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Liquid chromatography–tandem mass spectrometry assays for intracellular deoxyribonucleotide triphosphate competitors of nucleoside antiretrovirals

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Abstract

This study was aimed to apply an LC–MS–MS method previously developed for intracellular nucleoside reverse transcriptase inhibitors-triphosphate (NRTI-TPs) to the determination of natural deoxyribonucleotides (dNTPs) in human peripheral blood mononuclear cells. The LC–MS–MS method was directly used in assay of dATP and dTTP. Interferences by ribonucleotides (rNTPs) prevented direct application to the two other analytes: dGTP and dCTP. A periodate oxidation procedure was therefore optimized to remove rNTPs from the cell medium in order to quantitate dCTP and dGTP. The determination of the intracellular ratio of NRTI-TP/dNTP in HIV-infected patients now involves use of the same chromatographic system for simultaneous assay of several analytes.

Keywords: Deoxyribonucleotide triphosphate; Nucleosides

1. Introduction

Nucleoside reverse transcriptase inhibitors (NRTIs) constitute one of the main classes of antiretrovirals used against human immunodeficiency virus (HIV). These drugs are intracellularly metabolized to their triphosphorylated derivatives (NRTI-TPs), their active form, which inhibit the viral reverse transcriptase [1,2]. These NRTI-TPs therefore compete with natural 2′-deoxyribonucleoside-5′-triphosphates (dNTP5) such as 2′-deoxyadenosine-5′-triphosphate (dATP), 2′-deoxythymidine-5′-triphosphate (dTTP), 2′-deoxycytidine-5′-triphosphate (dCTP) and 2′-deoxyguanosine-5′-triphosphate (dGTP) for binding to reverse transcriptase and then stop viral replication. The chemical structure of each NRTI is related to that of a natural dNTP, without hydroxyl in the 3′ position. Thus, the activity and toxicity of NRTIs depend not only on the intracellular NRTI-TPs but also on the intracellular concentrations of dNTPs and mostly on the concentration ratio between the two entities. Therefore, the methods of quantification of dNTPs, as well as NRTI-TPs, require the highest possible

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sensitivity and selectivity in order to detect the smallest amounts of analytes present in human lymphocytes among the myriad of other ribonucleotides and deoxyribonucleotides.

In the past, three kinds of analytical methods have been developed to quantify intracellular dNTPs: an enzymatic assay using DNA polymerase and synthetic oligonucleotides [3–5], a radioimmunoassay (RIA) [6], and high-performance liquid chromatography (HPLC) methods with UV detection [7–12]. Indirect and direct measurements [13–15] have been described for intracellular NRTI-TPs (such as zidovudine-TP or AZT-TP, stavudine-TP or d4T-TP, lamivudine-TP or 3TC-TP and dideoxyadenosine-TP or ddA-TP). Indirect measurement of NRTI-TPs involves a multiplestep process including solid-phase extraction and dephosphorylation of the NRTI-TPs to NRTIs, which are then quantified by RIA [16–22], enzyme immunoassay (EIA) [23] or liquid chromatography–tandem mass spectrometry (LC–MS–MS) [24,25].

We previously developed LC–MS–MS for intracellular NRTI-TPs by means of ion-pairing HPLC compatible with MS–MS [15,26,27]. The best strategy for better precision and accuracy of this ratio determination (i.e., dTTP vs. AZT-TP and d4T-TP, dCTP vs. 3TC-TP, dATP vs. ddA-TP, dGTP vs. Carbovir-TP or CBV-TP), should be the use of only one analytical method to quantify the main dNTPs and their competitors (NRTI-TPS). LC–MS–MS seems to be the only method of achieving this and allows a priori the determination of the intracellular levels of all these compounds in one analysis.

The aim of this study was to apply a previously developed LC–MS–MS method for simultaneous assay of intracellular NRTI-TPs in treated patients to the four endogenous dNTPs, with a view to obtaining the NRTI-TP/dNTP ratio in HIV-infected and NRTI-treated patients. In addition, several validation results will be presented such as precision, accuracy, limit of quantification.

