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Tenofovir Diphosphate and Emtricitabine Triphosphate Concentrations in Blood Cells Compared with Isolated Peripheral Blood Mononuclear Cells: A New Measure of Antiretroviral Adherence?

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Abstract

Background—The active metabolites of tenofovir (TFV) and emtricitabine (FTC) in peripheral blood mononuclear cells (PBMCs) have been used as markers of long-term antiretroviral (ARV) adherence. However, the process of isolating PBMCs is expensive, complex, and not feasible in many settings. We compared concentrations of TFV-diphosphate (TFV-DP) and FTC-triphosphate (FTC-TP) in the upper layer packed cells (ULPC) obtained after whole blood centrifugation to isolated PBMCs as a possible alternative marker of adherence.

Methods—Ten HIV+ adults with HIV RNA <50 copies/mL on a TDF/FTC-containing regimen provided five paired PBMC and ULPC samples over 6h. TFV-DP and FTC-TP concentrations were analyzed by liquid chromatography/mass spectrometry (LC-MS/MS). Partial areas under the curve (AUC) were calculated using noncompartmental methods and Spearman Rank Correlations (rho) between PBMC and ULPC were determined.

Results—The median (25^{th}–75^{th} percentile) concentration of TFV-DP in PBMCs was 143 (103-248) fmol/10^6 cells and in ULPC was 227 (160-394) fmol/10^6 cells (rho=0.65;p <0.0001). The concentration of FTC-TP in PBMCs was 6660 (5650-10000) fmol/10^6 cells and in ULPC was 19.0 (12.0-27.8) fmol/10^6 cells (rho 0.55;p<0.0001). Compared to PBMCs, ULPC TFV-DP was 64% higher and FTC-TP was 99.7% lower. ULPC concentrations of TFV-DP and FTC-TP in 1

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Conflicts of Interest: KBP and ADMK have previously received grant support from Gilead. Gilead donated study product to FEM-PrEP (LV, KF, TC, IDB). JLA, CS, PM, HMAP have no COI.

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additional subject receiving a single dose of TDF/FTC were only 0.05% and 25%, of the other 10 subjects, respectively.

Conclusions—ULPC concentrations significantly correlated with PBMC concentrations. Preliminary single-dose data suggest some discrimination between intermittent vs. consistent dosing. ULPC concentrations of TFV-DP and FTC-TP should be further investigated as a simply-collected, surrogate measure of ARV adherence.

Keywords

tenofovir disphosphate; emtricitabine triphosphate; adherence; intracellular concentrations; red blood cells; total blood cells; peripheral blood mononuclear cells

Introduction

Recent studies of the fixed dose combination of oral tenofovir disoproxyl fumarate (TDF) and emtricitabine (FTC), marketed as Truvada®, for the prevention of HIV infection have had discrepant results. The iPrEX study of daily TDF/FTC in men who have sex with men demonstrated 44% protective efficacy overall, and 73% protective efficacy in subjects reporting >90% adherence.[1] The Partners PrEP study arm of daily TDF/FTC in the uninfected partners of serodiscordant heterosexual couples demonstrated 75% protective efficacy with 81% adherence as measured by detectable plasma drug concentrations.[2,3] Additionally, the TDF2 study of TDF/FTC in heterosexual men and women, demonstrated 62% efficacy with 80% adherence as measured by plasma drug concentrations.[4] In contrast, the FEM-PrEP study, which evaluated the efficacy of daily TDF/FTC in preventing HIV infection in high risk heterosexual women, was stopped early due to futility.[5] Although study subjects reported >90% adherence to study drug therapy, tenofovir (TFV) and FTC were detected in the plasma of only 21% of infected women at the visit in which she first had evidence of infection and only 26% at the last visit with no evidence of infection.[5] Only 37% and 35% of uninfected controls had detectable drug concentrations at these same visits.

These studies demonstrate that consistent adherence is critical for efficacy, and may particularly be for women. However, it is difficult to objectively measure adherence in real-time during clinical trials, and self-reported adherence often overestimates true adherence.[6,7] Concentrations of TFV and FTC in plasma, do not accumulate significantly over time (plasma half-lives are 17 and 10 hours respectively), and thus cannot be used to differentiate between single, intermittent and consistent dosing,[8] but can be used as measures of short-term adherence.

Conversely, TFV-DP and FTC-TP in peripheral blood mononuclear cells (PBMCs) have half-lives of 6.25 and 1.6 days, respectively.[9,10] Therefore, their accumulation has been considered a better measure of adherence, although no algorithms yet exist for interpretation of the results, and the isolation of PBMCs is both expensive and difficult. However, recently Rower et al demonstrated that the concentration of TFV-DP in the red blood cells (RBCs) of 5 subjects correlated with TFV-DP concentration in PBMCs.[11] Therefore, RBCs may be an alternate vehicle for measuring these compounds with long half-lives.

During the FEM-PrEP study, investigators collected monthly samples of “upper layer packed cells” (ULPC) for future virology investigations. The layer of blood cells that remained after centrifugation and plasma removal from a 10mL EDTA tube is estimated to contain approximately 10^{6-7} PBMCs and 10^{9-10} RBCs.[12] In order to determine whether these samples could be used to measure TFV-DP and FTC-TP as a surrogate for longer-term adherence, we conducted an investigation to determine the intra- and interindividual variability of TFV-DP and FTC-TP in ULPC samples, to evaluate the correlation between TFV-DP and FTC-TP concentrations in ULPC and PBMC samples, and to determine the stability of the ULPC samples as collected and processed at the FEM-PrEP study sites.

