**Plasma and Breast Milk Pharmacokinetics of Emtricitabine, Tenofovir and Lamivudine using dried blood and breast milk spots in Nursing African Mother-Infant Pairs**

Running title: Plasma and breast milk PK of FTC, TDF and 3TC

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**Synopsis**

*Background*

Breast milk transfer of first-line antiretroviral therapy (ART) from mother to infant is not fully understood.

*Objectives*

To determine the concentrations of lamivudine (3TC), emtricitabine (FTC) and tenofovir (TFV) in maternal blood, breast milk (BM) and infant blood from breastfeeding mother-infant pairs.

*Patients and Methods*

Intensive pharmacokinetic sampling of maternal dried blood spots (DBS), dried breast milk spots (DBMS) and infant DBS from 30 Ugandan and 29 Nigerian mothers receiving first-line ART and their infants was conducted. DBS and DBMS were collected pre-dose and at 5-6 timepoints up to 12 hours following observed dosing. Infant DBS were sampled twice during this period. 3TC, FTC and TFV were quantified using LC-MS/MS, with non-compartmental analysis to calculate key pharmacokinetic parameters.

*Results*

Peak concentrations in BM from women taking 3TC and FTC occurred later than in plasma (4-8h compared to 2h for 3TC, and 2-4h for FTC). Consequently, the milk-to-plasma (M:P) ratio of 3TC taken once-daily was 0.95 (0.82-1.15) for AUC0-12 , whereas for AUC12-20  this was 3.04 (2.87-4.16). 3TC was detectable in 36% (14/39) of infants (median 17.7 (16.3-22.7) ng/mL).

For FTC 200mg once daily, the median M:P ratio was 3.01 (2.06-3.38). Three infants (19%) had measurable FTC (median 18.5 (17.6-20.8) ng/mL)

For TFV 300mg once daily, the median M:P ratio was 0.015 (0-0.03) and no infant had measurable TFV concentrations.

*Conclusions*

FTC and 3TC accumulate in breast milk and were detected in breastfeeding infants. In contrast, TFV penetrates the breast milk to a small degree, but is undetectable in breastfeeding infants.

**Introduction**

In 2015, an estimated 1.4 million HIV-positive women became pregnant, of whom approximately 1 million received combination antiretroviral therapy (ART).1 Given that breastfeeding remains the only acceptable, feasible, affordable, sustainable and safe infant feeding option in low- and middle-income countries (LMIC),2 the number of infants exposed to antiretroviral drugs through pregnancy and breastfeeding will continue to increase as countries adopt WHO guidelines for universal, lifelong access to ART.3

In most countries where universal breastfeeding is recommended, first-line ART comprises the non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz (EFV), together with the nucleoside reverse transcriptase inhibitors (NRTI) tenofovir (TFV) and either lamivudine (3TC) or emtricitabine (FTC) in fixed-dose combination (FDC). It is important to understand the transfer of these drugs from mother to the breastfed infant, since it has been demonstrated that low infant concentrations of drugs predispose to the selection of HIV-drug resistance should HIV transmission occur, 4, 5 and there continue to be conflicting safety data, for example regarding the safety of tenofovir (TFV) on developing bone. 6 The pharmacokinetic (PK) profiles in paired maternal and infant plasma have been reported for EFV 7 and 3TC, 8 but only three studies have sought to measure TFV, 9-11 and a single study measured FTC in the breast milk of HIV-positive mothers. 9 These studies of TFV and FTC did not conduct intensive pharmacokinetic sampling or present paired mother and infant data for analysis.

This study aimed to describe the pharmacokinetic transfer of the current WHO-recommended NRTI backbone of first-line ART regimens from mother to breastfed infant. Intensive pharmacokinetic data of these drugs in dried blood spots (DBS) and dried breast milk spots (DBMS) from Ugandan and Nigerian mother-infant pairs are reported.