2. Experimental

2.1. Chemicals

dATP, dTTP, dCTP, dGTP and 2-chloroadenosine-5′-triphosphate (C1ATP) were from Sigma (St. Louis, MO, USA). Zidovudine-TP (AZT-TP), stavudine-TP (d4T-TP), lamivudine-TP (3TC-TP) and ddA-TP were synthesized by C. Guerreiro and R. Sarfati of the Unité de Chimie Organique, Institut Pasteur, Paris, France.

2.2. Materials and reagents

CR312 (Jouan, St. Herblain, France) and Sigma 2K15 (Bioblock Scientific, France) centrifuges were used to isolate peripheral blood mononuclear cells (PBMCs), using Histopaque-1077 (Sigma–Aldrich, reference H8889, St. Quentin-Fallavier, France) or cell preparation tubes (CPTs) (Becton Dickinson, Le Pont de Claix, France) for patients. Human blood from healthy subjects was obtained from EFS (Rungis, France). Cells were counted in Mallassez cells using a microscope with a 20/0.65 objective (glass model, Olympus BH2 microscope, Sony) and stained with 0.4% trypan blue solution from Sigma. *N′*,*N′*-Dimethylhexylamine (DMH) was from Sigma–Aldrich (Milwaukee, WI, USA). HPLC-quality acetonitrile was from SDS (Peypin, France), formic acid ammonium salt and analytical formic acid were from Sigma, and ultrapure water from a Milli-Q_{plus} 185 purifier (Millipore, France). Sodium periodate, rhamnose and 2′-deoxyguanosine were from Sigma and methylamine was from Sigma–Aldrich and analytical orthophosphoric acid from Labosi (Oulchy-Le-Château, France). Nitrogen HP45 was from Air Liquide (Paris, France). Eppendorf 1.5-ml safe lock cones were used to preserve samples.

2.3. Instrumentation

LC–MS–MS analysis was achieved with a HPLC system 1100 (Agilent Technology, Les Ulis, France) connected to an API 3000 tandem mass spectrometer with an electrospray source (Sciex, Applied Biosystems, France) operating in the negative mode. This instrumentation was monitored using Analyst version 1.1 data acquisition and treatment software (Applied Biosystems).

2.4. Standard stock solutions

Standard stock solutions of each dNTP (dATP, dTTP, dCTP, dGTP) and internal standard (2-chloroadenosine triphosphate or C1ATP) were prepared at

concentrations of 100 μg/ml in ultrapure water and stored at −20 °C for up to 1 year. The calibration standards (0.3, 1, 5, 10, 15, 20 pmol) and quality controls (QCs) (2, 8, 17 pmol) were prepared each day from these stock solutions by serial dilution (10, 1, 0.1 μg/ml). A 20-μl volume of the calibration standard and quality control was added to frozen PBMC pellets before cellular lysis by cold 0.05 *M* Tris–HCl–methanol (30:70, v/v).

2.5. Preparation of PBMCs and cell extraction procedures

PBMCs were separated from blood constituents and analytes were extracted according to the previously described procedure [26]. Forty 1-μl volumes of cell extract were injected directly into the LC–MS–MS system for dATP and dTTP and were injected after periodate oxidation in the case of dCTP and dGTP.

2.6. LC–MS–MS conditions

2.6.1. HPLC conditions

A Supelcolgel ODP-50, 150×2.1 mm, 5 μm particle size column (Supelco, St. Quentin-Fallavier, France) was used and the mobile phase was composed of two buffers A and B. Buffer A was composed of 50% 6 m*M* DMH dissolved in 20 m*M* ammonium formate, pH 5 (solution A) and 50% ultrapure water. A gradient was obtained with a second buffer (B) containing 50% solution A and 50% acetonitrile. The pH of the mobile phase was approximately 11. Two gradients were employed: gradient 1 was used for dATP, dTTP and dCTP analysis and gradient 2 for dGTP analysis. Gradient 1 was as follows: from $t=0$ to $t=2$ min, A–B (70:30), followed from $t=2$ to $t=\sim12$ min by a linear gradient from 70:30 to 35:65, then from $t=12$ to $t=13$ min by a linear gradient from 35:65 to 0:100. Column washing with 100% B was performed over 3 min and the equilibration time before the next analysis was set at 10 min so that the run time was 26 min. Gradient 2 was as follows: from $t=0$ to $t=2$ min A–B (70:30), followed from $t=2$ to $t=25$ min by a linear gradient from 70:30 to 35:65, then from $t=25$ to $t=26$ min by a linear gradient from 35:65 to 0:100. Column washing with 100% B was performed over 3 min and the equilibration time before the next analysis was set at 10 min so that the run time was 39 min. The flow-rate was set at 0.3 ml/min and the temperature was constantly kept at 30 °C. The internal standard was C1ATP.

