Journal of Chromatography B, 878 (2010) 621–627

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous quantification of emtricitabine and tenofovir nucleotides in peripheral blood mononuclear cells using weak anion-exchange liquid chromatography coupled with tandem mass spectrometry

Robert S. Jansen[a,*], Hilde Rosing[a], Wiete Kromdijk[a], Rob ter Heine[a], Jan HM Schellens[b,c], Jos H. Beijnen[a,c]

[a] Department of Pharmacy & Pharmacology, Slotervaart Hospital/The Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands
[b] Department of Clinical Pharmacology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[c] Science Faculty, Department of Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 5 August 2009
Accepted 7 January 2010
Available online 14 January 2010

Keywords:
Emtricitabine
Tenofovir
Nucleotides
LC–MS
Weak anion-exchange

ABSTRACT

Emtricitabine (FTC) and tenofovir (TFV) are widely used antiviral agents that require intracellular phosphorylation to become active. This article describes the development and validation of an assay for the simultaneous quantification of FTC mono-, di- and triphosphate (FTC-MP, -DP and -TP), TFV and TFV mono- and diphosphate (TFV-MP and -DP) in peripheral blood mononuclear cells. Reference compounds and internal standards were obtained by thermal degradation of FTC-TP, TFV-DP, stable isotope-labeled TFV-DP and stable isotope-labeled cytosine triphosphate. Cells were lysed in methanol:water (70:30, v/v) and the extracted nucleotides were analyzed using weak anion-exchange chromatography coupled with tandem mass spectrometry. Calibration ranges in PBMC lysate from 0.727 to 36.4, 1.33 to 66.4 and 1.29 to 64.6 nM for FTC-MP, FTC-DP and FTC-TP and from 1.51 to 75.6, 1.54 to 77.2 and 2.54 to 127 nM for TFV, TFV-MP and TFV-DP, respectively, were validated. Accuracies were within −10.3 and 16.7% deviation at the lower limit of quantification at which the coefficients of variation were less than 18.2%. At the other tested levels accuracies were within −14.3 and 9.81% deviation and the coefficients of variation lower than 14.7%. The stability of the compounds was assessed under various analytically relevant conditions. The method was successfully applied to clinical samples.

1. Introduction

Emtricitabine (2′,3′-dideoxy-5-fluoro-3′-thiacytidine; FTC; Fig. 1) and tenofovir (TFV; Fig. 1) are a nucleoside and a nucleotide reverse transcriptase inhibitor (NRTI and NtRTI), respectively. NRTIs and NtRTIs form the backbone of highly active antiretroviral therapy (HAART), which is used against human immunodeficiency virus (HIV) infections. A combination of both agents with the non-nucleoside reverse transcriptase inhibitor efavirenz has recently been approved as the first once-daily, single pill combination therapy against HIV.

Both analogs require intracellular phosphorylation to become active. In cells, the nucleoside analog FTC is first phosphorylated to its monophosphate (FTC-MP) by deoxycytidine kinase after which it is converted further to its di- and triphosphate (FTC-DP and FTC-TP; Fig. 1).

The acyclic nucleotide analog TFV, on the other hand, already contains a phosphonate moiety. To increase the bioavailability and cell penetration, this polar molecule is administered as a disoproxil fumarate prodrug (TDF) that first requires hydrolization to TFV. Subsequently, TFV is phosphorylated intracellularly to its mono- and diphosphate (TFV-MP and TFV-DP; Fig. 1) containing a phosphonate and one or two phosphate groups in total, respectively. FTC-TP and TFV-DP are the actual active metabolites that inhibit viral reverse transcriptase, thereby inhibiting viral replication.

The intracellular pharmacokinetics of the phosphorylated metabolites are different from the plasma pharmacokinetics of the parent compounds. The plasma half-lifes of FTC and TFV are, for example, 8–10 [1] and 14 [2] h, whereas intracellular half-lifes of 39 [1] and 150 [3] h have been observed for FTC-TP and TFV-DP, respectively. Moreover, metabolite levels are subject to inter-individual variation and intracellular drug-drug interactions [4]. Finally, low triphosphate levels can cause viral resistance, whereas high levels have been associated with treatment toxicity [5]. Thus, monitoring of intracellular FTC and TFV nucleotide levels will give more insight in the pharmacology of FTC and TFV.

FTC-TP and TFV-DP have indirectly been determined in peripheral blood mononuclear cells (PBMCs) after dephosphorylation, using high performance liquid chromatography with ultraviolet (HPLC–UV) [6] and mass spectrometric (LC–MS) detection [7].