**Materials and Methods**

**Study Design**

1. *Study Sites and Participants*

Thirty Ugandan mother-infant pairs were recruited from the Infectious Diseases Institute, Makerere University, Kampala, Uganda. Table 1 summarises the breakdown of number of participants on each ART regimen. In summary, 30 mother-infant pairs contributed to the 3TC and 18 to the TFV analyses. The study protocol and the material transfer agreement were approved by the University of Liverpool Research Ethics Committee, UK and the Joint Clinical Research Centre IRB, Uganda and the Uganda National Council for Science and Technology (HS1675).

Twenty nine mother-infant pairs were recruited from two hospitals in Benue State, Nigeria: Bishop Murray Medical Centre, Makurdi and St Monica’s Hospital, Adikpo. The ART regimens of each participant are summarised in Table 1, with nine mother-infant pairs contributing to the 3TC analysis, 27 to the TFV analysis and 16 to the FTC analysis. Ethical approval was obtained from the National Health Research and Ethics Committee (NHREC), Abuja and Obafemi Awolowo University Teaching Hospitals Ethics and Research Committee, Ife-Ife, Nigeria. This cohort has previously been described in detail. 12-14

In both cohorts, most infants were within 6 months of age and reportedly still being exclusively breastfed. All infants were fed on demand during the study to reflect real-life situations, with the time of most recent breastfeeding being recorded.

1. *Sampling Scheme*

Participants who were sampled prior to and at intervals after dosing were provided with a standard local meal followed by observed dosing of their usual medication. For patients who were sampled at later time points following a self-administered dose, no dietary restrictions were applied, in keeping with standard practice.

Participants receiving 3TC 150 mg twice daily had maternal DBS (mDBS) and DBMS sampling prior to and at 1, 2, 4, 6 and 8 hours post-dose (Uganda N=9) and prior to and at 0.5, 1, 2, 4, and 8 hours post dose (Nigeria, N=2). Participants receiving 3TC 300 mg once daily had mDBS and DBMS sampling at and prior to and at 0.5, 1, 2, 4 and 8 hours post dose (Nigeria, N=7), prior to and at 1, 2, 4, 6 and 8 hours post-dose (Uganda, N=3) or at 12, 16 and 20 hours post-dose (Uganda, N=18).

All participants receiving FTC 200 mg once daily were all at the Nigerian sites and had mDBS and DBMS sampling prior to, and at 0.5, 1, 2, 4, 8 and 12 hours post-dose.

Participants receiving TDF 300 mg once daily had mDBS and DBMS sampling performed prior to and at 0.5, 1, 2, 4, 8 and 12 hours post-dose (Nigeria, N=27), prior to and at 1, 2, 4, 6 and 8 hours post-dose (Uganda, N=3) and at 12, 16 and 20 hours post-dose (Uganda, N=18).

For all analytes, infant DBS was collected at 2 and 8 hours post-dose, and for the later time points sampled in Uganda, at 14 and 20 hours post dose.

1. *Sampling Methods*

In Uganda, mDBS were prepared by accurately spotting 50 µL blood onto the Whatman 903 Protein Saver card, and plasma was harvested from the remaining blood. DBS samples were collected from infants, after sterile skin cleaning and heel prick using a 2mm safety lancet (BD, Oxford, Oxfordshire, UK). All samples were stored locally at -80°C, transported to the Department of Molecular and Clinical Pharmacology, University of Liverpool, UK on dry ice and then kept at -80°C prior to analysis.

In Nigeria, whole blood samples from both mother and infant were collected as DBS after sterile skin cleaning and finger prick using a 2mm safety lancet (BD, Oxford, Oxfordshire, UK). The first drop of blood was discarded and subsequent blood drops were collected on the sample collection areas of Whatman 903 cards. Plasma was not harvested at this site due to logistic constraints.

At both sites, within 2 minutes of each DBS collection, about 5 mL of breast milk was manually expressed by the mother and 30 µL aliquots were spotted onto each circle on the Whatman 903 Protein Saver card.

