Doubly Hyphenated Method (LC-MS/MS-Fluorometric) for Determination of Nucleoside Triphosphates and Analogs in Peripheral Blood Mononuclear Cells (PBMCs)

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Introduction

- Quantitative determination of active drugs in cells containing the relevant drug target has become an important objective in drug development. For antiviral drugs, delineation of the pharmacokinetics of their active nucleoside triphosphates and analogs (TPs) in peripheral blood mononuclear cells (PBMCs) is now routinely used to assess drug efficacy and safety and to help set appropriate drug dosing regimens. For such purposes, determination of the number of PBMCs in a sample is required; TPs in PBMC samples are typically reported as mass of drug per a specified number of cells, from which an intracellular drug concentration can be calculated from a known cell volume.
- We previously described[1] the use of genomic DNA (gDNA) in place of extraction of reference PBMC DNA as calibration standards and Quality Control samples (QCs) in an extension of the method of Benech et al.[2] for determination of the number of PBMCs in isolated human PBMC samples, based on the same genomic DNA content of all PBMCs[3,4].
- We now report the validation of a hyphenated method that incorporates simultaneous determination of two nucleoside triphosphate analogs (TPs), emtricitabine triphosphate (FTC-TP) and tenofovir diphosphate (TFV-DP), by LC-MS/MS methods in a sample of PBMCs, along with determination of the PBMC count in the same sample by the gDNA calibration standard method.

Background

- Benech et al.[2] reported a method for determination of TPs in PBMCs that uses the DNA content extracted from reference PBMCs as the basis of counting PBMCs in a calibration standard or collected sample. DNA is quantified by extraction of the DNA from the standard or sample and treating the DNA extract with a fluorogenic agent (SYBR® Green). The observed fluorescence signal is proportional to the number of cells in the calibration standard or sample.
- A challenge we encountered in using the method of Benech was obtaining reference PBMC samples that were consistent and suitable for use as DNA standards. To address this we explored and successfully validated[1] the use of purified genomic DNA (gDNA) for preparation of calibration standards and utilized the fact that all human DNA-containing cells contain the same amount of DNA content. (Variations due to sex and mitochondrial DNA are <1.5% and therefore negligible.)

Objective

- Validate a hyphenated method for simultaneous determination of FTC-TP and TFV-DP in isolated human PBMC samples.
- Incorporate the validated method that uses purified gDNA as calibration standards for determination of PBMC counts into the overall method for the same sample in order to report TFV-DP and FTC-TP in units of ng/10^6 cells .

Methods: Reagents

- Blood Processing
  - NS: Normal saline (0.9% aqueous NaCl),
  - RBC (red blood cell) lysing solution (Qiagen , #158904 or equivalent)
  - Incurred sample PBMC aliquot pellets prepared by pooling isolated PBMCs from dosed subjects, apportioning the pooled PBMCs into aliquots, and generating PBMC pellets according to the PBMC processing method. Baseline concentration: mean from analysis of 4 replicate aliquots in each of 2 consecutive runs.
- LC-MS/MS
  - PBMC lysis buffer: 0.05 M aqueous Tris HCl, pH 7.4:methanol, (30:70, v:v)
  - Solvent 1: methanol:water (70:30, v:v)
  - Solvent 2 (Reconstitution solvent): methanol:water (20:80, v:v)
  - TFV-DP Internal Standard (IS): $^{13}C_5$-TFV-DP
  - FTC-TP Internal Standard (IS): $^{13}C^{15}N_2$-FTC-TP
  - IS Working Solution: $^{13}C_5$-TFV-DP (500 ng/mL) and $^{13}C^{15}N_2$-FTC-TP (5000 ng/mL) in water
  - Mobile Phase A: 10 mM ammonium acetate in acetonitrile:water (30:70, v:v), pH 5.5
  - Mobile Phase B: 5 mM ammonium acetate in acetonitrile:water (30:70, v:v), pH 10.0
  - Autoinjector Wash Solvents:
    - R0, R1, and R2: acetonitrile:water:ammonium hydroxide (20:80:0.2; v:v:v)
    - R3: acetonitrile:water (50:50, v:v)
- Cell Counting
  - 1 N NaOH: 1 N aqueous NaOH
  - KP buffer: 1 M aqueous KH2PO4, pH 7.4
  - KPNH buffer: KP buffer:1 N NaOH (34:1, v:v)
  - Commercially available stock solutions:
    - SYBR® Green
    - Genomic DNA Reference Standard
  - SYBR® WS: Commercial stock solution diluted 1:10,000 with water

