Quantitation of Intracellular Triphosphate of Emtricitabine in Peripheral Blood Mononuclear Cells from Human Immunodeficiency Virus-Infected Patients

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An analytical methodology combining solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) was developed to quantify the intracellular active 5'-triphosphate (TP) of β-L-2',3'-dideoxy-5-fluoro-3'-thiacytidine (emtricitabine) (FTC) in human peripheral blood mononuclear cells (PBMCs). The FTC nucleotides, including 5'-mono-, di-, and triphosphates, were successively resolved on an anion-exchange SPE cartridge by applying a gradient of potassium chloride. The FTC-TP was subsequently digested to release the parent nucleoside that was finally analyzed by HPLC with UV detection (HPLC-UV). Validation of the methodology was performed by using PBMCs from healthy donors exposed to an isotopic solution of [3H]FTC with known specific activity, leading to the formation of intracellular FTC-TP that was quantitated by an anion-exchange HPLC method with radioactive detection. These levels of FTC-TP served as reference values and were used to validate the data obtained by HPLC-UV. The assay had a limit of quantitation of 4.0 pmol of FTC-TP (amount on column from approximately 106 cells). Intra-assay precision (coefficient of variation percentage of repeated measurement) and accuracy (percentage deviation of the nominal reference value), estimated by using quality control samples at 16.2, 60.7, and 121.5 pmol, ranged from 1.3 to 3.3% and −1.0 to 4.8%, respectively. Interassay precision and accuracy varied from 3.0 to 10.2% and from 2.5 to 6.7%, respectively. This methodology was successfully applied to the determination of FTC-TP in PBMCs of patients infected with human immunodeficiency virus after oral administration of various dosing regimens of FTC monotherapy.

Nucleoside analogs are an important class of antiviral drugs for the treatment of both human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus (HBV) infections. HIV-1 and HBV, the causative viruses of AIDS and acute and chronic hepatitis, respectively, affect more than 100 million (HIV) and 300 million (HBV) people worldwide. Despite the availability of 14 drugs, including six nucleoside reverse transcriptase inhibitors (NRTIs), that have greatly increased treatment options for HIV-1 infections, there is still no cure for AIDS. Cross-resistance and tolerance issues warrant the search for new compounds with a superior antiviral activity and a better safety profile. Although a vaccine is available for HBV, treatments for both human immunodeficiency virus type 1 (HIV-1) and hepatitis B virus (HBV) infections (1). This compound possesses an unnatural β-L-2',3'-dideoxy-5-fluoro-3'-thiacytidine (emtricitabine) (FTC) is an analog of deoxycytidine that is currently under clinical development for the treatment of HIV-1 and HBV infections (1). This compound possesses an unnatural β-L-structure. It is an analog of deoxycytidine that is currently under clinical development for the treatment of HIV-1 and HBV infections (1). This compound possesses an unnatural β-L-structural configuration (Fig. 1) that has apparently conferred on it higher in vitro antiviral activity against both HIV-1 (50% effective concentration [EC50] = 10 to 20 nM) and HBV (EC50 = 10 to 40 nM) and lower toxicity than the natural β-D enantiomer (2, 5, 14, 20). The in vitro activity of FTC against HIV-1 is approximately 4- to 10-fold more potent than 3TC, a close analog that has been approved for the treatment of both HIV-1 and HBV infections (2). This in vitro finding was recently confirmed by the results of a phase I/II clinical trial of FTC at 25, 100, and 200 mg once a day (q.d.) compared with 3TC at 150 mg twice a day (b.i.d.) in HIV-infected patients (1). Similar to other nucleoside analogs, FTC requires multistep intracellular phosphorylation by various cellular kinases to its 5'-monophosphate (MP), diphosphate (DP), and the active triphosphate (TP) (5, 10). The FTC 5'-TP (FTC-TP) competitively inhibits HIV-1 reverse transcriptase and can also be incorporated into viral genome, causing termination in DNA chain elongation. HBV is a member of the hepadnavirus family whose replication cycle includes the reverse transcription of an RNA template (6). This reverse transcription process is the target of the anti-HBV nucleoside analogs, including FTC. One of the primary objectives of clinical pharmacologic research of antiretroviral agents is to define pharmacokinetic-pharmacodynamic relationships which could allow more rational selection of therapeutics regimens that are expected to maximally suppress viral replication while preserving safety. Efforts to correlate plasma pharmacokinetics of anti-HIV nucleoside analogs to their virologic and/or immunologic outcomes have had little success, with the exception of didanosine, for which a relationship between its plasma pharmacokinetics and the suppression of HIV p24 antigen has previously been documented in patients (3). Since NRTIs require intracellular
activation, it has previously been hypothesized that the intracellular level of their active 5′-TP anabolite may be an appropriate predictor of the virologic response (7, 21, 25, 26). Recent data have shown that a higher level of intracellular zidovudine 5′-TP was associated with a better suppression of plasma viral load and an increase in CD4+ cell count in HIV-infected individuals (4). Previous studies also strongly supported this hypothesis with the demonstration that intracellular concentrations of the active 5′-TP of lamivudine and stavudine, rather than levels of the parent nucleosides in plasma, correlated with virologic response in HIV-infected patients (18, 19). Intracellular pharmacokinetic analysis of antiviral nucleoside analogs in large-scale clinical trials has been made possible by recent advances in analytical techniques that allow specific and reproducible quantitation of subpicomole amounts of the nucleotides. Although nucleotide-specific enzymatic assays that directly measure the TP based on inhibition of reverse transcriptase activity are available (12), methodologies using chromatographic separation of intracellular phosphates, enzyme digestion followed by radioimmunoassay, or high-performance liquid chromatography (HPLC) with UV (HPLC-UV) or mass spectrometric detection are more appealing since TPs from different nucleosides can be simultaneously isolated (8, 11, 13, 15, 16, 22). This technique is especially suitable for samples obtained from AIDS clinical trials, where two or more NRTIs are often used in combination therapy. The separation processes of 5′-MP, DP, and TP are critical and have been initially performed by strong anion-exchange HPLC that was highly selective although time consuming (15). The separation method was recently improved by using an anion-exchange solid-phase extraction (SPE) cartridge (11), which is preferred for large clinical trials since multiple samples can be simultaneously processed. This latter technique was first applied to a zidovudine TP assay (11) and was recently used to quantitate intracellular nucleotides of lamivudine in AIDS patients (13, 16).

