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Validation and Clinical Application of a Novel LC-MS Method for Quantification of Dolutegravir in Breast Milk

J Gini[1,2], **SD Penchala**[1,2], **A Amara**[1,2], **E Challenger**[1,2], **D Egan**[1,2], **C Waitt**[1,3], **M Lamorde**[3], **C Orrell**[4], **L Myer**[5], **S Khoo**[1,2], **LJ Else**[1,2]

[1]Department of Molecular & Clinical Pharmacology, University of Liverpool, 70 Pembroke Place, Liverpool, L69 3GF, UK [2]Liverpool Bioanalytical Facility, Royal Liverpool Hospital, Prescot Street, Liverpool, L7 8XP, UK [3]Infectious Diseases Institute, Makerere University College of Health Sciences, Kampala, Uganda [4]Desmond Tutu HIV Foundation, Gugulethu Community Health Centre, Cape Town, South Africa [5]Centre for Infectious Diseases Epidemiology & Research, University of Cape Town, South Africa

Abstract

Background—A novel, sensitive and reproducible method for quantification of dolutegravir (DTG) in dried breast milk spots (DBMS) was developed and validated for use in clinical studies. Its application enabled measurement of DTG pharmacokinetics in breastfeeding mothers and their infants.

Results/Methodology—Sample extraction was by liquid-liquid extraction using TBME, with Dolutegravir–d5 as an internal standard. Dolutegravir was eluted on a reverse phase C_{18} Waters XBridge (3.5μm: 2.1 x 50 mm) column using a gradient mobile phase consisting of 0.1% formic acid in deionised water or methanol. The assay was validated over a calibration range of 10-4000 ng/mL.

Conclusion—Stability, inter and intra-assay variability were acceptable according to FDA and EMA bioanalytical method guidelines. The assay is robust, accurate, precise and can be reliably applied for analysis of clinical samples in trials from low resource settings.

Keywords

Dolutegravir; Antiretroviral; Breast milk; Validation; pharmacokinetics; HIV; mass spectrometry; clinical trial

Background

It is recommended globally that HIV-positive women receive antiretroviral therapy (ART) throughout pregnancy and breastfeeding, and for life, irrespective of clinical disease stage or CD4 count [1]. In untreated HIV-positive mothers, breastfeeding is associated with increased risk of mother-to-child transmission of HIV [2]; however exclusive breastfeeding under antiretroviral cover is recommended for HIV seropositive women in low and middle income countries [3–5] as formula feeding is associated with increased infant mortality. Significant drug transfer and excretion through breast milk (BM) can occur via the mammary epithelial

cells, especially during the colostral phase, around 3-4 days after delivery [6,7], which implies that medicated breastfeeding mothers may expose their babies to clinically significant antiretroviral (ARV) concentrations [8]. Furthermore, infants of mothers receiving ART who acquire HIV via BM have been shown to present with high rates of drug resistance, thus compromising their future treatment options [9–13]. It is therefore essential to determine ARV pharmacokinetics and transfer into BM in order to assess the potential risk of harmful exposure to the infant through breastfeeding, and limit the development of drug resistance.

The pharmacokinetics of ARV in BM and breast-fed infants can be determined by measuring drug concentrations in paired maternal plasma (MP), infant plasma (IP) and BM, which are then expressed as BM:MP and IP:BM ratios. BM transfer has been evaluated for a number of ARV, including the nucleoside (NRTI: abacavir, emtricitabine, lamivudine, stavudine, tenofovir, zidovudine) and non-nucleoside reverse transcriptase inhibitors (NNRTI: efavirenz, etravirine and nevirapine) and protease inhibitors (PI: atazanavir, indinavir, lopinavir, ritonavir) [14–16]. However, pharmacokinetic data on more recently licenced ARV are lacking [17–19].

Analytical methods used for measuring ARV in BM are limited. At the current time, there are a total of 19 published methods that quantify ARV in BM, however, the majority use HPLC-UV [20], with only a few studies employing the more sensitive and specific technique of LC-MS [20,21]. To date, only analytical methods for the following ARV have been developed: Lamivudine, stavudine, efavirenz, indinavir, lopinavir, nevirapine, ritonavir and zidovudine. However, most of these methods are in fact modifications of previously validated assays used for plasma and serum and provide limited detail on how the BM samples were collected and stored, the type of calibration matrix and extraction method used. Furthermore, data to support the stability of these drugs in BM following sample processing and long-term storage are limited, or completely lacking. The majority of BM methods published used structurally related (analogue) internal standards [22]; however, ideally stable isotopically labelled internal standards (SIL-IS) should be used to correct for matrix effects, as they share the same physicochemical properties and co-elute with the analyte of interest. The commercial availability of these compounds is continuing to increase, such that they are now routinely used for LC-MS analysis.

Quantification of drugs from BM presents a number of analytical challenges: BM can vary in fat content throughout the feeding time, and the nutritional intake of the mother may also cause variation in BM fat content [23]. Analytical methods previously described for determination of ARV in BM predominantly use liquid fractions of whole or skimmed milk which require more intensive and time consuming sample clean up procedures, such as Solid Phase Extraction (SPE) [24]. Physiochemical properties influence the partitioning of drug into BM, which contains variable lipid, aqueous and protein fractions. Indeed, significant differences in drug recoveries between whole and skimmed have been reported which may result in misleading results, especially as whole milk is more likely to reflect that ingested by the breastfeeding infant. For example, Shapiro and colleagues noted recoveries of drug between skimmed and whole BM to be 72% vs 95% for lamivudine, 103% vs 94% for

zidovudine and 99% vs 89% for nevirapine, and in light of this variability elected to report whole milk concentrations [25].