Methods

Study Design and Population

Ten HIV-infected adults taking FTC 200mg and TDF 300mg daily as a part of their antiretroviral regimen (either as Truvada® or in Atripla®) were recruited from the UNC Healthcare Infectious Diseases Clinic in Chapel Hill, NC. The study was conducted under a general blood draw protocol, approved by the UNC Biomedical Institutional Review Board and all study activities were carried out in accordance with the ethical standards of the Helsinki Declaration of 1975, as revised in 2000. Subjects with an undetectable viral load for 6 months and provider endorsed adherence were specifically chosen for recruitment.

Sample Collection and Processing

Subjects were admitted to the North Carolina Translational and Clinical Sciences (NC TraCS) Institute Clinical and Translational Research Center (CTRC). Blood was collected in 10ml K_2EDTA tubes (BD Diagnostics, Franklin Lakes, NJ) and 8ml CPT tubes (BD Diagnostics, Franklin Lakes, NJ) at 0, 1, 2, 4 and 6 hours following a witnessed dose, or 12, 13, 14, 16, and 18 hours following a subject verified dose taken the night prior to admission. Subjects took their doses of TDF/FTC within an hour of the time that they usually take their home dose.

Within 4 hours after collection, K_2EDTA tubes stored at room temperature were processed by centrifugation at 800xg at 21°C for 10 minutes, as was done at the urban FEM-PrEP study sites. The resultant plasma was centrifuged again and then aliquoted into labeled cryovials, and stored at −80°C. The total blood cells remaining were counted by trypan blue exclusion on a Countess Cell Counter (Invitrogen™) and then frozen at −80°C. To simulate conditions at a rural FEM-PrEP study site, at one time point per subject, the ULPC samples were split between two cryovials: one was immediately processed and cells stored at −80°C, and the other refrigerated for 14 hours prior to processing and storage at −80°C.

Within 2 hours after collection, CPT tubes were centrifuged at 1300xg for 30 minutes at room temperature with the brake off. The resulting PBMC-containing upper layer was removed, combined with 2mL of cold phosphate buffered saline (PBS) used to wash cells off the gel layer of the CPT tube, and centrifuged at 350xg for 10 minutes at 4°C. After discarding the supernatant, the cell pellet was re-suspended in red blood cell lysis buffer and allowed to sit at room temperature for 2 minutes then 10mL of cold PBS was added and the

cells were again centrifuged at 300xg for 5 minutes at 4°C. Cells were counted using Trypan blue exclusion and a Countess Cell Counter. After counting, cells were lysed with 300μL of 70:30 methanol:water solution and placed on ice for 15 minutes before storage at −80°C until analysis.

Since the majority of the PBMCs would be expected to reside within the top portion of the ULPC, further analysis was performed to determine if TFV-DP and FTC-TP concentrations would differ depending on where the sample was obtained. Therefore, aliquots of 0.5ml were taken from the top and bottom of an additional 10mL EDTA tube from a subject taking TDF/FTC.

Laboratory Analysis

The direct determination of TFV-DP and FTC-TP concentrations was performed in PBMC samples by protein precipitation followed by LC-MS/MS analysis. Calibration standards and quality control samples were prepared in PBMC lysate (70:30 methanol:water with $1*10^6$ cells/mL lysate). The stored PBMC samples were centrifuged and the methanolic extracts subjected to protein precipitation with 1:1 methanol:1mM ammonium phosphate solution containing the isotopically-labeled internal standard ^{13}C TFV-DP (Moravek Biochemicals, Brea, CA). Samples were evaporated to dryness under nitrogen and reconstituted with 1mM ammonium phosphate. Using a Shimadzu High Performance Liquid Chromatography (HPLC) system (Shimadzu, Columbia, MD), the analytes were eluted from a Thermo Scientific BioBasic AX (50 × 2.1mm 5μm particle size) column (Thermo Fisher Scientific, Waltham, MA) with 70:30 10mM ammonium acetate:acetonitrile (pH 5.55) and 75:25 10mM ammonium acetate:acetonitrile (pH 9.45) as the mobile phases. An API- 5000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA) operated in positive ion electrospray mode was used to detect analytes. Data were collected using AB Sciex Analyst Software, with m/z transitions of 448.0/270.0 (TFV-DP) and 488.0/130.1 (FTC-TP). The dynamic range of the assay was 1-2,500 ng/mL lysate; raw concentration values were normalized for cells counts (cells/mL lysate) and molecular mass of the analyte, with final concentration values reported as fmol/10^6 cells. All calibrators and quality control samples were within 15% of the nominal value for both within-day and between-day runs. Within-day and between-day precision was < 15%. Recoveries of TFV-DP, FTC-TP, and ^{13}C TFV-DP seen with this methodology were all approximately 100%.

