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Gini, Penchala, Amara *et al.* [Query-Q1: Vikas Sharda(CE) to Ali(AU)] It is our house style to italicize genes to differentiate them from proteins. Can you please check and confirm that any genes in your article are italicized correctly? [Reply By: Sujan Dilly Penchala(AU)] Not applicable

[Query-Q2: Vikas Sharda(CE) to Ali(AU)] It is our house style to leave all proteins, genes and growth factors in their abbreviated form only. Please could you ensure none of these have been defined (including the figures and tables of articles)? [Reply By: Sujan Dilly Penchala(AU)] Not applicable Validation & clinical application of a novel LC-MS method for quantification of dolutegravir in breast milk

[Query-Q3: Vikas Sharda(CE) to Ali(AU)] Please check all author names, in particular your co-author's names, and affiliation details are presented correctly (including in the running header at the top of the page). We will not change these once published in most cases. [Reply By: Sujan Dilly Penchala(AU)] Names & affiliations checked, all

 Methodology

Validation and clinical application of a novel LC-MS method for quantification of dolutegravir in breast milk

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Abstract

Aim: 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 Tert-butyl methy-ether, with DTG-d5 as an internal standard. DTG was eluted on a reverse phase C₁₈ Waters XBridge (3.5 µm: 2.1 × 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:

antiretroviral • breast milk • clinical trial • dolutegravir • HIV • mass spectrometry • pharmacokinetics • validation

Background

It is recommended globally that HIV-positive women receive antiretroviral (ARV) therapy 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 ARV 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 ARV concentrations [8]. Furthermore, infants of mothers receiving ARV therapy 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 (analog) internal standards (IS) [22]; however, ideally stable isotopically labeled 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 [24]. ~~Physicochemical~~Physicochemical 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 versus 95% for lamivudine, 103 versus 94% for zidovudine and 99 versus 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 (<50 µl) 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 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 DBMS, with application of the method in breastfeeding mother and infant pairs.

Materials & methods

Reagents & equipment

DTG and deuterated IS DTG-d5 were purchased from Toronto ~~research-chemicals~~Research Chemicals (ON, Canada). LC-MS grade Methanol (99.9%), formic acid, Propan-2-ol and Tert-butyl methyl 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 BM 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 C₁₈ Waters XBridge (3.5 µm: 2.1 × 50 mm) column (Waters Corporation, USA) set at 40°C oven temperature

and interfaced with a 2 μm C₁₈ Quest column saver (Thermo Fisher Scientific) was used to resolve the analytes. A Thermo Quantum Access mass spectrometer with a heated ESI source (Thermo Fisher Scientific) was used for quantification. TSQ Tune software was used for compound tuning and optimization, and LCQuan (version 2.7 Thermo Fisher Scientific) was used for data acquisition, data processing and reporting.

Chromatographic & mass spectrometry conditions

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

The mass spectrometer used ESI set in a positive ionization mode using selective reaction monitoring. The spray voltage was 29 V, the capillary temperature was 300°C and vaporization temperature 350°C, respectively. The sheath and ~~auxiliary~~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 $\mu\text{g}/\text{ml}$ in methanol via direct infusion into the mass spectrometer at a flow rate of 5 $\mu\text{l}/\text{min}$. The m/z transitions were 420.1 \rightarrow 277.1 m/z for DTG and 425.1 \rightarrow 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 s.

Preparation of stock solutions, standards, quality controls & IS of DBMS & DPS

A primary DTG stock solution (1 mg/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 $\mu\text{g}/\text{ml}$. The intermediate solutions were then used to spike into the blank human BM for preparation of the non-zero calibration standards (nine levels: 10, 25, 50, 200, 500, 1000, 2000, 3200 and 4000 ng/ml) and internal quality control samples (QC) (~~LQC~~low quality control, LQC = 30 ~~ng/ml~~ ng/ml; medium quality control, MQC = 455 ng/ml and high quality control, HQC = 3700 ng/ml). Exactly 30 μl of each calibrator and QC was spotted onto the middle of the 12 mm circle of the protein saver cards. The spots were then dried overnight at ambient temperature before sealing in a ~~ziplock~~zip-lock bag with desiccant sachets, and stored at -40°C until use.