**Drug Quantification and Analysis**

*DBS and DBMS*

3TC, FTC and TFV were quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS) using a method recently described. 15 In brief, the entire DBS or DBMS spot was removed using a 12 mm punch. Initial extraction of DBS was with 0.1% formic acid in water for 5 min prior to the addition of deuterated internal standard. 800 µL of acetonitrile was then added prior to centrifugation, evaporation and reconstitution in 100 µL water: acetonitrile (99:1 v/v). DBMS samples were extracted with 1 mL of acetonitrile: water (70:30, v/v) by tumbling for 30 minutes in the presence of internal standard, with evaporation and reconstitution as for DBS. A reverse phase Phenomenex column was used on a HLPC connected to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron Corporation, Hemel Hempstead, Hertfordshire, UK) equipped with a heated electrospray ionisation source. Xcalibur Software and LCquan (version 2.6.1, Thermo Fisher Scientific, Hemel Hempstead, UK) were used for method set up, data acquisition, data processing and reporting.

The assay was shown to yield consistent data across a range of haematocrits down to 22%. Cross-validation of 3TC and TFV concentrations was undertaken by analysis of paired maternal plasma and DBS obtained from the same blood draw. The lower limit of quantitation for all analytes in DBS was 16.6 ng/mL, and in BM was 16.6 ng/mL for 3TC and FTC and 4.2 ng/mL for TFV.

*Plasma*

3TC and TFV were quantitated from 100µl of plasma. 20 µl of internal standard mixture 3TC-IS (2.5µg/ml) and FTC-IS (2.5µg/ml) was added. 400µl of acetonitrile was added to precipitate the proteins followed by centrifugation at 4000 rpm for 10 min. 300µl of the above supernatant was removed and evaporated to dryness under a nitrogen steam. The dried residue was dissolved in 100µl of water: acetonitrile (99:1 v/v) and transferred into auto sampler vials. The samples were analysed on a validated in-house LC-MS/MS method. Chromatographic and mass spectrometer conditions were the same as detailed above for DBS and DBMS analysis.For plasma analysis, the calibration curve was linear from 5-5000 ng/ml for all anlytes, with a lower limit of quantitation of 5 ng/mL.

**Statistical Methods**

Non-compartmental analysis of pharmacokinetic data to obtain Cmax, Tmax and area under the concentration-time curve (AUC) was undertaken using data from both sites using WinNonLin (Phoenix, version 6.1; Pharsight Corp., Mountain View, CA).

To evaluate the agreement between maternal DBS (mDBS) and plasma concentrations of the drugs, the paired DBS and plasma samples from Ugandan mothers were correlated using linear regression. A correction factor was obtained to standardise mDBS concentrations to an estimated plasma value. This and Bland Altman analysis were undertaken using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego California USA).

Inadequacy of low temperature storage facilities in remote study locations in Nigeria precluded the collection of plasma samples from the Nigerian cohort who were receiving FTC-containing ART regimens. Therefore, the correlation between mDBS and plasma for TFV in this study was compared with that reported by Zheng et al who reported good correlation between plasma and DBS for both TFV and FTC16. If results were similar for TFV, a previously published correction factor for FTC was used to estimate plasma concentrations from the DBS data.

Since the milk-to-plasma (M:P) ratio varies over the dosing interval due to a number of factors, including delayed peak in breast milk concentration resulting from slower distribution into the breast compartment, 8,17 the ratio of the AUC for paired DBMS and estimated plasma was calculated arithmetically to summarise the relationship between drug exposure in the breast milk compared to the plasma over the entire dosing interval. This is presented M:P AUC ratio.

To estimate clinical relevance, the breast milk concentrations were interpreted as the percentage of the recommended infant dose that would be ingested by an exclusively breastfed infant. TDF is not recommended for children under the age of two years, and therefore this calculation was not performed for TFV concentrations. The standard assumption of 150 mL/kg/day milk intake was made.

**Results**

*Patient Populations*

Ugandan and Nigerian populations were similar in terms of maternal age, weight and CD4 count and infant age and weight (Table 1). All women had been commenced on ART prior to or during their recent pregnancy, and were therefore all at steady state by the time of sampling. All Ugandan and 80% of Nigerian women stated they were exclusively breastfeeding at the time of the study.