This report describes the development and validation of an analytical methodology that combines an anion-exchange SPE procedure with an HPLC technique for the separation and quantitation of FTC-TP in human peripheral blood mononuclear cells (PBMCs). Assay performance, including purity of extracted nucleotides, extraction recovery, and intra- and interassay variability, was evaluated. This method was successfully applied to determine the level of FTC-TP in PBMCs isolated from HIV-infected patients receiving various oral regimens of FTC monotherapy.

MATERIALS AND METHODS

Chemicals. Nonlabeled FTC (molecular weight = 247) was provided by Triangle Pharmaceuticals, Inc. (Durham, N.C.). [6-3H]FTC (5 Ci/mmol) was obtained from Moravek Biochemicals (Brea, Calif.). 2′,3′-Dideoxy-5-fluoroctidine (β-L-FddC; molecular weight = 229), used as an internal standard (IS), was kindly provided by J. L. Imbach and G. Gosselin (Montpellier, France). These standards were more than 98% pure as ascertained by the HPLC methods described below. Alkaline phosphatase (3.100 U/mg of protein) and phosphodiesterase I (31.0 U/mg [dry weight]) for enzyme digestion were purchased from Worthington Biochemical Corporation (Freehold, N.J.). HPLC-grade potassium chloride (KCl), potassium phosphate monobasic, orthophosphoric acid (85%), acetonitrile, and methanol were obtained from Fisher Scientific (Fair Lawn, N.J.). All other chemicals used were of analytical grade.

Preparation of FTC-TP standards. The unavailability of nucleotide standards of FTC led us to adopt an assay validation strategy that included the exposure of PBMCs from healthy human donors to tritiated FTC with known specific activity to produce intracellular phosphates of FTC in vitro. A classical anion-exchange HPLC technique was then used to accurately determine the FTC-TP level in a fraction of the total cell extracts based on its radioactivity and the specific activity of the probe. The rest of the cell extracts with hence known levels of FTC-TP were then used to prepare calibration standards or quality controls (QCs) to validate the nonradioactive HPLC method with UV detection. Briefly, human PBMCs were isolated from fresh whole blood. Blood was diluted with an equal volume of phosphate-buffered saline (PBS) and laid onto a Ficoll-Histopaque...
density gradient in 50-mL conical tubes. After centrifugation at 500 × g for 30 min at room temperature, the layer containing PBMCs was carefully recovered and washed three times with PBS. Isolated PBMCs were then suspended in RPMI 1640 medium supplemented with 2% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine and stimulated for 48 h with phytohemagglutinin (PHA) at a final concentration of 10 µg/mL. All cultures were maintained at 37°C, under an atmosphere of 5% CO2.

