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Tenofovir and emtricitabine concentrations in hair are comparable between individuals on tenofovir disoproxil fumarate versus tenofovir alafenamide-based ART

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Abstract

Tenofovir disoproxil fumarate (TDF) in combination with emtricitabine (FTC) is the backbone for both human immunodeficiency virus (HIV) treatment and pre-exposure prophylaxis (PrEP) worldwide. Tenofovir alafenamide (TAF) with FTC is increasingly used in HIV treatment and was recently approved for PrEP among men-who-have-sex-with-men. TDF and TAF are both metabolized into tenofovir (TFV). Antiretrovirals in plasma are taken up into hair over time, with hair levels providing a long-term measure of adherence. Here, we report a simple, robust, highly sensitive, and validated high-performance liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS)-based analytical method for analyzing TFV and FTC from individuals on either TDF/FTC or TAF/FTC in small hair samples. TFV/FTC are extracted from ~5 mg hair and separated on a column using a gradient elution. The lower quantification limits are 0.00200 (TFV) and 0.0200 (FTC) ng/mg hair; the assay is linear up to 0.400 (TFV) and 4.00 (FTC) ng/mg hair. The intra-day and inter-day coefficients of variance (CVs) are 5.39–12.6% and 6.40–13.5% for TFV and 0.571–2.45% and 2.45–5.16% for FTC. TFV concentrations from participants on TDF/FTC-based regimens with undetectable plasma HIV RNA were 0.0525 ±

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CONFLICT OF INTEREST
CAK reports grant support to the institution from the Gilead Research Scholars Program in HIV. LZB has been retained as a consultant for attorneys representing plaintiffs in a lawsuit against Gilead Sciences, Inc., related to TAF and TDF products. All others have no conflict of interests for this study.

ETHICS STATEMENT
Participants' hair samples were collected under the “Shaved Heads Study” procedures approved by the UCSF Institutional Review Board (IRB#14-13687).

CONSENT TO PARTICIPATE
All participants provided written informed consent prior to participation.

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0.0295 ng/mg, whereas those from individuals on TAF/FTC-based regimens were 0.0426 ± 0.0246 ng/mg. Despite the dose of TFV in TDF being 10 times that of TAF, hair concentrations of TFV were not significantly different for those on TDF versus TAF regimens. Pharmacological enhancers (ritonavir and cobicistat) did not boost TFV concentrations in hair. In summary, we developed and validated a sensitive analytical method to analyze TFV and FTC in hair and found that hair concentrations of TFV were essentially equivalent among those on TDF and TAF.

Keywords

emtricitabine (FTC); hair concentrations; tenofovir (TFV); tenofovir alafenamide (TAF); tenofovir disoproxil fumarate (TDF)

1 | INTRODUCTION

Tenofovir (TFV) is the most widely used antiretroviral (ARV) medication for treatment and prevention of human immunodeficiency virus (HIV) worldwide.[1-3] A nucleotide reverse transcriptase inhibitor (N(t)RTI), TFV is highly hydrophilic and polarized and exhibits poor oral bioavailability, leading to the development of tenofovir disoproxil fumarate (TDF) to improve bioavailability.[1,4] TDF is normally coadministered with emtricitabine (FTC), a nucleoside reverse transcriptase inhibitor (N(s)RTI), either as a backbone in antiretroviral treatment (ART) or for pre-exposure prophylaxis (PrEP).[1-3]

TDF/FTC is a very effective and well-tolerated drug for HIV treatment and, since 2012, for PrEP.[1-3,5] However, TDF exhibits some adverse drug reactions (ADRs), including loss of bone mineral density,[3,5] proximal tubular dysfunction,[1,2,6,7] and renal insufficiency.[8,9] A new TFV prodrug, tenofovir alafenamide (TAF), was approved after having demonstrated some benefit in safety profiles for bone and renal function. However, in recent years, TAF has demonstrated increased weight gain[10] and metabolic effects compared to TDF when used for ART or PrEP.[11]

TFV concentrations in the plasma with TAF are 4-fold lower than with TDF, although intracellular TFV-diphosphate levels with TAF are 10-fold higher than with TDF.[12] TAF is stable in the body and is activated intracellularly leading to greater TFV intracellular accumulation.[13] The lower TFV concentration in the plasma on TAF compared to TDF is putatively related to lower rates of renal and bone toxicity on the former than the latter, although the mechanism by which TAF is associated with excess weight gain is still under investigation.[2,14,15] Besides its use in treatment, TAF/FTC has been studied in men-who-have-sex-with-men (MSM) and transgender women as an alternative to TDF/FTC for PrEP and was approved by the US Food and Drug Administration (FDA) for those at risk of HIV for sexual exposures other than vaginal sex.

The efficacy of ART and PrEP is determined by adherence to daily medications. Self-reported adherence has limitations of recall and social desirability bias so objective metrics of adherence, where drug levels are measured in a biomatrix (e.g., plasma, urine, dried blood spots [DBS], hair) have been developed. Plasma and urine ARV concentrations (since TFV is eliminated in urine mainly unchanged), can be useful to objectively determine adherence.

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However, ARVs are normally eliminated from the body over 1–2 days so plasma/urine drug levels only assess adherence over a recent dosing window.[16,17] If the medication is processed intracellularly like TFV or FTC, drug levels in DBS can reflect adherence over a longer period of time since the half-life of these metabolites in red blood cells is longer than that in plasma.[18] However, blood collection is invasive, and both plasma and urine require cold chain shipping and storage.

Analyzing ARV levels in hair is an attractive alternative for monitoring longer-term adherence since drugs distribute into hair from the systemic circulation over a period (weeks to months).[16,17] Hair samples are collected non-invasively and can be shipped and stored at room temperature.[17] The University of California San Francisco-Hair Analytical Laboratory (UCSF-HAL) has quantified a number of ARVs in hair (e.g., atazanavir,[19] lopinavir,[20] ritonavir,[20] efavirenz,[20] nevirapine,[21] darunavir, raltegravir, dolutegravir, TFV/FTC,[22-29]) for research projects over the past 15 years. Hair concentrations of ARVs have been shown to be strong predictors of virologic response for a variety of ARVs, including TFV/FTC.[30-41]

In this article, we describe our validated quantitative analytical method for TFV and FTC in human hair samples from HIV patients using high-performance liquid chromatography tandem mass spectrometry (LC/MS/MS). We also perform quantitative comparison of TFV levels in hair from individuals on TAF/FTC- versus TDF/FTC-based ART.

2 | MATERIALS AND METHODS

2.1 | Chemicals

TFV and FTC reference compounds were obtained from United States Pharmacopeia (USP, Rockville, MD). Tenofovir-d_6 (TFV-d_6) and emtricitabine-$^{13}C,^{15}N_2$ (FTC-$^{13}C,^{15}N_2$) were purchased from Toronto Research Chemicals (Toronto, Canada). Ammonium acetate was obtained from Spectrum Chemical (New Brunswick, NJ). Ammonium phosphate dibasic and methanol (MeOH) were purchased from Thermo Fisher (Waltham, MA). Trifluoroacetic acid was obtained from Acros Organics (Thermo Fisher). Hydrazine dihydrochloride was purchased from MilliporeSigma (St. Louis, MO). All other reagents as well as solvents used for LC/MS/MS analysis were high-performance liquid chromatography (HPLC) or analytical grade. Water was deionized and filtered using a Barnstead NANOpure II water purification system (Boston, MA).

2.2 | Hair samples

Blank hair samples were used as a matrix for standards and controls and were acquired from healthy volunteers, who were HIV-negative and had never taken TDF, TAF, and FTC. Positive control hair samples were acquired from participants living with HIV, who were recruited from the “Shaved Heads Study” from the UCSF HIV clinic (“Ward 86”) at Zuckerberg San Francisco General Hospital.[19] The “Shaved Heads Study” procedures were approved by the UCSF Institutional Review Board (IRB#14-13687) and all participants provided written informed consent prior to participation. Participants enrolled in the current study were required to be on TDF- or TAF-containing ART for at least 6 months with good adherence as reported by the provider, the participant, and sustained documented virologic

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suppression (HIV RNA < 40 copies/ml). Eligible participants for the study had their hair cut down to 2 cm prior to having their hair shaved. The shaved scalp hair was then packaged in aluminum foil, placed into Ziploc® bags at room temperature, and transported to the laboratory to be used as "incurred" positive control hair samples.