* Corresponding author. Tel.: +31 20 5124073; fax: +31 20 5124753.
E-mail address: Robert.jansen@slz.nl (R.S. Jansen).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.01.002

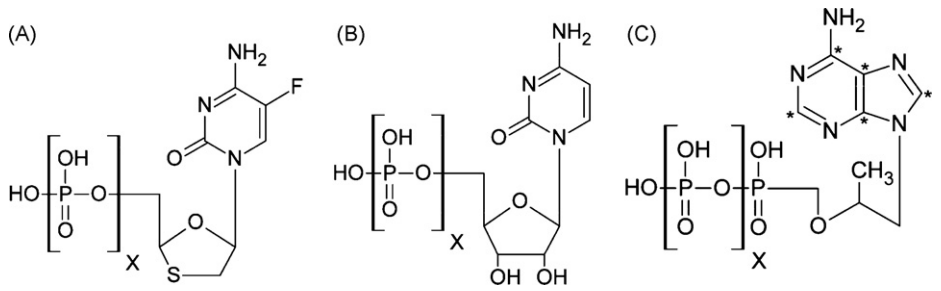

Fig. 1. Chemical structures of FTC (A), cytosine (B) and TFV (C) ($X=0$) and their mono- ($X=1$), di- ($X=2$) and triphosphate ($X=3$). The asterisks indicate ^{13}C-atoms in *TFV internal standards.

Direct LC–MS determination of FTC-TP and TFV-DP has been performed using the ion-pairing agents tetrabutylammonium [1,3,4,8–11] and dimethylhexylamine [12–16] and weak anion-exchange liquid chromatography (WAXLC–MS/MS) [17]. Although some of these methods also include TFV and/or TFV-MP [4,10–12], none include FTC-MP or FTC-DP.

In this paper we describe the development and validation of a method for the quantification of FTC-MP, -DP and -TP in combination with TFV, TFV-MP and TFV-DP in human PBMCs.

2. Materials and methods

2.1. Chemicals

FTC and TFV were purchased from Sequoia Research products (Pangbourne, UK). $^{13}C_9$,$^{15}N_3$(U)-labeled cytidine triphosphate (*CTP; Fig. 1) was purchased from Buchem BV (Apeldoorn, The Netherlands), whereas $^{13}C_5$-labeled TFV-DP (*TFV-DP, Fig. 1) was obtained from Moravek (Brea, CA, USA). Unlabeled FTC-TP and TFV-DP were kindly provided by Gilead Sciences Inc. (Foster city, CA, USA).

Methanol and acetonitrile were obtained from Biosolve Ltd. (Amsterdam, The Netherlands) and distilled water was obtained from B Braun (Melsungen, Germany). Ammonium acetate, aqueous ammonia 25%, glacial acetic acid, tetrabutylammonium dihydrogenphosphate, potassium dihydrogen phosphate were all from Merck (Darmstadt, Germany).

2.2. Instrumentation

HPLC–UV experiments were performed on an Agilent 1100 series liquid chromatograph system (Agilent technologies, Palo Alto, CA, USA) consisting of a binary pump, in-line degasser, autosampler, column oven and UV detector. Data were acquired using Chromeleon 6.50 software (Dionex Corp., Sunnyvale, CA, USA).

The mobile phases consisted of 10 mM tetrabutylammonium dihydrogenphosphate with 70 mM potassium dihydrogen phosphate (mobile phase A) and methanol (mobile phase B). A mobile phase mixture containing 7.5% (v/v) B was delivered isocratically to a Synergi hydro-RP column (150 mm × 2.0 mm ID, 4 μm particles; Phenomenex, Torrance, CA, USA) with a flow of 0.25 mL/min to separate the analytes. FTC and FTC-MP were only separated using 100% mobile phase A delivered to two Synergi hydro-RP columns (150 + 150 mm × 2.0 mm ID, 4 μm particles). A volume of 10 μL was injected using the autosampler thermostated at 4 °C. Absorption was measured at 280 nm (FTC) and 259 nm (TFV).

LC–MS/MS analyses were also performed on an Agilent 1100 series liquid chromatograph system consisting of a binary pump, in-line degasser and autosampler but with an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster city, CA, USA) for detection.

Table 1
HPLC stepwise gradient for the WAXLC–MS/MS experiments.

Time (min)	Flow rate (mL/min)	Mobile phase A[a] (%)	Mobile phase B[b] (%)
0	0.25	90	10
0.50	0.25	90	10
0.51	0.25	50	50
1.75	0.25	50	50
1.76	0.25	0	100
6.50	0.25	0	100
6.60	0.50	90	10
9.50	0.25	90	10
10.0	0.25	90	10

[a] 10 mM ammonium acetate in acetonitrile–water (30:70, v/v) pH 6.0.
[b] 1 mM ammonium acetate in acetonitrile–water (30:70, v/v) pH 10.5.