After centrifugation, cells were suspended in PHA-free medium at a cytocrit of 2 × 106 cells/mL. An isotopic solution of [3H]FTC (specific activity, 74.6 dpm/pmol) was added to the culture at a final concentration of 10 µM and incubated for 24 h with a final incubation volume of 50 µL in a 100-mL culture flask. Practically, 8 to 10 flasks were processed simultaneously. Following incubation, cells (around 100 × 10⁶ cells/flask) were pelleted by centrifugation and rinsed three times with cold PBS. Cell pellets from all flasks were pooled and extracted twice with 4 mL of 60% methanol at −70°C overnight. Cellular debris was then removed by centrifugation at 2,000 × g for 10 min. The resulting supernatant containing FTC nucleotides was collected and stored as 500-µL aliquots at −70°C until analysis. These aliquots were to serve as standards of the calibrating curve or as QC samples. Standards and QCs were from independent incubations using separate sources of isotope dilution of [3H]FTC. The quantity of FTC-TP was determined in triplicate from 100-µL aliquots of both. Forty HIV-infected PBMCs were aliquot therefore served as a reference value for the preparation of standards or QCs using the remaining authentic aliquots (15 500-µL aliquots for standards and QCs) for the validation of the HPLC-UV method. A five-point calibration curve was made at 8.1 (2.0), 20.2 (5.0), 40.5 (10.0), 81.0 (20.0), and 202.4 (50.0) pmol (equivalent amount of FTC in nanograms) of FTC-TP on column and three QCs at 16.2 (4.0), 60.7 (15.0), and 121.5 (30.0) pmol (ng) on column were prepared. The calibration standards and QCs were simultaneously processed by anion-exchange SPE followed by enzyme digestion to hydrolyze FTC phosphates. Fractions containing FTC were subsequently quantitated by reverse-phase HPLC with UV detection as described later in this report.

### Anion-exchange HPLC method

This methodology was applied to measure levels of FTC-TP in PBMCs from HIV-infected patients enrolled in a phase I/I clinical trial of FTC. Five groups of patients received, over 2 weeks, escalating doses of FTC at 25 mg b.i.d., 100 mg q.d., 100 mg b.i.d., 200 mg q.d., and 200 mg b.i.d. (23, 24). This study was approved by each of the six Institutional Review Boards of the participating sites. Forty HIV-infected patients were enrolled after giving written informed consent and were sequentially assigned to receive one of the regimens. At least 15 mL of blood was drawn into two Vacutainer CPT cell preparation tubes (Becton Dickinson, Franklin Lakes, N.J.) prior to and at 1, 3, 9, and 12 h after the first dose of FTC. The tubes were centrifuged at 1,500 × g for 20 min at room temperature. The upper layer, containing plasma and PBMCs, was recovered. A 500-µL aliquot was used to count PBMCs and the remaining portion was centrifuged at 500 × g for 10 min to pellet cells. Plasma was removed and 200 µL of 60% methanol was added to the cell pellet. Protein samples were shipped on dry ice and stored under −70°C until analyzed.

### Anion-exchange SPE

SPE was performed using anion-exchange cartridges (Sep-Pak Vac, 100-mg phase; Waters, Milford, Mass.). Cartridges were pre-conditioned with 500 µL of deionized water. Cell extracts (calibration standards, QCs, and patient samples) were loaded onto the cartridge and eluted under reduced pressure. The cartridge was then washed with 500 µL of water. This fraction combined with that obtained from the initial step represents the unchanged FTC nucleoside, and the cartridge was rinsed with 500 µL of water and 200 µL of 20 mM KCl. The nucleotides of FTC, including its 5’-MP, DP, and TP, were successively resolved and eluted by using a KCl gradient. Briefly, FTC-DP-choline was eluted with 400 µL of 60 mM KCl and the cartridge was rinsed with 100 µL of the buffer. The 5’-MP, -DP, and -TP were eluted with 300 µL of 100 mM KCl, 400 µL of 120 mM KCl, and 500 µL of 400 mM KCl, respectively. The cartridge was rinsed with 100 µL of the corresponding buffers between each step. Following SPE, purity of the unchanged drug and each nucleotide was checked by high-performance liquid chromatography (HPLC) as described below. The identity of the spotted nucleotides was assessed based on their relative retention time and enzyme digestion using alkaline phosphatase and/or phosphodiesterase.

