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Quantitation of tenofovir and emtricitabine in dried blood spots (DBS) with LC–MS/MS

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ABSTRACT

A reversed-phase high performance liquid chromatographic (LC), tandem mass spectrometry (MS/MS) assay for the determination of tenofovir (TFV) and emtricitabine (FTC) in dried blood spots (DBS) from human whole blood was developed and validated. Whole blood samples were spotted, dried, and a 3 mm punch was extracted with methanol for analysis by LC–MS/MS utilizing stable isotope labeled internal standards. The assay was validated over the range of 2.5–1000 ng/mL for TFV and 2.5–5000 ng/mL for FTC. The method was accurate (within ±15% of control) and precise (coefficient of variation ≤15%) for hematocrit concentrations ranging from 25% to 76%; using edge punches vs. center punches; and spot volumes of 10–50 μL. Analytes were stable for five freeze/thaw cycles and up to 6 days at room temperature, whereas long-term storage required −20 °C or −80 °C. Comparison of TFV and FTC in DBS vs. plasma yielded $r^2 \geq 0.96$, indicating that DBS can be used as a plasma alternative for pharmacokinetic analyses in vivo.

1. Introduction

Tenofovir (TFV) and emtricitabine (FTC) are widely-prescribed antiretroviral drugs used for the treatment and prophylaxis of HIV infection [1]. These agents are co-formulated as Truvada, among other co-formulation products. Both agents are also active against Hepatitis B virus (HBV) infection, although only tenofovir has an indication for HBV treatment [2,3]. Measurement of TFV and FTC in plasma has been used to monitor adherence to therapy, and to evaluate pharmacokinetics in special populations such as infants, children, pregnancy, and for drug–drug interaction studies [1,4–6]. Thus far, most assays have utilized plasma or serum matrices for these studies.

Plasma processing is time consuming, and it requires technical personnel and onsite or nearby centrifugation. Blood collection for harvesting plasma typically requires ≥4 mL of blood, which can add up to large volumes when conducting pharmacokinetic studies among infants or pediatric patients. The dried blood spot (DBS) blood sampling strategy offers advantages to plasma drawbacks: blood for DBS can be collected and processed at any clinic or research facility quickly and easily, and DBS requires only ∼25 μL of whole blood, making it suitable for pharmacokinetic studies in special patient populations [7]. Because of these advantages, many DBS methods have been developed for a variety of drugs, including antiretrovirals [7], but a DBS method for tenofovir and emtricitabine has not been developed, to our knowledge. An important consideration for tenofovir and emtricitabine in DBS will be the high accumulation of phosphorylated tenofovir in red blood cells [8]. We previously described preliminary analyses and the potential clinical application of DBS for adherence monitoring in subjects receiving tenofovir and emtricitabine, but we did not describe the method validation [8]. This communication provides the methodology and supporting analytical validation results for the measurement of TFV and FTC in DBS.

2. Methods

2.1. Chemicals and materials

TFV and FTC were acquired from the NIH AIDS Research & Reference Reagent Program (Germantown, MD, USA). TFV

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isotopic internal standard ($^{13}C_5$ TFV-iso, MW = 292.2) and FTC-isotopic internal standard ($^{15}N_2$,$^{13}C_1$ FTC-iso, MW = 250.2) were purchased from Moravek Biochemicals, Inc (Brea, CA, USA). Methanol (HPLC Grade) and formic acid were purchased from Fisher Scientific, (Fairlawn, NJ, USA), and acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). Whatman 903 Protein Saver Cards, desiccants, and humidity indicators were obtained from Fisher Scientific. Human blank whole blood with EDTA anticoagulant was obtained from Biological Specialty Corporation and from consented participants in human research protocols that were approved by the local institutional review board (IRB).

2.2. Preparation of stocks and standard calibrators, quality control and internal standard samples

Individual standard prep stocks (1 mg/mL) of TFV and FTC were prepared in ultra-pure water (UPH_2O). These were used to make the combined standard working stocks of TFV/FTC in UPH_2O. Twenty microliters of working stocks were combined with 480 microliters whole blood to arrive at final concentrations of TFV/FTC for standard calibrators (2.5/2.5, 5.0/5.0, 10/10, 25/25, 50/50, 100/100, 250/250, 500/500, 750/1000, 1000/5000 ng/mL). Separate quality control (QC) prep stocks (1 mg/mL) of TFV and FTC were prepared in UPH_2O. These were combined to prepare QC working stocks and the validation QC samples in the same way as standard calibrators. The concentrations of validation QC samples were prepared at 6 different levels: Level 1: 2.5/2.5, Level 2: 5.0/5.0, Level 3: 7.5/7.5, Level 4: 15/15, Level 5: 200/400, and Level 6: 800/4000 ng/mL. The combined working internal standard (IS) solution of TFV-iso and FTC-iso were made in UPH_2O yielding a final concentration of approximately 16.7 ng/mL. Purity and salt content were taken into consideration when preparing prep stocks. All solutions were stored at 4 °C in glass vials.