*Cross-validation between mDBS and Plasma*

Plasma and mDBS concentrations of 3TC and TFV showed a strong positive correlation (R2 = 0.97 and 0.88, respectively). Using a correction factor derived from the average plasma:mDBS ratio (y =0.88x for 3TC and y = 1.57x for TFV), Bland-Altman analysis indicated good agreement between the two methods (Figure 1), noting slightly lower agreement between the plasma and mDBS analyses at higher analyte concentrations for 3TC and TFV. However, it should be noted that the majority of data points were at concentrations that demonstrated good agreement.

This approach for standardising by a correction factor is similar to that employed by Zheng and colleagues in the development of a DBS assay for TFV and FTC. 16 Our study yielded a similar correction factor for TFV, but we were unable to compare plasma and DBS concentrations for FTC. Therefore we used the correction factor of y=0.8x as derived by Zheng to standardise FTC DBS data for plasma. Concentration-time profiles for each drug were obtained using the estimated plasma and breast milk concentrations.

***Pharmacokinetic Data***

The key pharmacokinetic parameters (Cmax, Tmax and AUC, and the M:P ratio) for all three analytes in plasma (estimated) and breast milk are presented in Table 2 and summarised in Figures 2-4. Using AUC as a summary measure of drug exposure, the findings for each drug are summarised below.

1. *3TC*

Of thirty-nine women who received 3TC-containing regimens, 28 took 300 mg once daily and 11 took 150 mg twice daily. Of these, 10 were administered their medication in the morning as part of a NVP-based regimen (and therefore had intensive PK from 0-8 h post-dose) and 18 women on EFV-based regimens took their medication in the evening, allowing sampling from 12-20h post dose. PK profiles are illustrated in Figure 2. For women taking regimens containing 150mg twice daily, the estimated plasma AUC0-8 was 3644 (2817-3783) ng.h/mL and in BM was 5937 (5263-6130) ng.h/mL, giving a median AUC0-8 M:P ratio of 1.65 (1.59-1.92). Among women taking 300mg once daily as part of a FDC, an AUC0-12 was 4541 (3160-5997) ng.h/mL and in BM was 4420 (2106-5502) ng.h/mL, giving an AUC0-12  M:P ratio of 0.95 (0.82-1.15). Women who took 300mg once daily and were sampled from 12-20 hours after dose had an estimated plasma AUC12-20 of 2510 (1803-3071) ng,h/mL, a BM AUC12-20 of 7600 (4913-10810) ng.h/mL and an AUC12-20 M:P ratio of 3.04 (2.87-4.16).

3TC was detectable in the DBS of 36% (14/39) of infants, at a median of 17.7 (16.3-22.7) ng/mL. Of these, detectable infant levels were found in 3/11 (27%) infants whose mothers were exposed to twice daily dosing, 7/10 (70%) infants whose mothers had 300mg dosing that morning and 4/18 (22%) of those whose mothers had dosed the previous evening.

1. *FTC*

Data were available from 16 mothers taking 200 mg FTC once daily, as part of FDC, with PK profiles in estimated plasma and DBMS illustrated in Figure 3. The estimated plasma AUC0-12 was 2371 (1276-3344) ng.h/mL, in BM was 4991 (4094-7179) ng.h/mL with a median AUC0-12 M:P ratio of 3.01 (2.06-3.38).

Three infants (19%) had measurable FTC concentrations, at 17.5, 18.8 and 19.4 ng/mL.

1. *TFV*

Forty-eight mothers received tenofovir-containing regimens, all with once daily dosing of 300 mg. Of these, 27 received their medication in the morning enabling PK sampling from 0 to 12 h, and 18 took their dose in the evening allowing measurement of PK from 12-20 h post-dose. Three Ugandan mothers had sampling from 0 to 8h post dose; given the small size of this group, these were excluded from the following analysis. PK parameters are illustrated in Figure 4.

The estimated plasma AUC0-12 was 1591 (1193-2234) ng.h/mL, in BM was 21.1 (0-36.2) ng.h/mL, with a median AUC0-12  M:P ratio of 0.015 (0-0.03). The estimated plasma AUC12-20 was 1413 (1144-1830) ng.h/mL and in BM was 0 (0-37.2) ng.h/mL, giving a median AUC12-20 M:P ratio of 0 (0-0.02).

No infant had measurable TFV concentration.

