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Simultaneous Quantification of Intracellular Natural and Antiretroviral Nucleosides and Nucleotides by Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

Nucleoside reverse transcriptase inhibitors (NRTI) require intracellular phosphorylation, which involves multiple enzymatic steps to inhibit the human immunodeficiency virus type 1 (HIV-1). NRTI-triphosphates (NRTI-TP) compete with endogenous 2'-deoxyribonucleosides-5'-triphosphates (dNTP) for incorporation by the HIV-1 reverse transcriptase (RT). Thus, a highly sensitive analytical methodology capable of quantifying at the low femtomoles/ 10^6 cells level was necessary to understand the intracellular metabolism and antiviral activity of NRTIs in human peripheral blood mononuclear (PBM) cells and in macrophages. A novel, rapid, and a reproducible ion-pair chromatography–tandem mass spectrometry (MS/MS) method was developed to simultaneously quantify the intracellular phosphorylated metabolites of abacavir, emtricitabine, tenofovir disoproxil fumarate, amdoxovir, and zidovudine, as well as four natural endogenous dNTP. Positive or negative electrospray ionization was chosen with specific MS/MS transitions for improved selectivity on all the compounds studied. The sample preparation, the ion-pair reagent concentration, and buffer composition were optimized, resulting in the simultaneous quantification of 13 different nucleotides in a total run time of 30 min. This novel method demonstrated optimal sensitivity (limit of detection 1–10 nM for various analytes), specificity, and reproducibility to successfully measure NRTI-TP and dNTP in human PBM cells and macrophages.

Nucleoside reverse transcriptase inhibitors (NRTI) remain key components of highly active antiretroviral therapy (HAART) for first line therapy for the treatment of human immunodeficiency virus type 1 (HIV-1). Understanding their uptake and metabolism in human peripheral blood mononuclear (PBM) cells and macrophages can provide insights on their potency as antiviral agents.^{1–4} Although plasma levels of nucleosides are important in terms of maximum concentration (C_{max}), which represents the loading dose, it is well established that the triphosphate (TP) of the NRTI, which interacts with the viral polymerase, is the active metabolite and more relevant for this class of drugs. Thus, accurate measurement of the intracellular concentration of nucleoside triphosphates is essential.^{5–8}

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SUPPORTING INFORMATION AVAILABLE Values of chromatography parameters (N_e , k' , T_f , and area) for 3TC, 3TC-MP, 3TC-TP, DXG, DXG-MP, DXG-TP, TFV, TFV-DP, ZDV, TP, (–)-FTC-TP, and CBV-TP. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The objective of this work was to develop and validate an accurate, rapid, and a highly sensitive method for the simultaneous quantification of the phosphorylated metabolites of NRTIs such as abacavir (ABC), emtricitabine [(-)-FTC], tenofovir disoproxil fumarate (TDF), amdoxovir (DAPD), and zidovudine (ZDV) as well as the natural endogenous 2'-deoxyribonucleosides-5'-triphosphates (dNTP). TDF, DAPD, and ABC-MP are bioconverted to tenofovir (TFV), 9- β -D-(1,3-dioxolan-4-yl)guanine (DXG), and (-)-carbovir-monophosphate (CBV-MP), respectively, prior to being converted to their NRTI-TP forms.⁹⁻¹¹ The discrimination between the successive intracellular metabolites resulting from the conversion of NRTI to NRTI-monophosphate (MP), NRTI-diphosphate (DP), and NRTI-TP was required, since the NRTI-TP are responsible for antiviral efficacy, while the NRTI-MP such as ZDV may be associated with toxicity.^{12,13} Separation was also essential since NTPs generate breakdown products in the electrospray probe, which are mainly the corresponding DP, MP, and nucleoside forms,¹⁴ which could lead to errors in the quantification of these metabolites. A high sensitivity of detection was also required, since the levels of natural endogenous dNTP in macrophages and in resting PBM cells are approximately 70 and 315 fmol/10⁶ cells, respectively.¹⁵ A weak anion-exchange (WAX) method was previously utilized by our group¹⁶ and others;^{17,18} however, the nucleosides were eluted in the void volume of the column and therefore the method had to be modified.