Mobile phase A (10 mM ammoniumacetate in acetonitrile/water (30:70, v/v) pH 6.0) and B (1 mM ammoniumacetate in acetonitrile/water (30:70, v/v) pH 10.5) were delivered to a Biobasic AX column (50 mm × 2.1 mm ID, 5 μm particles; Thermo Fisher Scientific Inc., Waltham, MA, USA) with a 10 mm guard cartridge as presented in Table 1. Before each injection the needle was washed with methanol for 40 s. Samples were kept at 4 °C in the autosampler tray and a 25 μL injection volume was applied. The switching valve directed the eluate to waste during the first 3 and last 2 min of the run. Ionization was performed using a turbo V ion source operated in the positive ionization mode at a voltage of 5500 V and 550 °C. The curtain gas (N_2) was set at 10, whereas gas 1 and 2 (zero air) were set at 70 and 50, respectively. Scans of 50 ms were performed at unit resolution. The compound dependent scan conditions are summarized in Table 2. Data were acquired and processed using Analyst 1.5 software (Applied Biosystems).

2.3. Preparation of reference and internal standard stock and working solutions

Aqueous solutions of FTC-TP, *CTP, TFV-DP and *TFV-DP were incubated in a water bath at 90 °C. The thermal degradation of

Table 2
Settings for the API4000 triple quadrupole mass spectrometer.

Compound	Precursor ion (m/z)	Product ion (m/z)	Declustering potential (V)	Collision energy (V)
FTC-MP	328	130	45	20
*CMP	336	119	45	20
FTC-DP	408	130	62	35
*CDP	416	119	62	35
FTC-TP	488	130	75	29
*CTP	496	119	75	29
TFV	288	176	126	35
*TFV	293	181	126	35
TFV-MP	368	270	76	27
*TFV-MP	373	275	76	27
TFV-DP	448	270	86	37
*TFV-DP	453	275	86	37

Table 3
Inter-assay performance data for FTC and TFV nucleotides in PBMC lysate.

Compound (calibration range)	Nominal concentration (nM)	Inter-assay accuracy (% DEV)	Inter-assay precision (% CV)
FTC-MP (0.727–36.4 nM)	0.727	−4.57	15.1
	2.27	−12.4	11.0
	4.54	0.793	13.2
	24.2	−0.634	8.37
FTC-DP (1.33–66.4 nM)	1.33	−0.902	10.8
	4.15	−3.37	7.99
	8.30	0.00803	6.92
	43.1	−0.139	10.1
FTC-TP (1.29–64.6 nM)	1.29	−1.79	12.2
	4.04	−11.4	10.2
	8.08	1.39	9.48
	43.1	−2.41	10.7
TFV (1.51–75.6 nM)	1.51	5.74	18.2
	4.73	−7.37	10.7
	9.46	−3.81	9.79
	50.4	−2.76	8.42
TFV-MP (1.54–77.2 nM)	1.54	−1.86	12.9
	4.82	−4.70	11.6
	9.65	−2.00	10.6
	51.4	−4.40	10.5
TFV-DP (2.54–127 nM)	2.54	6.33	12.6
	7.95	−4.82	11.1
	15.9	4.11	9.55
	84.8	−3.59	10.7

DEV: deviation from nominal.
CV: coefficient of variation.

the compounds into lower phosphates was monitored using the HPLC–UV system described in Section 2.2. Mixtures containing similar amounts of FTC, *C, TFV and *TFV nucleotides were obtained after 5, 4.25, 3 and 2 h, respectively. The content of these nucleotide mixtures was determined using HPLC–UV. Two FTC and two TFV reference solutions were prepared from separate weightings. The peak areas obtained by injecting these references solutions in three-fold were compared to those obtained by injecting the nucleotide solutions with unknown concentrations in three-fold. The mixtures of FTC and TFV nucleotides were finally mixed and diluted in water to obtain working solutions. Likewise, the *C nucleotides were mixed with the *TFV nucleotides and diluted in water to obtain the internal standard working solution. All solutions were stored at −70 °C.

2.4. Isolation and lysis of PBMCs

Blank PBMCs were isolated from human leukocyte buffy coat (Sanquin, Amsterdam, the Netherlands) as previously described [18]. Clinical samples were obtained by drawing 8 mL whole blood in a cell preparation tube (BD Vacutainer CPT; BD, Franklin lakes, NJ, USA). After centrifugation at 1500 × *g* for 30 min (20–25 °C) the PBMCs were collected, transferred to a 15 mL tube and washed twice with 14 mL cold phosphate buffered saline (PBS) in a cooled centrifuge (4 °C). The number of cells isolated was determined using a Cell-Dyn 4000 haematology analyzer (Abbott Diagnostics, Abbott Park, IL, USA). Blank PBMCs were directly lysed in methanol:water (70:30, v/v) (22.5×10^6 PBMCs/mL), whereas clinical samples were stored at −20 °C as a cell pellet, and lysed in 200 μL methanol:water (70:30, v/v) before analysis.