2.6.2. MS–MS conditions

After chromatographic separation, the mobile phase was directly introduced into the mass spectrometer via an electrospray ionzation (ESI) source operating in the negative mode. At the end of each analysis, ultrapure water–acetonitrile (50:50, v/v) was introduced using an additional LC pump to rinse the ESI source at 0.3 ml/min. Nitrogen was employed as nebulizing, curtain and collision gases. The acquisition mode used was multiple reaction monitoring (MRM). The dwell time for each transition was 0.15 s. The mass spectrometer parameters for each dNTP were adjusted by infusing a solution of each dNTP at the concentration 0.1 μg/ml prepared in a mixture of buffer A–buffer B (60:40, v/v). The MRM transitions were: 489.9→158.9, 465.9→158.9, 480.9→158.9, 505.9→158.9 and 540.2→158.9 for dATP, dCTP, dTTP, dGTP and C1ATP, respectively. Some mass spectrometer parameters were identical for all dNTPs. The nebulizing gas reached 1.23 l/min, the curtain gas 1.25 l/min and the collision gas 4 l/min. The ionspray voltage was set at −4000 V and the temperature at 450 °C. Cone voltage (CV), focusing potential (FP), collision energy (CE) and collision cell exit potential (CXP) were optimized for each dNTP. The cone voltages were set at −65, −60, −115, −80 and −75 V for dATP, dTTP, dGTP, dCTP and C1ATP, respectively. The values of the collision energy were −40, −40, −40, −35 and −45 V for dATP, dTTP, dGTP, dCTP and C1ATP, respectively. The collision cell exit potentials were −8, −10, −8, −8, −15 V for dATP, dTTP, dGTP, dCTP and C1ATP, respectively. The focusing potentials were −250, −230, −390, −310, −250 V for dATP, dTTP, dGTP, dCTP and C1ATP, respectively.

2.7. Periodate oxidation procedure

The periodate oxidation procedure for the degradation of rNTPs interfering with dGTP and dCTP was optimized from a previously described procedure [8]. A 65-μl volume of a 5 m*M* aqueous solution of deoxyguanosine and 45 μl of a 0.5 *M* aqueous solution of sodium periodate were added to

each cell extract (100 μl). The samples were gently mixed and incubated at 37 °C for 5 min. A 60-μl volume of a 4 *M* aqueous solution of methylamine (adjusted to pH 7.5 with phosphoric acid) and 20 μl of a 1 *M* aqueous solution of rhamnose were then added. The samples were gently mixed and incubated at 37 °C for 30 min. The samples were then injected into the LC–MS–MS system for dCTP and dGTP measurement.

2.8. Method validation

Calibration curves were established over a linear concentration range from 30 to 2000 fmol/10^6 cells, according to dNTP levels described in previous studies in vitro [7,8,28–31]. Because of the matrix effect, which occurs in most bioanalytical method such as LC–MS–MS assays, calibration curves were plotted using PBMCs. PBMC pellets, containing 10×10^6 cells, used for standards and QCs were assumed to contain the same amount of endogenous dNTPs because they were prepared from the same blood. These endogenous dNTP quantities were taken into account for clinical samples by subtracting the blank from the calibration curve. For each analyte, a calibration curve was constructed using calibration standards at six concentrations (30, 100, 500, 1000, 1500, 2000 fmol/10^6 cells). The lines of best fit were determined using regression analysis based on the ratios of the peak areas of the analytes to the internal standard (C1ATP). Accuracy was expressed as the absolute percent deviation from the theoretically determined concentration (% difference). Precision was evaluated as the relative standard deviation (RSD) of the mean expressed as a percent for each sample.

