Development, validation and clinical application of a novel method for the quantification of efavirenz in dried breast milk spots using LC-MS/MS

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Objectives: This manuscript describes the development, validation and clinical application of a novel method for the quantification of the antiretroviral drug efavirenz in dried breast milk spots using LC-MS.

Methods: Dried breast milk spots were prepared by spotting 30 μL of human breast milk on each circle of Whatman 903 Protein Saver cards. Chromatographic separation was achieved on a reverse-phase C18 column with 1 mM ammonium acetate in water/acetonitrile using a solvent gradient at a flow rate of 400 μL/min and detection was by TSQ Quantum Access triple quadrupole mass spectrometer equipped with a heated electrospray ionization source. The method was applied to characterize the breast milk pharmacokinetic profile of efavirenz in HIV-positive nursing mothers receiving regimens containing 600 mg of efavirenz once daily.

Results: The assay was validated over the concentration range 50–7500 ng/mL. Accuracy ranged between 95.2% and 102.5% and precision ranged between 1.05% and 9.53%. The average recovery of efavirenz from dried breast milk spots was 106.4% and the matrix effect was 8.14%. Stability of efavirenz in dried breast milk spots and processed samples at room temperature, −40°C and −80°C was demonstrated. In the pharmacokinetic study, the mean (SD) AUC0–24, Cmax and Cmin of efavirenz in breast milk were 59620 ng.h/mL (17440), 4527 ng/mL (1767) and 1261 ng/mL (755.9), respectively. The mean (range) milk-to-plasma concentration ratio over the dosing interval was 0.78 (0.57–1.26).

Conclusions: The dried breast milk spot method is simple, robust, accurate and precise, and can be used in settings with limited resources.

Keywords: liquid chromatography, mass spectrometry, antiretroviral

Introduction

The WHO recommends exclusive, ‘on demand’ breastfeeding starting within 1 h of birth, up to 6 months of age, and continued with gradual introduction of appropriate complementary foods up to 2 years of age or beyond. In addition to its nutritional benefits, ready availability and affordability, the health benefits of breastfeeding for both infant and mother have long been recognized.1–4 However, breastfeeding in the presence of maternal drug use is widespread despite the lack of safety data for either prescriptions or permissive statements. In fact, over 90% of nursing mothers take at least one drug during the first week after delivery, 17% take at least one drug until 4 months after delivery and 5% receive drugs for chronic conditions, giving rise to concerns over the presence of drugs in breast milk and their potential effects on the nursing infant.5

Making an informed decision requires an accurate evaluation of the potential risks versus benefits based on knowledge of the extent of the drug’s excretion in human breast milk.5 For instance, HIV-positive mothers breastfeed their babies while taking antiretroviral drugs (ARVs) started during pregnancy for their own health and for prevention of mother-to-child transmission (PMTCT) of HIV. Understanding the dynamics of HIV viral load in breast milk and the safety of maternal drugs for the breastfed infant requires understanding of the pharmacokinetics of ARVs in breast milk.7 Therapeutic drug concentrations in breast milk can prevent ongoing localized replication of viral reservoir and the development of drug-resistant virus,8 which may otherwise be passed to infants,
who become infected if PMTCT fails.9 On the other hand, high concentrations may lead to toxicity in exposed infants.

Very few bioanalytical methods have been described in the literature for the quantification of ARV drugs in breast milk. The complexity of breast milk makes available methods either difficult to validate because of inadequate sample clean-up or complicated due to multiple sample clean-up steps involving a combination of liquid–liquid and solid-phase extraction in a single method.10,11 In addition, lack of standardization makes cross-study comparisons difficult, particularly given differential drug accumulation within specific fractions of milk. For instance, studies reporting the excretion of abacavir, efavirenz, etravirine, lamivudine, lopinavir, nevirapine, zidovudine, tenofovir and emtricitabine in human breast milk have used methods validated only in plasma.12–17 A fully validated method was described for the quantification of lamivudine, lopinavir, nelfinavir, nevirapine, ritonavir, stavudine and zidovudine (7 out of 25 ARVs in use) in breast milk.18 However, the multiple extraction steps make the method time-consuming and expensive, which is a barrier to implementation in resource-limited settings. No fully validated method has been described for the quantification of other ARV drugs in breast milk, including efavirenz, an HIV NNRTI, which is recommended by the WHO as a regular component of first-line regimens across different populations.19