The extraction of TFV-DP and FTC-TP from ULPC samples was performed in an ice bath to maintain analyte stability. Calibration standards and quality control samples were prepared in packed red blood cells (Biological Specialty Corporation, Colmar, PA). Isotopically-labeled ^{13}C TFV-DP and lamivudine triphosphate (3TC-TP) were added as internal standards. Analytes were extracted with 70:30 dichloromethane:methanol. The upper aqueous layer was removed, evaporated under nitrogen, and reconstituted with 1mM ammonium phosphate. LC-MS/MS analysis was performed using similar conditions to those described for the PBMC samples. The same anion exchange column (Thermo Scientific BioBasic AX) was used for ULPC sample analysis, but the mobile phases used were 750mM ammonium acetate and 75:25 5mM ammonium actate:acetonitrile (pH9.50). The use of high salt (750mM ammonium acetate) removed carryover from the assay, but a divert

valve was required to redirect the flow to waste during the high salt portion of the gradient. In the ULPC analysis, m/z transitions of 448.0/350.0 (TFV-DP) and 488.0/130.1 (FTC-TP) were monitored. Using a 100uL extraction volume, the dynamic range of the assay was 10,000-15,000,000 fmol/mL (TFV-DP) and 5,000-7,500,000 fmol/mL (FTC-TP). Cell counts were then utilized to calculate concentrations in fmol/10^6 cells for comparison to PBMC values. The recoveries of TFV-DP and ^{13}C TFV-DP were approximately 20%, while the recoveries of FTC-TP and 3TC-TP were approximately 60%. All calibrators and quality controls samples were within 15% of the nominal value. Within-day and between-day precision was < 15%.

Pharmacokinetic and Statistical Analysis

Noncompartmental analysis was performed using Phoenix Win Nonlin v6.1 (Pharsight, Inc; Cary, NC). Partial area-under-the-concentration-time curves over 6 hours ($AUC_{0\text{-}6hr}$ or $AUC_{12\text{-}18hr}$) were determined using the trapezoidal rule (linear up/log down interpolation). Geometric Mean Ratios (GMR) with 90% confidence intervals were calculated to compare ULPC and PBMC concentrations. Summary statistics and Spearman Rank Correlation were calculated using SAS 9.2 (SAS Institute Inc; Cary, NC). Data are presented as median (25^{th}-75^{th} percentile) unless otherwise noted.

Results

Demographics

Six of the ten subjects were female and 80% were African American. The median age was 49.5 years (range 25-57 years). All participants had an undetectable viral load at their previous clinic visit (<40 copies/mL) and the median CD4 cell count at last draw was 911 cells/mL (range 572-1380 cells/mL). The HIV antiretroviral regimens varied, but all contained TDF and FTC. The other antiretrovirals were as follows: atazanavir/ritonavir (three subjects), lopinavir/ritonavir (two subjects), darunavir/ritonavir (one subject), efavirenz (two subjects), raltegravir (one subject), and a combination of raltegravir, maraviroc, and darunavir/ritonavir (one subject).

Plasma Concentrations

The median (interquartile range (IQR)) Cmax in plasma was 349 (314-433)ng/mL for TFV and 2970 (2020-3310)ng/mL for FTC in those subjects sampled from 0 to 6 hours post-dose. The median (IQR) Tmax in plasma was 1 (1-1.5) hours for TFV and 2 (1-2) hours for FTC. For those subjects that were sampled from 12 to 18 hours post-dose, the median (IQR) plasma concentration at C_{12} was 93.1 (77.1-102) ng/mL for TFV and 223 (184-584) for FTC.

ULPC Concentrations Compared to PBMC Concentrations

The median (IQR) concentration of TFV-DP in PBMCs was 143 (103-248) fmol/10^6 cells and in ULPCs was 227 (160-394) fmol/10^6 cells. The ULPC concentrations of TFV-DP were 64% higher on average than the PBMC concentrations with a GMR (90% CI) of 1.64 (1.39-1.94). The concentrations of TFV-DP in PBMCs and ULPCs were significantly correlated (rho=0.65; p<0.0001) (Figure 1a).

For FTC-TP, the median (IQR) concentration in PBMCs was 6660 (5650-10000) fmol/10^6 cells and in ULPCs was 19.0 (12.0-27.8) fmol/10^6 cells. The ULPC concentration of FTC-TP was 99.7% lower than the PBMC concentrations (Figure 1b). The GMR (90% CI) between ULPC and PBMC concentrations was 0.0026 (0.0022-0.0030). The FTC-TP PBMC and ULPC concentrations were significantly correlated (rho=0.55; p<0.0001). The ULPC concentrations of TFV-DP and FTC-TP were also highly correlated (rho=0.73; p<0.0001) (Figure 1c).

Individual subject pharmacokinetic profiles for TFV-DP and FTC-TP are shown in Figure 2, and demonstrate the differences in exposure between PBMCs and ULPCs. The intersubject variability (CV% (range)) of TFV-DP and FTC-TP in ULPC was 56.7 (34.5-69.3)% and 49.3 (41.2-73.2)%, respectively, when calculated in fmol/10^6 cells. Calculated from 5 samples collected over the 6 hour sampling interval, the intrasubject variability (CV% (range) for fmol/10^6 cells) for TFV-DP and FTC-TP was 25.8 (14.2-63.1)% and 28.0 (13.3-61.5)%, respectively. Comparisons to PBMC variability can be found in Table 1, as can average concentration and AUC comparisons. Concentrations are also reported in fmol/mL, as it is unlikely that clinical research sites will be able to perform cell counting on ULPC specimens.