**Discussion**

Full pharmacokinetic profiles of 3TC, FTC and TFV in plasma (DBS-derived) and breast milk across the dosing interval are presented in addition to infant DBS concentrations. 3TC and FTC both penetrate breast milk and whilst the relative ‘dose’ ingested by the infant was less than one percent of the recommended treatment dose for infants of similar weight, these drugs are detectable in a proportion of infants.

It is notable that the absorption of 3TC into the breast milk lags behind plasma, with a time to maximum concentration of 6 hours compared to 2 hours, respectively. It is then eliminated more slowly from breast milk than blood. Taken together, this means that the milk-to-plasma ratio increased from a median of 0.95 between 0 and 12 hours to a median of 3.04 from 12 to 20 hours after dose. This has previously been noted in a study investigating the PK profile of 3TC in blood and breast milk, which also reported similar AUC for both maternal plasma and BM. 8 Similarly, a recent study investigating blood and breast milk FTC concentrations among women taking FTC-TDF as HIV prevention found a M:P ratio of 0.63 and of 2.1 at 1-2 and 23-24 h post dose, respectively, 18 suggesting the differential pharmacokinetics of FTC may follow a similar pattern; that 96% of infants reportedly had detectable FTC at a median of 13.2 ng/mL indicates considerable exposure. In that study, whilst authors comment on ‘less variability’ in FTC concentrations in breast milk compared to plasma throughout the dosing interval, the selected time points suggest that study design may have missed the peak breast milk concentration. Our findings from undertaking intensive PK sampling in paired mDBS and DBMS suggest caution when interpreting M:P values from single, or few timepoints as this may result in misleading conclusions. For example, consideration of early timepoints alone might result in underestimation of the total exposure of the breastfed infant to the drug in question.

It is important to understand the clinical consequences of infant exposure to low concentrations of 3TC and FTC. Evidence regarding low-level drug exposure and emergence of HIV resistance drawn from the Kisumu Breastfeeding Study (KiBS) 5 and Breastfeeding, Antiretrovirals and Nutrition Study (BAN) 4 indicated NRTI resistance in cases where PMTCT failed to prevent infant infection. However, these studies involved ART regimens which are no longer recommended as first-line in pregnancy and breastfeeding and took place in an era before universal lifelong ART to this population was recommended. It is notable that whilst integrase strand transfer inhibitors (INSTI) are increasingly recommended in place of NNRTI in first-line regimens, and are being adopted in LMIC, the NRTI backbone will remain a key component. Larger longitudinal cohort studies including infant pharmacokinetics and clinical follow-up will be required to fully explore this question.

The finding of low TFV in breast milk is not unexpected since TFV is a dianion at physiological pH and suffers from poor membrane permeability; this is consistent with previous work. 9-11, 18 Furthermore, TFV has poor oral bioavailability, hence it is administered as the pro-drug TDF, or increasingly as the phosphonoamidate pro-drug tenofovir alafenamide (TAF). Therefore the finding that infants exposed to low concentrations of TFV in breast milk have undetectable TFV concentrations would be anticipated. However, this contrasts with a study undertaken in Malawi, assessing mothers and infants at 1 and 12 months postpartum where low concentrations of TFV (median [IQR] 24 [0-51.6] and 0 [0-29.9] ng/mL) were measurable at 1 and 12 months, respectively, although breast milk concentrations were low with a M:P ratio of less than 0.1.11

Whilst the summary data for the present study population are presented, inter- and intra-individual variability was considerable. Current work seeks to delineate the covariates influencing this and the clinical consequences thereof. Changes to WHO recommendations which will impact upon pregnant and breastfeeding women will need to be evaluated in suitable populations. Examples include the ongoing DolPHIN-1 study (NCT02245022) evaluating dolutegravir exposure in pregnant HIV mothers and their neonates and NCT02499874, evaluating EFV 400mg in pregnant women. Breast milk elimination of tenofovir alafenamide has not yet been reported.

*Limitations*

Due to the logistic challenges of undertaking intensive PK sampling in postpartum mothers and their infants, we were unable to perform a full 24 hour profile. However, by enrolling participants with similar characteristics for early (0-12 hour) and late (12-20 hour) sampling, we were able to consider the full dosing interval.