Dried Breast Milk Spots (DBMS), whereby a relatively small (<50μL) volume of BM is spotted directly onto an inert filter card, offer several advantages for drug quantification as compared with liquid BM. Use of DBMS enables quantification of drugs in whole BM, which is a direct reflection of the milk ingested by the breastfeeding infant. DBMS are also more suitable for collection and storage in low resource settings as they can be stored and shipped at ambient temperatures without the need for refrigeration or dry ice during transit. We have previously developed and validated dried breast milk spot (DBMS) methodologies for the NRTI (emtricitabine, lamivudine, tenofovir) and NNRTI (efavirenz and nevirapine) [26,27].

The integrase inhibitor dolutegravir (DTG) was licensed by the US food and drug administration (FDA) in 2013 for the treatment of HIV infection [1], and more recently, recommended as a first line treatment of HIV in the 2016 WHO guidelines [2]. Thus global uptake and the number of pregnant women and breastfeeding infants exposed to DTG in limited resource settings is predicted to increase significantly over the coming years.

Well-designed pharmacokinetic studies and robust analytical methods for quantifying DTG in BM are therefore needed to address these safety concerns in vulnerable patient populations. Our aim was to develop and validate an accurate, sensitive and robust LC-MS method for quantification of DTG in BM using dried breast milk spots (DBMS), with application of the method in breastfeeding mother and infant pairs.

Materials and methods

Reagents and Equipment

DTG and deuterated internal standard (IS) Dolutegravir-d5 (DTG-d5) were purchased from Toronto research chemicals (Toronto, ON, Canada). LC-MS grade Methanol (99.9%), formic acid, Propan-2-ol and Tert-butyl methy-ether (TBME) were obtained from Sigma Aldrich, UK. Deionised water (18.2 MΩ purity) was produced from Elga® Option S water purification units, Purelab® and Classic Ultraviolet water filtration (UVF) purifier (Elga LabWater, UK). Whatman 903 Protein Saver cards were obtained from GE Health Care, UK. Blank human breast milk was donated by volunteers through the Wirral Mothers' Milkbank and transferred to the lab for use with approval from Liverpool Women's Hospital.

The HPLC system consisted of an Accela autosampler (set at a tray temperature of 10°C) and an Accela pump (Thermo Scientific, Hemel Hempstead, UK). A reverse phase C_{18} Waters XBridge (3.5μm: 2.1 x 50 mm) column (Waters Corporation, U.S.A.) set at 40°C oven temperature and interfaced with a 2μm C_{18} Quest column saver (Thermo Fisher Scientific, Hemel Hempstead) was used to resolve the analytes. A Thermo Quantum Access mass spectrometer with a heated electrospray ionisation source (Thermo Fisher Scientific, Hemel Hempstead) was used for quantification. TSQ Tune software was used for compound tuning and optimisation, and LCquan (version 2.7 Thermo Fisher Scientific, Hemel Hempstead) was used for data acquisition, data processing and reporting.

Chromatographic and mass spectrometry conditions

Mobile phase A (0.1% formic acid in deionised water) and B (0.1% formic acid in methanol) were programmed at flow rate of 500 µL/min to generate a solvent gradient for chromatographic separation. The gradient program started with 80% mobile phase A which was held for 0.5 minutes and then decreased to 20% mobile phase A for 2.5 minutes. Subsequently the column was washed with 100% mobile phase B for 2 minutes and re-equilibrated back to the starting mobile phase conditions over a total run time of 9 minutes. The injection volume was 5 µL and needle was washed with IPA: water [1:1(v/v)] after each injection.

The mass spectrometer used electrospray ionisation (ESI) set in a positive ionization mode using selective reaction monitoring (SRM). The spray voltage was 29 V, the capillary temperature was 300°C and vaporisation temperature 350°C, respectively. The sheath and auxiliary gas pressures were set at 50 and 10 arbitrary units. Argon was used as a collision gas and delivered at a pressure of 1.5 mTorr. The compounds were tuned using a concentration of 1 µg/mL in methanol via direct infusion into the mass spectrometer at a flow rate of 5µL/min. The m/z transitions were 420.1 → 277.1m/z for DTG and 425.1 → 276.9 for DTG-d5 with collision energies of 29 and 27 V, respectively. The scan width was set at 0.01 m/z and the dwell time at 0.1 seconds.

Preparation of stock solutions, standards, QCs, and internal standards of DBMS and DPS

A primary DTG stock solution (1mg/mL) was prepared in methanol and stored in the refrigerator at 4°C until use. This stock was subsequently serially diluted with methanol and water [1:1 (v/v)] to obtain intermediate solutions of 200, 50, and 2.5 µg/ml. The intermediate solutions were then used to spike into the blank human BM for preparation of the non-zero calibration standards (9 levels: 10, 25, 50, 200, 500, 1000, 2000, 3200 and 4000 ng/ml) and internal QC (LQC = 30ng/mL, MQC = 455 ng/mL and HQC = 3700 ng/mL). Exactly 30µL of each calibrator and QC was spotted onto the middle of the 12mm circle of the protein saver cards. The spots were then dried overnight at ambient temperature before sealing in a ziplock bag with desiccant sachets, and stored at -40°C until use.