The geometric mean (coefficient of variance (CV%)) of the partial AUCs calculated over the 6 hour sampling period for TFV-DP in PBMCs was 966 (50.4) fmol*h/10^6 cells and in ULPC was 1590 (59.6) fmol*h/10^6 cells. These were significantly correlated (rho=0.88;p=0.0008). The geometric mean (CV%) of the partial AUCs for FTC-TP in PBMCs was 47700 (36.4) fmol*h/10^6 cells and in ULPC was 122 (45.2) fmol*h/10^6 cells. These were also significantly correlated (rho=0.78;p=0.0075).

Discrimination between Single and Multiple Dosing

One additional subject prescribed TFV and FTC in combination with didanosine, darunavir/ritonavir, and raltegravir, had undetectable concentrations of TFV-DP and FTC-TP in samples obtained just before, and 1 hour after, a witnessed dose of TDF/FTC. At 6 hours post-dose, the TFV-DP concentrations in ULPC were 100-fold lower, and FTC-TP concentrations were 5-fold lower, than the other 10 subjects (Figure 3). In PBMCs, this subject had TFV-DP concentrations below the limit of detection at all time points. FTC-TP concentrations were undetectable at time zero and increased by 4 hours to concentrations similar to other subjects following a witnessed dose. In plasma, the concentrations of both TFV and FTC for this subject were below the limit of detection at t=0, but similar to the other 10 subjects following a witnessed dose. These pharmacokinetic profiles are suggestive of exposure after a single dose, and demonstrate that ULPC samples may provide discrimination of this dosing pattern.

Stability and Uniformity of ULPC Concentrations

When the ULPC samples that had been immediately frozen at −80°C were compared to those that were refrigerated for 14 hours, TFV-DP concentrations decreased from a median (IQR) of 205 (123-334) fmol/10^6 cells to 192 (109-313) fmol/10^6 cells corresponding with a median (range) change of 7% (−19 to 9%). The FTC-TP concentrations increased from a

median (IQR) of 14.3 (10.5-24.1) fmol/10^6 cells to 26.9 (14.9-35.5) fmol/10^6 cells corresponding with a median (range) change of 52% (−18 to 123%) (Figure 4).

Samples taken from the top layer of the ULPC had 5% lower TFV-DP concentrations, and 15% higher FTC-TP concentrations, compared to those taken from the bottom of the tube. This is biologically plausible, as there is a greater concentration of PBMCs in the top layer, and FTC-TP is preferentially phosphorylated in PBMCs. Conversely, there is a greater concentration of RBCs in the bottom layer, and TFV-DP is preferentially phosphorylated in RBCs.

Discussion

Adherence is critically important to interpreting the true effectiveness of PrEP strategies. In the iPrEX study in men, only 9% of those who became HIV infected had drug detected in plasma, while 51% of those who remained uninfected had detectable drug.[1] Further adherence data from the iPrEX study indicated that men were >90% protected if their TFV-DP concentrations in cryopreserved PBMCs were >15.6 fmol/million cells. This was followed by modeling and simulation estimates of 76% (56-96%) protection when 2 doses of TDF/FTC are taken per week, 96% (90-99.9%) protection when 4 doses are taken per week and 99% (97-99.9%) protection when 7 doses are taken per week.[13] In Partners PrEP and TDF2, 81% and 80% of participants had detectable plasma concentrations of TFV indicating high levels of adherence and ultimately, high levels of protection.[3,4] In contrast, plasma analysis of the FEM-PrEP samples found <35% of samples with detectable TFV concentrations indicating poor adherence and failure to detect a protective effect.[5]

As was evident in previous PrEP studies, measuring drug concentrations is a more accurate measure of adherence than is self-report.[14,5] Yet TFV and FTC have plasma half-lives of 17 and 10 hours respectively.[8] Because of these short plasma half-lives, and subsequently minimal accumulation over time, it is difficult to differentiate consistent adherence from a participant who took doses only on the days of study visits (so-called “white coat” adherence). Indeed, we witnessed this in our subject with suspected non-adherence.

However, the intracellular phosphorylated metabolites of TFV and FTC, TFV-DP and FTC-TP, have documented intracellular half-lives of 6.25 days and 1.6 days, respectively.[9,10] This long half-life results in significant intracellular accumulation over time, allowing for the assessment of adherence over the past 2-4 weeks. But intracellular TFV-DP and FTC-TP are traditionally measured in PBMCs, the isolation of which is complex and costly and not often done at rural international study sites.

Previous data suggest that TFV is phosphorylated in red blood cells. A study published by Durand-Grassland, et al. evaluated concentrations of the phosphorylated nucleoside reverse transcriptase inhibitors (NRTIs): zidovudine, lamivudine, and tenofovir in isolated PBMCs compared to samples in which contaminating red blood cells had not been lysed.[14] TFV-DP concentrations were 20% higher in the samples containing RBCs. Additionally, 3TC-TP concentrations were 99.3% lower in the samples containing RBCs. Since 3TC and FTC are both deoxycytidine analogues, they undergo very similar phosphorylation pathways.[15]

Therefore the data found for 3TC-TP is similar to our findings of FTC-TP ULPC concentrations 99.5% lower than in PBMCs. Additionally, Rower, et al. recently demonstrated 70% higher TFV-DP exposure in RBCs compared to PBMCs[11] and Castillo-Mancilla et al. demonstrated the RBC half-life of TFV-DP to be 17 (13-22) days.[16]

We were able to measure both TFV-DP and FTC-TP in the ULPC samples, because this layer contains both RBCs (in which TFV-DP is preferentially phosphorylated), and PBMCs (in which FTC-TP is preferentially phosphorylated). TFV-DP and FTC-TP correlated well with the PBMC concentrations. TFV-DP concentrations were 64% higher in ULPC samples than in PBMCs, and FTC-TP concentrations were 99.7% lower in ULPC samples. Low intrasubject variability across the sampling period suggests ULPC sampling could occur at any time during the dosing interval to measure adherence.