2.3 | Drug analysis

LC/MS/MS analyses were performed using a pair of LC-10AS pumps (Shimadzu, Kyoto, Japan), a 717 Plus autosampler (Waters, Milford, MA) coupled to a Quattro Ultima triple quadrupole mass spectrometer (Waters) utilizing positive electrospray ionization. Samples were separated by reverse phase chromatography using a Synergi Polar-RP column (4 μm, 150 × 4.6 mm; Phenomenex, Torrance, CA) and a gradient mobile phase from 0% to 45% MeOH containing 5mM ammonium acetate, trifluoroacetic acid, and ammonium phosphate dibasic in water. Detection of TFV, TFV-d_6, FTC, FTC-$^{13}C,^{15}N_2$ was performed by multiple reaction monitoring of the $[M + H]^+$ transitions of 287.9→175.9 *m/z*, 293.8→181.8 *m/z*, 248.0→129.9 *m/z*, and 251.0→133.0 *m/z*, respectively. Data were processed and analyzed by MassLynx software (version 4.1, Waters).

2.4 | Method validation

2.4.1 | Standard curve and quality control solutions—Standard curve (SC) and quality control (QC) stock solutions were prepared for TFV, FTC, TFV-d_6, and FTC-$^{13}C,^{15}N_2$ at 200 μg/ml in 50% MeOH. SC and QC working solutions were prepared by diluting their respective stock solutions with 50% MeOH to obtain a high working solution with TFV and FTC concentrations of 0.100 and 1.00 μg/ml, respectively, and a low working solution with TFV and FTC of 5.00 and 50.0 ng/ml, respectively. The working internal standard (IS) solution was prepared by combining IS stock solutions of TFV-d_6 and FTC-$^{13}C,^{15}N_2$ and diluting to 50.0 and 500 ng/ml with 50% MeOH, respectively. All the stock and working solutions were stored at 4°C and were stable over at least 6 months.

2.4.2 | Sample preparation—SC samples were prepared by spiking high or low working solutions (containing both TFV and FTC) on approximately 5 mg (between 4.00 to 6.00 mg) blank human hair samples in glass test tubes. The range of SC was 0.00200–0.400 ng/mg hair for TFV and 0.0200–4.00 ng/mg hair for FTC. QC samples were prepared at 0.00600, 0.0400, and 0.320 ng/mg hair for TFV and 0.0600, 0.400, and 3.20 ng/mg hair for FTC. Participant hair samples (from those on TDF/FTC vs. TAF/FTC treatment) were cut to about 1 mm in length and approximately 5 mg of the hair sample was weighed into a glass test tube. Each participant sample was prepared in 10 replicates.

To each glass test tube of SC, QC, or participant samples described above, 0.250 ml of MeOH containing 1% trifluoroacetic acid was added and vortex-mixed followed by adding 0.250 ml of water containing 1% trifluoroacetic acid and 25 μl of 10% hydrazine dihydrochloride in water. All samples, except the double blank sample (blank hair without drug or IS), had 20 μl of IS solution added, were vortex-mixed and then centrifuged at 3000 rpm for 5 min. Each tube was then incubated in a 37°C shaking water bath overnight. After centrifugation at 3000 rpm for 15 min, the supernatant was transferred to a new glass test tube and evaporated to approximately 200 °l under a nitrogen gas stream. The test

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tubes were then centrifuged at 3000 rpm for 15 min, and supernatant was transferred to autosampler vials followed by centrifugation at 10,000 rpm for 5 min and then injected into the liquid chromatography coupled with tandem mass spectrometer (LC–MS/MS).

2.4.3 | Specificity—Six lots of blank human hair samples in duplicate together with one lot of blank human hair samples at the lower limit of quantitation (LLOQ, 0.00200 ng/mg hair for TFV and 0.0200 ng/mg hair for FTC) were weighed and assayed using the methods above. Any endogenous interference peak was assessed by comparing the endogenous peaks of the blank hair samples with the drug and IS peaks in the LLOQ sample.

2.4.4 | Linearity—Linearity was assessed by preparing a standard curve consisting of one double blank, one blank (blank hair with IS, but without drug), and a standard curve spiked at nine concentrations (0.00200, 0.00400, 0.00800, 0.0160, 0.0400, 0.0800, 0.160, 0.320, 0.400 ng/mg hair for TFV and 0.0200, 0.0400, 0.0800, 0.160, 0.400, 0.800, 1.60, 3.20, 4.00 ng/mg hair for FTC). Linearity was determined by the peak area ratio of the drug to the IS with a $1/x^2$ weighted regression and correlation coefficient. Normalized concentrations were calculated by dividing the calculated concentration from the standard curve by the actual weighed hair weight and multiplied by 5 (target weight: 5.00 mg). The standard curve acceptance criteria for accuracy and precision were ± 15.0% for percent of difference (%RE) and 15.0%[42] for percent of coefficient of variance (%CV) except for the LLOQ, which was ± 20.0% for %RE and 20.0% for %CV. By following the FDA Guidance for Bioanalytical Method Validation,[43] up to two standard curve points (not including the upper limit of quantitation or LLOQ) not meeting the acceptance criteria were excluded.

2.4.5 | Accuracy and precision—The inter-day accuracy and precision of the method were evaluated over 4 days by analyzing four sets of spiked QC samples (n = 4) at the three drug concentrations mentioned above, and four incurred QC samples that were weighed out from a pool bottle. Intra-day accuracy and precision of the method were evaluated on one day by analyzing six sets of spiked QC samples (n = 6) at the three drug concentrations mentioned above. The LLOQ (for accuracy and precision) and incurred QC (for precision) samples were also evaluated. Accuracy was defined as %RE between the mean and nominal concentrations. Precision was defined as %CV between the mean and standard deviation. The acceptance criteria for the LLOQ were ± 20.0% for %RE and 20.0% for %CV and for QC and incurred QC ±15.0% for %RE and 15.0% for %CV.

2.4.6 | Recovery—The recoveries of spiked TFV and FTC were obtained by comparing the peak area ratio of extracted to non-extracted QC samples at the three spiked QC concentrations (high, medium, and low) in triplicate. For the drug recovery test, IS was added to the samples after the samples were extracted. The recovery of IS was determined by comparing the peak area ratio of extracted to non-extracted IS in triplicate. For the IS recovery test, the medium QC was spiked into the sample after the samples were extracted. The acceptance criterion is that the percent difference between the average concentration and the nominal concentration may not be greater than ±30%.

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2.4.7 | Extraction efficiency—The extraction efficiency of incurred QC hair was examined by measuring the drug concentrations present after three extractions on the sample. For the first extraction, TFV and FTC incurred hair samples (n = 3), were extracted using the hair sample process above. After the first extraction, new solvent and IS was added to the residual hair tubes and reprocessed. Finally, the process was repeated for a third time. The drug levels were then compared from all three extractions.

2.4.8 | Matrix effect—The matrix effect was evaluated in six different lots of human hair. The matrix effect was measured by comparing the peak area of drug or IS spiked in duplicate in post-extracted sample (in presence of hair) to those spiked in pure solution (absence of hair). The matrix factor (MF) is the drug or IS peak area in presence of hair divided by the drug or IS peak area in absence of hair. The IS-normalized MF is the MF of the drug divided by the MF of the IS. The acceptance criterion requires that the %CV between the MF and the IS-normalized MF not be greater than 15.0%.

2.4.9 | Stability—TFV and FTC stock solution stability at room temperature and 4°C were evaluated. For determining stability at room temperature, TFV and FTC stock solutions were aliquoted in duplicate. One set of aliquots was left out on the benchtop at room temperature while the other set was kept at 4°C. After 6 h, both sets of aliquots were injected into the HPLC-UV system, with subsequent comparison of the peak areas. For assessing the stability of TFV and FTC stock solutions at 4°C, a set of stock solutions was stored at 4°C. After a year elapsed, a new set of stock solutions was prepared and concentrations of TFV and FTC were compared via HPLC-UV analysis between the old and new set. The acceptance criterion requires that the solution stabilities may not be greater than ±5.00%. The stability of the processed samples at 4°C was also evaluated. High and low QC samples were spiked in triplicate and processed, stored at 4°C for a week, and then analyzed with a freshly processed spiked standard curve. The acceptance criterion is that the spiked nominal concentration may not be greater than ±15.0%. The stability of TFV and FTC were evaluated in human hair over time. A pooled incurred hair sample cut into about 1–2 mm pieces was stored in a 20-ml scintillation vial at room temperature and was aliquoted in triplicate at specific time intervals (such as 1, 3, 6, 12, 24 months or longer). The samples were analyzed for TFV/FTC concentrations at each time point. The average nominal concentration at each time point should not deviate more than ±15.0% from the concentration in the initial analysis.