A 1mg/mL DTG-d5 stock solution was prepared in methanol and stored at 4°C. On the day of analysis a working internal standard solution (250 µg/mL) was prepared in 50:50 (v/v) methanol:water.

Sample pre-treatment

This method employed a liquid-liquid extraction process to release the drug from the DBMS and remove matrix interferences [28]. Prior to extraction, 20 µL of DTG-d5 (2.5 µg/mL) was spotted directly onto each 12 mm circle of the DBMS using an electronic pipette and allowed to air dry for 20 minutes. After drying the spots, the entire 12 mm circle of the DBMS were punched and folded into clean 7 mL screw capped glass tubes and pre-soaked in 500 µL of 1.8 mg/mL EDTA in water for 10 minutes.

2 mL of TBME was added to each sample and the tubes capped and tumbled for 30 minutes. The samples were centrifuged at 3398g for 10 minutes to separate the precipitated protein

and water from TBME containing drug. Using a cryobath, containing dry ice and methanol, the protein, water and paper were frozen out, and non-frozen supernatant decanted into clean 12x75mm glass tubes and evaporated to dryness using nitrogen drier for 1 hour. Finally, samples were reconstituted using 500 μL of mobile phase [80:20 methanol: water (v/v) in 0.1% formic acid] and vortexed for 5 seconds. 100 μL of reconstituted sample was transferred to 300 μL autosampler vials, sealed with crimp caps (11mm aluminium PTFE/ silicone natural) and loaded onto the autosampler tray for subsequent injection to the LC-MS system.

Method validation

The LC-MS method was validated according the stipulated FDA and EMA guidelines acceptance criteria for bioanalytical assay development and validation [29,30].

Selectivity

Selectivity was evaluated by comparing the amount of interference from six different blank DBMS in relation to the lowest calibrator (LLOQ). Area responses of interfering noise at the retention time of DTG were accepted if the interference was less than 20% of the mean response of the LLOQ (n = 6). For the internal standard, less than 5% of the mean response areas in the 6 LLOQ samples was deemed to be acceptable.

Carryover

Carryover was determined through injection of DBMS calibrators at the upper limit of quantification (ULOQ) followed by 4 blank DBMS on 7 separate occasions. Carryover was then expressed as a percentage of the LLOQ and ULOQ, as per the EMA guidelines, which should not be >20% of the LLOQ.

Precision and accuracy

Precision and accuracy was determined by analysing five different assay batches, consisting of a calibration curve and 6 LLOQ, LQC, MQC, and HQC (separate extractions), run over 5 days. Deviation of ≤ ±15% of measured analyte from nominal concentrations was accepted, except at LLOQ where ≤ ±20% was accepted. Accuracy was defined as the percentage deviation of measured analyte from nominal concentration, and precision as a percentage coefficient of variation (%CV). Greater than or equal to 75% of calibration standards and 67% of QC in each run were required to have a deviation of ≤±15% from their respective nominal concentrations.

Recovery and matrix effect

The percentage recovery and matrix effects were determined quantitatively in accordance to the recommendations of Matuszewski et al [31]. The peak area responses of six replicates of pre-extracted DBMS QC (C), post-extracted DBMS blanks spiked at an equivalent QC concentration (B) and DTG spiked directly in mobile phase (A) without matrix, were compared. The percentage (%) recovery (process efficiency; %PE) was derived by comparing the peak area response of pre-extracted DBMS to the peak area response of mobile phase spiked with DTG at an equivalent concentration (C/A*100). The % matrix

effect (%ME) was determined by comparing the peak area response of DTG spiked into blank DBMS extracts with the peak areas at an equivalent concentration in mobile phase (B/A*100). The IS-normalized recovery (%Analysis RE) was calculated by comparing the peak area ratios of the pre-extracted (C2) and post-extracted (B2) DBMS QC (C2/B2*100).

Given that DTG is known to bind to free metals, the effect of EDTA (a chelating agent) on DTG recovery from BM was evaluated by comparing recovery of drug from DBMS pre-soaked in 1.8mg/mL EDTA in water against DBMS pre-soaked with water without EDTA, prior to liquid liquid extraction. Furthermore, the impact of different IS addition procedures on DTG recovery from DBMS were assessed. The IS was pipette either directly onto the DBMS, or added to the extraction solution.

Dilution Integrity

Six replicates (n=6) of DBMS were prepared at approximately 1.75 times the assay ULOQ (approximately 7000 ng/mL). Blank DBMS were also extracted and used to dilute the extracted dilution integrity DBMS by a factor of two and four in order to derive a concentration that falls within the calibration range. The resulting values were then back-calculated with the appropriate dilution factor.

Stability

Stability of DTG in DBMS (QC samples; 6 per level) for different storage and processing conditions was evaluated, including bench top stability, heat treatment, autosampler stability, re-injection reproducibility, processed and long term stability.

Bench top stability (storage of DBMS at room temperature for 5 days) was evaluated for QC by comparing with freshly prepared samples. Heat stability was assessed by heating DBMS at 50°C for 1 hour before analysis, and autosampler stability was assessed by injecting samples after 24 hours of storage in the autosampler. The stability of processed (extracted) samples was assessed by keeping the processed samples in the fridge (4°C) for 24 hours before analysis. Long term stability was evaluated by storing DBMS at ambient conditions for up to one year. In order to assess stability of the DBMS under the various conditions described above, the QC samples were read off a freshly prepared calibration curve and compared to their respective nominal value. Re-injection reproducibility was assessed by re-injecting samples left in the autosampler for 72 hours and results of the two runs compared for reproducibility.