The additional subject, for whom the TFV-DP and FTC-TP concentrations were significantly lower than the other 10 subjects, is consistent with intermittent adherence. The plasma concentrations of TFV and FTC for this subject were below the limit of detection at t=0, but similar to the other subjects at the time points following a witnessed dose, suggesting the potential ability to discriminate between “white coat adherence”: dosing just before a clinic visit. However, this will need confirmation in a larger group of subjects.

We also investigated the stability of ULPC intracellular concentrations when treated in a similar fashion to those collected in rural African FEM-PrEP study sites. We did not find significant drug degradation under refrigeration (4°C) for up to 14 hours, suggesting that samples can be kept cold for an extended period of time prior to freezing. Given the large concentrations of TFV-DP found in ULPC samples, the small percentage change in concentrations after refrigeration would not significantly alter interpretation of the results.

The small differences found in the ULPC concentrations taken from the top versus the bottom of the tube, are consistent to what we would expect with slightly higher concentrations of TFV-DP in the bottom where there is a greater proportion of red blood cells and slightly higher concentrations of FTC-TP at the top of the tube where there are greater proportion of PBMCs. These differences are not large enough to bias the clinical utility of results interpreted for adherence purposes. Therefore, it is possible to obtain samples for both virologic and pharmacologic measures from the same sample.

Conclusions

ULPC concentrations of both TFV-DP and FTC-TP were significantly correlated with PBMC concentrations with low inter- and intra-subject variability. Preliminary data suggest that these samples may discriminate between intermittent and consistent adherence, but more investigation is required to develop an adherence algorithm. Based on these results TFV-DP and FTC-TP concentrations are being evaluated in ULPC samples from the FEM-PrEP clinical study to characterize adherence.

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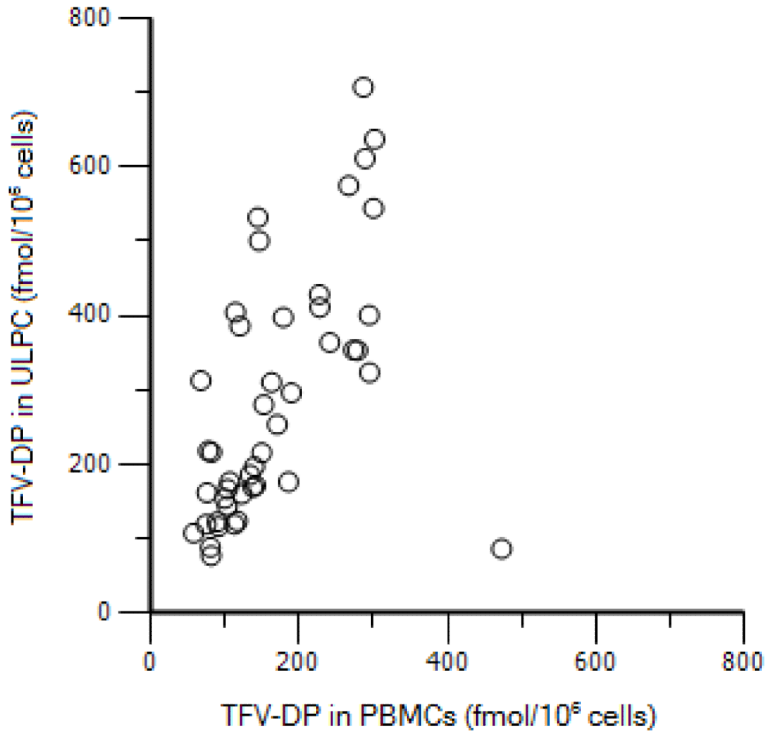