The use of dried matrix spots in the bioanalysis of drugs is increasingly becoming acceptable. This approach is characterized by several advantages compared with the traditional methods: low sample volume, ease of collection, biosafety, room-temperature storage, low-cost shipping, enhanced stability of some analytes20 and the potential for application in intensive pharmacokinetic studies in special populations. This technique has been described for whole blood21 and transparent matrices such as plasma,22 CSF,23 urine24 and synovial fluid.25 However, drug quantification in dried breast milk spots has not been reported. Here we describe a novel and simple method for the quantification of efavirenz in total (unfractionated) breast milk extracted from dried filter paper using LC-MS. Using this method, we describe for the first time the pharmacokinetics of efavirenz in human breast milk over a full dosing interval.

**Materials and methods**

Reference standard of efavirenz was obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada) and hexobarbital, used as an internal standard, was obtained from Sigma-Aldrich (Gillingham, Dorset, UK). LC-MS-grade acetonitrile was obtained from Fisher Scientific (Loughborough, Leicestershire, UK) and methanol from VWR International (Lutterworth, Leicestershire, UK). Water was produced from a Purelab Classic UVF (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 breast milk samples were obtained (with ethics approval) from Wirral Mothers’ Milk Bank, Clatterbridge Hospital, Wirral, UK and whole blood was obtained from drug-free healthy volunteers.

**LC-MS/MS systems**

The LC system consisted of a variable loop Accela autosampler and an Accela LC-Pump (Thermo Electron Corporation, Hemel Hempstead, Hertfordshire, UK). A reverse-phase Fortis™ C18 column: 3 μm, 100 mm × 2.1 mm (Fortis Technologies Ltd, Neston, Cheshire, UK) was used to resolve analytes, using a 2 μm C18 Quest column-saver (Thermo Electron Corporation, Hemel Hempstead, Hertfordshire, UK) as a guard column. The HPLC was connected to a TSQ Quantum Access triple quadrupole mass spectrometer (Thermo Electron Corporation, Hemel Hempstead, Hertfordshire, UK) equipped with a heated electrospray ionization source. TSQ tune software (Thermo Electron Corporation, Hemel Hempstead, Hertfordshire, UK) enabled the optimization of tuning parameters while LC Quan software (Thermo Electron Corporation, Hemel Hempstead, Hertfordshire, UK) was used for data acquisition and processing.

**LC-MS/MS conditions**

Chromatographic separation was achieved using a solvent gradient programme at a flow rate of 400 μL/min. The two mobile phases consisted of 1 mM ammonium acetate in water (mobile phase A) and 1 mM ammonium acetate in acetonitrile (mobile phase B). The gradient started with 70% mobile phase A, decreasing to 10% over 3 min. This was maintained for 1 min, followed by column equilibration to the initial conditions over 2 min. The total run time was 5 min. Injection volume was 10 μL and the needle was washed twice with 3 mL of methanol/water (1:1, v/v) between injections. The mass spectrometer was operated in negative ionization mode to produce characteristic fragmentation patterns and precursor ions ([M–H]−) for efavirenz and internal standard, which were then monitored by selective reaction monitoring.