The measurement of intracellular phosphorylated NRTI by liquid chromatography–tandem mass spectrometry (LC–MS/MS) remains difficult. The first obstacle is the high polarity induced by the phosphate moieties of nucleotides rendering their retention by reverse-phase chromatography a challenge. Generally, a volatile solvent is preferred to avoid ion suppression in the mass spectrometer. Thus, two specific end-capped columns such as PFP propyl and Hypersil GOLD-AQ were selected for their ability to retain polar and basic compounds, including phosphopeptides, without utilizing nonvolatiles buffers or extreme pH.^{19,20} It is interesting to define whether these columns would allow the separation of a wide range of compounds with different polarity and molecular weight similar to previously reports by ion-pair chromatography.²¹ The second obstacle is the interferences produced by endogenous nucleotide triphosphates and/or other components. For example, ATP, dGTP, and ZDV-TP have the same molecular weight (507 g/mol) and similar fragmentation pattern in negative ionization mode,²² and therefore, can interfere with respective measurements.²³ Similarly, lack of MS/MS specificity occurs between ZDV-MP, AMP, and dGMP. Thus, it was necessary to optimize the ionization mode, the column temperature, the composition and the pH of the mobile phase to obtain optimal separation and selectivity to avoid overlapping signals. Our approach applied the specificity offered by the positive ionization mode for most compounds, as suggested by Pruvost et al.²⁴ while using the specific fragmentation of ZDV-TP in the negative ionization mode as described previously by Compain et al.²³ This switch in polarity from positive to negative ionization was made possible by a highly optimized separation. This optimization was essential, mainly because several antiretroviral combination modalities, such as Combivir include ZDV. The third obstacle resides in the ability of the phosphate groups of the nucleotides to interact with stainless steel, causing a peak tailing on chromatograms.²⁵ For this reason, all stainless steel devices were generally replaced by PEEK [poly(aryl-ether-ether-ketone)] materials in the instruments. However, we found that this substitution could not be undertaken in the electrospray probe, where the metal needle has proved to enhance detection sensitivity. Asakawa et al.²⁵ showed that the use of ammonium hydrogen carbonate in the mobile phase prevented peak tailing and was compatible with LC–MS analysis. Furthermore, this buffer is suitable for the analysis of basic compounds.²⁶ Ammonium phosphate²⁷⁻²⁹ and ammonium hydroxide³⁰ were successfully used to prevent the interactions between stainless steel and the phosphate groups of nucleotides. Ammonium phosphate buffer is known to be semivolatile, causes ion-suppression, and should be used at low concentration. Moreover, this buffer must be combined with the appropriate ion-pair reagent. Alkylamines,

tetraalkylammonium salt or tetrabutyl ammonium hydroxide have been successfully used for the quantification of nucleosides and nucleotides particularly with an ammonium phosphate buffer, despite their incompatibility with MS detection.^{27,31} Among the available alkylamines: 1,5-dimethylhexylamine (1,5-DMHA)²⁴ was preferred to *N,N*-dimethylhexylamine (*N,N*-DMHA)³² for enhanced ionization in the positive mode.²⁴ Hexylamine (HMA) was also successfully used in association with the negative ionization mode.^{33,34} By testing these different conditions, we were able to optimize and validate an LC–MS/MS method for the simultaneous quantification of 13 endogenous nucleoside/nucleotides and analogues.

EXPERIMENTAL SECTION

Chemicals and Reagents

Ammonium phosphate [(NH₄)₃PO₄] and *N,N*-DMHA were purchased from Sigma Aldrich, (St. Louis, MO). Hexylamine (HMA) and ammonium formate (NH₄COOH) were purchased from Acros Organics (Morris Plains, NJ). HPLC-grade methanol, acetonitrile, ammonium hydroxide (NH₄OH), and ammonium acetate (NH₄CH₃COOH) were obtained from Fisher Scientific International, Inc. (Pittsburgh, PA). Ultrapure water was generated from Elga Ultrapurelab equipped with U.S. filters. Formic acid (HCOOH) and ammonium hydrogen carbonate (NH₄HCO₃) were purchased from Fluka (St. Louis, MO). High-pressure nitrogen and ultrahigh-purity-high-pressure argon were purchased from Nexair, LLC (Suwannee, GA). Nucleotides were obtained commercially from Sigma Aldrich (St. Louis, MO) or were synthesized in our laboratory. Isotopically labeled nucleotides, [¹³C¹⁵N]dATP, [¹³C¹⁵N]dGTP, [¹³C¹⁵N]dCTP, and [¹³C¹⁵N]TTP used for endogenous dNTP quantification were purchased from Sigma Aldrich (St. Louis, MO). Nucleosides and nucleotides were at least 98% pure.