2.5. Preparation of calibration, validation and stability samples

Six non-zero calibration standards (CALs) and validation samples (VSs) at the lower limit of quantitation (LLOQ), low, mid and high level (Table 3) were freshly prepared in blank PBMC lysate before each analytical run.

Stability samples (SSs) were prepared at the low and high level by spiking blank PBMC lysate with working solution (1.5% or less of the total volume). Aliquots of 100 μL of these solutions were stored at the test conditions and treated as unknowns before processing and analysis.

2.6. Sample processing

Unknowns and blank lysates were vortex mixed for 10 s. To 90 μL of the suspensions, 10 μL of working solution (CALs and VSs) or water (unknowns) was added. Internal standard working solution (5 μL) was then added at a concentration of approximately 3 nM for *CMP, *CDP and *CTP and of 10–15 nM for *TFV, *TFV-MP and *TFV-DP. Finally, the samples were again vortex mixed, centrifuged for 5 min at 23,100 × *g* and transferred to an autosampler vial.

2.7. Validation procedures

2.7.1. Linearity

The calibration standards were prepared and analyzed in duplicate in three separate analytical runs. Deviations of the back-calculated concentrations from the nominal concentrations should be within ±20% at the LLOQ and within ±15% at the other levels. No more than one third of the calibration standards were allowed to be rejected from the calibration curves.

2.7.2. Accuracy and precision

Validation samples at 4 levels were analyzed in five-fold in 3 analytical runs. For each compound the accuracy and precision values were assessed. The differences between the nominal and the determined concentration were used to calculate the intra- and inter-assay accuracies. The coefficients of variation (CV) were calculated to assess the precision of the method. Deviations and precisions should be within ±15% and less than 15%, respectively, except at the LLOQ where they should be within ±20% and less than 20%, respectively.

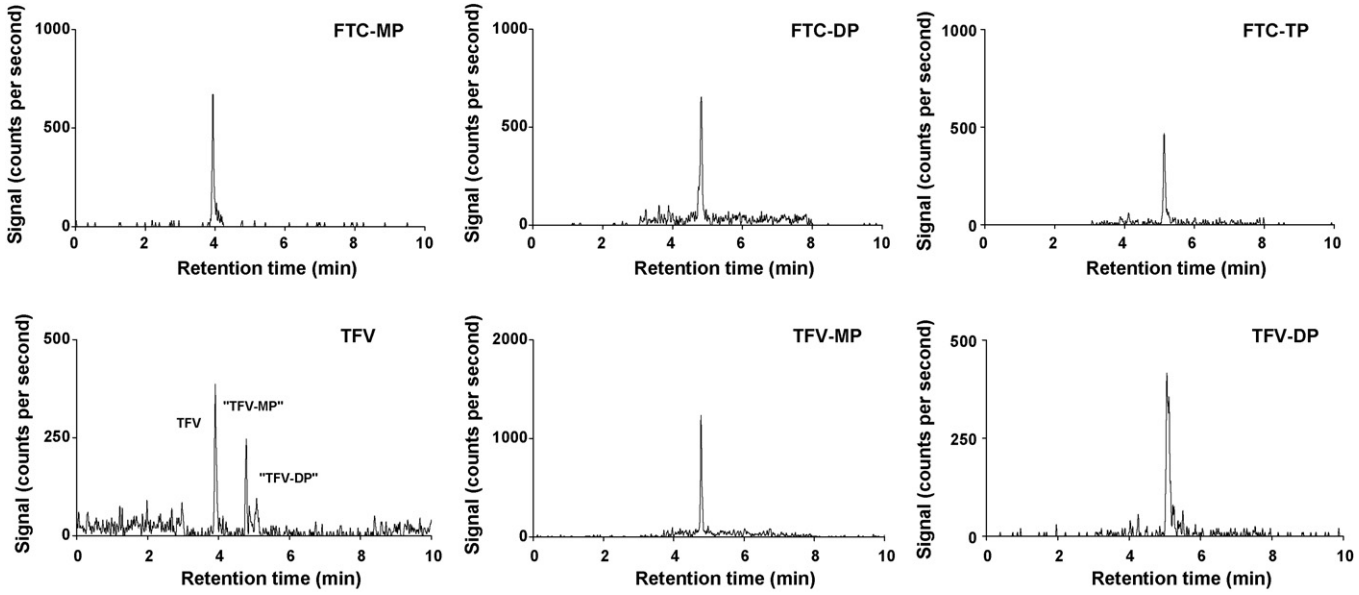

Fig. 2. Chromatograms of a sample spiked at the LLOQ level, containing 0.727 (FTC-MP), 1.33 (FTC-DP), 1.29 (FTC-TP), 1.51 (TFV), 1.54 (TFV-MP) and 2.54 nM (TFV-DP). "TFV-MP" and "TFV-DP" indicate the peaks in the TFV mass transition caused by up-front fragmentation of TFV-MP and TFV-DP to TFV.