Clinical Application

The validated method is currently being utilised for determination of DTG in DBMS, obtained as part of the ongoing DOLPHIN 1 clinical trial (NCT02245022) which seeks to assess the safety and pharmacokinetics of DTG in African HIV-infected pregnant mothers and their neonates. HIV-infected pregnant women are randomised at a number of sites, including the Infectious Disease Institute Makerere University, Kampala Uganda and the Desmond Tutu HIV Foundation, Gugulethu Community Health Centre, Cape Town South Africa, to receive either a DTG-based regimen or a standard of care regimen (not containing an integrase inhibitor).

Paired maternal plasma (MP) and BM samples were collected at two time points (one timed relative to a feed at the anticipated maternal T_{max}, and one at a random time point relative to maternal dosing) from breastfeeding mothers (1 Ugandan; 1 South African) at approximately 2 weeks post-partum. Paired plasma samples were also collected from the infant (IP) on two occasions to evaluate neonatal DTG exposure. In addition, paired MP, IP and BM samples were collected following the mother's discontinuation of DTG (and transfer to standard of care) in order to evaluate the persistence of DTG in these compartments after drug cessation.

BM samples were collected in EDTA containers and exactly 30 μL spotted on Whatman 903 protein saver cards. The cards were subsequently air-dried and sealed in individual ziplocked bags with desiccant. Plasma and DBMS were shipped on dry ice to the University of Liverpool Bioanalytical Facility for analysis. DBMS were stored at -40°C analysed according to the above sample pre-treatment method. DTG concentrations in plasma (MP and IP) were analysed using a previously described and fully validated LC-MS methodology [32]. Ratios between DTG concentrations measured in breast milk and maternal and infant plasma samples were calculated arithmetically and expressed as BM:MP and IP:BM ratios.

Results and Discussion

Method Development

Chromatographic and mass spectrometry conditions—DTG and DTG-d5 were eluted from the reverse phase column at approximately 1.8 minutes, respectively. Representative chromatograms obtained from extracted DBMS (blank and LLOQ) are shown in Figure 1. The m/z transitions were 420.1 → 277.1m/z for DTG and 425.1 → 276.9 for DTG-d5 with collision energies of 29 and 27 V, respectively. The scan width was set at 0.01 m/z and the dwell time at 0.1 seconds.

Sample pre-treatment—Due to the small volume of BM sample (30μL) applied to each circle, the entire spot was punched and subsequently extracted in order to ensure a high signal intensity on the LC-MS. Punching the entire circle after spotting with known volume also ensures reliable comparison with an equivalent volume of liquid sample, making it more suitable for use in therapeutic drug monitoring (TDM) and PK studies. However, a limitation of using the entire DBMS, as opposed to a sub-punch, is that the bioanalyst is solely reliant on the expertise of the technician at the site/clinic to accurately pipette the required volume on the filter card. This poses an additional challenge in Resource Limited Settings, where volumetric pipettes are unlikely to be maintained and calibrated to same standards as accredited research laboratories. Furthermore, regional variability, inter-operator differences in pipetting technique and on-site training of staff at investigator sites was shown to impact on the reproducibility of drug (efavirenz) quantification from Dried Blood Spots (DBS), as was observed in a global multicentre trial conducted across diverse health resource settings (ENCORE 1) [33].

Taking a DBMS sub-punch of a pre-defined diameter (e.g. 3-6 mm) is advantageous as study personnel do not need to apply an accurate volume to the card, so the technique is less reliant on operator skill. However, during early method development it was found that sub-

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punches and smaller BM volumes (<30μl) did not provide a high enough response at the assay LLOQ (10 ng/ml or 0.3 ng/DBMS sample). Furthermore, BM is colourless on the card, therefore, punching the entire circle ensures that the entire sample is utilised to measure the drug.

Method Validation

Selectivity—The background interference from extracted blank DBMS (n=6) at the retention time of DTG was on average <20% of the signal intensity of the assay LLOQ. Similarly, the background noise in blank DBMS was <5% of the signal intensity of the internal standard (DTG-d5) for all 6 batches tested.

Carryover—Mean percentage carryover into the extracted blanks (n=7) after injection of a ULOQ sample (4000 ng/mL) was 62.74% and 0.17%, 23.32% and 0.06%, 19.32% and 0.05% and 14.03% and 0.04% of the LLOQ and ULOQ after injection of first, second, third and fourth extracted blanks, respectively. Due to the observed carryover, four extracted blanks were incorporated after every injection of ULOQ or QCs and two blanks inserted between patient samples as precaution to prevent cross contamination between incurred samples. The relatively high percentage carryover is believed to be attributed to both the machine auto sampler and a column memory effect. As part of our previously developed LC-MS method for quantification of dolutegravir in plasma carryover from the HPLC column was mitigated by including an additional wash step (100% mobile phase B; 0.1% formic acid in methanol) in the gradient program [32].

Precision and accuracy—Five independent validation batches were run over 5 days, with each assay run consisting of a zero blank, 9 calibration standards (in duplicate) and 6 QC (LQC, MQC and HQC). The calibration was quadratic with a weighing factor of $1/X^2$, and linearity maintained from 10 ng/mL to 4000 ng/mL. The Mean regression coefficient (r^2 ±SD) was 0.9962 ± 0.0018. Inter- and intra-day accuracy and precision were within ±15% for all QC levels and ±20% for the LLOQ, as shown in Table 1.