Cell Isolation and Cell Treatment Procedure

Human PBM cells were isolated from buffy coats derived from healthy donors using a Histopaque technique. Cells were cultured in flasks for 18 h, 37 °C, 5% CO₂. Suspension cells (PBM cells) were removed and phytohemagglutinin (PHA) stimulated (6 µg/mL) for 72 h prior to treatment with NRTI. Adherent cells (monocytes) were exposed to 1 000 U/mL monocyte colony stimulating factor (m-CSF) for 18 h to confer differentiation. Macrophages were maintained for 7 days before treatment with NRTI. Cells were exposed to media containing 10 µM of DXG, TDF, ZDV, (–)-FTC, or ABC for 4 h at 37 °C. Cells were washed with 1× PBS and centrifuged at 350g for 10 min at 4 °C and counted using a Vi-cell XR counter (Beckman Coulter, Fullerton, CA; viability >98%). Cell pellets were resuspended in 1 mL of ice cold 70% methanol and subjected to vortexing for 30 s. The tubes were then kept at –20 °C for at least 1 h to allow for nucleotide extraction. A total of 10 µL/10⁶ cells of lamivudine (3TC), 3TC-MP, and 3TC-TP (internal standard, IS) were added to the suspension in order to obtain a final concentration of 100 nM for all three IS upon sample reconstitution.

Calibration Curve Preparation and LC–MS/MS Analysis

Calibration standards covering the range of 0.5–1 000 nM were prepared by adding appropriate volumes of serially diluted stock solutions to a nontreated cell pellet. Six calibration concentrations (0.5, 1, 5, 10, 50, and 100 nM) and three quality control standards (1, 10, and 100 nM) were used to define the calibration curve and for partial assay validation, respectively for DXG, DXG-MP, DXG-TP, TFV, TFV-DP, (–)-FTC-TP, and CBV-TP. Six calibration concentrations (5, 10, 50, 100, 500, and 1 000 nM) and three quality control standards (10, 100, and 1 000 nM) were used to define the calibration curve and for partial assay validation, respectively, for ZDV-MP, ZDV-TP, [¹³C¹⁵N]dATP,

[$^{13}\text{C}^{15}\text{N}$]dGTP, [$^{13}\text{C}^{15}\text{N}$]dCTP, and [$^{13}\text{C}^{15}\text{N}$]TTP. Calibration curves were calculated using the ratio of the analyte area to the internal standard area by linear regression using a weighting factor of $1/x$. The samples were centrifuged for 10 min at 20 000g to remove cellular debris, and the supernatant was evaporated until dry under a stream of air. Prior to analysis, each sample was reconstituted in $100\ \mu\text{L}/10^6$ cells of mobile phase A and centrifuged at 20 000g to remove insoluble particulates.

Instrumentation

The HPLC system was a Dionex Packing Ultimate 3000 modular LC system and comprised of two ternary pumps, a vacuum degasser, a thermostatted autosampler, and a thermostatted column compartment (Dionex Corp., Sunnyvale, CA). A TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron Corp., Waltham, MA) was used for detection. Thermo Xcalibur software version 2.0 was used to operate the HPLC and the mass spectrometer and to perform data analysis.

Chromatography Columns

Three columns were evaluated: PFP (pentafluorophenyl) propyl, 50 mm $\times$ 1 mm, 3 μm particle size, (Restek, Bellefonte, PA); Hypersil GOLD-AQ (aqueous), 100 mm $\times$ 1 mm, 3 μm particle size; and Hypersil GOLD-C18, 100 mm $\times$ 1 mm, 3 μm particle size (Thermo Electron Corp., Waltham, MA).