The electrospray voltage was set at 3.0 kV, the capillary temperature at 270 °C and vaporizer temperature at 350 °C. The sheath and auxiliary gas pressures were set to 60 and 15 arbitrary units, respectively. Argon was used as the collision gas at a pressure of 1.5 mTorr. Product ion characterization was done by directly infusing 1 μg/mL solutions of efavirenz and hexobarbital separately into the MS using a syringe at a flow rate of 5 μL/min. The transitions were m/z 313.979 → 214.991 and 244.025 for efavirenz and 235.071 → 42.449 for hexobarbital with optimal collision energies of 20 and 19 and tube lenses of 76 and 84, respectively. The scan width was set at 0.01 m/z and the scan time at 0.05 s. The peak width settings for Q1 and Q3 were set at 0.7 μm.

**Stock solutions, calibration standards and quality controls (QCs)**

Stock solutions of efavirenz and the internal standard were prepared from the pure compounds in 100% methanol to obtain a final concentration of 1 mg/mL and refrigerated at 4 °C until use. A 10 μg/mL working stock of efavirenz in breast milk was prepared by adding a predetermined volume of efavirenz stock solution to drug-free breast milk, tumbled for 60 min and used the same day. Nine calibration standards in the range of 50–7500 ng/mL, together with low QC (LOQ; 100 ng/mL), medium QC (MQC; 1500 ng/mL) and high QC (HQC; 6000 ng/mL), were prepared from separate working stocks by serial dilution with drug-free breast milk. Working solution of efavirenz at concentrations equivalent to the QCs and 100 μg/mL of the internal standard were prepared in methanol/water (50:50, v/v).

**Dried milk spot calibration standard and QC preparation**

Calibration standards and QCs were prepared by carefully spotting 30 μL of spiked and well-mixed breast milk on each circle of Whatman 903 Protein Saver cards. Spotted cards were left to dry at room temperature overnight and stored with desiccant sachets in ziplock bags. QC samples for stability testing were stored at room temperature, −40 °C and −80 °C.
Sample pretreatment

In each case, the entire spot was removed using a 13 mm hole punch, folded into a 7 mL screw cap tube and extracted with 1 mL of methanol by tumbling for 30 min in the presence of 20 µL of the internal standard. The extract was centrifuged at 4000 rpm for 5 min; supernatant was transferred into a 5 mL tube and evaporated to dryness in a centrifugal rotary vacuum evaporator (Thermo Electron Industries, Chateau Gontier, France) operated at 40°C. The residue was reconstituted in 500 µL of mobile phase A and B (50:50, v/v) and aliquots were transferred into autosampler vials. Each calibration standard level was prepared in duplicate for each of the six breast milk samples. The overall recovery was evaluated by storing extracted QC samples at room temperature and in the autosampler (4°C) for 24 h and over the weekend (72 h). Short-term stability of processed patient samples (n = 10) from an accepted validation assay run was also evaluated. For long-term stability at room temperature, −40°C and −80°C, QC samples were stored at these temperatures for 6 months. The concentrations of the stored samples were determined using calibration standards and QCs prepared with a freshly made efavirenz stock solution. To assess re-injection reproducibility in the event of instrument interruption, an accepted validation assay run was re-injected after 24 h in the autosampler.

Application in a pharmacokinetic study

The method was applied in a preliminary study to evaluate the breast milk pharmacokinetic profile of efavirenz in HIV-positive nursing mothers (n = 5) receiving regimens containing 600 mg efavirenz once daily. Patients were recruited from Bishop Murray Medical Centre, Makurdi, Nigeria and written informed consent was obtained prior to enrolment. Mothers taking antimalarial, antituberculosis or other drugs known to interact with efavirenz were excluded. Breast milk was manually expressed by the mothers mid-feed into 5 mL tubes, 0.5, 1, 2, 4, 8, 12 and 24 h after an observed evening dose of 600 mg efavirenz. To reflect real-life situations, patients took a standard local meal about 30 min before drug administration. A 30 µL aliquot was immediately spotted on each circle of Whatman 903 cards, dried and stored as previously described. Within 2 min of breast milk collection, whole blood samples were collected as dried blood spots (DBS) after sterile skin cleaning and finger prick using a 2 mm safety lancet (BD, Oxford, Oxfordshire, UK). The first drop of blood was discarded and subsequent blood drops were collected on Whatman 903 cards, dried and stored as described above. Samples were shipped at room temperature to the Department of Molecular and Clinical Pharmacology, University of Liverpool, Liverpool, UK for analysis. Efavirenz in dried breast milk spots was quantified using the method described above and efavirenz in DBS was quantified using a method described elsewhere. Plasma efavirenz concentrations were estimated using 