2.5 | Statistical analysis

All data are shown as calculated average concentration (mean) and standard deviation (SD). %RE was calculated from ([nominal concentration − target concentration]/target concentration) × 100, and %CV was calculated from (SD/mean) × 100. The Student *t* test was applied when comparing two groups. One-way analysis of variance (ANOVA) followed by post hoc Tukey method was performed when more than three groups were compared. A p value less than 0.05 was required to define statistical significance when evaluating differences between groups.

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3 | RESULTS

3.1 | Specificity

The specificity of the current analytical method for TFV and FTC was tested in six lots of blank hair from HIV-negative, healthy volunteers. A set of example chromatograms (double blank, blank, and LLOQ) are shown in Figures 1a for TFV and 1b for FTC. Areas of all the endogenous peaks found in six lots of blank human hair were less than one fifth of LLOQ area of TFV and of FTC. This indicated that the current analytical method is specific for the analysis of TFV and FTC in human hair samples.

3.2 | Linearity

The linearity of the standard curve was examined by plotting the nominal concentrations versus back-calculated concentrations and is shown in Figure 2. The %CV of the back-calculated concentrations ranged from 2.27% to 10.3% for TFV and 1.10% to 3.20% for FTC. Over the four validation days, the average correlation coefficients were 0.997 for TFV and 0.999 for FTC. The current method showed good linearity for both TFV and FTC.

3.3 | Precision and accuracy

The precision and accuracy of the method were tested through one intra-day and four inter-day sample preparations. The summary of the data is shown in Table 1. The intra-day precision (%CV) ranged from 5.39% to 12.6% for TFV and 0.571% to 2.45% for FTC. The intra-day accuracy (%RE) ranged from +4.38% to +8.00% for TFV and −0.500% to +1.87% for FTC. The inter-day precision (%CV) ranged from 6.40% to 13.5% for TFV and 2.45% to 5.16% for FTC. The inter-day accuracy %RE ranged from −2.25% to +1.00% for TFV and −0.250% to +4.06% for FTC. All %CV and %RE values were less than ±15%, which met acceptance criteria. Precisions of intra-day and inter-day LLOQ were 16.7% and 3.21% for TFV and 11.6% and 1.18% for FTC. Accuracies of intra-day and inter-day LLOQ were +2.00% and +4.00% for TFV and 14.5% and 3.50% for FTC. The %CV and %RE for the LLOQ were all less than 20%. The incurred QC data are summarized in Table 2. The normalized concentration was calculated from nominal concentration divided by actual hair weight and multiplied by target hair amount (5.00 mg). The Intra-day precision of incurred QC samples ranged from 3.76% to 7.52% for TFV and 1.52% to 3.63% for FTC. The inter-day precision of incurred QC samples was 6.14% for TFV and 2.99% for FTC. All %CV values for incurred QC samples were less than ±15%. These results show that the current method for TFV and FTC is precise, accurate, and reproducible.

3.4 | Recovery

The recovery of spiked drug and IS was tested in triplicate at three QC concentrations, and the data are summarized in Table 3. The overall mean recovery (average of recovery from all the QC samples) for TFV, FTC, TFV-d_6, and FTC-^{13}C,$^{15}N_2$ was 106%, 98.9%, 94.2%, and 96.6%, respectively.

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3.5 | Extraction efficiency

We tested the extraction efficiency of TFV and FTC from an incurred hair sample over three extractions in triplicate. As shown in Figure 3, 91.4 ± 0.9% of TFV and 99.1 ± 0.1% of FTC were extracted during the first extraction, then 6.89 ± 0.81% of TFV and 0.762 ± 0.028% of FTC during the second extraction followed by 1.76 ± 0.12% of TFV and 0.117 ± 0.040% of FTC during the third extraction. We performed only the first extraction in our method.

3.6 | Matrix effect

The matrix effect, which represents ion suppression or enhancement based on a drug's ionization properties, was tested on six lots of human hair at three QC concentrations. The mean IS-normalized MF was 0.937 for TFV and 1.02 for FTC. The %CV was 9.77% for TFV and 1.80% for FTC, so both of these values were less than 15%. There was no significant matrix effect for TFV and FTC when analyzed in the different lots of blank human hair.

3.7 | Stability

Stock solutions of TFV, TFV-d_6, FTC, FTC-^{13}C,$^{15}N_2$ were stable (>95.0%) for at least 6 h at room temperature, and stock solutions of TFV and FTC were stable (>95.0%) for at least 1 year at 4°C. TFV and FTC in the processed samples were shown to be stable (>95.0%) for a week when stored in a refrigerator at 4°C. TFV and FTC were stable in hair samples for at least 18 months at room temperature (deviation was less than ±15%), which indicated TFV and FTC were preserved stably in hair. The stability tests showed that TFV and FTC were stable under all conditions tested.

3.8 | Comparison of TFV and FTC hair concentrations in hair samples from patients on TDF versus TAF

We analyzed TFV and FTC hair concentrations in 16 participants from the “Shaved Heads Study”. This sample included 10 participants on TDF/FTC and 6 participants on TAF/FTC, with all participants documented to be on the same ART regimen for at least 6 months with stable virologic suppression. Representative chromatograms of TFV and FTC and average concentrations of these two ARVs are shown in Figures 4a (TDF/FTC participants) and 4b (TAF/FTC participants). In Table 4, we tabulate TFV and FTC hair concentrations of those on TDF/FTC (Table 4A) and TAF/FTC (Table 4B)-based regimens. Each TFV and FTC concentration represents the average of 10 replicates. Figure 5 summarizes TFV (a) and FTC (b) hair concentrations from participants who were using TDF/FTC versus TAF/FTC. Circles represent each patient's hair concentration. Average TFV hair concentrations were not significantly different between those on TDF- or TAF-containing ART regimens (0.0553 ± 0.0318 vs. 0.0379 ± 0.0139 ng/mg hair, $p = 0.229$). Similarly, FTC hair concentrations were not significantly different between individuals on TDF versus TAF (0.846 ± 0.659 vs. 1.04 ± 0.83 ng/mg, $p = 0.619$).

In Figure 6, we demonstrate the effects of pharmacokinetic boosters on TFV and FTC hair concentrations. TFV and FTC hair concentrations (for individuals on both TDF and TAF-containing ART) from individuals on concomitant boosters (cobicistat [COBI] or ritonavir [RTV]) were 0.0525 ± 0.0295 and 0.930 ± 0.601 ng/mg hair, respectively, whereas

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concentrations for those not on booster drugs were 0.0426 ± 0.0246 and 0.898 ± 0.925 ng/mg hair, respectively. There were no statistically significant differences in either TFV and FTC hair concentrations between participants on boosted and non-boosted ART with either TDF/FTC or TAF/FTC as the backbone.

Finally, we separately compared the effect of each booster-drug on TFV and FTC hair concentrations for participants on TDF/FTC and TAF/FTC-based ART regimens. The results are shown in Figure 7. For individuals on TDF-based ART, TFV concentrations in patients receiving COBI were 0.0844 ± 0.0357 ng/mg hair; in patients receiving RTV, TFV concentrations were 0.0388 ± 0.0146 ng/mg hair. Combining both sets of participants receiving boosters (COBI or RTV), TFV concentrations were 0.0584 ± 0.0336 ng/mg hair compared to TFV concentrations in participants not receiving boosters of 0.0483 ± 0.0327 ng/mg hair (Figure 7a). FTC concentrations with COBI or RTV were 1.03 ± 0.70 (COBI or RTV), 1.26 ± 0.59 (COBI) and 0.851 ± 0.807 (RTV) ng/mg hair, respectively, whereas FTC concentrations without any booster were 0.428 ± 0.343 ng/mg hair (Figure 7b). There were no statistically significant differences in concentrations between any of the groups (one-way ANOVA). With individuals on TAF-based ART, TFV and FTC hair concentrations without booster were 0.0370 ± 0.0185 and 1.37 ± 1.16 ng/mg hair, respectively, and those with booster (COBI or RTV) were 0.0389 ± 0.0117 and 0.705 ± 0.233 ng/mg hair, respectively (Figure 7c,d). No significant concentration differences were found for any measurement between patients receiving boosters and those not.

