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Application of an intracellular assay for determination of Tenofovir-diphosphate and Emtricitabine-triphosphate from erythrocytes using Dried Blood Spots

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Abstract

This communication describes the application of an existing intracellular methodology to the quantitation of tenofovir-diphosphate (TFV-DP) and emtricitabine-triphosphate (FTC-TP) from erythrocytes using dried blood spots (DBS). Concentrations were determined from a 3mm DBS punch extracted into a 70:30 methanol:water solution (lysed cellular matrix). This extraction solution was then subjected to a previously validated analytical procedure for lysed cellular matrix. Experiments for DBS validation used replicate samples from study participants to demonstrate acceptable reproducibility with spot volumes ranging from 10µL to 50µL and punch location either from the edge or center of the spot. Analysis of paired DBS with purified red blood cells showed that a 3mm DBS punch contained an average of 11.9 million cells for the observed hematocrit range of the participants (35% to 50%). Numerous stability tests were completed showing that whole blood in an EDTA vacutainer could sit for 24 hours at room temperature prior to spotting, and DBS could remain at room temperature for up to five days including shipment at ambient using 2-day delivery. DBS stability in storage was acceptable up to 18 months at –20°C or –80°C and DBS could undergo 4 Freeze/Thaw cycles. The described method was applied to HIV prophylaxis studies, demonstrating powerful associations with HIV acquisition through its ability to discriminate gradients of adherence.

Graphical Abstract

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Keywords

Analytical validation; dried blood spot; intracellular nucleoside analog; antiretroviral therapy; HIV-prevention; Adherence

1. Introduction

Adherence is the key factor in the success of tenofovir disoproxil fumarate-emtricitabine (TDF/FTC)-based therapy for both HIV infection and pre-exposure prophylaxis (PrEP) [1-3]. A central obstacle that prevents improvements to adherence is the absence of a reliable, quantitative, convenient way to measure it [4, 5]. Current methods (pill counts, patient self-report, Medication Event Monitoring Systems (MEMS), etc) have not made a lasting impact because of various weaknesses such as lack of objective evidence of drug ingestion, dependence upon patient participation/engagement, expense, and difficulty implementing into routine practice [2, 6].

Tenofovir (TFV) and emtricitabine (FTC) exhibit unique pharmacology that can be leveraged to assess adherence objectively and quantitatively [7, 8]. These nucleos(t)ide analogs undergo intracellular phosphorylation to TFV diphosphate (TFV-DP) and FTC triphosphate (FTC-TP), which are trapped intracellularly leading to long half-lives depending on the cell-type [9]. The half-life of TFV-DP in red blood cells is 17 days and the accumulation with repeated dosing is 25-fold, which lends itself to assess cumulative TFV dosing (adherence) in the preceding 1-2 months.[8, 10]. On the other hand, FTC-TP exhibits a 1 day half-life in red blood cells so that concentrations in the quantifiable range indicate recent dosing in the preceding ~48 hours [11]. However, purification and counting of red blood cells is labor-intensive and expensive. Therefore, this communication describes the validation details for quantifying intracellular TFVDP and FTC-TP from erythrocytes using DBS.

2. Materials and Methods

2.1 Clinical study samples

Blood was collected by venipuncture into EDTA vacutainer tubes (BD, Franklin Lakes NJ, USA) from studies approved by local Institutional Review Boards. Whole blood (25μL/spot) was pipetted onto Whatman 903 Protein Saver Cards (Fisher Scientific, Fairlawn, NJ, USA) and the cards were allowed to dry at room temperature for 2-24 hours before storage in plastic bags with a humidity indicator card and desiccant (Fisher Scientific). The DBS were stored at -20°C .