\[
\text{[DBS/EFV]} = \frac{(1 - HCT) \times f_{\text{hap}}}{f_{\text{fpr}}} ,
\]

where [DBS/EFV] is efavirenz concentration in DBS, HCT is the patient specific haematocrit and \( f_{\text{fpr}} \) is the fraction of efavirenz bound to plasma protein.

Results

LC-MS/MS conditions

The total run time was 5 min and retention times of efavirenz and the internal standard were 2.27 and 1.67 min, respectively. Representative chromatograms are presented in Figure S1 (available as Supplementary data at JAC Online).

Linearity, accuracy and precision

The method was linear, accurate and precise in the range of 50–7500 ng/mL. Mean regression coefficient (r²) was 0.9994 and the average relative standard deviation of the internal standard response was 3.7%. Accuracy (percentage bias) was between 95.2% and 102.5% and precision (CV) was between 1.05% and 9.53% (Table 1). These values are within bioanalytical method validation acceptance criteria as per FDA and EMA guidelines.

Recovery, matrix effect and dilution integrity

The method was applied in a preliminary study to evaluate the breast milk pharmacokinetic profile of efavirenz in HIV-positive nursing mothers (n = 5) receiving regimens containing 600 mg efavirenz once daily. Patients were recruited from Bishop Murray Medical Centre, Makurdi, Nigeria and written informed consent was obtained prior to enrolment. Mothers taking antimalarial, antituberculosis or other drugs known to interact with efavirenz were excluded. Breast milk was manually expressed by the mothers mid-feed into 5 mL tubes, 0.5, 1, 2, 4, 8, 12 and 24 h after an observed evening dose of 600 mg efavirenz. To reflect real-life situations, patients took a standard local meal about 30 min before drug administration. A 30 µL aliquot was immediately spotted on each circle of Whatman 903 cards, dried and stored as previously described. Within 2 min of breast milk collection, whole blood samples were collected as dried blood spots (DBS) after sterile skin cleaning and finger prick using a 2 mm safety lancet (BD, Oxford, Oxfordshire, UK). The first drop of blood was discarded and subsequent blood drops were collected on Whatman 903 cards, dried and stored as described above. Samples were shipped at room temperature to the Department of Molecular and Clinical Pharmacology, University of Liverpool, Liverpool, UK for analysis. Efavirenz in dried breast milk spots was quantified using the method described above and efavirenz in DBS was quantified using a method described elsewhere. Plasma efavirenz concentrations were estimated using 

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The study protocol and the material transfer agreement were approved by the National Health Research and Ethics Committee, Abuja and Obafemi Awolowo University Teaching Hospitals Ethics and Research Committee, Ile-Ife, Nigeria.

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Recovery, matrix effect and dilution integrity

The average (CV) recoveries of efavirenz from dried breast milk spots at LQC, MQC and HQC were 101.6% (7.40), 109.4% (2.58)
and 108.2% (2.97), respectively. Matrix effect was within ±15% for each of the six different matrices, with an average (CV) of 9.05% (4.94), 8.15% (5.35) and 7.27% (4.17) at LQC, MQC and HQC, respectively. The average efavirenz concentrations in the 10000 ng/mL dried breast milk spots diluted 2× and 4× were 10147 and 10893 ng/mL, representing 101.5% and 108.9% dilution integrity, respectively.