4 | DISCUSSION

4.1 | Analyzing TFV and FTC in human hair samples to assess drug adherence

Adherence is crucial for the effectiveness of ART both as treatment and prevention (undetectable equals untransmittable, U = U), as well as for PrEP.[44] Objective metrics of ARV adherence have been routinely measured in research studies since the limitations of self-reported adherence became manifest. UCSF-HAL has assayed drug levels in hair for a variety of HIV treatment and prevention studies around the world for over a decade. We describe here the analytical method we use for assaying TFV and FTC in hair. Our TFV/FTC hair analytical method has high sensitivity, specificity, reproducibility, accuracy, and precision. This method has previously been peer-reviewed and approved by the National Institute of Health's Division of AIDS (DAIDS) supported Clinical Pharmacology and Quality Assurance (CPQA) program.[45] After CPQA approval, we have analyzed TFV and FTC in hair samples for a variety of PrEP projects,16,22-25,27,46-50 as well as in pregnant and breastfeeding women on ART.[41] However, we have not described this validated analytical method in a publication prior to this report.

4.2 | Method development and validation for TFV and FTC quantification in hair

TFV is a hydrophilic (ALogP = −0.05, $LogD_{pH7.4}$ = −4.06)[51] and polarized acidic drug (acidic pK_a = 1.61)[51] containing a phosphate group. FTC is also a hydrophilic (ALogP = −0.46, $LogD_{pH7.4}$ = −0.27)[51] but less polarized neutral drug (acidic pK_a = 13.83, basic pK_a = 2.4).[51] TFV requires a highly aqueous mobile phase for its bioanalysis and also

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needs additives to ameliorate peak-tailing induced by the phosphate group in the nucleotide analogue structure.[52]

Because TFV/FTC concentrations in the biomatrix of hair were very low in a pilot study, we required a highly sensitive analytical method. Methods have been reported for TFV bioanalysis in a variety of biological materials, such as plasma,[53-58] urine,[59,60] peripheral blood mononuclear cells (PBMCs),[12,61,62] DBS,[18,63-65] cerebrospinal fluid (CSF),[56] dried breast milk spots,[65] and cervicovaginal lavage[66] from individuals with HIV using LC/MS/MS-based systems. In those methods, an LLOQ of TFV was reported as 0.5 ng/ml in plasma,[56] 10 ng/ml in urine,[60] 1 nM in PBMC,[67] 0.5 nM in DBS,[68] 0.1 ng/ml in CSF,[56] and 0.5 ng/ml in cervicovaginal lavage.[66]

Due to its high polarity, extraction of TFV from biological materials is relatively difficult. Liquid–liquid extraction for TFV/FTC showed low recovery. Solid-phase extraction, such as Waters Oasis® HLB (TFV: 104.35%[57]) or Oasis® MCX (TFV: 78.3–86.0%,[53,55,58,61,69] FTC: 68.5–83.8%[58,69]) could successfully extract TFV or FTC from biological fluids. Here, we simply extracted TFV and FTC from hair into acidified MeOH-water and evaporated out the MeOH.

Our current method has very high sensitivity in terms of TFV analysis compared with most other analytical methodologies. We used an ammonium acetate - trifluoroacetic acid based mobile phase and eluted TFV followed by FTC with a slow gradient of 45% MeOH. Considering that the final volume was 200 μl after evaporation of MeOH and 30 μl of sample was injected, our LLOQ of TFV was 0.0100 ng/ml (0.300 pg/injection) and FTC was 0.100 ng/ml (3.00 pg/injection). The lowest reported TFV LLOQ we found in the literature was 2 nM (0.574 ng/ml) with 8 μl sample injection after 2.5 times sample concentration (11.5 pg/injection).[70]

The assay reproducibility of our method was high (Table 5). The percent difference between the original- and reanalyzed-results met the acceptance criteria of the Food and Drug Administration guidelines for Bioanalytical Method Validation,[71] thus demonstrating that our method is reproducible in terms of drug extraction and quantification from hair. Our method also follows the European Medicines Agency guidelines on bio-analytical method validation.[72,73]

4.3 | Comparison of TFV and FTC hair concentrations among individuals on TDF/FTC-versus TAF/FTC-based regimens

We analyzed TFV and FTC hair concentrations from patients living with HIV on either TDF- or TAF-based ART with good adherence and long-standing virologic suppression. Average TFV hair concentrations were not statistically different between individuals on TDF versus TAF, although TFV levels in hair from those on TAF were slightly lower (−31.5%) than those on TDF (Figure 5a). FTC hair concentrations were slightly higher, but not significantly different, in individuals on TAF versus TDF (22.9% higher in TAF) (Figure 5b).

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TAF-based ART demonstrates noninferior HIV treatment activity to TDF but the lower plasma TFV concentrations with TAF versus TDF have been invoked as a mechanism to explain why renal[8] and bone[74] toxicities are lower with TAF. Ruane et al.[75] reported the maximum average plasma concentration (C_{max}) and area under the plasma concentration versus time curve (AUC) of TFV after TDF and TAF administration. Compared with TDF (300 mg), C_{max} of TFV from TAF (25 mg) and TAF (8 mg) was decreased 93.6% and 98.3%, and AUC was decreased 86.0% and 96.6%, respectively. Furthermore, Podany et al.[12] reported the change in TFV pharmacokinetics after switching from TDF (300 mg) to TAF (10 mg). C_{max} of TFV decreased 89.8% and intracellular TFV concentration increased 141% after switching from elvitegravir (EVG)/COBI/TDF/FTC to EVG/COBI/TAF/FTC. These results demonstrate that TAF-based treatment decreases TFV exposure by approximately 90% in plasma compared with TDF-based treatment. Finally, the AUC of TFV-diphosphate (TFV-DP, active form of TFV) in PBMCs in those on TAF was 613% (25 mg) and 16.7% (8 mg) higher than individuals on TDF.[75] Intracellular TFV-DP levels were 652% higher for darunavir/COBI/TAF/FTC[76] and 5.3-fold higher for EVG/COBI/TAF/FTC[77] than levels without boosting. The results of pharmacokinetic studies have shown that boosters increase the bioavailability and exposure to TFV with both TDF and TAF. Recently, Haaland et al.[78] reported on urine concentrations of TFV and FTC from MSM on TDF (TDF/FTC) and TAF (TAF/FTC/COBI/EVG). Median urine TFV concentrations from those on the TAF-based regimen were approximately 14 times lower than those on TDF, although the median FTC concentrations were not different between regimens. Since TFV is mainly eliminated into the urine, urinary TFV concentrations essentially parallel plasma concentrations.

In terms of why TFV and FTC hair concentrations did not differ among those on TDF- and TAF-based regimens, a drug will distribute from blood into the hair papilla along with other nutrients followed by movement into hair matrix cells, such as melanocytes and keratinocytes.[79] Considering the 17 times lower plasma C_{max} and 10 times lower urinary TFV concentration from 25 mg TAF compared with 300 mg TDF, TFV hair concentrations were expected to be lower among those on TDF versus TAF.

One possibility why TFV hair levels were similar between TDF- and TAF- based ART would be differences in adherence. However, hair samples in our study were obtained from patients with provider and patient-reported high adherence, with undetectable viral loads. Indeed, the average TFV hair concentration from TDF in our study was very close to the median concentration derived when we previously administered TDF/FTC to HIV-noninfected volunteers via directly-observed methods 7 days a week (0.038 ng/mg hair).[17] Furthermore, FTC hair concentrations were also similar between those in the TDF and TAF groups. A second possibility would be that there is active transport of TFV into the hair follicle. Hair follicle cells are one of the most frequent cell types in the body, and it is possible that some forms of uptake transporters, like organic cation transporters,[2] could mediate uptake of TFV. However, no previous report has described TFV uptake transporters in hair follicles to date.