Ion-Pair Method Development Using Hypersil GOLD-C18 Column

Ammonium phosphate buffer (1 and 2 mM) and ammonium hydrogen carbonate (10 and 20 mM) were assayed by preparation of four separate buffers containing 3 mM HMA. The pH 7, 8, 9.2, and 10 were tested using four separate buffers containing 10 mM ammonium hydrogen carbonate and 3 mM HMA. To obtain pH 7 and 8 and pH 10, the buffer was adjusted with formic acid and ammonium hydroxide, respectively. No adjustments were required to obtain pH 9.2. Finally, the HMA concentration at 0, 3, 6, 9, and 12 mM was optimized using a buffer consisting of 1 mM ammonium phosphate. Standard calibration solutions prepared at 50 nM were used. The factors that were considered to discriminate the general quality of the chromatography for all analogues were the tailing factor ($T_f = A_{5\%} + B_{5\%}/2A_{5\%}$, where A and B are the left and the right peak width at 5% of height, respectively), the peak capacity ($k' = t_r - t_o/t_o$, where t_r is the retention time of the compound of interest and t_o is the retention time of an unretained compound), the effective plate number [$N_e = 5.54(t_r - t_o/w_{1/2})$, where $w_{1/2}$ is the peak width at half height] and the peak area.

Optimized Ion-Pair Method Used for Partial Validation

A linear gradient separation was performed on the Hypersil GOLD-C18 column (Thermo Electron, Waltham, MA). The mobile phases A and B consisted of 2 mM ammonium phosphate buffer containing 3 mM HMA and acetonitrile, respectively. The flow rate was maintained at 50 $\mu\text{L}/\text{min}$, and a 45 μL injection volume was applied. The autosampler was maintained at 4 $^{\circ}\text{C}$ and the column at 30 $^{\circ}\text{C}$. The gradient used for separation started with 9% acetonitrile and reached 60% in 15 min. The return to initial conditions was achieved without the ramp, and the equilibration time was 14 min.

Mass Spectrometry Conditions

The mass spectrometer was operated in the positive or negative ionization mode with spray voltages of 3.0 and 4.5 kV, respectively, and at a capillary temperature of 390 $^{\circ}\text{C}$; sheath gas was maintained at 60 (arbitrary units), ion sweep gas at 0.3 (arbitrary units), auxiliary gas at 5 (arbitrary units). The collision cell pressure was maintained at 1.5 mTorr with a 0.01 s

scan time, 0.1 scan width ($\Delta m/z$) and 0.7 full width half-height maximum resolution (unit mass) for both quadrupoles (Q1 and Q3) at all transitions. The intensity of selected product ion in the MS/MS spectrum of each compound was optimized using direct infusion of the analytes in the corresponding mobile phase at a concentration of 25 $\mu\text{g/mL}$, which was loaded separately into the instrument using a syringe pump at 5 $\mu\text{L/min}$. These selected reaction-monitoring (SRM) transitions were further optimized for each compound at the exact proportion of mobile phase/acetonitrile in the source using LC-MS/MS injection. The following scan parameters were utilized: precursor ion m/z , product ion m/z , collision energy, tube lens offset, and ionization mode (Table 1). All nucleosides and nucleotides were analyzed in positive mode from 2 to 11 min, except for ZDV-TP, which was analyzed between 11 and 13 min in negative mode. The chromatography method developed had optimal separation between the last two eluted nucleotides, namely, CBV-TP and ZDV-TP to enable a switch in polarity (from positive to negative) at 11 min. Before 2 min and after 13 min, the effluent from the column was diverted to waste and a cleaning solution was sprayed on the ion source to avoid loss of signal. Multiple cleaning solutions were assessed with different percentages of acetonitrile (20%, 25%, and 50%) and with different percentages of formic acid (0%, 0.1%, and 0.4%). The combination of acetonitrile/water: 80/20 (v/v) without formic acid demonstrated the greatest sensitivity over time.