90% of mother-infant pairs were enrolled when the infant was less than six months of age, and it was stated to the investigators that they were exclusively breastfeeding their infants, in accordance with WHO and national policies. However, it was not possible to verify this with certainty, and it is possible that some of the study participants may have introduced mixed feeding. Whilst this may have resulted in lower infant concentrations of drug due to lower volume of ingested milk, it is unlikely to have impacted the maternal plasma and breast milk pharmacokinetic profiles.

It was infeasible to harvest plasma from the Nigerian participants to enable direct comparison of mDBS and plasma concentrations for FTC, hence we used a previously published correction factor to standardise between the two matrices. However, such analysis was undertaken for 3TC and TFV among the Ugandan cohort, yielding TFV results comparable with published data.

**Conclusion**

3TC and FTC accumulate in breast milk with M:P ratios ranging from 0.82 to 4.16 and 2.06 to 3.38, respectivelyover the dosing interval, resulting in detectable concentrations in a high proportion of infants. TFV is detectable at low concentrations in breast milk, but was not measurable in any of forty-seven infants sampled.

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**Transparency Declaration**

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| --- | --- | --- |
|  | Uganda (N=30) | Nigeria (N=29) |
| *Regimen* | | |
| NVP twice daily + AZT-3TC twice daily | 9 | 1 |
| NVP twice daily + TDF-3TC once daily | 3 | 6 |
| NVP twice daily + TDF- FTC once daily | 0 | 7 |
| EFV-TDF-3TC once daily | 18 | 1 |
| EFV-TDF-FTC once daily | 0 | 13 |
| EFV once daily + AZT-3TC twice daily | 0 | 1 |
| *Key Patient Characteristics* | | |
| Maternal age (yr; mean, range) | 30 (21-37) | 30 (20-38) |
| Maternal weight (Kg; mean, range) | 61 (43-87) | 59 (46-79) |
| CD4 count (cells/mm3; median, IQR) | 527 (345-608) | 689 (552-938) |
| Infant age (days; mean, range) | 101 (81-146) | 143 (80-215) |
| Infant weight (Kg; mean, range) | 5.9 (4-6.8) | 6.2 (3-10) |

Table 1: Patient characteristics

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Estimated Plasma | | | DBMS | | | M:P Ratios |
| **Drug** | **Dose** | **tmax (h)** | **Cmax (ng/mL)** | **AUC0-12 (ng.h/mL)** | **tmax (h)** | **Cmax (ng/mL)** | **AUC0-12 (ng.h/mL)** | **M:P ratio**  **(AUC0-12)** |
| FTC | 200mg | 2  (0.5-4) | 384  (269 - 530) | 2371  (1276 - 3344) | 4  (2-8) | 843  (702-1132) | 4991  (4094-7179) | 3.01  (20.6-3.38) |
| 3TC\* | 150mg bd | 4  (2-4) | 674  (637-715) | 3644  (2817-3783) | 6  (4-6) | 908  (772-1015) | 5937  (5263-6130) | 1.65  (1.59-1.92) |
| 3TC | 300mg od | 2  (1.5-3) | 915  (635-1118) | 4541  (3160-5997) | 6  (4-8) | 663  (445-890) | 4420  (2106-5502) | 0.95  (0.82-1.15) |
| TDF | 300mg od | 1  (0.5-2) | 293  (176-391) | 1591  (1192-2234) | 4  (1-6) | 5.98  (0-8.05) | 21.1  (0-36.2) | 0.015  (0-0.03) |

Table 2: Summary of pharmacokinetic parameters

All results are presented as median (IQR)

Figure 1: Correlation plots for concentration of 3TC (A) and TFV (B) from maternal plasma and mDBS

Figure 2: Pharmacokinetic profiles of 3TC in breast milk and estimated plasma

Figure 3: Pharmacokinetic profiles of FTC in breast milk and estimated plasma

Figure 4: Pharmacokinetic profiles of TFV in breast milk and estimated plasma

Figure 1



Figure 2

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Figure 3



Figure 4