To evaluate the quantification limit, the endogenous presence of dNTPs in the blank cells must be taken into account. The limit of quantification was therefore assumed to be the smallest quantity of analyte that could be differentiated from the endogenous quantity. Therefore, a 95% confidence interval was calculated ($CI=m\pm t_\alpha\sigma/\sqrt{n}$) for the ratio of the peak areas of the analytes to the internal standard (dNTP/I.S.) in blank cells ($n=5$). The highest limit value of this confidence interval was compared to that of the peak area ratio of dNTP/IS in PBMC pellets spiked with 0.3 pmol, arbitrarily assumed to be the dNTP limit of quantification. Six spiked samples at the limit of quantification were then quantified with a calibration curve and QCs to determine the precision of the quantification limit for each dNTP.

3. Results and discussion

3.1. Application of LC–MS–MS to the analysis of dNTPs

In order to establish the retention time of each dNTP, standards were prepared in PBMCs and injected into the LC–MS–MS system. Cone voltage, focusing potential, collision energy and collision cell exit potential were optimized for each analyte. The mobile phase, comprising DMH as ion-pairing reagent, was selected at high pH in order to improve the sensitivity in the negative mode. Despite the previous use of DMH in the positive mode by others [14], this possibility was not tested by us. The retention times of dATP, dTTP, dCTP, dGTP and C1ATP were 10.2, 10.5, 8.5, 10.1 and 11.7 min, respectively. The chromatograms of each dNTP are shown in Fig. 1. No interfering peak was noted when the previously developed LC–MS–MS [15,26] was applied to dATP and dTTP. However, interference in the MRM of dCTP and dGTP prevented their measurement. The chromatographic peak of dCTP is disturbed (broad, split peak) and that of dGTP is fully masked by an endogenous compound. This compound was identified as adenosine triphosphate or ATP (data not shown) by virtue of its mass spectrum and retention time. dGTP and ATP have the same molecular mass (507 g/mol) and consequently the same major daughter ion as a triphosphate nucleotide (m/z 159). Moreover, this ribonucleotide is one hundred times more concentrated than the dNTPs and their chromatographic behaviors are very similar when an ion pairing agent is used in the mobile phase. A sample treatment was therefore required to eliminate the ATP interference. Two kinds of sample treatments to remove rNTPs from cell extracts have been described: periodate oxidation [7,8,10,32–34] and boronate affinity chromatography [35]. Boronate affinity chromatography was tested. However, direct coupling with mass spectrometry

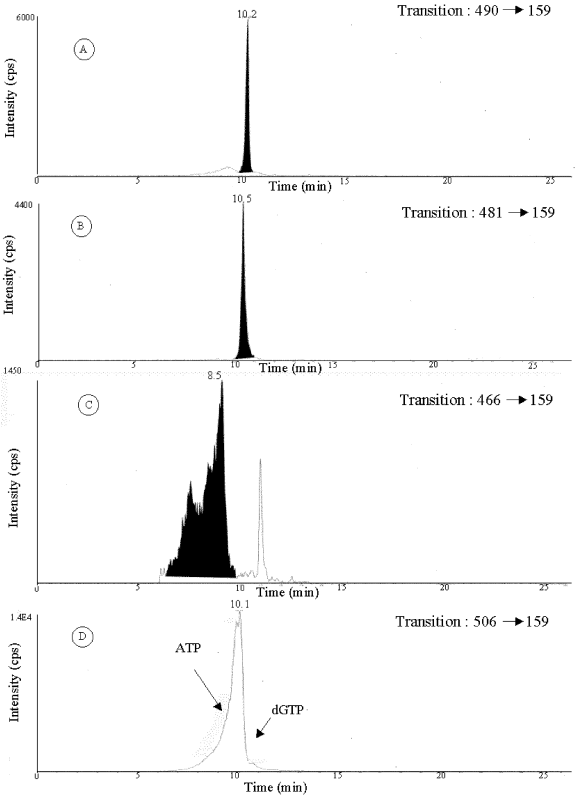

Fig. 1. Chromatograms of (A) dATP, (B) dTTP, (C) dCTP and (D) dGTP in PBMCs (10×10^6 cells/ml) after direct transposition of the LC–MS–MS method developed for antiretrovirals NRTI-TP.

was not possible because of the high salt concentration of the mobile phase [36–39]. Consequently, the periodate oxidation was chosen to optimize the ATP destruction procedure.