A 1 mg/ml DTG-d5 stock solution was prepared in methanol and stored at 4°C. On the day of analysis a working IS solution (250 $\mu\text{g}/\text{ml}$) was prepared in 50:50 (v/v) methanol:water.

Sample pretreatment

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 $\mu\text{g}/\text{ml}$) was spotted directly onto each 12 mm circle of the DBMS using an electronic pipette and allowed to air dry for 20 min. 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 presoaked in 500 μl of 1.8 mg/ml EDTA in water for 10 min.

A total of 2 ml of TBME was added to each sample and the tubes capped and tumbled for 30 min. The samples were centrifuged at 3398 $\times g$ for 10 min 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~~non-frozen supernatant decanted into clean 12 \times 75 mm glass tubes and evaporated to dryness using nitrogen drier for 1 h. 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 s. 100 μl of reconstituted sample was transferred to 300 μl autosampler vials, sealed with crimp caps (11 mm 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 or the lower limit of quantitation (LLOQ). [Query-Q4: Vikas Sharda(CE) to Ali(AU)] Please define the following terms: LLOQ, HQC, MQC, and LQC. [Reply By: Sujun Dilly Penchala(AU)] Terms have been defined and included in the text below. 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 IS, less than 5% of the mean response areas in the six 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 four blank DBMS on seven 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 & accuracy

Precision and accuracy was determined by analyzing five different assay batches, consisting of a calibration curve and six LLOQ, LQC, MQC and HQC (separate extractions), run over 5 days. Deviation of $\leq \pm 15\%$ of measured analyte from nominal concentrations was accepted, except at LLOQ where $\leq \pm 20\%$ 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 $\leq \pm 15\%$ from their respective nominal concentrations.

Recovery & 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 ($C/A \times 100$). 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 ($B/A \times 100$). 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 ($C2/B2 \times 100$).

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 presoaked in 1.8 mg/ml EDTA in water against DBMS ~~pre-soaked~~ presoaked 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 pipetted 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 (quality control (QC) samples; six 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.

Given that DBMS are to be collected and processed from mothers in Sub-Saharan Africa, where higher temperatures are to be expected, we sought to verify the stability of the DBMS at a range of temperatures up to 50°C. Therefore, heat stability was assessed by heating DBMS at 50°C for 1 h before analysis. Autosampler stability was assessed by injecting samples after 24 h of storage in the autosampler. Bench top stability (storage of DBMS at room temperature for 5 days) was evaluated for QC by comparing with freshly prepared samples. The stability of processed (extracted) samples was assessed by keeping the processed samples in the fridge (4°C) for 24 h before analysis. Long-term stability was evaluated by storing DBMS at ambient conditions for up to 1 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 with their respective nominal value. Re-injection reproducibility was assessed by re-injecting samples left in the autosampler for 72 h and results of the two runs compared for reproducibility.

Clinical application

The validated method is currently being utilized 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 randomized 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 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.