2.3. Preparation of dried blood spot sample

DBS were prepared by spotting 25 μL of whole blood that contained either standard or QC onto 903 Protein Saver Cards (except for testing the effect of spot volumes which were spotted using serial volumes of 5–50 μL). The pipette tip was not allowed to touch the paper. After spotting, the cards were allowed to dry for at least for 2 h and up to overnight. Once dried, cards were placed in plastic bags and stored in a sample box with desiccant and humidity indicators at room temperature (RT), 4 °C, −20 °C, and/or −80 °C for the storage condition tests.

2.4. Sample extraction from DBS

One 3 mm diameter disk was punched with a Harris micropunch (Sigma–Aldrich, St. Louis, MO, USA) from the blood spots for extraction. An additional punch from a clean card was performed between each DBS sample punch to minimize the potential for carry-over. The punched discs were placed in a micro-centrifuge tube and TFV and FTC were extracted with 200 μL of 100% methanol, and 20 μL of working IS. Samples were sonicated for 10 min and centrifuged for 1 min. Supernatants were then dried and reconstituted in 100 μL UPH_2O for LC–MS/MS analysis.

2.5. Clinical study samples

DBS samples were collected from consented study subjects participating in IRB approved protocols with a DBS component [8]. Blood was collected in EDTA Vacutainers and 25 μL were spotted as described above. Samples were collected from subjects at variable times post dose and variable durations of TFV/FTC therapy. Samples from human subjects were used to complement QC samples to evaluate punch location, spot volume, and stability testing. To evaluate TFV/FTC DBS concentrations vs. plasma concentrations, a total of 30 selected DBS samples with paired plasma samples were analyzed, as described previously [8].

2.6. LC–MS/MS instrumentation

Quantification of TFV and FTC and their respective internal standards was performed with high performance liquid chromatography tandem mass spectrometry (LC–MS/MS) with an approach similar to previously published methods [9,10]. The instrumentation was a Thermo Scientific TSQ Vantage® triple quadrupole mass spectrometer coupled with a Thermo Scientific Accela® UHP pump (Thermo Scientific, San Jose, USA) and CTC Analytics HTC PAL® auto sampler. The Vantage® system utilized the Ion Max HESI II® electrospray ionization (ESI) probe, which was operated in ESI + mode. The selective (SRM) and highly selective reaction monitoring (HSRM) $[precursor/product]^+$ transitions (m/z) were: TFV (288.04/176.11), TFV-IS (293.04/181.11), FTC (248.10/130.00), FTC-IS (251.10/133.00). 20 μL of extracted DBS solution was injected onto the system and analytes were separated with an isocratic mobile phase (0.1% Formic Acid in 0.5% Acetonitrile: 99.5% UPH_2O) with a flow rate of 250 μL/min. The analytical column was Synergi Polar RP 2.5 μM, 100A, 2.0 × 100 mm, purchased from Phenomenex (Torrance, CA, USA). The retention times were approximately 2.3 min for TFV and 4.6 min for FTC. Data were captured and analyzed with Xcalibur™ 2.0.7 SP1 software (Thermo Scientific San Jose, USA).

2.7. Validation strategy

DBS is regarded as an alternative matrix bio-analytical methodology with no standard validation guidelines. Therefore, accepted bio-analytical methodology validation practices were used as a guide to evaluate accuracy and precision [11,15]. Validation was demonstrated if inter-assay and intra-assay accuracy (% deviation from nominal) and precision (% coefficient of variation (CV)) were within 15% at all QC levels, except the lower limit of quantitation (LLOQ) where within 20% was allowed. Additionally, literature references for DBS methodologies were utilized to determine other important factors that should be considered when validating a method for a DBS matrix [7,12–14]. These included effects of punch location, spot volume, hematocrit, and dilution/punch-stacking. Acceptance criteria for tested conditions were within ±15% deviation from the control and/or nominal and ≤15% CV for replicate analysis.

The validation also included the use of human subject samples to assess validation factors that cannot be imitated by spiking TFV/FTC into whole blood and spotting. This was especially important for TFV/FTC because these drugs are phosphorylated in red blood cells [8,18]. For TFV, this phosphorylation leads to substantial accumulation of phosphorylated anabolites with repeated dosing. Because these phosphorylated anabolites are susceptible to hydrolysis when released from the intracellular compartment, it was important to include samples from various durations of therapy in the validation. In order to evaluate samples at different levels of drug accumulation, samples were utilized from initial dosing (day 1) and accumulated dosing (day 20 and/or day 30) from human subjects receiving a daily TDF/FTC regimen.

3. Validation results and discussion

3.1. Accuracy and precision

3.1.1. Standard performance

A single calibration curve was run with each of 5 analytical runs that assessed accuracy and precision. The calibration curve