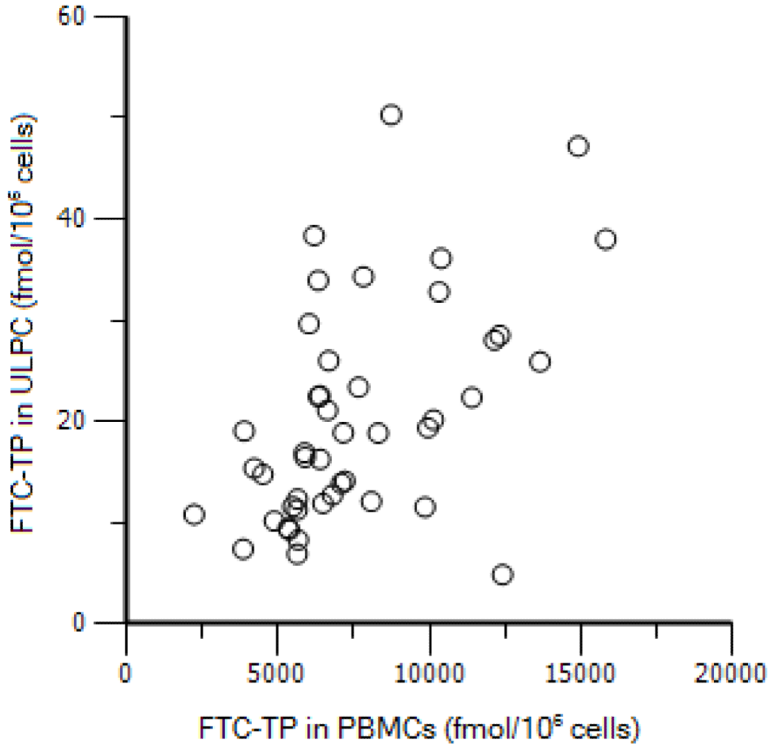

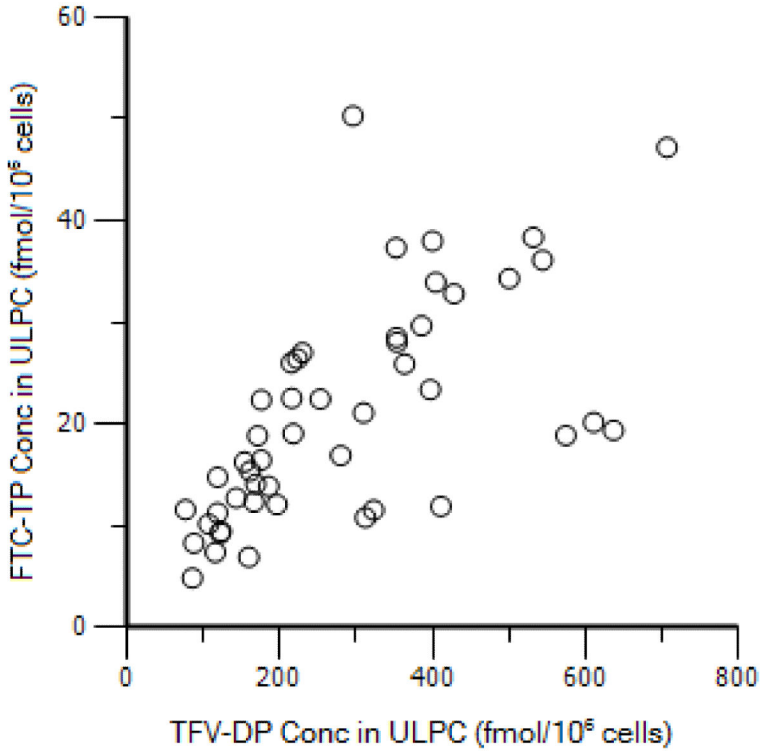

Figure 1.

Individual concentration/time points plotted for TFV-DP (figure 1a) and FTC-TP (figure 1b) concentrations in ULPC and PBMCs. A significant ($p<0.0001$) correlation between the two matrices is noted for TFV-DP (rho=0.65) and FTC-TP (rho=0.55). In figure 1c, a significant ($p<0.0001$) correlation is also noted between TFV-DP and FTC-TP in ULPC samples (rho=0.73).