3.2. Optimization of periodate oxidation procedure

The periodate oxidation procedure leads to the oxidation of rNTPs by sodium periodate and to its degradation products by methylamine [40–42]. Because the internal standard is an rNTP which may be degraded by this procedure [8], all the remaining sodium periodate must be inactivated before the addition of the internal standard. Rhamnose, which inactivates the remaining sodium periodate, was therefore also added. One of the main drawbacks of this sample treatment is a partial loss of dGTP, since some dicarbonyl compounds were formed by the periodate oxidation and reacted with dGTP [8]. The addition of deoxyguanosine may circumvent this problem because it could compete with dGTP in this type of reaction. Each reactant was optimized to eliminate most of the ATP and to maintain dGTP levels constant. The decrease in ATP was quantified according to the ratio of the ATP peak area to the internal standard. The result of this optimization is illustrated by the chromatogram in Fig. 2. The best result was obtained with a mixture containing 45 μl of a 0.5 *M* aqueous solution of sodium periodate, 65 μl of a 5 m*M* aqueous solution of deoxyguanosine, 20 μl of a 1 *M* aqueous solution of rhamnose and 45 μl of a 4 N aqueous solution of methylamine. After the periodate oxidation, the quantity of ATP dropped by a factor of 3000.

Because of the addition of 65 μl of a 5 m*M*

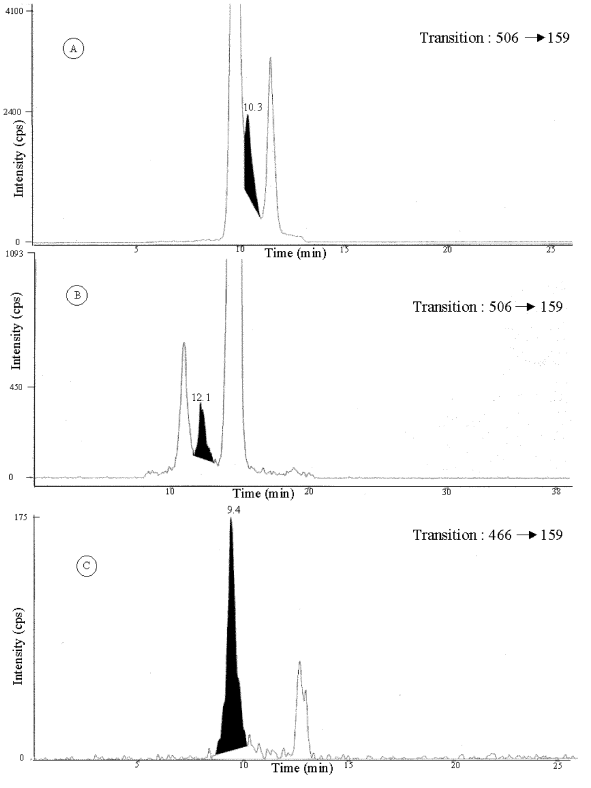

Fig. 2. Chromatograms after chemical treatment with periodate oxidation of (A) dGTP analyzed with gradient 1, (B) dGTP analyzed with gradient 2 and (C) dCTP analysed with gradient 1 in PBMCs (10×10^6 cells/ml).

aqueous solution of deoxyguanosine, the intracellular dGTP was not degraded and its quantity in PBMCs remained constant. Thus, the peak of endogenous dGTP could be detected in PBMC samples. However, the resolution [calculated by $R=2(Tr_2-Tr_1)/(w_1+w_2)$] between the remaining ATP (0.03%) and dGTP was insufficient (0.64) (Fig. 2). It was therefore necessary to enhance the chromatographic resolution between the dGTP and ATP peaks. One possibility among others (pH modification, counterion concentration) was to decrease the proportion of acetonitrile in buffer B. In this case, the retention of the analytes was increased and, consequently, their chromatographic separation was improved. To optimize the resolution, several gradients were tested in comparison to gradient 1, associated with a run time as short as possible. The best resolution was achieved with gradient 2 (Fig. 2), with which the resolution increased from 0.64 to 0.95. This was the best compromise between a satisfactory resolution and the run time (39 min). Only dGTP was analyzed with gradient 2 because an increase in resolution was not necessary for the other three endogenous dNTPs (dATP, dCTP, dTTP), for which the LC–MS–MS conditions were the same as for NRTI-TPs [26]. To allow complete elimination of ATP from the dGTP chromatogram, the volume of reagent can be fine tuned using the sequential simplex methods (MultiSimplex 2.1 software from Grabitech Solutions, Sweden). The first results obtained with this software indicate that ATP should be totally eliminated using slightly modified sodium periodate and methylamine volumes. This needs to be clarified and eventually optimized.

