%)	
	Intra	Inter	Intra	Inter
1.4	1.41 ± 0.05	1.41 ± 0.08	3.5	5.7
14	14.6 ± 0.47	14.4 ± 0.66	3.2	4.6
140	140.2 ± 5.75	138.8 ± 11.1	4.1	8.0
600	583.0 ± 6.76	588.3 ± 34.2	1.2	5.8

The intra- and inter-assay data represent the mean $\pm$ S.D. of ten and 36 observations, respectively.

Table 2
TTP freeze/thaw stability

Freeze/thaw no. cycles[a]	QC (14 ng/ml)	QC (140 ng/ml)	QC (600 ng/ml)
	Mean calc. conc.		
1	13.9	141.7	606.3
2	14.1	130.3	602.3
3	12.1	115.6	486.4
Change in concentration from cycle 1 to 3 (%)	−12.9	−18.4	−19.8

[a] $n = 6$ samples for each cycle freeze/thaw.

Table 3
Intracellular TTP concentrations of HIV-infected patients on ZDV 300 mg (bid)

Patient no.	Gender	Sampling time (h) after ZDV dose	TTP conc. in PBMC[a] (pmol/million)
1	Male	1	122.7
2	Male	3	63.5
3	Male	6	36.6
4	Female	7	64.6

[a] Intracellular concentrations are means of two measurements.

very small quantities of analytes. This is well illustrated by the results presented in this paper where TTP concentrations as low as 0.8 ng/ml can be measured quantitatively. In HPLC-UV determinations the best LOQ for TTP was 48 ng/ml [6]. Further, the absence of interference from residual endogenous intracellular components or from other concomitantly administered drug analytes is a valuable aspect of LC/MS/MS. This is facilitated by scanning unique ion pairs in preference to single ion monitoring available with LC/MS. The absence of interference from other analytes was clearly seen in the analysis of a TTP buffer sample, which was spiked with thymidine, thymidine mono- and diphosphate, and two nucleotide analogues, 2′-deoxyuridine triphosphate and ZDVTP.

An additional advantage is the number of cells (5×10^5 cells) required, a number significantly lower than that required by HPLC-UV methods ($1–2 \times 10^7$). For the TTP assay, this translates into a blood volume requirement of 1–2 ml compared to 10–20 ml previously reported as necessary for HPLC-UV methods. Therefore this method is applicable to samples collected from pediatric patients.

PBMC were extracted with 70% methanol and the extracts were directly injected onto the system after addition of internal standard, without need of any further complicated and time-consuming sample preparation procedure. To prepare blank matrix for this method, endogenous TTP was decomposed by heating the PBMC extract at 90 °C in hot water for 12–14 h until TTP was undetectable by LC/MS/MS assay.

TTP stability studies showed that there was 12.9–19.8% decrease in TTP concentration over three cycles of freeze/thaw (Table 2). TTP was found to be stable up to 4 months at temperature of −70 °C. Similar results were also obtained from stability studies of ZDVTP, an analog of thymidine nucleotide [12].

This method has been successfully used to determine intracellular TTP concentrations in PBMC samples taken from patients managed with ZDV. Preliminary data are included in Table 3. Considerable variability in TTP concentration is observed among these subjects and may be influenced by the dose of ZDV.

In summary, significant advantages of this method include high selectivity due to absence of interference from other nucleotides, good accuracy and recovery combined with a low LOQ for TTP.

Furthermore the rapid and simple sample preparation, short run time of 4 min and the need for only one million cells as compared to 10 to 20 million cells for HPLC methods are added advantages.

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