a. Correlation between TFV-DP Concentrations in ULPC and PBMCs

b. Correlation between FTC-TP Concentrations in ULPC and PBMCs

c. Correlation between TFV-DP and FTC-TP Concentrations in ULPC

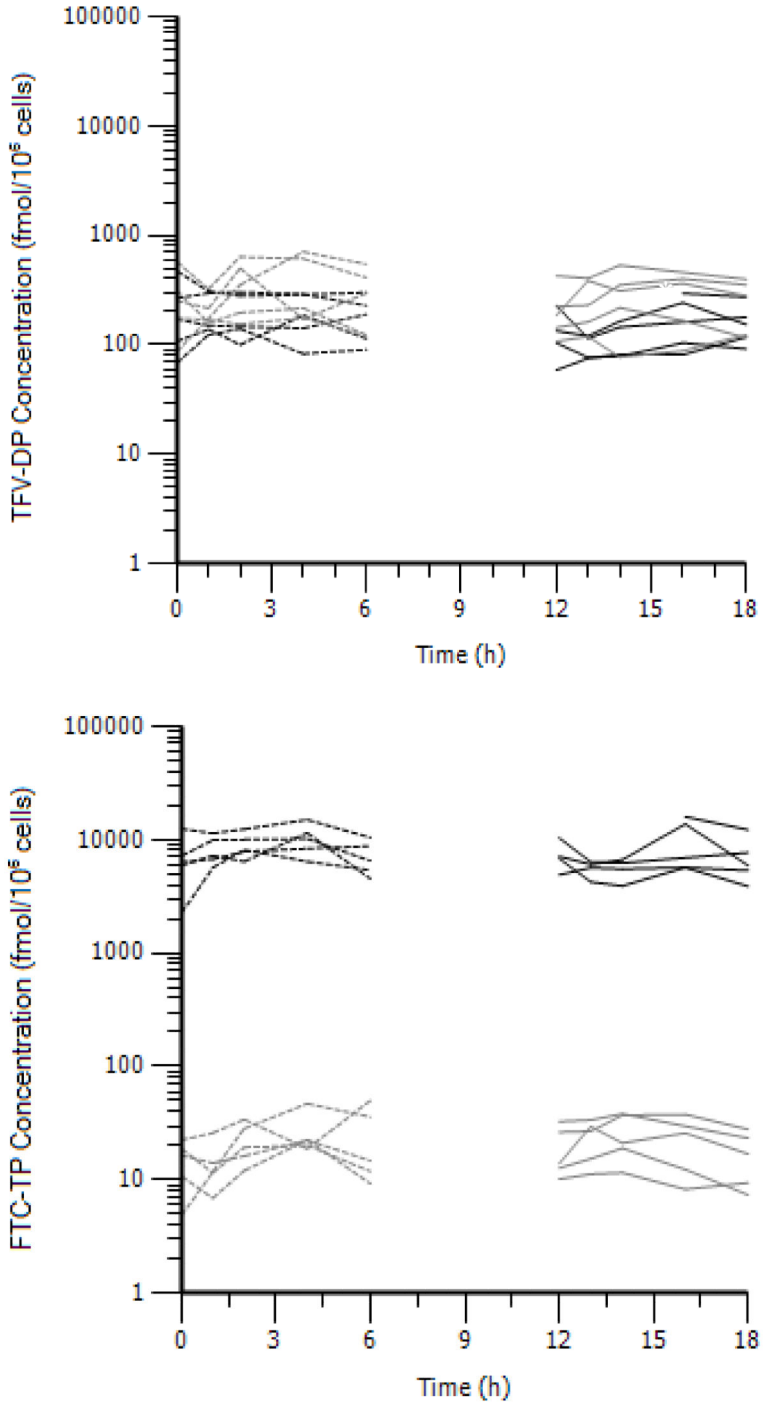

Figure 2.

Individual subject concentration/time profiles for TFV-DP (figure 2a) and FTC-TP (figure 2b) in both PBMCs and ULPC. The subjects that sampled 0-6 hours following a dose are separated by time from the subjects that sampled 12-18 hours following a dose. *For this subject, PBMC samples obtained at 12h, 13h, and 14h post-dose were compromised, and consequently not use in the analysis.

a. Individual Subject Pharmacokinetics of TFV-DP in ULPC and PBMCs
b. Individual Subject Pharmacokinetics of FTC-TP in ULPC and PBMCs

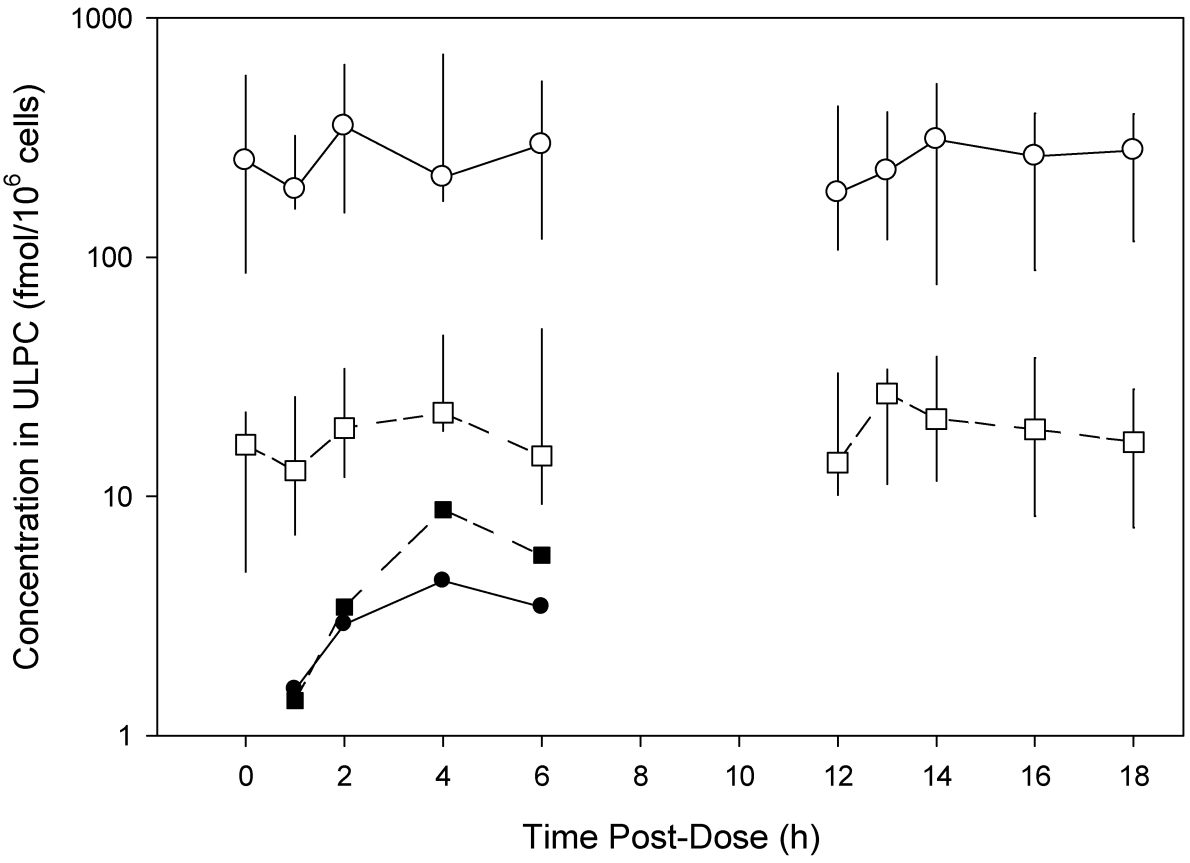

Figure 3.