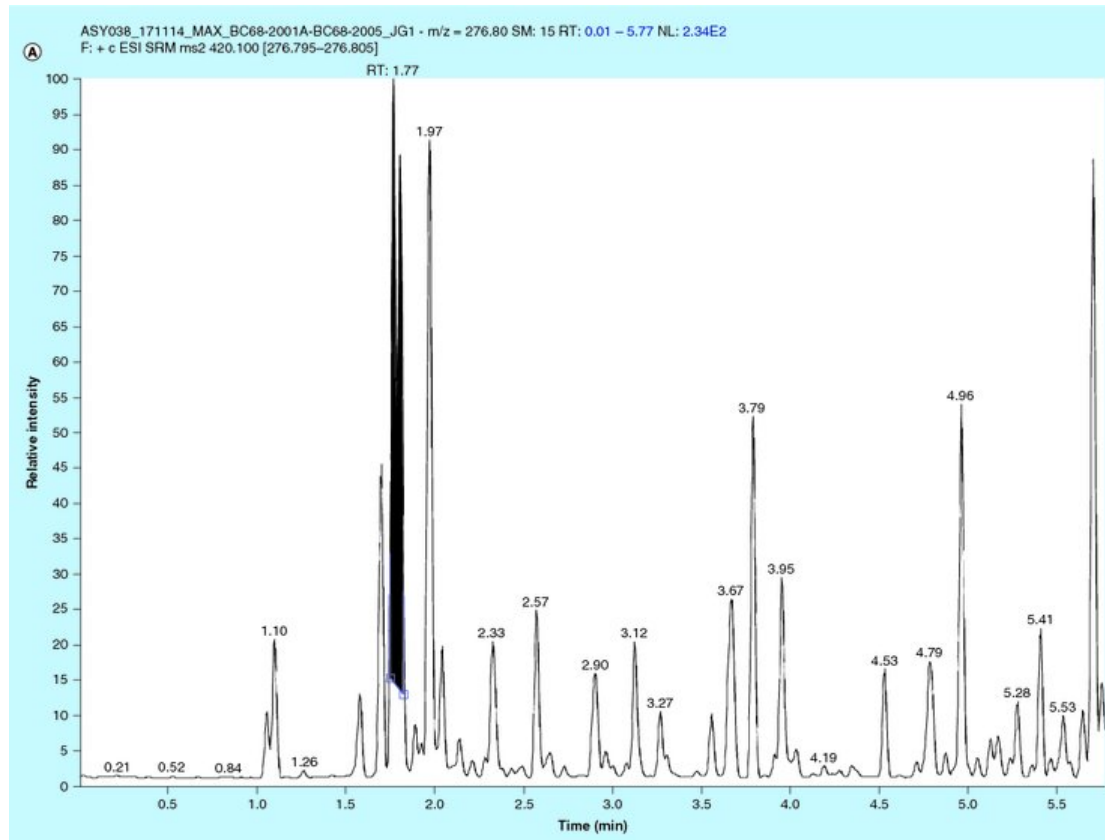
BM samples were collected in EDTA containers and exactly 30 μ l spotted on Whatman 903 protein saver cards. The cards were subsequently air dried and sealed in individual ziplocked bags with desiccant. Plasma and DBMS were shipped on dry-ice to the University of Liverpool Bioanalytical Facility for analysis. DBMS were stored at -40°C analyzed according to the above sample pretreatment method. The DBMS obtained from HIV-infected mothers were not heat inactivated before analysis as the drying process on the filter card renders the HIV virus inactive, thus DBMS samples were processed/extracted as per universal precautions. DTG concentrations in plasma (MP and IP) were analyzed using a previously described and fully validated LC-MS methodology [32]. Ratios between DTG concentrations measured in BM and maternal and IP samples were calculated arithmetically and expressed as BM:MP and IP:BM ratios.

Results & discussion

Method development

Chromatographic & mass spectrometry conditions

DTG and DTG-d5 were eluted from the reverse phase column at approximately 1.8 min, respectively. Representative chromatograms obtained from extracted DBMS (blank and LLOQ) are shown in Figure 1. The m/z transitions were $420.1 \rightarrow 277.1$ m/z for DTG and $425.1 \rightarrow 276.9$ 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 s.



