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Development, validation and clinical application of a method for the simultaneous quantification of lamivudine, emtricitabine and tenofovir in dried blood and dried breast milk spots using LC–MS/MS

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ABSTRACT

Objectives: To present the validation and clinical application of a LC–MS/MS method for the quantification of lamivudine (3TC), emtricitabine (FTC) and tenofovir (TFV) in dried blood spots (DBS) and dried breast milk spots (DBMS).

Methods: DBS and DBMS were prepared from 50 and 30 μL of drug-spiked whole blood and human breast milk, respectively. Following extraction with acetonitrile and water, chromatographic separation utilised a Synergi polar column with a gradient mobile phase program consisting of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Detection and quantification was performed using a TSQ Quantum Ultra triple quadrupole mass spectrometer. The analytical method was used to evaluate NRTI drug levels in HIV-positive nursing mothers-infant pairs.

Results: The assay was validated over the concentration range of 16.6–5000 ng/mL for 3TC, FTC and TFV in DBS and DBMS except for TFV in DBMS where linearity was established from 4.2–1250 ng/mL. Intra and inter-day precision (%CV) ranged from 3.5–8.7 and accuracy was within 15% for all analytes in both matrices. The mean recovery in DBS was > 61% and in DBMS > 43% for all three analytes. Matrix effect was insignificant.

Median $AUC_{0\text{-}8}$ values in maternal DBS and DBMS, respectively, were 4683 (4165–6057) and 6050 (5217–6417) ng h/mL for 3TC, 3312 (2259–4312) and 4853 (4124–6691) ng h/mL for FTC and 1559 (930–1915) and 56 (45–80) ng h/mL for TFV. 3TC and FTC were quantifiable (> 16.6 ng/mL) in DBS from 2/6 and 1/6 infants respectively whereas TFV was undetectable in all infants.

Conclusions: DBS and DBMS sampling for bioanalysis of 3TC, FTC and TFV is straightforward, robust, accurate and precise, and ideal for use in low-resource settings.

1. Introduction

It is internationally recommended that HIV-positive women receive triple antiretroviral therapy (ART) throughout pregnancy until the end of breastfeeding or for life irrespective of clinical disease stage or CD4 count [1]. As breastfeeding remains the only acceptable, feasible, affordable, sustainable and safe infant feeding option in many parts of the world [2], the number of infants exposed to antiretroviral drugs through pregnancy and breastfeeding will continue to increase.

First-line ART comprises efavirenz (EFV), tenofovir diproxil fumarate (TDF) and either lamivudine (3TC) or emtricitabine (FTC) used preferably as a fixed-dose combination. It is important to understand the breast milk transfer of these drugs since low infant levels predispose to HIV-drug resistance should HIV transmission occur [3,4], and there is conflicting data regarding the effects of tenofovir (TFV) on developing bone [5]. The pharmacokinetic profiles in paired maternal and infant plasma (calculated from dried blood spots [DBS]) and breast milk (BM) have been reported for EFV [6] and 3TC [7], but only three studies have sought to measure TFV [8–10] and a single study FTC in the BM of HIV-positive mothers [8], and these did not present intensive pharmacokinetic profiles and paired mother and infant data.

Furthermore, a systematic review of antiretroviral measurement in

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BM noted marked methodological differences with regard to the collection and storage of samples, the matrix used to prepare the standards and quality controls, the fraction of milk analysed, the extraction method and the type of internal standard used [11]. We have recently developed and validated dried blood spot (DBS) and dried breast milk spot (DBMS) methodology for EFV [12] and nevirapine (NVP) [13]. In addition to quantifying drugs in whole BM, these techniques have the advantage of being suitable for collection and storage in low resource settings. We now report the DBS and DBMS LC–MS/MS method for accurate simultaneous quantitation of TFV, 3TC and FTC, with application of the method in breastfeeding mother-infant pairs.

2. Materials and methods

Lamivudine (3TC) and internal standard (IS) lamivudine-$^{15}N_2$,^{13}C (3TC-IS), emtricitabine (FTC) and emtricitabine-^{13}C,$^{15}N_2$ (FTC-IS) and tenofovir (TFV) were obtained from TRC Canada (North York, Ontario). LC–MS grade acetonitrile was obtained from Fisher Scientific (Loughborough, Leicestershire, UK), methanol from VWR International (Lutterworth, Leicestershire, UK), formic acid from Sigma-Aldrich (Gillingham, Dorset, UK) and water was produced from an Elga Option 4 water purifier (Elga LabWater, High Wycombe, Buckinghamshire, UK) and was further purified to 18.2 MΩ with a Purelab Classic UVF (Elga LabWater, High Wycombe, Buckinghamshire, UK). Whatman 903 Protein Saver cards were obtained from Scientific Laboratory Supplies (Hessle, East Yorkshire, UK). Blank whole blood was collected into EDTA tubes from drug-free healthy volunteers and blank BM samples were obtained from the Wirral Mothers' Milk Bank, Clatterbridge Hospital, Wirral, UK; the University of Liverpool Research Ethics Committee approved these processes.