2.7.3. Specificity and selectivity

Specificity and selectivity were determined in PBMC lysate from 5 different individuals. Double blank (no internal standard added) samples were checked for analyte and internal standard interferences. Samples spiked at the LLOQ level were analyzed and the deviation from the nominal concentration should be within ±20%.

2.7.4. Matrix effect and recovery

Lysate containing 0, 25, 50, 75, 100 and 125 × 10^6 PBMCs/mL (corresponding to 0, 5, 10, 15, 20 and 25 × 10^6 PBMCs per sample) was prepared in methanol:water (70:30, v/v) using PBMCs isolated from a single donor buffy coat. These suspensions were used to prepare validation samples at the mid level as described in Section 2.6. Each PBMC concentration was processed and analyzed in triplicate.

2.7.5. Carry over

Carry over was assessed by injecting a blank sample after the highest calibration standard. Carry over of the analytes should be less than 20% of the peak area of a sample at the LLOQ. Carry over of the internal standards should be less than 5% of the internal standard peak area in a spiked sample.

2.7.6. Stability

Stability of the stock solutions was assessed after 5 h at 20–25 °C using the HPLC–UV system. Stock solutions were considered stable if the determined concentrations did not deviate more than ±5% from the concentration determined at time zero. Stability in lysate was determined after 6 h at 20–25 °C. Moreover, we assessed the reinjection reproducibility of the processed samples by reinjecting the calibration samples and the validation samples at the low and high level after 24 h in the autosampler at 4 °C. Analytes were considered stable in biomatrix if the determined concentrations did not deviate more than ±15% from the concentration determined at time zero. All stability experiments were performed in triplicate.

2.8. Application of the assay

Using the described method, PBMCs were isolated from a patient treated with 200 mg FTC and 245 mg TDF once-daily for 3 years, who switched to a single once-daily tablet also containing 200 mg FTC and 245 mg TDF. Samples were collected 4, 8 and 12 months after therapy switch. The analytical result, expressed as nM in PBMC lysate, was finally multiplied with the lysate sample volume to obtain the absolute amount of the analytes in a sample. This amount was then divided by the number of cells present in the sample to obtain the amount of analyte per 10^6 PBMCs.

3. Results and discussion

3.1. Reference standards

Because some analytes were not commercially available we thermally degraded FTC-TP, *CTP, TFV-DP and *TFV-DP into mixtures of their lower phosphates. Since the chromophores of a nucleoside and its nucleotides are identical we could quantify the obtained mixtures on the isocratic HPLC–UV systems, using reference FTC and TFV material. The thus obtained solutions contained 32.7, 59.8 and 58.2 μM (FTC-MP, -DP and -TP, respectively), 68.1, 69.4 and 115 μM (TFV, TFV-MP and -DP, respectively). The *TFV internal standards did not cause a signal in the mass transitions of their unlabeled variants, indicating sufficient isotopic purity. Stable isotope-labeled FTC nucleotides were not commercially available. Therefore we used the nucleotides of the stable isotope-labeled structural analog cytosine.

3.2. WAXLC–MS/MS

Up-front fragmentation causes the degradation of higher phosphates into lower phosphates before entering the mass analyzers. Therefore, separation between mono-, di- and triphosphates is necessary for their correct quantification. We have previously developed a method for the separation of nucleoside mono-, di- and triphosphates, which we now applied to these analytes [18,19]. Separation was achieved by applying a pH gradient to a weak anion-exchange column, thereby decreasing the anion-exchange capacity of the column and separating the nucleotides [20]. Fig. 2 shows typical chromatograms of a sample spiked at the LLOQ. The effect of the up-front fragmentation is clearly visible as peaks in the mass transition of TFV at the retention time of TFV-MP and TFV-DP. Reducing the declustering potential to decrease up-front fragmentation did not result in an increased TFV-MP and -DP signal intensity. The sensitivity was similar using the negative and the positive ioniza-

Table 4
Stability data of the analytes in working solutions and biomatrix.