Partial Method Validation

Interday reproducibility was assessed by four injections each at low, medium, and high concentrations at 1, 10, and 100 nM of DXG, DXG-MP, DXG-TP, (–)-FTC-TP, TFV, TFV-DP, CBV-TP and 10, 100, and 1 000 nM of ZDV-MP, ZDV-TP, [$^{13}\text{C}^{15}\text{N}$]dATP, [$^{13}\text{C}^{15}\text{N}$]dGTP, [$^{13}\text{C}^{15}\text{N}$]dCTP, and [$^{13}\text{C}^{15}\text{N}$]TTP in a matrix containing 10^6 cells/100 μL of mobile phase on four consecutive days. Intraday reproducibility was evaluated by five consecutive injections each of the same concentrations noted above. The accuracy was calculated as a percentage of the difference between the theoretical value and the calculated value from the experiment against the theoretical value. Precision was expressed as % relative standard deviation (RSD).

RESULTS AND DISCUSSION

Column Selection

The methodology using the column Hypersil GOLD-C18 with ion-pair reagent displayed the highest performance. The PFP propyl and the Hypersil-AQ columns designed for the separation of polar compounds with the use of volatile buffers that are compatible with mass spectrometry detection failed to discriminate the components of interest without the use of an ion pair reagent. Several buffers were assessed, such as ammonium formate and ammonium acetate, with concentrations ranging from 5 to 50 mM adjusted to a pH range from 4 to 8. At pH 4 and 8, no retention was achieved: all nucleoside triphosphates were eluted into the void volume. At pH 5 and 6, retention and separation were observed but the symmetry of the peaks obtained, the retention capacity, and the sensitivity were not sufficient for quantification purposes. Neither an increase in organic phase nor in ammonium salt improved these parameters. Thus, the development of an ion-pair methodology using the most efficient Hypersil GOLD-C18 column became the main focus.

Temperature Selection

The column compartment temperature was evaluated between 20 and 35 $^{\circ}\text{C}$, and a significant improvement was observed at 30 $^{\circ}\text{C}$, which was chosen as the optimal column temperature.

Ion-Pair Method Development

The values of chromatography parameters (N_e , k' , T_f , and area) for 3TC, 3TC-MP, 3TC-TP, DXG, DXG-MP, DXG-TP, TFV, TFV-DP, ZDV-TP, (-)-FTC-TP, and CBV-TP were used as a guide to select the optimal pH, HMA concentration, and buffer composition (Table S1 in the Supporting Information).

HMA Concentration Effect—HMA at 12 mM gave the lowest peak tailing (Figure 1a), the highest effective plate number, and the greatest peak area among all concentrations assessed; however, the ion source was clogged following 10 injections and the sensitivity was decreased without significant improvements even after source cleaning for 15 min between each injection. Interestingly, the decrease in peak tailing observed at 9 or 12 mM HMA suggests that HMA might possess a silanol-masking activity. Generally, phosphorylated metabolites and pairing ions (HMA) undergo a partition mechanism between the mobile phase and the stationary phase.³⁵ This mechanism can be described by an equation: $\text{HMA}^+ (\text{mobile}) + \text{P}^- (\text{mobile}) \leftrightarrow \text{HMA}^+\text{P}^- (\text{stationary})$, where hexylamine (HMA^+) is the cation and the phosphorylated nucleotides (P^-) are the anions. Thus, enough HMA should be available in the mobile phase for this reaction to occur. Optimized HMA concentration at 3 mM was sufficient to obtain a symmetric peak, while avoiding clogging the source and maintaining ionization over time.