The median (range) concentration/time profiles of TFV-DP and FTC-TP in ULPC with comparison to the concentration/time profiles of the additional non-adherent subject. The open shapes represent the median (range) concentrations for each time point for the 10 adherent subjects included in the analysis and the solid shapes represent the concentrations for each time point for the additional non-adherent subject. The circle data points connected by a solid line represent TFV-DP concentrations and the square data points connected by a dashed line represent FTC-TP concentrations. Data from the additional subject are consistent with single versus multiple dosing. Subjects that sampled from 0-6 hours following a dose are seperated by time from the subjects that sampled 12-18 hours following a dose.

Median (range) ULPC Concentrations of TFV-DP and FTC-TP with Additional Subject

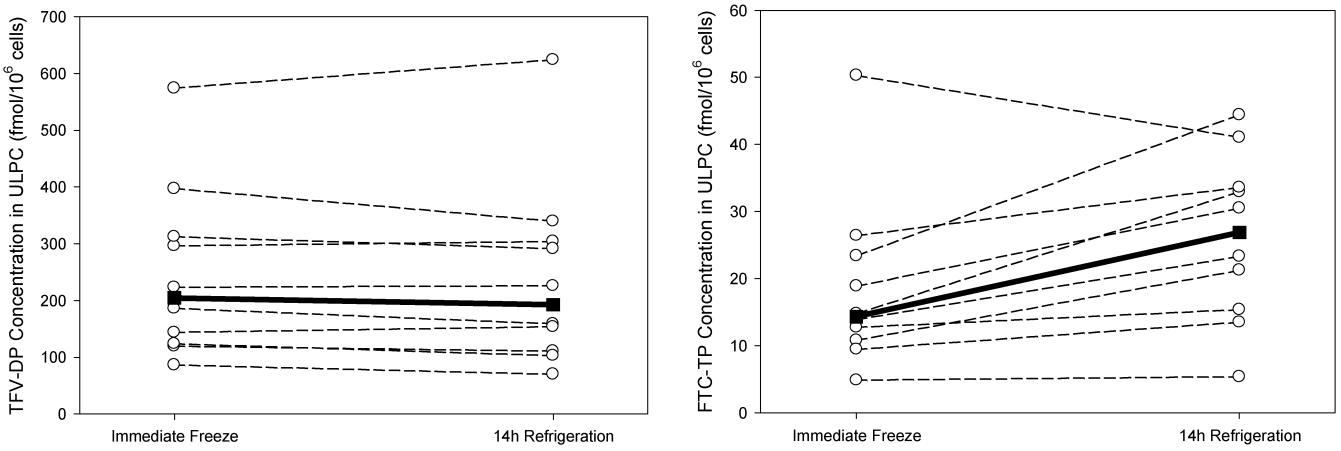

Figure 4.

ULPC Concentrations of TFV-DP or FTC-TP in ULPC at a single time point per subject immediately frozen at −80°C compared to that same time point kept refrigerated at 4°C for 14 hours prior to freezing. The dashed lines represent changes to individual subject samples and the solid black line represents the median change of all subject samples.

ULPC Concentrations (fmol/106 cells) in TFV-DP and FTC-TP after Immediate Freezing and 14 hour Refrigeration Prior to Freezing

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Table 1

Pharmacologic Measures of TFV-DP and FTC-TP in ULPC and PBMCs (Reported as median (range))

	PBMC TFV-DP Concentration (fmol/10^6 cells)	**ULPC TFV-DP Concentration (fmol/10^6 cells)**	**ULPC TFV-DP Concentration (fmol/ml)**	**PBMC FTC-TP Concentration (fmol/10^6 cells)**	**ULPC FTC-TP Concentration (fmol/10^6 cells)**	**ULPC FTC-TP Concentration (fmol/ml)**
$AUC_{0\text{-}6h}$	929(619-1830)	1670 (957-3170)	1.39×10^7 (1.08×10^7-2.42×10^7)	47000 (37000 75900)	106 (82.7-192)	1.20×10^6 (7.76×10^5-1.51×10^6)
$AUC_{12\text{-}18h}$	943 (508-1740)	1950 (588-2730)	1.61×10^7 (6.65×10^6-1.92×10^7)	42200 (28400 89200)	136 (59.6-200)	1.18×10^6 (6.82×10^5-1.62×10^6)
$C_{avg\ 0\text{-}6h}$	155 (103-305)	279 (159-529)	2.32×10^6 (1.80×10^6-4.03×10^6)	7830 (6160-12700)	17.6 (13.8-32.0)	2.01×10^5 (1.29×10^5-2.51×10^5)
$C_{avg\ 12\text{-}18h}$	157 (84.7-289)	324 (97.9-455)	2.68×10^6 (1.11×10^6-3.20×10^6)	7030 (4740-14900)	22.7 (9.93-33.3)	1.97×10^5 (1.14×10^5-2.70×10^5)
Intersubject variability CV%	46.1 (25.0-74.3)	56.7 (34.5-69.3)	38.0 (34.4-45.6)	31.2 (16.8-53.6)	49.3 (41.2-73.2)	39.2 (22.4-50.3)
Intrasubject variability CV%	25.2 (5.23-29.5)	25.8 (14.2-63.1)	6.16 (3.30-14.0)	22.5 (5.93-41.6)	28.0 (13.3-61.5)	19.0 (10.7-46.1)