### Enzyme digestion

The phosphates of FTC resolved from the SPE step were then subjected to enzyme digestion to release the nucleoside. Fractions containing the phosphates were incubated with alkaline phosphatase (50 U/fraction) at 37°C overnight. Following digestion, acetonitrile (3 volumes) was added to precipitate proteins. Supernatant was recovered after centrifugation and dried under nitrogen. The residue was then dissolved in 150 µL of water containing 50 µg (218 pmol) of β-FdA as an IS and analyzed by reverse-phase HPLC as described later in this section.

### Anion-exchange HPLC with radioactive detection

Due to the unavailability of FTC-TP standards, we developed an assay validation strategy that included the use of PBMCs from healthy human donors to produce intracellular FTC nucleotides in vitro. A concentration of 10 µM FTC was physiologically relevant and expected to lead to formation of FTC nucleotides comparable to that obtained in FTC-treated patients. A similar strategy to validate an assay quantitating 3TC phosphates in human PBMCs has been previously applied (16). The levels of the intracellular TP of FTC were then measured by anion-exchange HPLC with radioactive detection and served as references to prepare calibration standards and QCs. These standards and QCs were then used to validate the nonradioactive method by an HPLC assay with UV detection for the measurement of intracellular FTC-TP in human PBMCs. Figure 2 depicts a typical phosphorylation
profile of FTC in human PBMCs, illustrating the formation of its 5'-MP, DP, TP, and DP-choline derivatives. The identity of FTC derivatives was assessed by their relative retention times and was further ascertained by enzyme digestion using alkaline phosphatase and alkaline phosphatase plus phosphodiesterase for the DP-choline derivative. Unlike 3TC, which had a substantial formation of intracellular DP-choline derivative (16), FTC-DP-choline constituted a rather low percentage (~3%) of total phosphate in PBMCs from all donors (n = 7) and therefore this metabolite was not further analyzed.

Purity of FTC nucleotides following anion-exchange SPE. Following SPE, the four fractions representing unchanged FTC and its 5'-MP, DP, TP, and DP-choline derivatives were separately injected onto the anion-exchange HPLC for a purity check. As shown in Fig. 2, only a single peak was obtained in each case and was identified as the expected phosphate by comparing peak retention time, indicating that the SPE procedure led to the resolution of pure FTC nucleotides. Figure 3 shows a representative SPE profile of FTC phosphates.

Recovery of FTC-TP. The overall recovery of FTC-TP was assessed by comparing its amount as measured by reverse-phase HPLC with UV detection using a standard curve prepared from FTC in water to the nominal amount of the calibration standards and QCs. Authentic replicates (three for QCs at 16.2, 60.7, and 121.5 pmol of FTC-TP and five for calibration standards at 8.1, 20.2, 40.5, 81.0, and 202.4 pmol) of the aliquots of cell extracts obtained after exposure of human PBMCs to 10 μM FTC were analyzed by HPLC-UV following SPE and enzyme digestion. The standard curve was obtained by directly injecting 8.1, 20.2, 40.5, 81.0, and 202.4 pmol of FTC in water onto the chromatograph. The amount of FTC-TP in the calibration standards and QCs prepared from cell extracts was calculated by using this standard curve. The overall recovery of FTC-TP was high, ranging from 95.0 to 108.1% (Table 1).

Selectivity of HPLC-UV. Triple or quadruple combination therapy, often involving two nucleoside analogs, is becoming a standard regimen for the treatment of HIV infections. Therefore, it appeared necessary to evaluate potential chromatographic interference by nucleoside antiretroviral agents and natural analogs. Under the specified chromatographic conditions (see Materials and Methods), FTC exhibited a retention time of 10.4 ± 0.2 min, while retention times for β-L-FddC (IS), ddI, d4T, 3TC, and ddA were 7.0, 4.0, 11.5, 7.9, and 5.7 min, respectively. The retention times for zidovudine, abacavir, and carbovir were greater than 13 min. Analysis of a predose PBMC sample (blank) showed no interference, demonstrating a high specificity of the HPLC-UV method (Fig. 4).