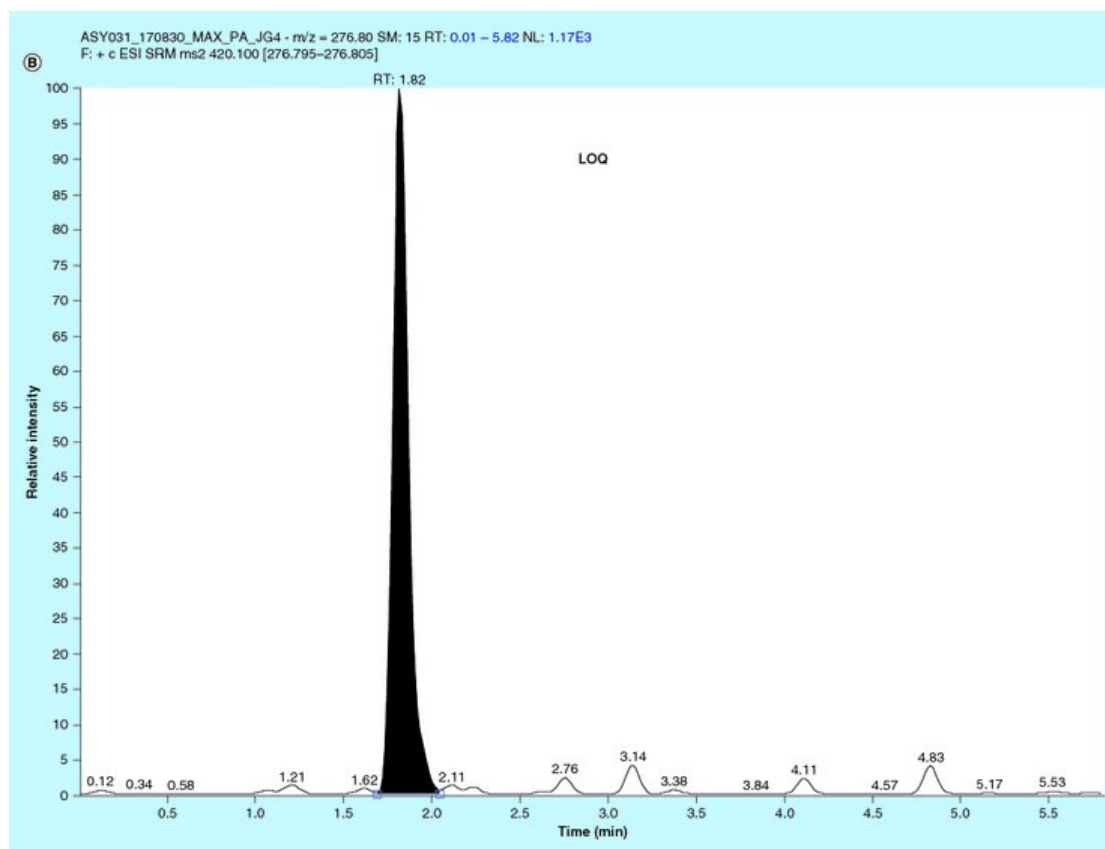


Figure 1. **Representative LC-MS/MS ion chromatograms of dolutegravir.** Chromatograms of (A) a blank DBMS and (B) the assay lower limit of quantification (LLOQ). [Query-Q6: Vikas Sharda(CE) to Ali(AU)] Please provide a general/main title for Figure 1. [Reply By: Sujan Dilly Penchala(AU)] included in the text above [low quality control, LQC; medium quality control, MQC; high quality control, HQC and LLOQ lower limit of quantification. [Reply By: Sujan Dilly Penchala(AU)] Title added. Representative LC-MS/MS ion chromatograms of dolutegravir.

Sample pretreatment

Due to the small volume of BM sample (30 μ l) 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**, 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 predefined 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-punches and smaller BM volumes (<30 μ l) did not provide a high enough response at the assay LLOQ (10 ng/ml or 0.3 ng/DBMS sample). Furthermore, BM is **colorless** on the card, therefore, punching the entire circle ensures that the entire sample is utilized 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 IS (DTG-d5) for all six batches tested.

Carryover

Mean percentage carryover into the extracted blanks ($n = 7$) after injection of a ULOQ sample (4000 ng/ml) was 62.7 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 DTG 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 & accuracy

Five independent validation batches were run over 5 days, with each assay run consisting of a zero blank, nine calibration standards (in duplicate) and six 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 ($r^2 \pm SD$) was 0.9962 ± 0.0018 . Inter- and intra-day accuracy and precision were within $\pm 15\%$ for all QC levels and $\pm 20\%$ for the LLOQ, as shown in Table 1.