**Stability and re-injection reproducibility**

Processed QC and incurred patient samples were stable at room temperature and in the autosampler (4°C) for 24 h and over the weekend (72 h) with percentage deviations from nominal concentrations (for QC samples) or from the original assay results (for patient samples) within ±15%. The stability of efavirenz in dried breast milk spots after storage for 6 months ranged from 92% to 101% at room temperature, from 105% to 111% at 240°C and from 110% to 113% at 280°C. Reinjection reproducibility was demonstrated in a repeat analysis of an accepted validation batch with all assay validation parameters within acceptable limits.

**Application in a pharmacokinetic study**

The method was successfully applied to investigate the pharmacokinetics of efavirenz in human breast milk. Median (range) age of mothers included was 29 years (25–34) and weight was 55 kg (45–71). All five mothers started an efavirenz-containing regimen before delivery and samples were collected at steady-state, 146 days (108–194) after delivery. As shown in Figure 1(f), the breast milk profile approximately paralleled the plasma profile. As with the plasma, breast milk concentrations also exhibited considerable interindividual variability (Figure 1a–e). The mean (SD) AUC, C_max and C_min were 59620 ng.h/mL (17440), 4527 ng/mL (1767) and 1261 ng/mL (755.9), respectively, in breast milk. Corresponding values in plasma were 74230 ng.h/mL (25610), 4711 ng/mL (1083) and 2571 ng/mL (1092), respectively (Figure 1). Interestingly, breast milk efavirenz concentration exceeded plasma concentration at some point during the dosing interval in all five patients. Also, breast milk concentration was predominantly higher than plasma concentration in patient 1. The average (range) milk-to-plasma (M/P) ratio over the dosing interval was 0.78 (0.57–1.26). The M/P ratio was 0.83 at 4 h post-dose.

**Discussion**

To our knowledge, this is the first description of a drug quantification method in dried breast milk spots. Additionally, the full pharmacokinetic profile of efavirenz in human breast milk has been described for the first time. The simplicity, accuracy and precision of the developed method will facilitate interlaboratory transfer. The stability of efavirenz in dried breast milk spots at room temperature will facilitate studies in settings with inadequate cold storage facilities and shipping without the need for dry ice, thus extending its utility in resource-limited settings. Other advantages include patient acceptability, and reduction in perceived or actual risks to the patient resulting from multiple sampling of large volumes. The low sample volume required per assay may also extend application of the method to pharmacokinetic studies in animals in which adequate breast milk volumes may be difficult to obtain because of low rate of milk production.

As a result of the complexity of human breast milk, previously described methods involve complicated extraction procedures with a combination of liquid–liquid extraction and solid-phase extraction for sample clean-up. Some authors have used skimmed milk for validation, which has the potential for underestimating breast milk concentrations and hence the M/P ratio of drugs that are highly bound to breast milk proteins and lipids. The use of a dried matrix spot allows quantification of drug concentrations in whole milk because the cellulose material entraps milk proteins and lipids on drying, allowing selective extraction of analytes.

The ARV efavirenz, which is an essential component of first-line regimens and frequently used by HIV-positive nursing mothers in