Another possibility could be that the majority of TFV detected in the hair of participants on TAF came from TAF, in contrast to TFV in those on TDF coming directly from the

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plasma. Liu et al.[17] reported that TFV hair concentrations increased monotonically with the amount of dose administered to HIV-noninfected volunteers. This would imply that TFV hair concentrations correlated with blood TFV exposure. TAF has increased lipophilicity to improve its bioavailability and is structurally quite stable in the blood circulation until converted to TFV in the tissues that have cleavage enzymes such as cathepsin A[13,80] and carboxylesterase 1.[80,81] If the hair follicle has a cleavage enzyme, it makes sense that the hair concentrations of TFV from those on TAF are subsequently high, since TFV concentrations in PBMCs are similarly high, even if TFV plasma concentrations are low.[75-77] Although further study is needed, this suggests the same cut-offs can be used to estimate adherence thresholds with the two drugs.

4.4 | Effect of booster drugs on TFV and FTC hair levels

To boost plasma concentrations of some ARVs that are extensively metabolized by cytochrome P450 (CYP) 3A, RTV or COBI are routinely co-administered. In addition to CYP3A, RTV and COBI inhibits other CYPs[82,83] as well as organic anion transporting polypeptides[82,83] and efflux transporters.[82,83] Conversely, multidose RTV[82] is known to induce metabolizing enzymes such as CYP3A, CYP1A2, CYP2Cs, and UDP-glucuronosyl transferases.

Since both TDF and TAF are substrates of efflux transporters in the gut, boosters will be expected to increase plasma concentrations of TFV. Mills et al.[76] reported that C_{max} and AUC of TFV were, respectively, 413 ng/ml and 3737 ng·h/ml from TDF with darunavir/COBI/FTC and 18.8 ng/ml and 339 ng·h/ml from TAF with darunavir/COBI/FTC. Compared with TFV parameters among those on TDF, the C_{max} and AUC of TFV in those on TAF-based regimens were decreased 95.5% and 90.9%, respectively. Sax et al.[77] also reported that plasma exposure of TFV was 91% lower in participants who took EVG/COBI/TAF/FTC compared to EVG/COBI/TDF/FTC.

TFV has been associated with ADRs in the kidney[9] and bone.[74] TDF with boosters is reported to be associated with a higher risk of bone and renal toxicity, especially with a single dose tablet formulation.[15] In TDF to TAF switching studies that involve a booster (such as the darunavir/COBI/TDF/FTC to darunavir/COBI/TAF/FTC switch study: EMERALD[84]), non-inferiority in terms of virologic suppression was reported between groups even when the TAF dose was 10 mg. Moreover, there was a slight improvement in renal parameters and bone measurements with the switch to TAF. These results support the TFV pharmacokinetic differences between TDF and TAF.[76]

Our study compared TFV and FTC hair concentrations between boosted (10 participants) versus non-boosted (6 participants) ARV regimens. There was no statistical difference in TFV and FTC hair concentrations between those in the non-boosted and boosted ART groups (Figure 6), even though those on boosters demonstrated slightly higher TFV concentrations (Figure 7). We further analyzed the effects of boosters on TFV and FTC hair concentrations in each TAF and TDF regimen separately, finding no statistical differences in hair concentrations between boosted and non-boosted TDF- or TAF-based regimens (Figure 7). Comparing plasma AUC of TFV between non-boosted[75] and boosted[76] regimens, an AUC of TFV was 1.95 times higher for TDF and approximately 5 times higher for

TAF co-administrated with darunavir/COBI. These changes did not match our TFV hair concentration differences. It may be that the distribution of TFV or TAF into hair is not parallel to plasma distribution or there is another mechanism (e.g., transporters), involved in drug distribution into hair follicles and cells. Further investigation is needed.

4.5 | Limitations

This study has some limitations. First, a matrix effect of hair samples would depend on the amount and quality of hair. Even though we used six lots of blank hair for the matrix effect study, these were not representative of all types (e.g., straight, wavy, curly, coily) and colors (e.g., black, blonde, brown, red, gray) of hair. Also, they represented hair that was clean, devoid of chemical products, and not chemically processed. We weighed 2, 5, and 10 mg of hair and tested the relationship between the amount of hair and the matrix effect. These results indicated that more hair could affect the assay result. In this study we did not wash the hair samples before drug extraction. Because external contamination (e.g., sweat, sebum) might affect hair ARV concentrations, a hair washing evaluation study may be required. However, since we used deuterated ISs for this assay, we believe this compensated for the matrix effect. Second, we did not take into account potential variables that may be caused by hair care products in this method, such as hair dyes, shampoos, conditioners, bleach, hair products (e.g., hair gel, hair spray, baby powder) and other hair treatments (e.g., straighteners/relaxer, permanent wave chemicals). Although all of these hair care products can potentially cause matrix effects, deuterated ISs should also compensate for the matrix effect since eluted hair care products will co-exist with TFV/FTC and ISs in the extraction solution. We are currently carrying out studies to elucidate the effects of hair care products on ARV levels in hair. Third, we did not check all crosstalk between other ARVs as well as other concomitant medications. We have tested effects of HIV non-nucleoside reverse transcriptase inhibitors, protease inhibitors, and integrase strand transfer inhibitors, and did not see crosstalk between these drugs and TFV/FTC (data not shown). Finally, we had a limited number of samples for evaluating booster effects on TFV/FTC concentrations. However, all the participants had excellent adherence as demonstrated by their persistently undetectable viral loads; therefore, the TFV and FTC hair concentrations discussed above would reasonably assess TFV/FTC concentration differences between TDF and TAF.

5 | CONCLUSION

We describe here the development of a robust, validated analytical method for assaying TFV and FTC concentrations in human hair using LC/MS/MS. The method is highly sensitive and reproducible for TFV and FTC quantitation, and approved by the Division of AIDS' supported CPQA. We have applied this method to analyze TFV and FTC concentrations in hair from participants who are on TDF/FTC and TAF/FTC, finding similar levels despite differences in TFV exposure in plasma. Finally, boosters did not appreciably increase TFV levels in hair with either TDF or TAF. Further investigation is required to explain relative differences in TFV concentrations in hair and plasma on TDF versus TAF. However, our robust analytical method is now fully validated and has been applied to many research studies examining adherence or exposure to TAF- or TDF-based ART and, increasingly, PrEP.

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Abbreviations:

ARV	antiretroviral
ART	antiretroviral therapy
TDF	tenofovir disoproxil fumarate
TAF	tenofovir alafenamide
TFV	tenofovir
TFV-DP	tenofovir diphosphate
FTC	emtricitabine
LC/MS/MS	high-performance liquid chromatography coupled with tandem mass spectrometry
PrEP	pre-exposure prophylaxis.

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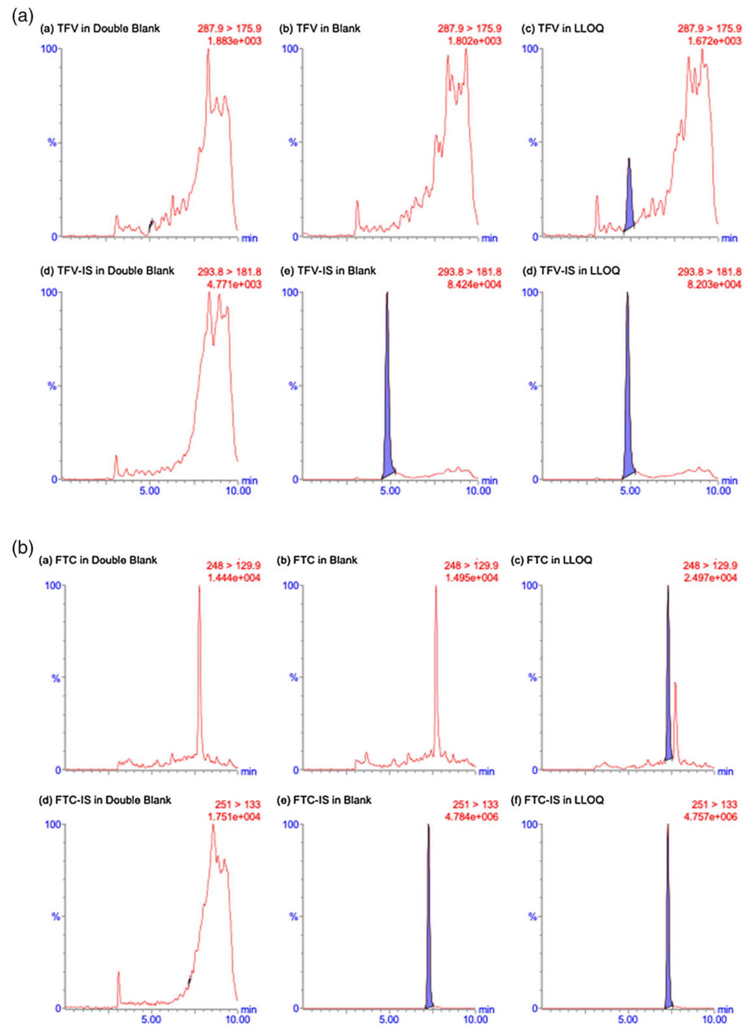

FIGURE 1.