Methods

Blood Sample Collection and Processing to a PBMC Pellet

- Standard processes (See Benech) are used to generate a PBMC sample and include steps for washing, for lysis of red blood cells and removal of their contents, and for collection of the PBMC sample by centrifugation as a cell pellet, which is immediately frozen at -70 °C for storage.
  - PBMCs are isolated from blood samples (8 mL) collected in Cell Preparation Tubes (CPTs) with sodium citrate as anticoagulant.
  - Immediately after blood collection, CPTs are centrifuged (18-25 °C, 1800 RCF, 20 min). Each PBMC layer is immediately transferred by pipette to a separate tube and washed with ~14 mL of 4 °C NS, centrifuged (ambient temperature, 400 RCF, 10 min); supernatant is removed by pipette.
  - Each PBMC sample is then treated with 2 mL of 4 °C RBC lysing solution for 2-5 min at ambient temperature, followed by NS (10-12 mL, 4 °C), inversion to mix (3-4 times), centrifugation (ambient temperature, 400 RCF, 10 min), and supernatant decantation by pipette to produce a PBMC pellet.
  - Each PBMC pellet is then treated with NS (2 mL, 4 °C) by gently pipetting up/down to achieve cell resuspension.
  - Each PBMC sample is centrifuged (ambient temperature, 3100 RCF, 5 min), followed by removal of the supernatant by pipette to generate a a PBMC pellet that is then flash frozen on dry ice.
  - Total processing time from blood draw to freeze is ≤2.5 h.

Methods Cont'd

PBMC Pellet Processing for Sequential LC-MS/MS and Cell Count Analysis

- For the initial analysis steps, the method follows the procedure of Benech to thaw and lyse the PBMC pellet with PBMC lysis to extract the TPs, but not the sample DNA, which remains in a "debris pellet" upon centrifugation.
- The debris pellet is then treated with 0.200 mL of 1 N aqueous NaOH with sonication, followed by overnight ambient temperature incubation, to solubilize the sample DNA and break it into smaller fragments, as in the Benech method. However, DNA Calibration Standards and QCs are prepared by spiking known amounts of reference standard gDNA into blank PBMC pellet debris samples and then following the same procedure (addition of 0.200 mL of 1 N NaOH) as for samples.
- gDNA Calibration Standard concentrations and corresponding cell counts:

Conc. (µg/µL)	0.0665	0.166	0.333	0.416	0.499	0.665	0.831	1.17
Cell Count (× 10^6 cells)	2.00	5.00	10.0	12.5	15.0	20.0	25.0	35.0

- gDNA Calibration Standard concentrations and corresponding cell counts:

Conc. (µg/µL)	0.0665	0.200	0.499	0.800	1.17
Cell Count (× 10^6 cells)	2.00	6.00	15.0	24.0	35.0

LC-MS-MS Procedure (Detailed Steps)

- Prepare spiking solutions containing both analytes (TFV-TP and FTC-TP)
- Thaw frozen PBMC samples and blank PBMC pellets (for Calibration Standards and QC samples) on wet ice (5-30 min).
- Add 1 mL of ice-cold Solvent 1 to each tube and mix (vortex) vigorously; use pipette tip to assist pellet dispersion if needed.
- Add 20 µL of appropriate spiking solutions of TFV-DP/FTC-TP to blank PBMC tubes to prepare Calibration Standards and QC samples.
- Add 20 µL of IS solution to all tubes and vortex mix at high speed 1 min.
- Also prepare appropriate blank controls with just buffer or IS solution.
- Centrifuge all samples, standards and controls (14000 rpm, 30 min).
- Transfer supernatants to separate tubes and dry under N_2 (40 °C).
- Add 240 µL of Solvent 2 to each tube to reconstitute; vortex mix at low speed 2 min; centrifuge (3000 rpm, 5 min).
- Transfer resultant supernatants to microvials of a sample plate; centrifuge plate (3000 rpm, 5 min).
- Inject 20 µL of each sample supernatant into an LC-MS-MS system

LC Settings, Column, Parameters

- Column: BioBasic AX, 2.1 x 50 mm, 5 µm, Thermo Scientific
- Gradient (% Solvent B in Solvent A)