Matrix	Condition	Analyte	Initial concentration (nM/μM)	Found concentration (nM/μM)	Deviation (%)
Water (stock solution)	5 h, 20–25 °C	FTC-MP	*32.7*	*33.3*	1.83
		FTC-DP	*59.8*	*60.7*	1.51
		FTC-TP	*58.2*	*59.1*	1.55
		TFV	*68.1*	*70.9*	4.11
		TFV-MP	*69.4*	*71.9*	3.60
		TFV-DP	*115*	*115*	0.00
PBMC lysate	6 h, 20–25 °C	FTC-MP	2.29	2.21	−3.35
			21.2	23.8	12.4
		FTC-DP	4.00	3.77	−5.59
			41.3	38.9	−5.65
		FTC-TP	3.54	3.05	−13.9
			37.9	38.0	0.264
		TFV	3.78	4.45	10.7
			43.7	48.3	10.7
		TFV-MP	4.57	5.01	9.62
			46.7	48.0	2.79
		TFV-DP	7.31	8.18	11.9
			74.9	71.3	−4.81
Final extract	24 h reinjection, autosampler (4 °C)	FTC-MP	2.02	2.21	8.58
			24.2	21.3	−13.5
		FTC-DP	3.97	3.58	−10.9
			41.6	47.7	12.7
		FTC-TP	3.39	3.38	−0.494
			40.0	44.2	9.58
		TFV	4.20	3.84	−9.38
			50.1	48.3	−3.73
		TFV-MP	4.62	4.22	−9.64
			50.0	51.8	3.41
		TFV-DP	7.21	6.94	−3.89
			86.5	76.5	−13.1

tion mode for all analytes except TFV, which was ionized more efficiently in the positive ionization mode. Although the detection of these phosphate-containing analytes is intuitively expected to be more sensitive in the negative ionization mode, we and others showed the effective ionization and superior selectivity in the positive ionization mode [14,19]. For the nucleotides of the pyrimidines FTC and *C, the protonated base was the most abundant fragment for each nucleotide. The loss of a mono- and diphosphate moiety most likely results in the m/z 270 fragment of TFV-MP and TFV-DP, whereas cleavage of the ether bond resulted in a fragment including the base (m/z 176) for TFV.

3.3. Validation procedures

3.3.1. Linearity

The calibration curves were constructed using linear regression of the analyte-internal standard ratio with a $1/x^2$ weighting factor. The deviations of the mean back-calculated concentrations from nominal were between −1.98 and 3.89%, −3.58 and 4.94% and −3.51 and 3.79% for FTC-MP, FTC-DP and FTC-TP, respectively. Likewise, the deviations were between −1.56 and 1.43%, −1.47 and 2.01% and −3.15 and 2.89% for TFV, TFV-MP and TFV-DP, respectively. The coefficients of variance were lower than 13.1% and correlation coefficients (r) were better than 0.990 for all analytes.

3.3.2. Accuracy and precision

In Table 3 the inter-assay performance is summarized for the tested concentration levels. Likewise, the intra-assay accuracies and precisions were between −10.3 and 16.7% deviation and lower than 17.8% CV for all analytes at the LLOQ level, whereas the intra-assay accuracies and precisions were between −14.3 and 9.81% deviation and lower than 14.7% CV for all analytes at the other concentrations. Therefore, the accuracy and precision of the method comply to commonly accepted criteria for bioanalytical method validation [21].

3.3.3. Specificity and selectivity

No interferences were observed in the chromatograms from blank PBMC lysate at the retention times of the analytes or internal standards. Moreover, the accuracies of the samples spiked at the LLOQ ranged from −18.0 to 19.7% deviation from nominal. Thus, the specificity and selectivity of this assay are satisfactory.

3.3.4. Matrix effect and recovery

The number of PBMCs isolated can vary per sample. Moreover, this factor is very important for the matrix effect and recovery [22]. Therefore, the matrix effect and recovery was determined at several cell concentrations including the normal range of 5–20 × 10^6 PBMCs per sample [22].

Fig. 3 shows the matrix effect and recovery for FTC-TP. Similar results were obtained for all other analytes. As expected, the absolute analyte signals decrease at higher cell amounts. This can be due to absorption to cell debris (recovery) or ion-suppression caused by

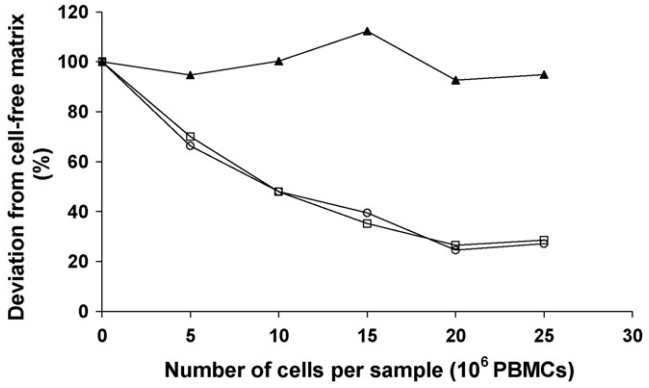

Fig. 3. Matrix effect plus recovery for FTC-TP (□) and *CTP(○), and the analyte-internal standard ratio(▲) in samples containing different amounts of PBMCs.