(a) LC/MS/MS chromatograms of TFV and TFV-IS obtained by applying the present method. (b) LC/MS/MS chromatograms of FTC and FTC-IS obtained by applying the present method

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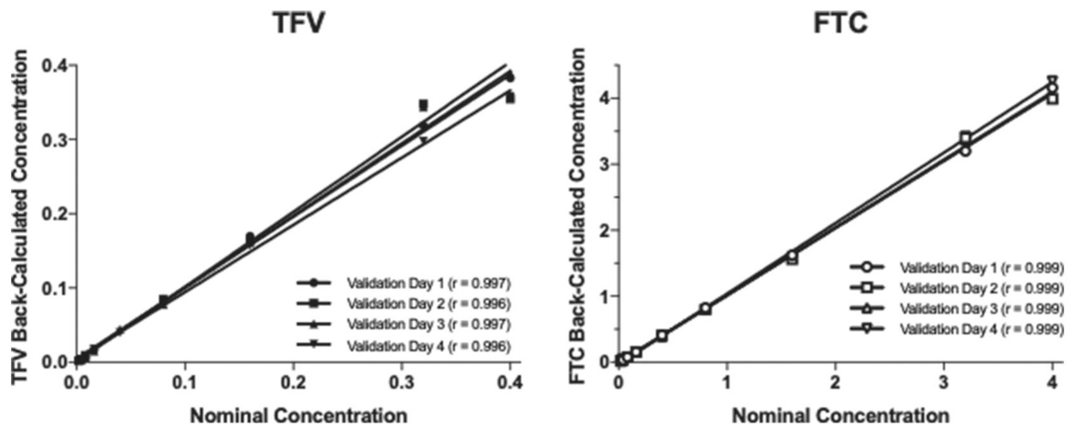

FIGURE 2.
Standard curve of TFV and FTC

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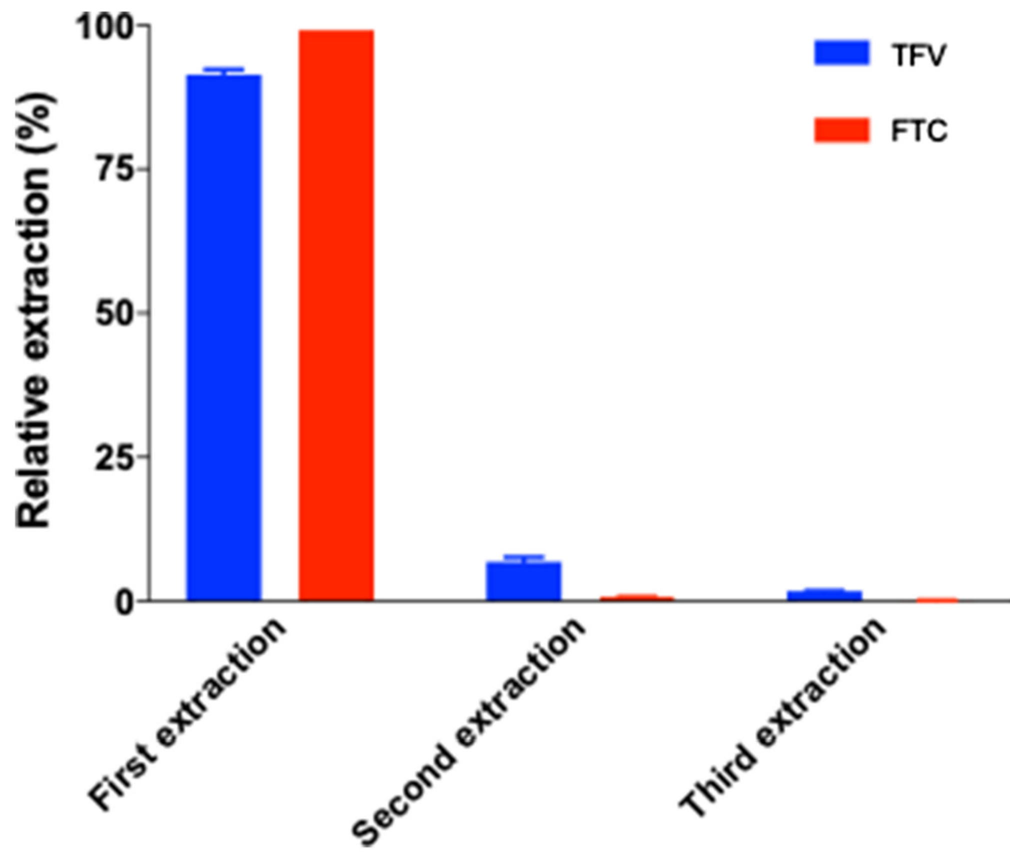

FIGURE 3.
Extraction efficiency of TFV and FTC from participant hair samples

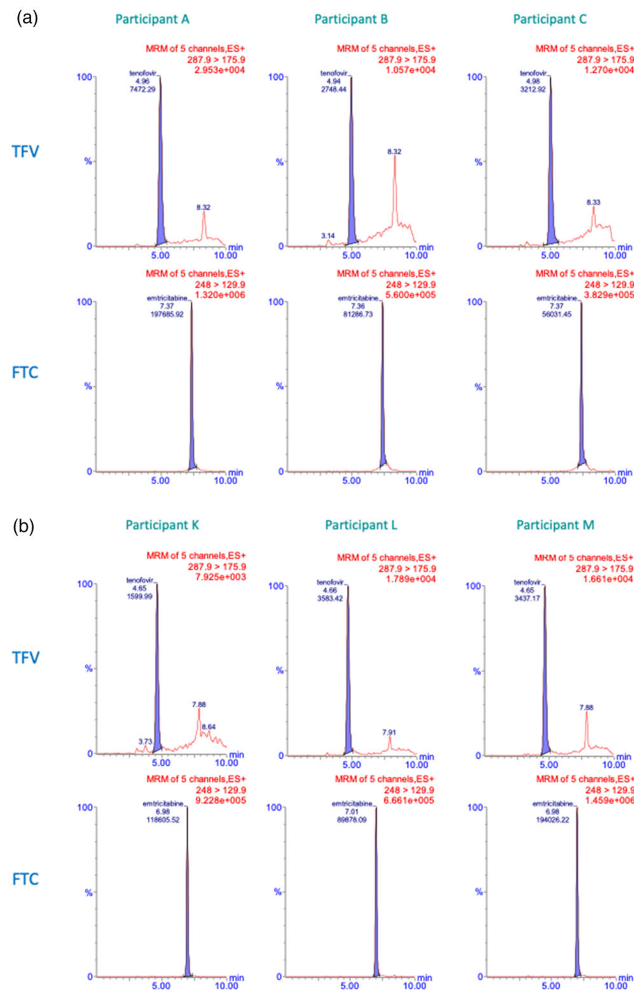

FIGURE 4.