### Table 1. Overall recovery of FTC-TP after anion-exchange SPE and enzyme digestion

<table>
<thead>
<tr>
<th>Nominal amount (pmol on column)</th>
<th>n</th>
<th>Measured amount (pmol on column)</th>
<th>Recovery (%)</th>
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</thead>
<tbody>
<tr>
<td>QCs</td>
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<tr>
<td>16.2</td>
<td>5</td>
<td>17.4 ± 0.8</td>
<td>107.2</td>
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<tr>
<td>60.7</td>
<td>5</td>
<td>57.9 ± 8.9</td>
<td>95.0</td>
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<tr>
<td>121.5</td>
<td>5</td>
<td>116.2 ± 8.1</td>
<td>95.6</td>
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<tr>
<td>Calibration standards</td>
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<tr>
<td>8.1</td>
<td>3</td>
<td>8.1 ± 0.8</td>
<td>101.4</td>
</tr>
<tr>
<td>20.2</td>
<td>3</td>
<td>21.9 ± 2.4</td>
<td>108.1</td>
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<tr>
<td>40.5</td>
<td>3</td>
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<td>99.7</td>
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<tr>
<td>81.0</td>
<td>3</td>
<td>78.9 ± 5.7</td>
<td>97.5</td>
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<tr>
<td>202.4</td>
<td>3</td>
<td>195.1 ± 16.2</td>
<td>96.4</td>
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</table>

* Means ± standard deviations.
more, the same number of cells does not lead to the formation of FTC-TP and its metabolites. In order to standardize the quantification of FTC-TP, we determined the limit of detection (LOD) and the limit of quantitation (LOQ) of the HPLC-UV methodology. The LOD was characterized by an accuracy of 0.2% and a precision ranging from 1.3 to 3.3%. Interassay performance was assessed over a period of 2 months using both FTC-TP QCs (six independent experiments) and calibration standards (four separate experiments). As shown in Table 3, interassay performance was characterized by an accuracy of 0.4 to 6.7% and a precision of 0.9 to 10.2%.

**Application to biological samples.** Using this new combined HPLC and SPE methodology, levels of FTC-TP were determined in PBMCs of HIV-infected patients receiving oral doses of either 25 mg b.i.d., 100 mg q.d., 100 mg b.i.d., 200 mg q.d., or 200 mg b.i.d. as part of a phase I/II study of FTC mono-therapy (23, 24). Six blood samples were obtained prior to and up to 12 h after the first dose of FTC, and PBMCs were isolated and processed as described in Materials and Methods. Results from this study demonstrated that high intracellular levels of FTC-TP were associated with a better suppression of plasma HIV-1 RNA levels (24). Representative chromatograms of FTC-TP, reduced to FTC after enzyme digestion, in all PBMC samples from a patient are depicted in Fig. 4. Quantities of FTC-TP in the 1, 3, 6, 9, and 12 h PBMC samples in this patient were 8.0, 55.8, 123.0, 116.4, and 119.0 pmol, respectively. When normalized to the number of PBMCs of the samples used in the assay, the levels were 0.20, 1.48, 2.02, 2.26, and 1.52 pmol/10^6 cells, respectively.

In summary, an analytical methodology combining anion-exchange SPE and HPLC with UV detection was developed and validated for the quantitation of intracellular 5'-TP of FTC in human PBMCs. Although the validation was performed only on the TP, this methodology can easily be applied to measure the other phosphates of the compound, as was recently reported for 3TC-MP and DP (16), since, as demonstrated in Fig. 2, purely resolved 5'-MP and DP of FTC can be obtained. Analysis of these intermediate anabolites should lead to a better understanding of the different steps and the kinetics involved in the activation of NRTIs and to an evaluation of the contribution of these nucleotides to antiviral effects and/or toxicity. In conclusion, despite its complexity, this methodology has successfully been applied to quantify intracellular FTC-TP in HIV-infected patients enrolled in a phase I/II clinical trial. Evaluation of intracellular pharmacokinetics of the active TP of the drug in conjunction with virologic and immunologic response should allow the establishment of pharmacokinetic-pharmacodynamic relationships in antiviral therapy with the drug.

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**REFERENCES**