Time (min):	0	1.0	1.5	3.0	3.5	5.0
% Solvent B:	15	50	100	100	15	15

- Injection Volume: 5 µL; Autoinjector Temperature: 4°C; Column Oven Temperature: 35 °C
- Flow rate: 0.5 min; Cycle time: 5.5 min
- Retention times: TFV-DP and $^{13}C_5$-TFV-DP: ~2.00 min; FTC-TP and $^{13}C^{15}N_2$-FTC-TP: ~2.02 mi

Mass Spectrometer Settings

- Source temperature (TEM): 650 °C; Collision Gas (CAD): 10 psig N_2; Curtain Gas (CUR):40 psig N_2; Ion Source Gas 1 (GS1): 65 psig N_2; Ion Source Gas 2 (GS2): 65 psig N_2; Ion Spray Voltage (IS V): 5200 %; Entrance Potential (EP) 10 V; Scan Duration: 3.0 min

Compound	Ionization Mode	Dwell ime (msec)	Declustering Potential (V)	Collision energy (eV)	Collision Exit Potential (V)	Transition (m/z)
TFV-DP	TIS+	200	80	58.0	20.0	448.1→ 176.1
$^{13}C_5$-TFV-DP	TIS+	100	80	58.0	20.0	453.1→ 181.1
FTC-TP	TIS+	200	65	24.0	15.0	488.1→ 130.1
$^{13}C^{15}N_2$-TFV-DP	TIS+	100	65	24.0	15.0	491.1→ 133.1

LC-MS-MS Procedure: Calibration Standard and QC Concentrations and Standard Curve Regression

- Calibration standards and QCs were prepared by spiking 1 mL of blank PBMC pellet lysate with 20 µL of appropriate aqueous spiking solutions that contained both analytes.
- Calibration Standard TFV-TP and FTC-TP Concentrations

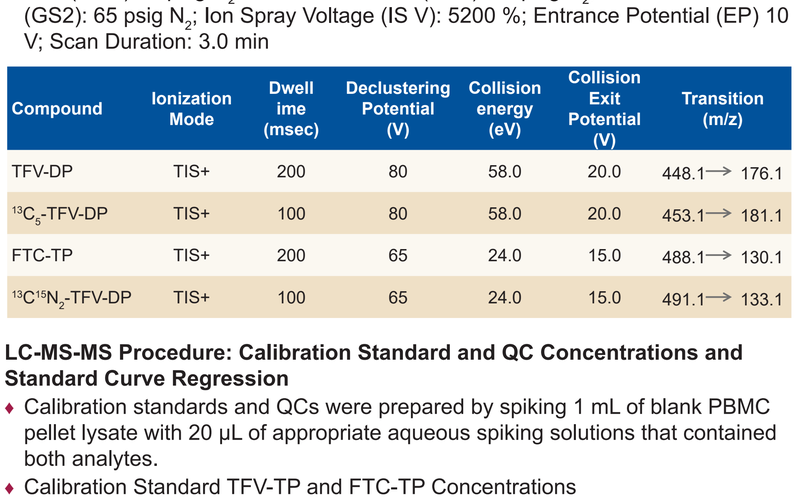
TFV-DP Conc. (ng/mL)	2.00	4.00	10.0	50.0	100	500	900	1000
FTC-TP Conc. (ng/mL)	20.0	40.0	100	500	1000	5000	9000	10000

- QC TFV-TP and FTC-TP Concentrations

TFV-DP Conc. (ng/mL)	2.00	6.00	100	400	800
FTC-TP Conc. (ng/mL)	20.0	60.0	1000	4000	8000

- Standard curve (Peak area ratio vs concentration):
  - Linear regression, weighted by 1/(nominal concentration)^2 for both analytes
  - Concentration converted to ng/sample

Representative Standard Curves



gDNA Cell Counting Procedure (Detailed Steps)

- Add 500 µL of KP buffer to each overnight-incubated sample, calibration standard, and QC sample tube, and then dilute each 1:10 in KPNH buffer; vortex briefly after each addition.
- Transfer 10-µL aliquots of the diluted samples, standards, and controls to wells of a 96-well plate.
- Incubate the plate for 15 min at ambient temperature.
- Add 100 µL of SYBR® WS to each well; incubate plate contents for 1 h at ambient temperature protected from light.
- Read fluoresence signal of plate wells on a Molecular Devices Gemini EM Spectrophotometer (λex 465 nm; λem 530 nm)
- Standard Curve (Signal units vs µg DNA per mL of sample): Linear regression, weighted by 1/nominal concentration
- Convert µg DNA per mL of sample to cell count per sample by factor of 6.65 pg of DNA per cell:
  - 166 µg of DNA per 25 x 10^6 cells, and 0.978 x 10^9 base pairs per pg of DNA (Doležel et al.[3])
  - An average of 3.25 x 10^9 base pairs in the male and female genome4, and 2 pairs of chromosomes per human genome.