2.2 DBS Sample Extraction and LC-MS/MS instrumentation

One 3mm punch from a DBS using a Harris Micro-punch (Fisher Scientific) was extracted in a micro centrifuge tube with 0.5mL of a 70:30 methanol:water (v/v) lysing solution. The resulting lysing solution was then subjected to analysis as published previously [12]. Briefly, TFV-DP and FTC-TP were purified via Waters (Milford, MA, USA) QMA Solid Phase Extraction (SPE) cartridges followed by dephosphorylation to the molar equivalent parent moieties (TFV and FTC, respectively) with acid or alkaline phosphatase. The sample was then desalted and concentrated on a Phenomenex (Torrance, CA, USA) Strata-X SPE cartridge. Separation of analytes was achieved on a Phenomenex Synergi Polar RP column ($2.1 \times 100\text{mm}$, $2.5\mu\text{m}$) with isocratic mobile phase (0.5% acetonitrile in water with 0.1% formic acid) at 0.25mL/min flow rate. A Thermo Scientific (San Jose, CA, USA) TSQ Vantage[®] triple quadrupole mass spectrometer detector was used in the ESI (+) mode and the analyte [precursor/product]⁺ transitions (m/z) were: TFV (288.04/176.11), stable-labeled TFV-internal standard (293.04/181.11), FTC (248.10/130.00), stable-labeled FTC-internal standard (251.10/133.00). The quantifiable linear range for TFV was 2.5-2000 fmol/sample and for FTC was 0.1-200 pmol/sample [12].

2.3 Validation methods

The feasibility of measuring TFV-DP from erythrocytes using DBS was published previously including preliminary stability information and TFV-DP correlations between purified erythrocytes versus DBS [8]. In the present communication, full validation details are presented including that for FTC-TP. It was not possible to prepare traditional DBS quality controls (drug-triphosphate cannot be injected into erythrocytes nor can it be added to whole blood because of degradation due to phosphatases), so validation experiments used replicate DBS samples from clinical study participants who were taking TDF-FTC based therapy. Validation assessments included inter- and intra-run precision of the DBS extraction, inter-card precision and accuracy (expressed as percent difference), stability testing of various factors affecting the DBS processing and storage, and the effect of spot volume, punch location, and subject hematocrit. Acceptance criteria for tested conditions were 15% coefficient of variation (%CV) for precision and $\pm 15\%$ deviation from control. Incurred sample reanalysis required $\pm 30\%$ deviation from the initial analysis and at least half the values being within acceptance.

3. Results and Discussion

3.1 Precision of the DBS Extraction

Intra- and inter-run precision was assessed by replicate extraction (n=3 DBS punches) from 5 DBS cards each spotted from a different study subject. Analysis was performed on five separate analytical run dates. Results are shown in Table 1. Concentration results from the 5 different DBS cards were 110 to 757 fmol/punch for TFV-DP and 0.170 to 0.283 pmol/punch for FTC-TP, which provided the range of levels for testing DBS extraction precision. The intra- and inter-extraction precision was 11.6% for TFV-DP and 12.9% for FTC-TP. These data show acceptable precision results for the extraction and analysis of TFV-DP and FTC-TP from a DBS erythrocyte matrix.

3.2 Inter-Card Precision and Accuracy

Inter-card precision and accuracy was assessed by utilizing replicate cards spotted from the same blood draw (n=5 study subjects; 2 cards spotted, Card A and Card B). Card A and Card B had 3 replicate DBS punches extracted and analyzed on five separate runs. Results are shown in Table 2. The inter-card precision and accuracy (%Diff) were within acceptable range (13.5% and 13.9%) for TFV-DP and FTC-TP, respectively. These data demonstrate acceptable performance between DBS cards for the analysis of TFV-DP and FTC-TP from erythrocytes using DBS.

3.3 Effect of Spot Volume and Punch Location

The effect of spot volume was evaluated by spotting 10, 30, and 50 μ L volumes and comparing with the standard 25 μ L spotted volume (control). This was performed with two different subject blood draws that resulted in low and high control values for TFV-DP (220 and 820 fmol/punch) and FTC-TP (0.167 and 0.240 pmol/punch). When comparing the resulting mean concentrations (n=3) from the tested volumes (10, 30, 50 μ L) to the control spot volume, all were within the acceptable range ($\pm 15\%$ Diff) except the 50 μ L spotted sample from the FTC-TP low concentration DBS (+15.9%). The %CV was 13.9% for all tested volumes. To assess the effect of punch location, drug concentrations from DBS punched in the center versus the edge of the DBS were compared from three different participants each at 3 different concentrations to represent a low, medium, and high concentration range. TFV-DP exhibited a mean (range) %Diff of 1.4% (–12.1% to 10.0%) across all samples and 8 of 9 FTC-TP were acceptable; mean %Diff of 1.6% (–11.1% to 7.5%; +16.7%). Together these data show that spot volumes between 10-50 μ L and punches from either the center or edge of the spot are acceptable for TFV-DP and FTC-TP DBS analysis.