Table 1. Precision and accuracy for dolutegravir dried breast milk spots.			
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
HQC: xxxx high quality control; LLOQ: xxxx lower limit of quantitation; LQC: xxxx low quality control; MQC: xxxx medium quality control.			

Recovery & matrix effect

Mean (\pm CV %), percentage recovery (%RE), process efficiency (%PE) and matrix effect (%ME) of DTG from DBMS are summarized in Table 2: The mean % RE, PE and ME (%CV) of DTG from DBMS presoaked 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 (30 μ l) 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].

Table 2. Recovery and Matrix Effect for dolutegravir dried breast milk spots following liquid-liquid extraction with and without addition of EDTA, and the internal standard spiked in the extraction solution or directly onto the dried breast milk spots filter paper.					
Method	Nominal QC concentration (ng/ml)	%ME (SD) (B/A*100)	%RE (SD) (C/B*100)	%PE (SD) (C/A*100)	%Analysis RE (SD) (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.

DBMS: dried breast milk spot; HQC: ~~xxxx~~ high quality control,; IS: Internal standard; LLOQ: ~~xxxx~~ lower limit of quantitation; LQC: ~~xxxx~~ low quality control,; MQC: ~~xxxx~~ medium quality control,.

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 pretreated 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 optimize drug recovery were use of 1.8 mg/ml EDTA in water to presoak 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 BM 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 IS 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 presoak 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 presoak 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 summarized in Table 3. DTG was stable in DBMS after 5 days on the bench at room temperature, when refrigerated for 24 h at 4°C, during processing, and when stored in the autosampler for up to 24 h. DTG was stable after heating DBMS at 50°C in oven for 1 h and results were reproducible when samples were re-injected after 72 h (~~reinjection~~ re-injection 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 $\pm 15\%$ 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.

Table 3. Short and long term stability of dolutegravir dried breast milk spots.

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 h)	LQC	10.47	1.26
	HQC	7.05	1.26
Heat treatment (50°C; 1 h)	LQC	4.78	8.96
	MQC	10.41	-4.78
	HQC	5.65	6.78
Autosampler stability (10°C; 24 h)	LQC	5.79	-10.29
	HQC	4.04	-9.61
Long term stability (RT; 12 months)	LQC	9.32	2.47
	HQC	3.11	16.53
Re-injection reproducibility (72 h)	LQC	12.20	-2.84
	MQC	6.35	-5.73

Clinical application

DTG concentrations from paired MP and BM samples collected at two time points from two breastfeeding mothers (1 Ugandan; 1 South African) are described in order to support the clinical utility of this method, which was developed primarily for analysis of DBMS from the DolPHIN-1 clinical trial. The final results from DolPHIN-1, which includes a total of 29 MP:BM pairs (taken on three occasions), has recently been presented at the International AIDS Conference 2018 [37].

A chromatogram from a clinical DBMS sample, taken at 4 h 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 h 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 and 75.5 ng/ml at 4 and 24 h 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 h 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 h 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.