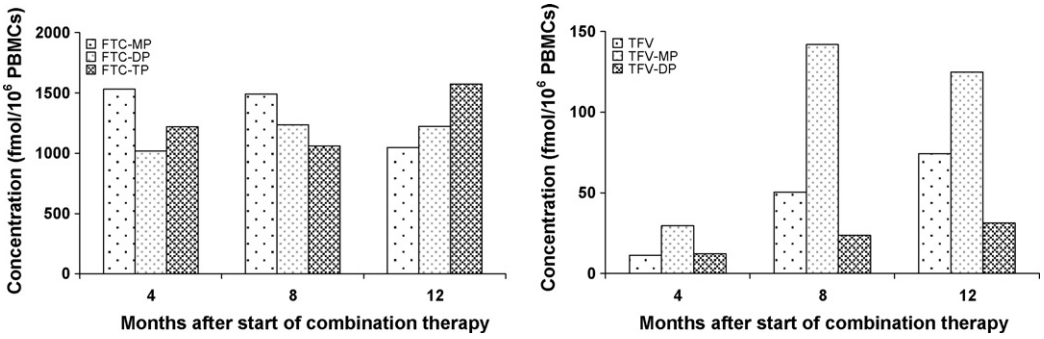

Fig. 4. Analyte levels in PBMCs from a patient on treatment with once-daily truvada (containing 200 mg FTC and 245 mg TDF).

co-eluting cell constituents eluting at the same time as the analytes (matrix effect). The internal standards, however, corrected well for this effect. The analyte-internal standard ratios determined in cell-containing samples were between 88.7 and 112% of those determined in the cell-free samples, showing the robustness of the assay towards number of isolated cells.

3.3.5. Carry over

Carry over was less than 17.1% of the area of an LLOQ sample for all analytes and less than 4.35% of the internal standard area of a spiked sample for all internal standards. Therefore, carry over of the analytes and internal standards was acceptable.

3.3.6. Stability

The stability data are presented in Table 4. The table shows the concentrations determined at time zero and after the indicated periods under several conditions. The concentrations determined after storage under the test conditions did not deviate more than ±5% for the stock solutions and not more than ±15% for the biological samples indicating acceptable stability.

3.4. Application of the assay

The determined nucleotide levels are depicted in Fig. 4. All analytes were detected in the samples. The levels of FTC-TP were low, but in the range of previously reported values [1]. The sparse data on FTC-MP and FTC-DP show comparable FTC-MP, FTC-DP and FTC-TP levels, although FTC-MP and FTC-DP levels are relatively high in these samples [6].

Like the FTC nucleotide levels, the levels of TFV and its metabolites were low compared to previously reported values [3,7,16]. TFV and its metabolites have, to the best of our knowledge, never been determined simultaneously in clinical samples, but *in vitro* and macaque experiments by others showed several different TFV:TFV-MP:TFV-DP ratios at similar concentrations [4,10,11,23]. TFV-MP levels that are higher than TFV-DP levels have, however, never been reported. In contrast to our results, these ratios were determined after short exposure to the drugs.

Interestingly, the FTC nucleotides do not show a trend in time, whereas the levels of TFV and its mono and diphosphate increase over the sampling period of 8 months, indicating slow intracellular accumulation.

The relatively low FTC-TP and TFV-DP levels in combination with relatively high levels of their lower phosphates raise the possibility that degradation might have taken place during sample collection and pretreatment. The methods used for sample collection and processing were, however, very similar to previously published methods. Cell isolation has been performed using CPT-tubes [3,7,16] or ficoll paque [1,4,23], followed by washing steps using a cooled saline solution [3,15,16,23]. Since TFV can also be phosphorylated in red blood cells, some have performed a red blood cell lysis step [15,23]. The lack of such a lysis step in our procedure does, however, not explain the relatively low analyte levels. Cell lysis using 70% methanol is widely used and has been validated by others based on the levels of endogenous nucleotides [23,24]. These levels have been monitored in clinical samples, serving as a control for reproducible sample pretreatment [23]. Thus, although the analytes show a pattern that suggests degradation, the fact that widely accepted and validated methods were used, makes degradation unlikely.

4. Conclusions

A sensitive method for the simultaneous quantification of FTC-MP, -DP and -TP, and TFV, TFV-MP and -DP has been developed and validated. Assuming a mean of 10 million PBMCs per sample the LLOQ of the method is 14.5, 26.6 and 25.9 fmol/10^6 PBMCs for FTC-MP, FTC-DP and FTC-TP and 30.3, 30.9 and 50.9 fmol/10^6 PBMCs for TFV, TFV-MP and TFV-DP, respectively. The method requires minimal sample pretreatment and is the first for the simultaneous quantitation all 6 analytes.