3.4 Stability

3.4.1 Stability in whole blood prior to spotting—Three EDTA vacutainer tubes were drawn from each of six participants and processed immediately (T=0 hr, control), one at 24 hrs (t=24 hrs), and one at 48 hrs (t=48 hrs). As summarized in table 3, section A1, the mean %Diff at t=24 hr was –10.2% for TFV-DP (3 of 6 passing) and 3.3% for FTC-TP (but only 2 of 6 passing). All t=48 hr samples failed low for both analytes, suggesting erythrocyte lysis by this time. A second experiment was performed to assess shorter time durations (t= 6 hr and t=24 hr) with n=5 samples (table 3, section A2). Again, TFV-DP passed at both times, but FTC-TP did not. Further investigation of the relationship between FTC-TP in DBS with FTC in plasma (from the same blood draw) showed that increased FTC in plasma was associated with increasing FTC-TP in erythrocytes as blood sits at room temperature (Figure 1). This suggests that extracellular FTC in plasma is taken up into erythrocytes and is converted to FTC-TP in this scenario. Thus, FTC-TP should be used as a qualitative marker of adherence; quantitation serves as a marker of recent dosing (within ~48 hours) whereas levels below the limit of quantification suggest no recent dose [11]. In summary, blood for DBS can be spotted up to 24hr after the blood draw for assessment of TFV-DP and FTC-TP for adherence.

3.4.2 Storage stability of DBS sample—DBS stability at room temperature (RT) storage was evaluated by comparing TFV-DP and FTC-TP concentrations from 5 separate DBS cards following varying times of RT storage (e.g. 1, 3-5, 6-8, 9-11, 12-15, and 16-18 days) versus control that was extracted immediately after drying. The control concentration range was 106 to 826 fmol/punch for TFV-DP and 0.163 to 0.279 pmol/punch for FTC-TP from the tested DBS cards. Results are summarized in table 3, section B, showing that RT storage was acceptable through day 5 for TFV-DP (%Diff $\pm 5.8\%$) and through day 8 for FTC-TP (%Diff $\pm 9.0\%$). Therefore, RT storage should not exceed 5 days for DBS.

Long term stability of TFV-DP in DBS was compared at RT, 4°C and –20°C versus –80°C, for storage up to 538 days, as shown in Figure 2. Samples stored at RT degraded in the first weeks of storage. Samples at 4°C were comparable with –80°C for several months, while samples stored at –20°C were comparable to –80°C for up to 538 days. An additional incurred analysis of DBS samples was conducted after approximately 1 year (range: 314 to 392 days) at the fore mentioned storage conditions. Results are shown in table 3, section C, which further indicates that analytes are stable long term in DBS when maintained at either –20°C or –80°C.

3.4.3 Stability Following Freeze/Thaw—Analyte stability following freeze-thaw was assessed with DBS samples stored at –20°C and/or –80°C by comparing concentrations following 4 freeze/thaw cycles versus control without undergoing freeze/thaw cycles. A freeze/thaw cycle consisted of removing the card from –20°C/–80°C storage, thawing at room temperature for 2~3 hours, then returning to –20°C/–80°C storage. The observed control concentration ranges were 112 to 835 fmol/punch for TFV-DP and 0.177 to 0.290 pmol/punch for FTC-TP. As shown in table 3, section D, DBS samples were stable for up to four freeze/thaw cycles when stored at either –20°C or –80°C.

Given the relatively short RT stability, it was unknown whether ambient or dry ice shipping would be required. To test this, pairs of DBS samples (n= 57) that had been stored at –20°C were shipped on dry ice (control) versus ambient temperature from 3 different USA locations (San Francisco, CA; Washington DC; or Miami, FL to Aurora CO, USA) [13]. The study spanned both summer and winter months. The %Diff (mean) versus control were –3.6% for TFV-DP and 0.9% for FTC-TP with more than 90% passing acceptance criteria. This indicates that DBS samples may be shipped over two days at ambient temperature, which decreases shipping costs and simplifies the process.