→ Report overall result for a sample as ng of analyte per million (10^6) cells.

Results

Table 1. LC-MS/MS Method Validation Precision and Accuracy

QC Nominal Conc. (ng/mL)	TFV-DP		FTC-TP	
	LLOQ 2.00	Others 6.00, 100, 400, 800	LLOQ 20.0	Others 60.0, 1000, 4000, 8000
Intraday Precision (%CV)	6.1 to 10.7	0.7 to 3.9	9.2 to 14.1	1.8 to 10.8
Intraday Accuracy (%RE)	-6.5 to 10.7	-2.7 to 2.0	-10.0 to 7.2	-12.0 to 1.0
Interday Precision (%CV)	9.1	0.8 to 3.4	13.1	3.8 to 8.6
Interday Accuracy (%RE)	-1.0	-0.5 to 1.8	-3.2	-7.3 to -2.0

Table 2. LC-MS/MS Method Validation Results

Parameter	TFV-DP	FTC-TP
Calibrated Range in PBMC Lysate	2.00 to 1000 ng/mL	20.0 to 10,000 ng/mL
Stock and Working Solution Stability -70 °C	≥970 days	≥55 days
Ambient Temperature	≥7 hours	≥8 hours
Dilution Integrity (2-fold dilution)	Pass (1200 ng/mL)	Pass (12,000 ng/mL)
Post-Extraction Sample Stability (4 °C)	≥166 hours	≥166 hours
Selectivity (Analyte ≤20.0% of LLOQ; IS ≤5.0%)	Pass	Pass
QC Spiked to Different Cell Counts	10 x 10^6 and 20 x 10^6 are equivalent	10 x 10^6 and 20 x 10^6 are equivalent
Long-Term Stability: Incurred Sample	≥742 days at -70 °C	≥742 days at -70 °C

Table 3. gDNA Cell Count Method Validation Precision and Accuracy

QC Nominal DNA Conc. (µg/µL)	LLOQ 0.0665	Others 0.200, 0.499, 0.800, 1.17
Intrarun Precision (%CV)	1.6 to 3.2	0.2 to 3.6
Intrarun Accuracy (%RE)	-11.0 to 11.0	-14.8 to 11.6
Interrun Precision (%CV)	6.4	3.2 to 11.0
Interrun Accuracy (%RE)	-4.8	-4.3 to 0.5

Table 4. gDNA Cell Count Method Validation Results

Parameter	Result
Calibrated Range in PBMC Lysate: µg DNA/µL Cells/Sample	 0.0665 to 1.17 2.0 x 106 to 35.0 x 106
Benchtop stability of gDNA in QC sample matrix, spiked PBMC pellet samples, pellet debris samples, and pellet debris lysate samples	≥24 hours at room temperature for all sample types
Freeze/thaw stability of gDNA in QC sample matrix, spiked PBMC pellet samples, pellet debris samples, and pellet debris lysate samples	At least 5 cycles (-70 ° C freeze) for all sample types At least 5 cycles (-270 ° C freeze) for PBMC pellet samples
Long-term stability of gDNA in: QC Sample matrix Spiked PBMC pellet samples Pellet debris samples Pellet debris lysate samples	 ≥342 days (-70 °C) ≥224 days (at -20 °C and at -70 °C) ≥223 days (-70 °C) ≥224 days (-70 °C)
Dilution linearity (1.66 µg/µL in QC sample matrix diluted 2-, 4-, 8-, and 16-fold)	Pass (all dilutions)

Conclusions

- A hyphenated method for simultaneous determination of FTC-TP and TFV-DP in isolated human PBMC samples has been validated.
- The validated method that uses purified gDNA as calibration standards for determination of PBMC counts in PBMC samples after a lysis step to remove TFV-DP and FTC-TP has been incorporated into the method
- Overall results are reported in units of ng/10^6 cells.

References & Acknowledgements

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