2.1. LC–MS systems and conditions

The LC–MS system consisted of a Synergi polar-RP column (80A, 150 *2.0 mm and 4 μ; Phenomenex, Macclesfield, UK) with a 2 μm C_{18} Quest column-saver (Thermo Electron Corporation, Hemel Hempstead, Hertfordshire, UK) on a HPLC connected to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron Corporation, Hemel Hempstead, Hertfordshire, UK) equipped with a heated electrospray ionisation source (H-ESI). Xcalibur Software and LCquan (version 2.6.1, Thermo Fisher Scientific, Hemel Hempstead, UK) were used for method setup, data acquisition, data processing and reporting.

A solvent gradient programme (flow rate of 400 μL/min) with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) was used for chromatographic separation. The gradient programme started with 95% mobile phase A, and held for 0.2 min. Mobile phase A was decreased to 70% over 0.5 min. This was maintained for 3.0 min, followed by column equilibration to the initial conditions. The total run time was 6.0 min. The Injection volume was 25 μL and the needle was washed twice with 2 mL water: acetonitrile (95:5) between injections. The MS was operated in positive ionisation mode to produce characteristic fragmentation patterns. The electrospray voltage was set at 4.0 kV, the capillary temperature at 300 °C and the vaporizer temperature at 350 °C. The sheath and auxiliary gas pressures were set of 50 and 10 arbitrary units, respectively. Argon was used as the collision gas at a pressure of 1.5 mTorr. Product ion characterisation and tuning was done by directly infusing 1 μg/mL solutions of all the three analytes and their internal standards separately into the MS using a syringe at a flow rate of 5 μL/min. The most sensitive mass transitions (m/z) were monitored in selective reaction monitoring.

2.2. Stock solutions, calibration standards and quality controls (QC)

3TC and FTC stock solutions were prepared from their respective reference standards in 100% methanol, and TFV in 100% water to obtain a final concentration of 1 mg/mL and refrigerated at 4 °C until use. Similarly, 3TC-IS and FTC-IS 1 mg/mL stock solutions were prepared and frozen at −40 °C; working stock solutions of 250 ng/mL were prepared in methanol-water (50:50 v/v) and refrigerated at 4 °C prior to use. At the time of validation, the cost to purchase a stable isotope labelled internal standard for TFV was beyond our budget. 3TC-IS, FTC-IS and an analog IS (2-Chloroadenosine) were evaluated as potential IS for TFV in pre-validation experiments. FTC-IS was selected on the basis of it exhibiting a consistent response. A whole blood working stock containing all three analytes at 10 μg/mL was prepared, tumbled for 60 min and used to make nine whole blood standards in the range of 16.6–5000 ng/mL by serial dilution, including a blank sample of blood alone. Whole blood low quality control (LQC, 40 ng/mL), medium quality control (MQC, 400 ng/mL) and high quality control (HQC, 4000 ng/mL) samples were similarly prepared from a 10 μg/mL working stock. Working stocks in human breast milk were similarly prepared, with the difference that the calibration range for tenofovir was 4.2–1250 ng/mL with LQC 10 ng/mL, MQC 40 ng/mL and HQC 400 ng/mL respectively due to the lower anticipated concentrations of this analyte in clinical specimens [9].

2.3. DBS and DBMS standard and QC preparation

DBS standards and QCs were prepared by carefully spotting exactly 50 μL of whole blood standards and QCs on each circle of Whatman 903 Protein Saver Cards. DBMS standards and QCs were similarly prepared by spotting 30 μL of breast milk standards and QCs. Spotted cards were left to dry at room temperature overnight and stored with dessicant sachets in ziplock bags at −80 °C.

2.4. Sample pre-treatment

The entire DBS or DBMS spot was removed using a 12 mm hole punch and folded into a 7 mL screw cap tube. For DBS, initial extraction was with 200 μL 0.1% formic acid in water for 5 min prior to the addition of 3TC-IS and FTC-IS. IS was added to the extraction solvent [14] since spotting directly onto the card was not feasible for samples collected under field conditions. Then 800 μL acetonitrile was added to each tube and after vortexing, tubes were centrifuged at 4000 rpm for 10 min. 850 μL of each sample was carefully pipetted into a 5 mL tube, before evaporation to dryness under a stream of nitrogen. Finally, samples were reconstituted in 100 μL water: acetonitrile (99:1 v/v) and transferred into autosampler vials.

DBMS samples were extracted with 1 mL of acetonitrile: water (70:30, v/v) by tumbling for 30 min in the presence of 3TC-IS and FTC-IS. 800 ul was then transferred to a 5 mL glass tube, before evaporation to dryness under a stream of nitrogen. Finally, samples were reconstituted in 100 μL water: acetonitrile (99:1 v/v) and transferred into autosampler vials.

2.5. Calibration curves, accuracy and precision

A calibration curve consisting of a zero blank, nine standards in the range of 16.6–5000 ng/mL (and 4.2–1250 ng/mL in the case of DBMS for TFV) (n = 2 separate extractions for each level) and QCs (n = 6 separate extractions for each level) were run for each of DBS and DBMS. Calibration curves were constructed using a linear regression equation of analyte/IS peak area ratios versus nominal concentrations with a 1/concentration weighting. Accuracy was defined as percentage deviation of measured concentration from the nominal value and precision was defined as the percentage coefficient of variation (%CV). Not less than 75% of all standards and 67% of all QCs (50% at each level) in any batch were required to have a percentage deviation within ± 15%.

2.6. Recovery, matrix effect and dilution integrity

The percentage recovery and matrix effect were determined