(a) Representative chromatograms of TFV and FTC from three participants who were under TDF/FTC-based regimens. (b) Representative chromatograms of TFV and FTC from three participants who were under TAF/FTC-based regimens

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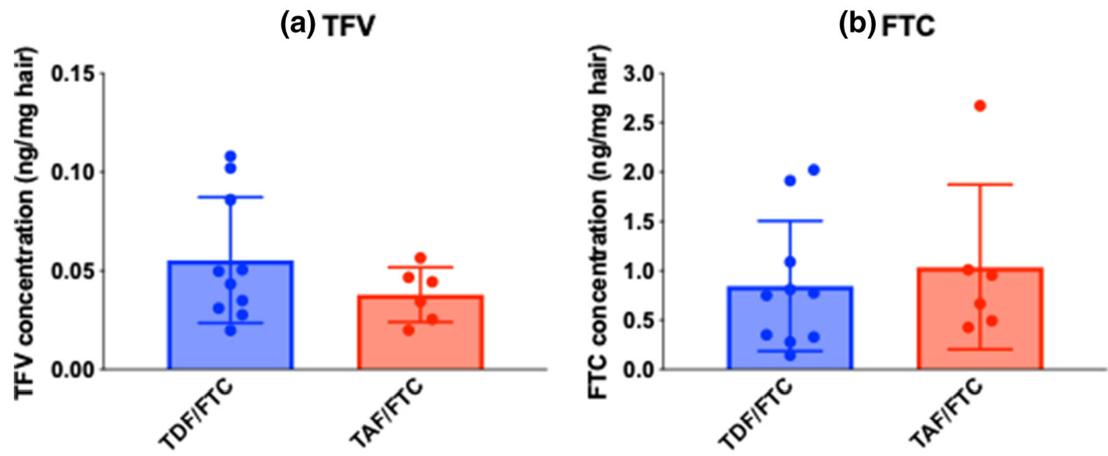

FIGURE 5.

Difference of TFV (a) and FTC (b) hair concentrations between TDF/FTC- and TAF/FTC-based regimen participants

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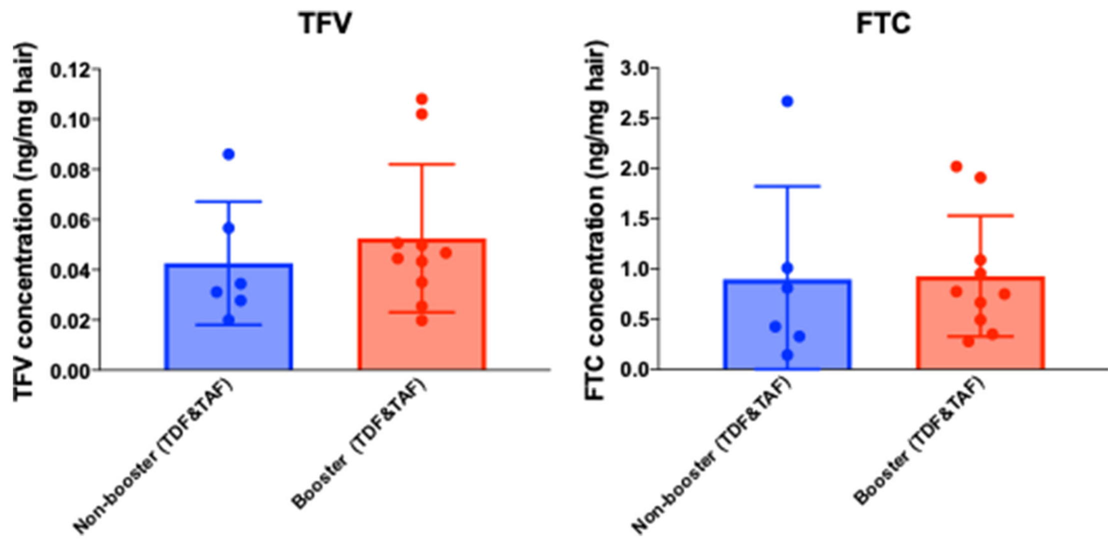

FIGURE 6.

Difference in TFV and FTC hair concentrations between boosted (co-administered with cobicistat or ritonavir) and non-boosted regimens

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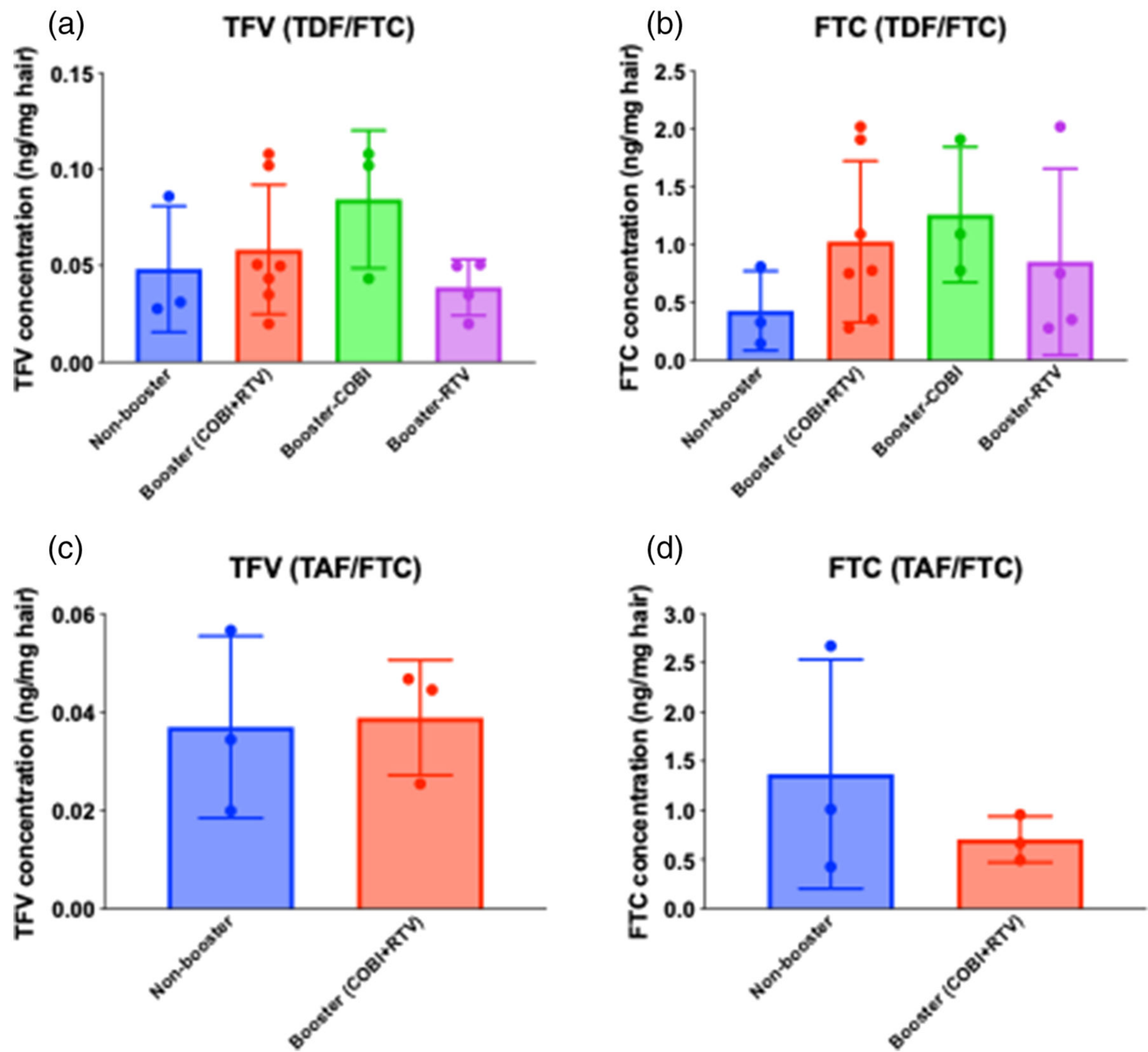

FIGURE 7.
Evaluation of TFV (a and c) and FTC (b and d) hair concentrations on boosted and non-boosted regimens with TDF/FTC and TAF/FTC formulations

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TABLE 1

Accuracy and precision of quality control concentrations calculated by standard curve