Buffer Effect—Ammonium phosphate at 1 and 2 mM and ammonium hydrogen carbonate at 10 and 20 mM were compared. Ammonium hydrogen carbonate caused an increase in peak tailing, a decrease in effective plate numbers for all analytes except for ZDV-TP (Figure 1b) at both 10 and 20 mM, and worsened the peak symmetry of all analytes at 20 mM. Ammonium phosphate at 2 mM, but not 1 mM, increased the effective plate number of all the analytes tested, except for DXG-TP (Figure 1b) and appeared to be a superior buffer for running samples over several days, without the peak shift observed with the ammonium hydrogen carbonate buffers. The improved properties obtained by the use of 2 mM ammonium phosphate versus ammonium hydrogen carbonate could be explained by its rank in the Hofmeister series, where the carbonate ions are placed lower than phosphate ions.^{36,37} Carbonate ions would have a lower propensity to retain protonated amines, such as HMA, than phosphate ions, explaining the necessity to raise the concentration of ammonium hydrogen carbonate. Carbonate ions would also increase the chaotropic effects, which could destabilize folded proteins, decrease their solubility, and give rise to salting-out behavior inside the column.³⁷ This was consistent with our observations with 20 mM ammonium hydrogen carbonate wherein a loss in chromatography quality was seen possibly due to clogging of the column (Figure 1b). In addition, ammonium hydrogen carbonate decomposes, which results in higher variability of the buffer composition and is not suitable for high-throughput analysis.³⁸ Finally, ammonium phosphate buffer is a silanol masking agent and is a potent buffer at high pH especially when a high volume of samples are injected and when the pK_a of the compound is ≥ 8 ,³⁸ which is often the case for nucleotides,³⁹ producing a better peak shape and making it the optimal buffer for nucleotide analysis.

pH Effect—With the application of pH 7, 8, 9.2, and 10, there was no noticeable change in the number of effective plates. The peak tailing decreased as the pH increased, and the peak capacity was stable from pH 7 to 9.2 but dropped at pH 10. The peak area and the signal intensity were decreased at pH 7, 8, and 10 and were increased at pH 9.2, making pH 9.2 optimal for all nucleotides (Figure 1c). Also, it was found that increasing the HMA concentration (pK_a 10.56) raised the pH and produced an improvement of signal intensity. Interestingly, when the pH was adjusted to 10 with ammonium hydroxide, a decrease in the signal intensity occurred (Figure 1c). Thus, both the pH and the silanol-masking effect of

HMA might enhance the positive ionization of nucleotides. Furthermore, the addition of ammonium hydroxide or formic acid to the mobile phase could lower the sensitivity as first described by Vela et al.²⁷ Additionally, the pH influenced the ionization of the nucleotides. At low pH and up to 9.5, ZDV-TP was better ionized in the negative mode than in the positive mode, due to the inability of the thymine base to accept an extra proton unless conditions are extremely basic (thymidine nucleoside and nucleotide $pK_a > 9.5$).³⁹ The pH chosen, 9.2, justifies the use of negative mode ionization for ZDV-TP along with a specific transition m/z 506 $\rightarrow$ 380.²³

Internal Standards Selection

It was critical to choose three internal standards compatible with the chemistries and the retention time of each of the three classes of compounds measured, nucleoside, MP, and TP. 3TC, 3TC-MP, and 3TC-TP were assessed. However, dCTP was measured by monitoring the ion transitions m/z 468 $\rightarrow$ 112, which slightly differed from the one for 3TC-TP m/z 470 $\rightarrow$ 112. Thus, an additional peak was detectable on the 3TC-TP reconstituted ion chromatogram, which represented the dCTP isotopic distribution. The separation of the two compounds by over 2 min alleviated the risks of dCTP interference over the peak area of 3TC-TP. 3TC, 3TC-MP, and 3TC-TP demonstrated a symmetrical peak shape and a lack of interference with endogenous compounds by injections of cell blanks compared with spiked standards and standards in reconstitution solvent.