3.5 Effects of hematocrit

TFV-DP and FTC-TP measurement in DBS is normalized per 3mm punch [8]. To determine the expected number of RBC in a 3mm punch, a previous analysis of paired DBS versus purified erythrocytes from the same blood draw among 18 participants was expanded from 29 to 132 paired samples [8]. The mean number of erythrocytes per DBS punch from this analysis was 11.9×10^6 erythrocytes/punch (95% CI 10.9, 12.9). The hematocrit range of the 18 participants was 35% to 50%. As expected, hematocrit had a small but statistically significant effect on erythrocytes/punch ($y = 4.12 + 0.187x$, where y is erythrocytes/punch and x is hematocrit; $P=0.05$). Using the point estimates from this regression formula, the

predicted erythrocytes/punch for a hematocrit of 35% was 10.7 million versus 13.5 million for a hematocrit of 50%, which were -10% and +12% from the mean value of 11.9 million, respectively. This hematocrit range encompasses 95% of the adult population [14]. Taken together, 11.9 million RBC/punch is a reasonable expectation for most DBS samples analyzed.

3.6 Considerations for clinical application

Modeling studies suggest that the expected TFV-DP in DBS for 100% adherence to daily dosing at steady-state was approximately 1560 fmol/punch; 700 fmol/punch represented four or more tablets per week, 350–699 fmol/punch represented two or three tablets per week, below limit of quantitation (BLQ) to 349 fmol/punch represented fewer than two tablets per week, and BLQ represented no doses [8, 15]. In the iPrEx open label extension study of daily TDF-FTC for HIV prevention among 1603 men who have sex with men, DBS were collected every 12 weeks for 72 weeks [15]. The corresponding HIV infection rate (infections per 100 person years) was 4.7 if TFV-DP suggested no dosing, 2.3 for fewer than two tablets per week, 0.6 for two to three tablets per week, and 0.0 for four or more tablets per week ($p < 0.0001$). [15] Thus, TFV-DP in DBS distinguished gradients of adherence and it was a powerful predictor of HIV outcomes for PrEP. FTC-TP was not part of this iPrEx analysis, but the present study indicates that FTC-TP in the quantifiable range suggests a recent dose (within ~48 hours) [11]. An ongoing study (DOT-DBS, [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02022657) identifier NCT02022657) will define intra- and inter-individual variability in TFV-DP/FTC-TP in DBS using directly observed dosing with different levels of adherence (33%, 67%, and 100% of daily dosing), including DBS spotting from finger-stick versus venipuncture.

4. Conclusion

This study validated that an existing analytical intracellular methodology could be applied for the determination of TFV-DP and FTC-TP in erythrocytes from a 3mm DBS punch. The approach was shown to be sensitive, precise, and robust considering spot volumes (10 to 50 μ L), punch location (edge versus center), hematocrits in the normal range (35% to 50%), and analyte stability (5 day stability at RT and 18 month stability at -20°C). Given the convenience of the DBS matrix for sample processing, storing, and shipping, this assay has been implemented in numerous HIV prevention studies and shown to be a powerful predictor of HIV prevention efficacy through its ability to assess adherence.

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Abbreviations

TFV-DP	Tenofovir-diphosphate
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FTC-TP	Emtricitabine-triphosphate
PrEP	Pre-exposure HIV prophylaxis
iPrEx	Iniciativa Profilaxis Pre-exposicion (PrEP initiative) study
DOT	Direct observed therapy
TDF-FTC	tenofovir disoproxil fumarate-emtricitabine

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Highlights

Validation for TFV-DP and FTC-TP in erythrocytes using dried blood spots (DBS)

Precise extraction demonstrated using replicate clinical samples

Analyte stability over 1 year at -20°C to -80°C and up to 5 days at ambient

Measurement not impacted by punch location, spot volumes, and hematocrit range tested

The method has been implemented as a biomarker of adherence to TDF-FTC therapy

Figure 1.

Blood was allowed to sit in EDTA whole blood for 24 hours prior to spotting versus immediate spotting (within 1 hour) for DBS. The difference in FTC-TP in DBS from 24 hour spotting versus control (y-axis) was associated with the FTC in plasma (x-axis), $Y = -13.1 + 0.07 * X$ ($P = 0.0001$).