	TFV				FTC			
	LLOQ	Low	Medium	High	LLOQ	Low	Medium	High
Theoretical concentration	**0.00200 (ng/mg hair)**	**0.00600 (ng/mg hair)**	**0.0400 (ng/mg hair)**	**0.320 (ng/mg hair)**	**0.0200 (ng/mg hair)**	**0.0600 (ng/mg hair)**	**0.400 (ng/mg hair)**	**0.320 (ng/mg hair)**
(A) Intra-day statistics								
Mean ± SD	0.00204 ± 0.00034	0.00633 ± 0.00050	0.0432 ± 0.0055	0.334 ± 0.018	0.0229 ± 0.0027	0.0597 ± 0.0011	0.402 ± 0.010	3.26 ± 0.02
%CV	16.7	7.82	12.6	5.39	11.6	1.76	2.45	0.571
%RE	2.00	5.50	8.00	4.38	14.5	−0.500	0.500	1.87
n	6	6	6	6	6	6	6	6
(B) Inter-day statistics								
Mean ± SD	0.00208 ± 0.00007	0.00606 ± 0.00082	0.0391 ± 0.0041	0.314 ± 0.020	0.0207 ± 0.0002	0.0599 ± 0.0031	0.399 ± 0.010	3.33 ± 0.13
%CV	3.21	13.5	10.6	6.40	1.18	5.16	2.45	3.87
%RE	4.00	1.00	−2.25	−1.88	3.50	−0.167	−0.250	4.06
n	4	16	16	16	4	16	16	16

TABLE 2

Precision of incurred sample concentrations calculated by standard curve

			TFV		FTC				TFV	FTC
Run no.	**Sample no.**	**Weight of hair (mg)**	**Nominal concentration (ng/mg hair)**	**Normalized concentration (ng/mg hair)**	**Nominal concentration (ng/mg hair)**	**Normalized concentration (ng/mg hair)**	**Run no.**	**Intra-day statistics**	**Normalized concentration (ng/mg hair)**	
1	1	5.04	0.141	0.140	1.96	1.94	1	Mean	0.133	1.89
	2	5.02	0.127	0.126	1.89	1.88		SD	0.010	0.04
	3	5.01	0.143	0.143	1.89	1.89		%CV	7.52	2.12
	4	5.00	0.124	0.124	1.85	1.85		n	4	4
2	1	5.00	0.141	0.141	1.93	1.93	2	Mean	0.134	1.93
	2	5.02	0.124	0.124	1.92	1.91		SD	0.010	0.07
	3	5.03	0.144	0.143	2.04	2.03		%CV	7.46	3.63
	4	5.02	0.128	0.127	1.87	1.86		n	4	4
3	1	5.00	0.120	0.120	1.92	1.92	3	Mean	0.123	1.95
	2	5.02	0.127	0.126	1.94	1.93		SD	0.003	0.05
	3	5.00	0.121	0.121	1.93	1.93		%CV	2.44	2.56
	4	5.05	0.126	0.125	2.05	2.03		n	4	4
4	1	5.04	0.138	0.137	1.95	1.93	4	Mean	0.133	1.98
	2	5.00	0.135	0.135	2.00	2.00		SD	0.005	0.03
	3	5.04	0.127	0.126	2.01	1.99		%CV	3.76	1.52
	4	5.00	0.133	0.133	1.98	1.98		n	4	4
Inter-day statistics							**Intra-day ranges**			
Theoretical concentration										
Mean				0.131		1.94		Mean	0.123–0.134	1.89–1.98
SD				0.008		0.06		SD	0.003–0.010	0.03–0.05
%CV				6.11		3.09		%CV	3.76–7.52	1.52–3.63
N				16		16		n	4	4

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TABLE 3

Recovery of spiked samples of TFV and FTC

QC sample	TFV spiked concentration (ng/mg hair)	Peak area ratio		Recovery (%)	Percent different between QC sample		
		Unprocessed sample	Processed sample		High	Medium	Low
(A) Recovery of TFV							
High	0.320	0.902 ± 0.022	0.894 ± 0.054	99.1	-	−1.90	−18.9
Medium	0.0400	0.103[a]	0.104 ± 0.006	101	1.90	-	−17.0
Low	0.00600	0.0193 ± 0.0008	0.0228 ± 0.0022	118	18.9	17.0	-
Internal standard	2.00	9.93 ± 2.16	9.35 ± 2.48	94.2	NA	NA	NA
(B) Recovery of FTC							
High	3.20	0.565 ± 0.005	0.555 ± 0.010	98.2	-	−3.80	1.60
Medium	0.400	0.0709 ± 0.0012	0.0724 ± 0.0026	102	3.80	-	5.40
Low	0.00600	0.0103 ± 0.0009	0.00995 ± 0.00038	96.6	1.60	−5.40	-
Internal standard	2.00	14.7 ± 0.3	14.2 ± 0.3	96.6	NA	NA	NA

[a] This was an average of two replicates because one sample was omitted because of an unacceptable TFV chromatogram.

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TABLE 4

Analysis of TFV and FTC from TDF- or TAF-based ART regimens

Patient ID	TFV concentration (ng/mg hair)	FTC concentration (ng/mg hair)	Regimen	Co-administered drug(s)
(A) TDF-based regimen				
A	0.102	1.91	TDF/FTC	EVG/COBI
B	0.0498	0.279	TDF/FTC	DRV/RTV
C	0.0433	1.09	TDF/FTC	EVG/COBI
D	0.108	0.776	TDF/FTC	EVG/COBI
E	0.0350	0.352	TDF/FTC	DRV/RTV
F	0.0277	0.145	TDF/FTC	NVP
G	0.0506	2.02	TDF/FTC	DRV/RTV
H	0.0198	0.752	TDF/FTC	DRV/RTV
I	0.0860	0.810	TDF/FTC	EFV
J	0.0311	0.329	TDF/FTC	DTG
Average	0.0553	0.846		
SD	0.0318	0.659		
%CV	57.5	77.9		
(B)TAF-based regimen				
K	0.0254	0.666	TAF/FTC	EVG/COBI
L	0.0445	0.494	TAF/FTC	LPV/RTV
M	0.0467	0.956	TAF/FTC	EVG/COBI
N	0.0566	2.67	TAF/FTC	DTG
O	0.0344	1.01	TAF/FTC	DTG
P	0.0199	0.426	TAF/FTC	DTG
Average	0.0379	1.04		
SD	0.0139	0.83		
%CV	36.7	79.8		

Abbreviations: COBI, cobicistat; DRV, darunavir; DTG, dolutegravir; EVG, elvitegravir; FTC, emtricitabine; LPV, lopinavir; NVP, nevirapine; RTV, ritonavir; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate.

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TABLE 5

Reproducibility results for TFV and FTC concentrations in clinical hair samples by LC/MS/MS-based method[a]

	TFV normalized average concentration (ng/mg)				FTC normalized average concentration (ng/mg)			
Patient ID	**Original concentration (ng/mg hair)**	**Repeated concentration (ng/mg hair)**	**Average concentration (ng/mg hair)**	**Difference (%)[b]**	**Original concentration (ng/mg hair)**	**Repeated concentration (ng/mg hair)**	**Average concentration (ng/mg hair)**	**Difference (%)[b]**
A	0.102	0.0926	0.0973	−9.66	1.91	1.80	1.86	−5.91
F	0.0277	0.0300	0.0289	+7.96	0.145	0.147	0.146	+1.37
I	0.0860	0.0840	0.0850	−2.35	0.810	0.869	0.840	+7.02
J	0.0311	0.0297	0.0304	−4.61	0.329	0.367	0.348	+10.9
K	0.0254	0.0293	0.0274	+14.2	0.666	0.721	0.694	+7.93
L	0.0445	0.0490	0.0468	+9.62	0.494	0.509	0.502	+2.99
M	0.0467	0.0533	0.0500	+13.2	0.956	1.02	0.988	+6.48
N	0.0566	0.0829	0.0698	+37.7	2.67	2.85	2.76	+6.52
O	0.0344	0.0406	0.0375	+16.5	1.01	1.09	1.05	+7.62
P	0.0199	0.0172	0.0186	−14.5	0.426	0.398	0.412	−6.80

[a]FDA recommends incorporating incurred sample reanalysis table.

[b]Difference (%) = (Repeated concentration − Original concentration)/(Average concentration) × 100.