The efficacy of the dCTP chromatographic peak (Fig. 2) was improved by the periodate oxidation, which suggests that the chromatographic separation of dCTP was previously disturbed by endogenous rNTPs whose determination is necessary to allow an accurate determination of this compound.

It is crucial to verify the influence of the sample treatment by periodate oxidation on the other endogenous (dATP and dTTP) or exogenous (ddA-TP, 3TC-TP and d4T-TP) analytes. With this aim in mind, PBMCs were spiked with ddA-TP, 3TC-TP and d4T-TP and this experiment was repeated twice. One sample was treated by periodate oxidation and the other not. The results are presented in Table 1.

Table 1
Influence of periodate oxidation on dNTPs (dATP and dTTP) and some NRTI-TPs (3TC-TP, ddA-TP, d4T-TP)

Compound	Difference between with and without periodate oxidation (%)
dATP	−9.5
dTTP	−58.5
ddA-TP	−22.9
3TC-TP	−73.9
d4T-TP	−42.2

The LC–MS–MS signal intensity of each analyte (except dCTP) was influenced by this chemical treatment. The difference between the treated sample and the control varied from −10% for dATP to −74% for 3TCTP. These results differ from those described by Garrett and Santi [7] since they indicate that all dNTPs were stable during periodate-methylamine treatment. To avoid this loss of analytes and thus a loss of sensitivity, the cell extract was divided in two parts: one part was directly injected into the LC–MS–MS system for dATP, dTTP and NRTI-TP measurement and the other was treated by periodate oxidation for dGTP and dCTP before the injection into the LC–MS–MS system. Then, dGTP was analyzed with gradient 2 and dCTP with gradient 1. It has also been reported that periodate oxidation forms oxidized products that have the same retention time as analytes and could interfere with dNTP peaks in HPLC–UV [7,29]. The sample extract was therefore purified by means of a miniature low-pressure strong anion-exchange (SAX) column to solve this problem [7,8,19,32–34]. The selectivity of our method (lack of oxidized product peaks) was such that no additional step was necessary.

3.3. Validation results

Calibration curves for dATP, dTTP, dCTP and dGTP were well described by unweighted linear regression analysis. The average slopes of the calibration curves with $n=4$ were [mean (RSD)]: 0.092 (15.4%), 0.0996 (6.4%), 0.0618 (5.8%) and 0.0362 (66.5%) for dATP, dTTP, dCTP and dGTP, respectively. Over the range 0.3–20 pmol dNTP, the regression coefficients r^2 of the calibration curves for the four dNTPs were better than 0.99.

QCs were prepared at three concentrations: 200, 800, 1700 fmol/10^6 cells. Five QC replicates were analyzed for intra- and inter-day precision and accuracy. All precision and accuracy results are summarized in Table 2. They are calculated with five replicates×three concentrations. These results are within the recommended ranges (RSD<15%), except for the dTTP inter-day precision and dCTP intra-day precision.

The limit of quantification (LOQ) was measured to be 0.3 pmol (0.03 pmol/10^6 cells) for dTTP, dCTP and 0.4 pmol (0.04 pmol/10^6 cells) for dATP. The precision was 7.6% for dTTP, 15.7% for dCTP and 6.0% for dATP and the precision of dGTP is still to be determined. The sensitivity of this method was superior to that of DNA polymerase assay (0.1 pmol/10^6 cells) [5] and RIA (1.3 pmol/10^6 cells with a minimum of 1×10^4 PBMCs) [6] and avoids the use of radiolabeled reagents. Moreover, this analytical method allows the simultaneous quantification of NRTI-TPs and dNTPs from one sample, which is useful for accurate determination of the NRTI-TP/dNTP ratio.