References

[1] L.H. Wang, J. Begley, C.R. St III, J. Harris, C. Wakeford, F.S. Rousseau, AIDS Res. Hum. Retroviruses 20 (2004) 1173.
[2] P. Barditch-Crovo, S.G. Deeks, A. Collier, S. Safrin, D.F. Coakley, M. Miller, B.P. Kearney, R.L. Coleman, P.D. Lamy, J.O. Kahn, I. McGowan, P.S. Lietman, Antimicrob. Agents Chemother. 45 (2001) 2733.
[3] T. Hawkins, W. Veikley, R.L. Claire III, B. Guyer, N. Clark, B.P. Kearney, J. Acquir. Immune. Defic. Syndr. 39 (2005) 406.
[4] K. Borroto-Esoda, J.E. Vela, F. Myrick, A.S. Ray, M.D. Miller, Antivir. Ther. 11 (2006) 377.
[5] D.J. Back, D.M. Burger, C.W. Flexner, J.G. Gerber, J. Acquir. Immune. Defic. Syndr. 39 (Suppl. 1) (2005) S1.
[6] A. Darque, G. Valette, F. Rousseau, L.H. Wang, J.P. Sommadossi, X.J. Zhou, Antimicrob. Agents Chemother. 43 (1999) 2245.
[7] T. King, L. Bushman, J. Kiser, P.L. Anderson, M. Ray, T. Delahunty, C.V. Fletcher, J. Chromatogr. B 843 (2006) 147.
[8] R.L. Claire III, Rapid Commun. Mass Spectrom. 14 (2000) 1625.
[9] S. Bartley, J.A. Begley, T.N. Clark, Proceedings of the 55th ASMS Conference on Mass Spectrometry and Allied Topics, 2007.
[10] W.E. Delaney, A.S. Ray, H. Yang, X. Qi, S. Xiong, Y. Zhu, M.D. Miller, Antimicrob. Agents Chemother. 50 (2006) 2471.
[11] A.S. Ray, F. Myrick, J.E. Vela, L.Y. Olson, E.J. Eisenberg, K. Borroto-Esodo, M.D. Miller, A. Fridland, Antivir. Ther. 10 (2005) 451.
[12] T. Lynch, G. Eisenberg, M. Kernan, Nucleosides Nucleotides Nucleic Acids 20 (2001) 1415.
[13] Z. Kuklenyik, A. Martin, C. Pau, G. Garcia-Lerma, W. Heneine, J. Pirkle, J. Barr, Proceedings of the 56th ASMS Conference on Mass Spectrometry and Allied Topics, 2008.
[14] A. Pruvost, F. Theodoro, L. Agrofoglio, E. Negredo, H. Benech, J. Mass Spectrom. 43 (2008) 224.
[15] L. Durand-Gasselin, D. Da Silva, H. Benech, A. Pruvost, J. Grassi, Antimicrob. Agents Chemother. 51 (2007) 2105.
[16] A. Pruvost, E. Negredo, H. Benech, F. Theodoro, J. Puig, E. Grau, E. Garcia, J. Molto, J. Grassi, B. Clotet, Antimicrob. Agents Chemother. 49 (2005) 1907.
[17] D. Delinsky, B. Hernandez-Santiago, R. Schinazi, Proceedings of the 54th ASMS Conference on Mass Spectrometry and Allied Topics, 2006.

[18] S.A. Veltkamp, M.J. Hillebrand, H. Rosing, R.S. Jansen, E.R. Wickremsinhe, E.J. Perkins, J.H. Schellens, J.H. Beijnen, J. Mass Spectrom. 41 (2006) 1633.
[19] R.S. Jansen, H. Rosing, C.J. de Wolf, J.H. Beijnen, Rapid Commun. Mass Spectrom. 21 (2007) 4049.
[20] G. Shi, J.T. Wu, Y. Li, R. Geleziunas, K. Gallagher, T. Emm, T. Olah, S. Unger, Rapid Commun. Mass Spectrom. 16 (2002) 1092.
[21] U.S Food and Drug Administration: Centre for Drug Evaluation and Research, Guidance for Industry: Bioanalytical Method Validation, 2001.
[22] F. Becher, A. Pruvost, J. Gale, P. Couerbe, C. Goujard, V. Boutet, E. Ezan, J. Grassi, H. Benech, J. Mass Spectrom. 38 (2003) 879.
[23] L. Durand-Gasselin, K.K. Van Rompay, J.E. Vela, I.N. Henne, W.A. Lee, G.R. Rhodes, A.S. Ray, Mol. Pharm. 6 (2009) 1145.
[24] J.E. Vela, L.Y. Olson, A. Huang, A. Fridland, A.S. Ray, J. Chromatogr. B 848 (2007) 335.