Recovery and Matrix effect—Mean (±CV %), percentage recovery (%RE), process efficiency (%PE) and matrix effect (%ME) of DTG from DBMS are summarised in Table 2: The mean % RE, PE and ME (%CV) of DTG from DBMS pre-soaked with EDTA, and IS spotted directly onto the spot was 105.07 (11.23), 101.52 (11.09) and 96.83 (8.18), respectively. The high % recoveries of DTG and limited effect of the sample matrix are likely attributed to the inherent properties of the filter cards and the fact that only a small volume (30μL) of sample matrix is applied. One suspected explanation is that complex biopolymers are formed between the cellulose of the protein saver cards and the proteins, peptides and polysaccharides in BM, which stick together to essentially “hold” the matrix components within the card thereby making extraction of free drug and sample processing much easier [34,35].

However, use of protein saver cards are known to be associated with challenges in bioanalysis such as a heterogeneous drug/matrix distribution on the cards, variable drug quantification and potential chemical interference from pre-treated cards [18–20]. Here, such

issues were circumvented by punching the entire circle of the DBMS, and adjusting reconstitution volume of the processed samples to reduce matrix interference. Other measures employed to optimise drug recovery were use of 1.8mg/mL EDTA in water to pre-soak punched cards, which was shown to increase recovery by approximately 50% (Table 2). Reasons for this observation is not certain, but binding of integrase inhibitors to polyvalent metals (Mg^{2+}, Mn^{2+}, Fe^{3+}, Al^{3+}, Zn^{2+}) rich in breast milk is well documented [21,22]. EDTA chelates polyvalent metals by forming complexes with the metals. Therefore, addition of EDTA to DBMS samples during processing likely increases the availability of free drug and recovery of free drug from the matrix. Further studies are required to understand the clinical implication of how BM rich in free metals affects the absorption and disposition of DTG in the breastfeeding infant. Addition of internal standard to liquid BM before spotting on cards is the optimal method for correcting variability and ensuring process efficiency during sample preparation and analysis [36]. However, such an approach is challenging in resource limited countries, particularly in a field based setting, due to lack of standard calibrated pipettes and potential operator variability. Thus the only feasible option was to either spot the IS directly on card immediately prior to extraction or add the IS in the pre-soak solution before extraction. The addition of IS directly onto the DBMS was found to improve the overall recovery of DTG and reproducibility of the method compared with adding IS into the pre-soak solution (Table 2).

Dilution Integrity—Result of dilution integrity of DTG DBMS method after 1:2 and 1:4 dilution of samples >175% of ULOQ (approximately 7000 ng/mL) with extracted blank DBMS was within 15% of anticipated concentration with % CV <3%.

Stability—Stability of DTG extracted from DBMS was determined under multiple conditions, which are summarised in Table 3. DTG was stable in DBMS after 5 days on the bench at room temperature, when refrigerated for 24 hours at 4°C, during processing, and when stored in the autosampler for up to 24 hours. DTG was stable after heating DBMS at 50°C in oven for 1 hour and results were reproducible when samples were re-injected after 72 hours (reinjection reproducibility). Long-term stability was also demonstrated; DTG DBMS were shown to be stable when stored at ambient temperature for a period of 1 year. All evaluated conditions for standards and QC were within ±15% of their respective nominal concentrations. These stability data indicate that DBMS are stable at high temperatures and over a long period of time. Therefore, DBMS are ideal in middle and low-income countries where stable power cannot be guaranteed, since refrigerators or freezers are not necessary for storage of DBMS.

Clinical Application—A chromatogram from a clinical DBMS sample, taken at 4 hours post the DTG dose, is depicted in Figure 2. Steady state DTG concentrations in BM and MP taken at 2 weeks post-partum in a Ugandan mother, were 154.2 and 3786.2 ng/mL and 40.9 and 1210.7 ng/mL at 4 and 24 hours post dose, resulting in BM:MP ratios of 0.05 and 0.04, respectively. Time matched IP concentrations in the breastfeeding infant were 67.8 ng/mL and 75.5 ng/mL at 4 and 24 hours post dose, resulting in IP:BM ratios of 0.44 and 1.85. Similarly, steady state BM and MP collected at post-partum in a South African mother at 3 and 24 hours post dose were 116.3 and 3029.5 ng/mL and 17.7 and 603.3 ng/mL, with

BM:MP ratios of 0.04 and 0.03, respectively. The IP concentration at 24 hours post the DTG dose was 16.3 ng/mL (IP:BM =0.92). In both women, DTG BM concentrations fell below the assay limit of quantification (<10 ng/ml) when sampled 2 days (Ugandan mother) and 9 days (South African mother) after the women stopped DTG therapy, whereas corresponding MP (103.8 ng/ml) and IP (58.6 ng/ml) concentrations remained detectable in the Ugandan mother and her infant for up to 2 days post DTG treatment cessation. These results should be interpreted with caution due to small number of samples evaluated. Transfer of DTG from the mother's circulation to BM is potentially governed by its high (>98%) protein binding and thus sole passage of the unbound (free) form [37,38]. The influence of transporters and enzymes on DTG transfer across the mammary epithelium to the breastfeeding infant is unknown[18], however immature metabolic pathways of UGT1A1 and CYP enzymes may be responsible for the relatively high levels of DTG seen in the infants [39]. These preliminary data support that of a recent publication from the US IMPAACT P1026s study team investigating DTG pharmacokinetics during pregnancy and post-partum. The study revealed high DTG concentrations in infants at the time of delivery (cord:MP ratio = 1.25), suggesting significant placental transfer; moreover, the elimination of DTG in the newborns was prolonged, even in the absence of breastfeeding, with all non-breast fed infants having quantifiable (>5.0 ng/mL) DTG concentrations up to 5-9 days after delivery [40]. These data indicate that significant DTG concentrations in infants may be attributed to a combination of high drug transfer in-utero, transfer through the BM of medicated mothers, and reduced UGT1A1 metabolism in the infant shortly after birth.