The stability of nucleotide triphosphates, such as dNTPs or NRTI-TPs, is mainly related to the triphosphate group. The preparation and storage of nucleotides contained in biological samples has been studied in terms of stability, and it is reported that nucleotides must be stored either in frozen whole cells or in cell lysis extract [44] at −80 °C. During our previous experiments on d4T-TP and ddA-TP, we showed that nucleotides are stable before (in the blood stored at 4 °C) and during the analytical process (cell washing at 4 °C) and in the LC autosampler (at 4 °C) [15,43]. The same precautions were followed during sample processing for dNTP measurement. This needs to be formally confirmed to comply with international recommendations [45].

3.4. dNTP and NRTI-TP concentrations in cell extracts from patients infected by HIV and treated with NRTIs

Cell samples were collected from eight patients infected by HIV and treated with NRTIs (all subjects gave their informed consent for participation in this study) and analyzed by the use of this method to determine dNTP and NRTI-TP levels and the NRTI-TP/dNTP ratio.

Only one of the eight patients analyzed was treated with a single NRTI. The other patients were receiving two or three NRTIs. The results are presented in Table 3 and the intracellular dGTP concentrations were also determined even if all these patients were not treated by abacavir (its competitor). The dGTP values varied from 0.40 to 0.89 pmol/10^6 cells. The four dNTP levels found are the first described for in vivo samples, as previous data were established using cell culture extracts [29,30]. The ratios are also the first available for d4T-TP/dTTP, ddATP/dATP, AZT-TP/dTTP in patients. The value of the 3TC-TP/dCTP ratio in treated patients has previously been calculated as around 10 [46], which is in agreement with our results (see Table 3). Intracellular 3TC-TP concentrations were over 1000-times more concentrated than the active metabolites of other NRTIs. This might suggest a lower affinity of the reverse transcriptase for a comparable therapeutic efficacy. Consequently, the 3TC-\TP/dCTP ratio was near to 10 even though the other ratios were close to 0.01 (Table 3). NRTI-TPs therefore competed effectively with the dNTPs in binding to the reverse transcriptase. These data should, of course, be confirmed using samples from more patients.

In conclusion, the assays described here offer significant advantages over the previous methods for

Table 2
Precision and accuracy of the analytical method for each dNTP (n=5 replicates×3 concentrations)

Precision	dGTP	dATP	dTTP	dCTP
Intra-day precision (mean RSD, %)	10.3	11.4	11.0	15.5
Inter-day precision (mean RSD, %)	12.8	11.4	17.3	9.1
Intra-day accuracy (mean, %)	113	109	105	96
Inter-day accuracy (mean, %)	111	122	114	103

Table 3
Determinations of dNTPs and of the NRTI-TP/dNTP ratio in samples from HIV-infected patients

	Patient	INTI-TP (pmol/10^6 cells)	dNTP (pmol/10^6 cells)	Ratio
Patients treated with d4T	2	0.0876	3.90	0.022
competitor: dTTP	5	0.0543	3.20	0.017
Patients treated with AZT	4	0.052	1.80	0.029
competitor: dTTP	8	1.24	6.70	0.186
Patients treated with 3TC	2	49.2	1.79	27.4
competitor: dCTP	4	28.3	2.40	11.8
	5	36.1	2.87	12.6
	6	2.5	0.55	4.5
	7	4.95	0.66	7.5
	8	30.5	1.71	17.9
	9	35.8	3.97	9.0
Patients treated with ddI	4	0.0597	1.40	0.041
competitor: dATP	6	0.023	2.60	0.009
	7	0.0495	3.90	0.013

the quantification of endogenous dNTPs. This analytical method is sensitive for clinical quantification of dNTPs. Both dATP and dTTP can be quantified simultaneously with three competitors of the reverse transcriptase of HIV (NRTI-TP5). dCTP is analyzed using the same chromatographic system but after a chemical treatment of the sample. dGTP needs to be submitted to the chemical treatment and is analyzed using a slightly modified HPLC gradient program. The NRTI-TP/dNTP ratio, which should accurately reflect the toxicity and efficacy of NRTIs, can therefore now be easily determined.

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