Method Sensitivity and Partial Validation

Intraday and interday criteria for all nucleosides and nucleotides studied were within the range of acceptance, precision $<20\%$, and $100 \pm 25\%$ accuracy (Table 2). A reconstituted ion chromatogram illustrates the separation obtained for all the calibration standards (Figure 2). ZDV-MP was eluted 1 min after an interfering peak, which could be dGMP, AMP, or a coelution of both. Lower limits of quantification were 1 nM for DXG, DXG-MP, DXG-TP, TFV, TFV-DP, (-)-FTC-TP, and CBV-TP and 10 nM for [$^{13}\text{C}^{15}\text{N}$]dATP, [$^{13}\text{C}^{15}\text{N}$]dGTP, [$^{13}\text{C}^{15}\text{N}$]dCTP, and ZDV-TP, and 100 nM for [$^{13}\text{C}^{15}\text{N}$]TTP and ZDV-MP, which was sensitive enough to quantify all NRTI-TP in PBM cells and in macrophages (Figure 3) as well as endogenous dNTP. Sensitivity and specificity of the method developed was achieved by the intensity and the specificity of the MS/MS product formed in the positive ionization mode.²⁴ This method was subsequently applied for quantitative analysis of intracellular NRTI-TP and endogenous dNTP levels in human PBM cells and in macrophages. The values found following NRTI incubations were in the expected range for ZDV-TP, CBV-TP, TFV-DP, and DXG-TP. However, the (-)-FTC-TP levels were 4-fold higher than those previously reported, which may be due to variable assay condition (Table 3).^{40,41} Endogenous dNTP levels were within the range of 0.33–11.9 and 0.08–5.8 pmol/ 10^6 cells in PBM cells and in macrophages, respectively. These values are within the previously reported range.¹⁵

CONCLUSIONS

This novel analytical method provides an accurate, rapid, and selective quantification of most clinically relevant nucleoside and nucleotide analogues simultaneously with successful application in primary human PBM cells and macrophages. This highly sensitive LC–MS/MS method was developed for simultaneous quantification of intracellular nucleotide concentrations of DAPD, TDF, ZDV, (-)-FTC, ABC, and endogenous dNTP but also provides a flexible foundation that can allow for the quantification of other NRTI with slight modifications. The developed method can also be applied for the accurate simultaneous determination of nucleosides and their intracellular metabolites from human samples in the

context of pharmacokinetic evaluations since combination treatments are used for the treatment of HIV-1 infection.

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Figure 1.

Chromatography parameters of NRTI-TP represented by the symbols: +, 3TC-TP; ×, DXG-TP; ◆, TFV-DP; □, CBV-TP; △, (-)-FTC-TP; and ○, ZDV-TP: (a) tailing factor (Tf) at five different HMA concentrations; 0, 3, 6, 9, and 12 mM. (b) Effective plate number (Ne) at four different buffer compositions; 1 and 2 mM ammonium phosphate; 10 and 20 mM ammonium hydrogen carbonate. (c) Peak area at pH 7.0, 8.0, 9.2, and 10.0.

Figure 2.

Typical chromatograms displaying all the standards used for the partial validation spiked in a PBM cells matrix containing 1×10^6 cells/100 μL of mobile phase. The RIC shows the peaks obtained for DXG, DXG-MP, DXG-TP, TFV, TFV-DP, ZDV-MP, ZDV-TP, (-)-FTC-TP, CBV-TP, $[^{13}\text{C}^{15}\text{N}]$ dATP, $[^{13}\text{C}^{15}\text{N}]$ dGTP, and $[^{13}\text{C}^{15}\text{N}]$ dCTP at 100 nM and $[^{13}\text{C}^{15}\text{N}]$ TTP at 500 nM. Internal standards, 3TC, 3TC-MP, and 3TC-TP were at 100 nM.

Figure 3.

(a) Typical chromatograms for NRTI-TP, obtained from macrophages. Five drugs (ZDV, ABC, TDF, (-)-FTC, and DXG) were incubated separately at 10 μ M for 4 h and analyzed in five different LC-MS/MS runs. The left chromatograms represent five background signals obtained from a single injection of untreated macrophage extract; these chromatograms are marked as “blank”. The right chromatograms represent the five signals obtained from five separate injections of treated macrophages extracts. The sample incubated with TDF was diluted 20 times. (b) Typical chromatograms of endogenous dNTP obtained from a single macrophage extract.

Table 1

Parent Compounds m/z , Product Compounds m/z , Collision Energy, and Tube Lens Values for All Nucleosides, Nucleotides, NRTI, and NRTI-TP

	parent m/z	product m/z	collision energy (V)	tube lens (V)	ionization mode
3TC	230	112	15	70	+
3TC-MP	310	112	27	70	+
3TC-TP	470	112	27	90	+
DXG	254	152	15	70	+
DXG-MP	334	152	26	90	+
DXG-TP	494	152	30	130	+
TFV	288	176	20	70	+
TFV-DP	448	270	27	130	+
ZDV-MP	348	81	35	90	+
ZDV-TP	506	380	18	90	-
(-)-FTC-TP	488	130	30	130	+
CBV-TP	488	152	35	130	+
dATP	492	136	20	116	+
dGTP	508	152	30	110	+
dCTP	468	112	30	90	+
TTP	483	81	20	90	+
[¹³ C ¹⁵ N]dATP	507	146	20	116	+
[¹³ C ¹⁵ N]dGTP	523	162	30	110	+
[¹³ C ¹⁵ N]dCTP	480	119	30	90	+
[¹³ C ¹⁵ N]TTP	495	134	20	90	+