Figure 2. TFV-DP stability when duplicate DBS were stored at room temperature (RT), 4°C, and -20°C versus a DBS replicate stored at -80°C (n=60 pairs of -80°C with other conditions). The thin dashed line represents no difference and the thick dashed lines represent 15% above and 15% below the -80°C value.

Table 1

Precision Assessment of DBS Extraction

	TFV-DP	FTC-TP
	fmol/punch	pmol/punch
Concentration range (n=5 DBS cards)	110 to 757	0.170 to 0.283 ^a
Intra-Extraction Precision (n=5 runs) (%CV)	0.2 to 10.5	1.1 to 12.9 ^a
Inter-Extraction Precision (n=75) (%CV)	3.5 to 11.6	4.2 to 10.8 ^a

^a n=71; 4/75 FTC-TP values were <0.100 pmol/punch and excluded from analysis.

Table 2

Inter-Card Precision and Accuracy

	TFV-DP	FTC-TP
Concentration range (n=5 replicate DBS cards)	fmol/punch	pmol/punch
Card A	102 to 846	0.158 to 0.291 ^b
Card B	100 to 865	0.150 to 0.312 ^b
Inter-Card Precision Card A (%CV)	2.1 to 8.5	1.1 to 4.1 ^b
Card B (%CV)	1.6 to 13.5	5.1 to 13.9 ^b
% Diff (Card A vs. Card B)	± 9%	± 12.4 ^b

^b 1/15 FTC-TP values were <0.100 pmol/punch for Card A and Card B and excluded from analysis.

Table 3

Stability Assessments

	TFV-DP		FTC-TP	
<i>a1. in Whole blood (n=6 samples)</i>	Mean %Diff to control	% Acceptable	Mean %Diff to control	% Acceptable
24 hr. (n=6)	-10.2	50 (3/6)	3.3	33 (2/6)
48 hr. (n=6)	-54.5	0 (0/6)	-43.4	0 (0/6)
<i>a2. in Whole blood (n=5 samples)</i>				
6 hr. (n=5)	3.4	100 (5/5)	21.7	40 (2/5)
24 hr. (n=5)	-6.7	100 (5/5)	49.9	20 (1/5)
b. Time allowable at RT Storage (n=5)	Mean %Diff to control	% Acceptable	Mean %Diff to control	% Acceptable
day 1	-5.8	75 (3/4)	-5.1	100 (4/4)
day 3-5	-5.7	60 (3/5)	-5.8	100 (5/5)
day 6-8	-15.1	60 (3/5)	-9.0	60 (3/5)
day 9-11	-15.7	60 (3/5)	-13.7	40 (2/5)
day 12-15	-5.8	80 (4/5)	-5.9	80 (4/5)
day 16-18	-20.2	33 (1/3)	-12.8	67 (2/3)
<i>c. Incurred Samples n=30</i>				
Elapsed storage time between runs #1 and #2 ranged from 314-392 days				
Mean % Difference (n=30)	TFV-DP (pass rate)	FTC-TP (pass rate)		
RT% Diff Run #2 vs #1	-70.3 (0/30)	-69.5 (12/30)		
4°C% Diff Run #2 vs #1	-20.9 (27/30)	-4.5 (29/30)		
-20°C % Diff Run #2 vs #1	-13.5 (27/30)	2.0 (28/30)		
-80°C % Diff Run #2 vs #1	-10.4 (29/30)	1.8 (30/30)		
<i>d. Freeze/Thaw (n=5 samples for 4 cycles)</i>				
% CV at -20°C / -80°C	10.5 / 7.3	12.9 / 10.4		
% Diff to control at -20°C / -80°C	$\pm 13.5^a / 12.3^b$	$\pm 13.2^a / 13.5$		
% Diff (-20°C vs. -80°C)	$\pm 10\%$	$\pm 10.6\%$		

^aF/T cycle 1 for TFV-DP DBS sample 4 (+19.8%), F/T cycle 4 for FTC-TP DBS sample 2 (-16.7%) at -20°C, all other $\pm 15\%$.

^bF/T cycle 1 for TFV-DP DBS sample 5 (-20.5%) at -80°C, all other $\pm 15\%$.