Conclusion

A sensitive, specific and reproducible electrospray ionization LC–MS methodology has been developed and validated for the accurate measurement of DTG in dried breast milk spots (DBMS). Assay validation experiments such as extraction recovery, matrix effects and short- and long-term stability have been discussed. The method has been used for the quantification of DTG in DBMS as part of an ongoing clinical trial investigating the safety and pharmacokinetics of DTG in pregnant and breastfeeding mothers and their infants. DBMS samples from two breastfeeding mothers (Uganda and South Africa) paired with mother and infant's plasma concentration, were analysed.

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Future Perspective

Dolutegravir is emerging globally as an acceptable first line treatment of HIV. It was recommended by WHO guideline for treatment of HIV in 2016[14], and has been rolled out in many western countries, while efforts are being made to make it available in low and middle-income countries. The high prevalence of HIV among women of reproductive age in resource limited countries [15], requires sufficient safety and pharmacokinetic data to ensure protection of neonatal and paediatric patients from adverse events, such as toxicity or acquired drug resistance, that may occur during breastfeeding. Robust analytical methods for measurement of drugs in localised body fluids like BM are a significant component of pharmacokinetic studies that aim to characterise antiretroviral distribution into anatomical sites relevant to HIV transmission. This assay will provide a tool for evaluating DTG secretion in BM and implication in neonatal safety and prevention of vertical transmission during breastfeeding.

The preparation and extraction of liquid breast milk is generally cumbersome and often requires solid phase extraction due to the high amount of fats, polysaccharides and proteins in BM [17]. In contrast, this method requires only 30μL of BM spot and samples can be easily shipped and stored at ambient temperatures. Although automated volumetric absorptive microsampling technologies are fast replacing conventional methods of sampling and analysis, it will take a long time for these to be made available in resource limited countries [16].

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Executive summary

Background

- Prevention of mother to child transmission of HIV is crucial in preventing and reducing HIV related deaths in children.
- To evaluate antiretroviral transfer into breastmilk (BM) in HIV-infected mothers, an LC-MS method for quantification the integrase inhibitor dolutegravir (DTG) in dried breast milk spots (DBMS) was developed.

Materials and Methods

- A liquid-liquid extraction technique was used to extract DTG from the DBMS (Whatman 903 Protein Saver cards).
- A stable isotope labelled internal standard DTG-d5 was used to stabilise the assay.
- The method was validated according to the most recent FDA and EMA guidelines on method validation.

Results and Discussion

- The method requires only a small volume of BM (30μL/DBMS)
- Use of a chelating agent (EDTA) and spotting of the IS directly on the card improved DTG recovery and overall reproducibility and precision of the assay.
- DTG was stable in DBMS at ambient conditions over a period of one year. Thus, storage and transportation challenges are avoided since DBMS can be transported at ambient conditions and do not require refrigeration.

Conclusion

- The method is currently being utilized in an ongoing clinical study (DOLPHIN-1) investigating the safety and pharmacokinetics of DTG in pregnant and breastfeeding African women and their neonates.