| Table 1. Accuracy and precision for the quantification of efavirenz in dried breast milk spots |
|---------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Concentration (ng/mL) | Inter-day mean | Inter-day SD | Inter-day precision (%CV) | Inter-day accuracy (%) | Intra-day mean | Intra-day SD | Intra-day precision (%CV) | Intra-day accuracy (%) |
| Calibration standards | | | | | | | | |
| 50 | 50.4 | 2.78 | 5.51 | 100.8 | 51.0 | 3.48 | 6.83 | 101.9 |
| 100 | 98.2 | 3.04 | 3.09 | 98.2 | 98.6 | 1.54 | 1.56 | 98.7 |
| 200 | 198.9 | 10.05 | 5.05 | 99.5 | 193.5 | 2.92 | 1.51 | 96.8 |
| 500 | 500.8 | 11.59 | 2.31 | 100.2 | 495.7 | 5.19 | 1.05 | 99.1 |
| 1000 | 1017.7 | 41.40 | 4.07 | 101.8 | 1045.4 | 35.31 | 3.38 | 104.5 |
| 2000 | 2012.4 | 36.51 | 1.81 | 100.6 | 2013.8 | 27.55 | 1.37 | 100.7 |
| 3500 | 3473.9 | 23.90 | 0.69 | 99.3 | 3464.4 | 27.00 | 0.78 | 99.0 |
| 5000 | 4961.2 | 75.80 | 1.53 | 99.2 | 4918.8 | 23.01 | 0.47 | 98.4 |
| 7500 | 7539.5 | 87.62 | 1.16 | 100.5 | 7574.4 | 83.04 | 1.10 | 100.0 |
| QC | | | | | | | | |
| LQC (100) | 99.5 | 9.48 | 9.53 | 99.5 | 101.7 | 6.29 | 6.19 | 101.7 |
| MQC (1500) | 1427.7 | 72.73 | 5.09 | 95.2 | 1492.2 | 40.98 | 2.75 | 99.5 |
| HQC (6000) | 6125.1 | 313.10 | 5.11 | 102.1 | 6087.1 | 330.04 | 5.37 | 102.5 |

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developing countries, is not licensed for use in children < 3 months old or < 3.5 kg because optimal dosing and safety have not been fully evaluated in this age group. In the first report of its excretion in breast milk by Schneider et al., single-point breast milk and plasma samples were collected 3–4 h post-dose and the M/P ratio was 0.54, compared with the M/P ratio of 0.83 at 4 h post-dose in the present study. This difference may be due to the use of skimmed milk instead of whole milk in the former study, resulting in underestimation of the breast milk concentrations of efavirenz, which in plasma is 99.5% protein bound. In addition, changes in milk production, composition and infant feeding patterns may cause variations in the M/P ratio during the dosing interval, making single-point estimates inaccurate and often misleading. Breast milk is more lipophilic than plasma; efavirenz may preferentially partition into this compartment and accumulate after maternal dose before the next infant feed. In fact, we

Figure 1. Concentration–time profiles of efavirenz in breast milk and plasma of five HIV-positive nursing mothers (a–e) taking regimens containing 600 mg of efavirenz once daily. The mean (SD) of all five patients is shown in (f). The mean (range) M/P ratio over the dosing interval was 0.78 (0.57–1.26).
observed intra- and interindividual variations in the M/P ratio during the dosing interval in the present study. In addition to the factors highlighted above, genetic factors may also play some role. 12 Using our method, M/P ratio ranged from 0.57 to 1.26 with an average of 0.78 over the 24 h dosing interval.

Uncertainties surrounding the use of many drugs during lactation may result in suboptimal adherence to essential pharmacotherapy or premature recourse to formula feeding by nursing mothers. In the context of HIV/AIDS, this will not be affordable, feasible, acceptable, sustainable or safe for the majority of the world’s HIV-positive women living in resource-limited countries. The availability of validated bioanalytical methods for the quantification of drugs in human breast milk is an important step towards resolving some of these uncertainties and clarifying the pharmacokinetics of drugs in this compartment. The dried milk spot method presented here is simple, accurate and precise and further studies to investigate applications for other drugs that may be used during lactation are warranted. A useful approach will be to modify methods already validated for the drug of interest in other matrices. Additional validation steps to establish a suitable extraction procedure, optimal recovery and drug stability in dried breast milk spot will be needed as per FDA guidelines. 25

In the present study, a potential limitation is that a cross-validation comparing the dried breast milk spot method with the traditional liquid breast milk method was not conducted. Also, unlike DBS, which can be collected directly from patients after a finger prick, the dried breast milk method relies on accurate pipetting from expressed breast milk and spotting on sample collection cards. It is also essential that spotting be limited to the marked sample collection areas on the cards since breast milk is colourless.

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Transparency declarations
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Supplementary data
Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References
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