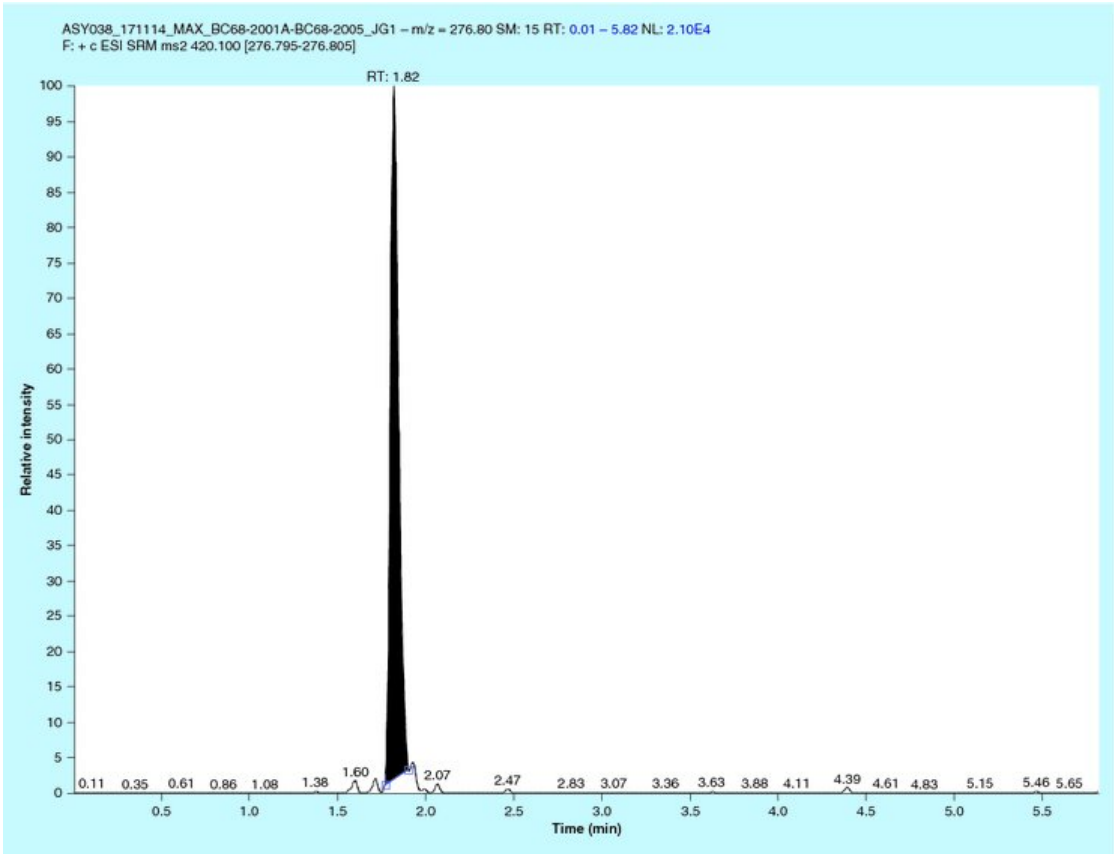


Figure 2. Chromatogram of patient dried breast milk spots sample taken at 4 h post the dolutegravir dose.

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 [38,39]. 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 [40].

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 nonbreastnon-breast fed infants having quantifiable (>5.0 ng/ml) DTG concentrations up to 5–9 days after delivery [41]. 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 ESI LC–MS methodology has been developed and validated for the accurate measurement of DTG in 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 analyzed.

Future perspective

DTG 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 pediatric patients from adverse events, such as toxicity or acquired drug resistance, that may occur during breastfeeding. Robust analytical methods for measurement of drugs in localized body fluids such as BM are a significant component of pharmacokinetic studies that aim to characterize ARV 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 BM 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 30 µl 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].

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 & methods

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

Results & discussion

- The method requires only a small volume of BM (30 µl/DBMS).
- Use of a chelating agent (EDTA) and spotting of the internal standard 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 1 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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Financial & competing interests disclosure [Query-Q7: Vikas Sharda(CE) to Ali(AU)] Please check that the financial disclosure is correct. If you are NIH funded and would like your article to be deposited on the NIHMS system/PMC you must state that here and on your copyright form. Please note that as per PMC's policy, the NIHMS/PMC deposit scheme does not apply if your article is not peer reviewed. [Reply By: Sujan Dilly Penchala(AU)] Financial disclosure is correct.

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Ethical conduct of research

Ethical approval to source blank human breast milk from the Wirral Mothers' Milk Bank, Clatterbridge Hospital, Wirral, UK; was granted by the University of Liverpool Research Ethics Committee. DOLPHIN -1: Ethical approvals were obtained from all institutions involved (University of Liverpool Research Ethics committee, Joint Clinical Research Centre Uganda, Ugandan National Council of Science and Desmond Tutu HIV Foundation, Gugulethu Community Health Centre, Cape Town Ethics and Research Committee). 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).

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