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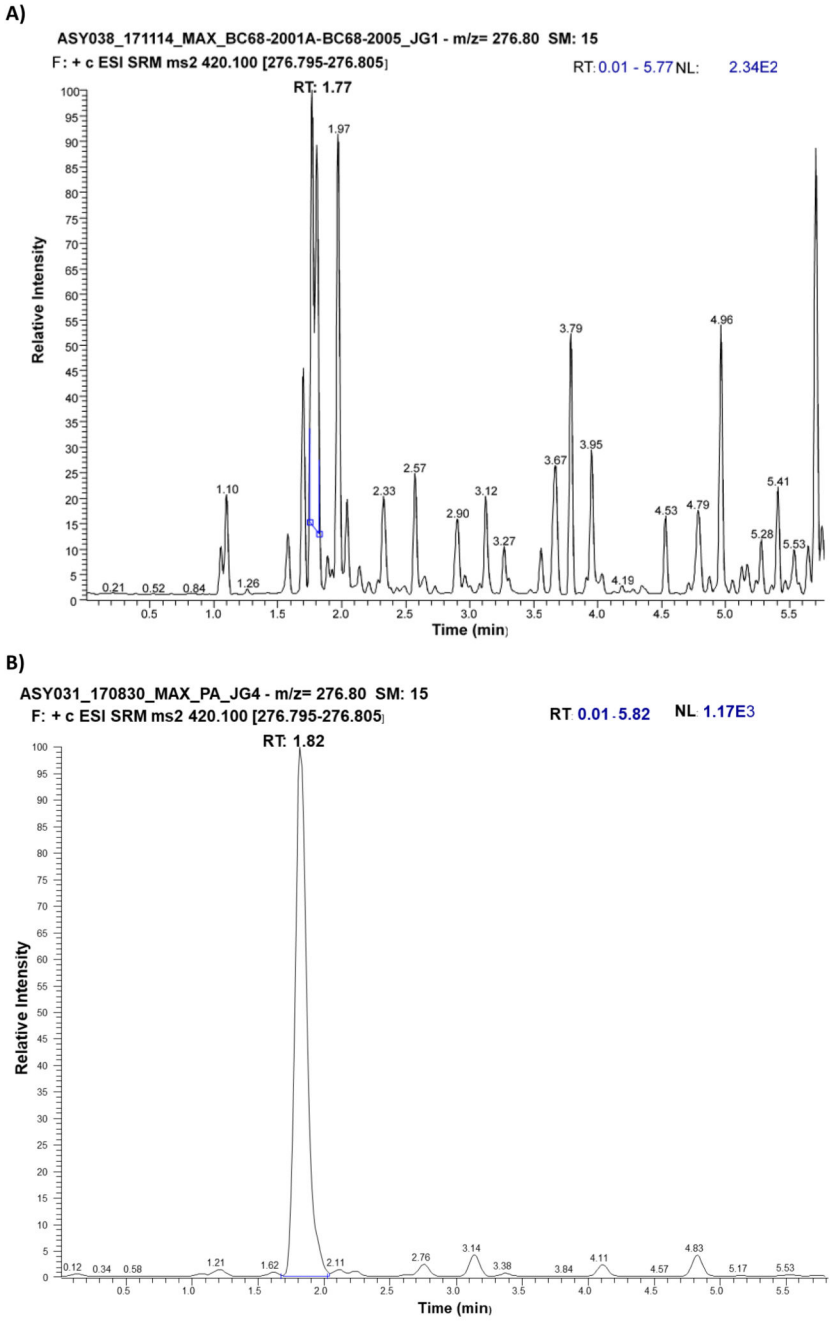

Figure 1.

Chromatograms of (A) a blank DBMS and (B) the assay lower limit of quantification (LLOQ)

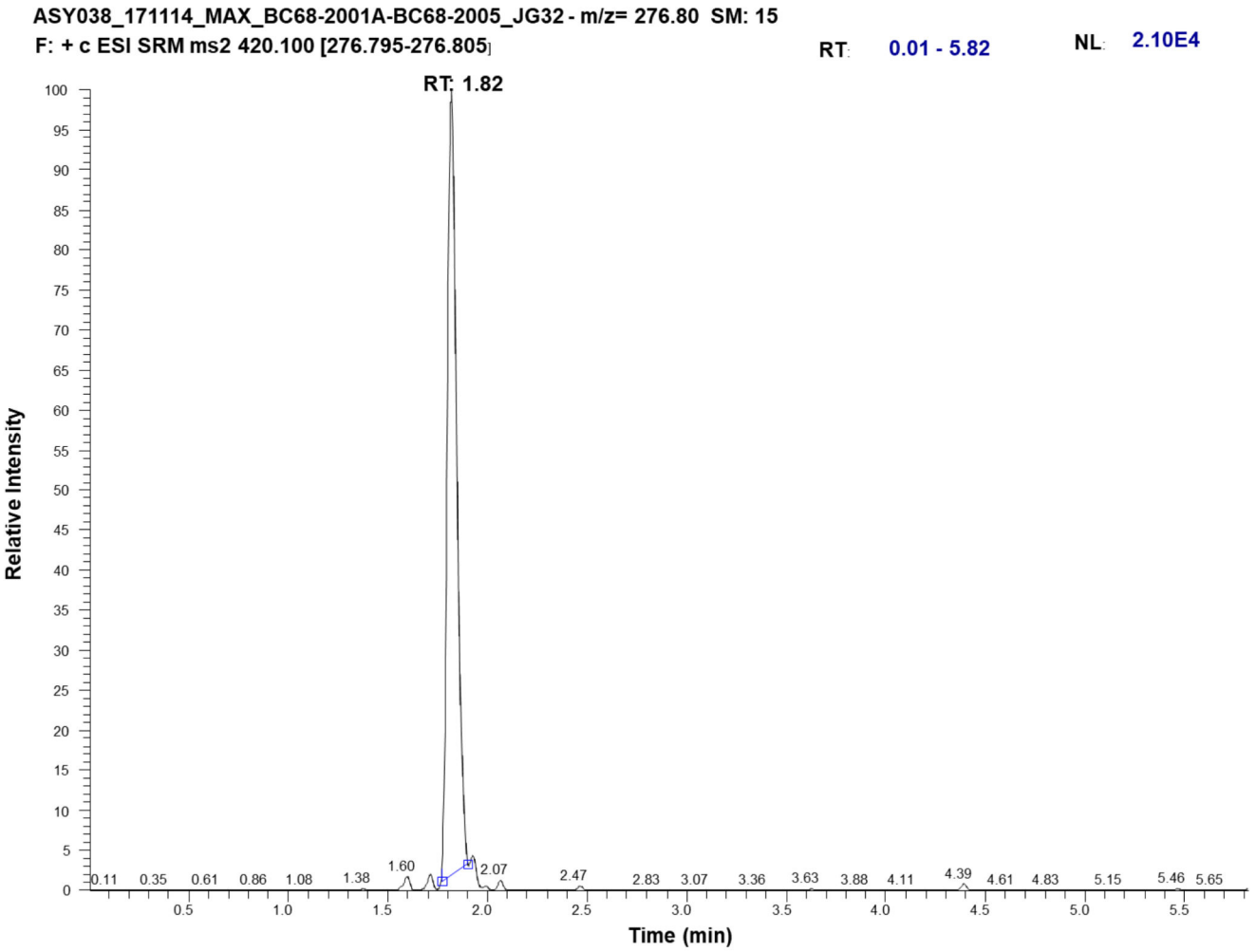

Figure 2.
Chromatogram of patient DBMS sample taken at 4 hours post the dolutegravir dose