Table 2

Intraday and Interday Accuracy and Precision for DXG, DXG-MP, DXG-TP, TFV, TFV-DP, ZDV-MP, ZDV-TP, (-)-FTC-TP, CBV-TP, and [$^{13}\text{C}^{15}\text{N}$]dNTP

	concn (nM)	interday ($n = 4$)		intraday ($n = 5$)	
		precision (%)	accuracy (%)	precision (%)	accuracy (%)
DXG	1	3.4	96.1	8.7	94.7
	10	5.3	91.7	3.4	93.7
	100	6.8	108.5	2.4	93.0
DXG-MP	1	10.9	105.4	11.2	102.0
	10	11.9	96.8	4.1	98.7
	100	0.2	102.2	5.4	96.8
DXG-TP	1	3.6	99.6	16.4	113.3
	10	7.5	109.7	3.6	110.4
	100	5.9	109.9	2.0	103.3
TFV	1	6.8	101.8	4.9	96.5
	10	3.6	100.6	2.4	88.7
	100	6.3	106.4	6.0	102.4
TFV-DP	1	14.3	108.8	6.8	96.5
	10	6.9	99.8	6.2	94.7
	100	9.5	93.2	3.3	97.0
ZDV-MP	10				
	100	14.1	105.1	13.1	110.1
	1000	6.5	98.8		
ZDV-TP	10	19.8	91.1	13.6	89.2
	100	19.2	86.5	19.4	92.8
	1000	6.2	104.8	11.4	98.3
(-)-FTC-TP	1	10.7	91.7	7.4	98.7
	10	13.5	99.9	13.0	112.9
	100	12.9	95.7	3.6	110.8
CBV-TP	1	12.3	94.5	6.0	111.2
	10	9.8	115.4	8.1	99.2
	100	20.0	97.7	4.2	95.8

	concn (nM)	interday (n = 4)			intraday (n = 5)		
		precision (%)	accuracy (%)		precision (%)	accuracy (%)	
$[^{13}\text{C}^{15}\text{N}]d\text{ATP}$	10	16.9	87.0		19.2	89.4	
	100	13.6	106.6		6.6	102.6	
	1000	5.4	97.5		3.9	95.6	
$[^{13}\text{C}^{15}\text{N}]d\text{GTP}$	10	11.9	100.9		11.4	99.1	
	100	10.5	108.4		5.1	103.7	
	1000	2.6	97.8		2.9	96.0	
$[^{13}\text{C}^{15}\text{N}]d\text{GTP}$	10	10.1	108.6		10.6	108.3	
	100	16.4	101.6		5.3	74.8	
	1000	5.9	103.1		2.9	108.8	
$[^{13}\text{C}^{15}\text{N}]d\text{TTP}$	10						
	100	16.4	114.1		5.3	103.8	
	1000	6.0	104.0		3.0	125.0	

Table 3

NRTI-TP Levels (Picomoles/ 10^6 Cells) in 72 h-PHA Stimulated Human PBM Cells, Following a 4 h Incubation of 10 μ M NRTI, in Comparison with Previously Published Data Using Similar Assay Conditions

	NRTI-TP levels	previous publications
ZDV-TP	1.13 ± 0.27	$\sim 1.1^{42}$
CBV-TP	0.16 ± 0.002	0.12 ± 0.07^{43}
TFV-DP	0.05 ± 0.03	$0.038\text{--}0.11^{40}$
(-)-FTC-TP	~ 16	$2.74\text{--}4.14^{40}$
DXG-TP	0.05 ± 0.03	$\sim 0.015^{44}$