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Table 1

Precision and Accuracy for DTG DBMS

Nominal Concentrations (ng/mL)	Mean (SD)	Precision (CV%)	Accuracy (%)
Inter-assay			
LLOQ	10.39 (1.09)	10.53	3.88
LQC (30)	26.00 (2.77)	10.64	-13.34
MQC (455)	440.14 (27.41)	6.23	-3.27
HQC (3700)	3762.67 (213.45)	5.67	1.69
Intra-assay			
LLOQ	10.05 (0.69)	6.83	0.53
LQC (30)	25.8 (1.93)	7.50	-13.95
MQC (455)	432.45 (13.08)	3.03	-4.96
HQC (3700)	3703.91 (169.50)	4.58	0.11

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Table 2

Recovery and Matrix Effect for DTG DBMS following liquid-liquid extraction with and without addition of EDTA, and the Internal Standard spiked in the extraction solution or directly onto the DBMS filter paper.

Method	Nominal QC Concentration (ng/mL)	%ME (SD)	%RE (SD)	%PE (SD)	%Analysis RE (SD)
		(B/A*100)	(C/B*100)	(C/A*100)	(C2/B2*100)
DBMS extracted with IS added to the extraction solvent	**30 (LQC)**	109.48 (7.80)	35.816 (4.15)	39.21 (5.99)	37.38 (7.94)
	455 (MQC)	129.63 (7.55)	50.53 (1.53)	65.51 (3.30)	55.66 (7.085)
	3700 (HQC)	142.95 (10.32)	39.41 (2.69)	56.33 (4.01)	41.33 (3.41)
	Overall Mean (%CV)	127.35 (13.24)	41.92 (7.96)	53.68 (13.35)	42.07 (25.67)
DBMS pre-soaked with EDTA, and IS added to the extraction solvent	**30 (LQC)**	90.37 (3.07)	102.23 (9.56)	92.38 (11.50)	98.64 (8.59)
	455 (MQC)	74.27 (7.49)	104.81 (8.46)	77.842 (6.48)	64.74 (5.63)
	3700 (HQC)	85.29 (6.93)	99.26 (11.97)	84.67 (11.49)	71.15 (9.73)
	Overall Mean (%CV)	83.31 (8.12)	102.10 (11.92)	84.97 (13.58)	78.18 (23.03)
DBMS pre-soaked with EDTA, and IS spotted directly onto the spot	**30 (LQC)**	97.10 (4.02)	92.94 (10.27)	90.24 (12.11)	102.10 (12.87)
	455 (MQC)	88.46 (8.62)	114.82 (14.33)	101.56 (11.08)	86.84 (10.58)
	3700 (HQC)	104.93 (12.03)	107.47 (15.18)	112.77 (7.45)	78.15 (10.12)
	Overall Mean (%CV)	96.83 (8.18)	105.07 (11.23)	101.52 (11.09)	89.03 (13.62)

A=Mean peak area response of analyte in mobile phase; B= Mean peak area response of analyte spiked after extraction of matrix (Spiked post extraction of blank DBMS); C= Mean peak area response of analyte spiked prior to extraction (spiked pre-extraction); %ME= Matrix effect, defined as ratio of mean peak area of analyte spiked post extraction (B) to mean peak area of analyte in mobile phase (A) X 100; %RE= Extraction yield, derived by dividing the mean peak area response of analyte spiked pre-extraction (C) with mean peak area response of analyte spiked post extraction (B) and multiplying by 100; %PE= Process efficiency calculated by dividing mean peak area response of analyte spiked pre-extraction (C) by mean peak area of analyte in mobile phase (A) and multiplying by 100; B2 is defined as response ratio of analyte spiked post extraction and internal standard; C2 is the response ratio of analyte spiked pre-extraction and internal standard; %Analysis RE calculated by dividing the mean response ratio of analyte spiked pre-extraction (C2) to mean response ratio of analyte spiked post extraction (B2), multiply by 100;

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Table 3

Short and long term stability of DTG DBMS

Stability	QC	Precision (%CV)	Accuracy (%bias)
Bench top (RT; 5 days)	LQC	4.70	0.37
	HQC	3.90	-7.59
Processed stability (4°C; 24 hr)	LQC	10.47	1.26
	HQC	7.05	1.26
Heat treatment (50°C; 1 hr)	LQC	4.78	8.96
	MQC	10.41	-4.78
	HQC	5.65	6.78
Autosampler stability (10°C; 24 hr)	LQC	5.79	-10.29
	HQC	4.04	-9.61
Long term stability (RT; 12mths)	LQC	9.32	2.47
	HQC	3.11	16.53
Re-injection Reproducibility (72 hr)	LQC	12.20	-2.84
	MQC	6.35	-5.73
	HQC	3.67	-0.89

RT = room temperature