Journal of Mass Spectrometry and Advances in the Clinical Lab 34 (2024) 1–7

Contents lists available at ScienceDirect

Journal of Mass Spectrometry and Advances in the Clinical Lab

journal homepage: www.sciencedirect.com/journal/journal-of-mass-spectrometry-and-advances-in-the-clinical-lab

Research Article

Rapid and sensitive liquid chromatographic–tandem mass spectrometric methods for the quantitation of dolutegravir in human plasma and breast milk

Ashley R. Rackow [a], Aashish Pandey [b], Amelia L. Price [b], Mark A. Marzinke [a,b,*]

[a] Division of Clinical Chemistry, Department of Pathology, Johns Hopkins University, United States
[b] Division of Clinical Pharmacology, Department of Medicine, Johns Hopkins University, United States

ARTICLE INFO

Keywords:
HIV
Dolutegravir
LC–MS/MS
Antiretroviral
Pharmacokinetics
Validation

ABSTRACT

Background: Dolutegravir (DTG) is part of a first-line antiretroviral therapy (ART) for HIV management in drug-naïve individuals and is recommended for the treatment of HIV during pregnancy. Robust analytical tools to quantify DTG are necessary to support clinical trials that characterize its multi-compartment drug distribution.
Methods: Potassium EDTA (K_2EDTA) plasma or whole breast milk was spiked with DTG and an isotopically labeled internal standard. Samples were prepared via protein precipitation prior to LC–MS/MS analysis. The assays were validated in accordance with regulatory recommendations.
Results: Analytical measuring ranges for DTG quantitation in plasma and breast milk were 100–10,000 ng/mL and 0.500 to 1000 ng/mL, respectively. Inter-assay precision and accuracy were 2.73 % to 3.41 % and −10.6 % to −5.37 % for plasma, and 4.24 % to 12.4 % and −5.63 % to 7.49 % for breast milk, respectively. DTG was stable for three freeze–thaw cycles and for at least 72 h at room temperature in matrix (plasma or breast milk). Additionally, whole blood was stable for 24 h at room temperature and 2 h under conditions of extended heat and humidity. Matrix effects for DTG in plasma and breast milk ranged from 101 % to 108 % and 78.2 % to 99.3 %, respectively. Quantitation in remnant plasma samples yielded measurable concentrations within the primary linearity of the assay.
Conclusions: Methods to quantify DTG in human plasma and breast milk have been developed and validated. These assays were designed to satisfy all criteria for implementation in clinical and clinical trial settings.

1. Introduction

In 2021, the Centers for Disease Control and Prevention (CDC) estimated that 1.2 million people aged 13 years and older were living with HIV in the United States [1], while a 2018 Joint United Nations Programme on HIV/AIDS (UNAIDS) report estimated a global prevalence of 37.9 million, representing 2.9 % of the population [2]. The incidence of HIV varies based on region; a birth cohort in Brazil found an HIV prevalence of 0.4 % during pregnancy [3], whereas a national meta-analysis of data in Nigeria estimated that 7.2 % of pregnant persons were living with HIV [4]. There are significant risks to maternal and fetal health if an HIV diagnosis remains untreated, including increased morbidity and mortality [5–8]. Early initiation of antiretroviral therapy (ART) decreases HIV viral load and is a successful modality in the prevention of mother-to-child (vertical) viral transmission.

Dolutegravir (DTG) is an integrase strand transfer inhibitor (INSTI) that blocks the insertion of viral cDNA into host cells. It is generally well tolerated with minimal side effects and is a common component of fixed-dose ART formulations [9]. DTG is also available as a generic formulation, which reduces treatment costs and makes it a feasible option in

Abbreviations: AMR, analytical measuring range; ART, antiretroviral therapy; CDC, Centers for Disease Control and Prevention; C_{max}, maximum concentration; C_{trough}, trough concentration; %CV, coefficient of variation; DTG, Dolutegravir; INSTI, integrase strand transfer inhibitor; K_2EDTA, potassium EDTA; LLOQ, lower limit of quantitation; MPA, mobile phase A; MPB, mobile phase B; SD, standard deviation; SRM, selective reaction monitoring; UNAIDS, Joint United Nations Programme on HIV/AIDS; WHO, World Health Organization; %DIF, percent difference; %DEV, percent deviation; LC-MS/MS, liquid chromatographic-tandem mass spectrometric; QC, quality control; ULOQ, upper limit of quantitation.

* Corresponding author at: Johns Hopkins University School of Medicine, 1800 Orleans Street, Sheikh Zayed Tower, Room B1-1020F, Baltimore, MD 21287, United States.
E-mail address: mmarzin1@jhmi.edu (M.A. Marzinke).

https://doi.org/10.1016/j.jmsacl.2024.09.001
Received 5 August 2024; Received in revised form 13 September 2024; Accepted 18 September 2024
Available online 19 September 2024

resource-limited settings. Among pregnant individuals, viral suppression was achieved a median of 28 days after the initiation of DTG, representing a significant improvement over efavirenz-containing regimens (84 days) [10]. In 2019, the World Health Organization (WHO) recommended DTG as a first-line therapy for all populations, including during the pregnant period [11].

There is a need for rapid and sensitive methods to detect DTG in biological matrices to support clinical trials and to better understand drug exposures during the pregnant and postpartum periods. Notably, pregnant persons exhibit lower plasma DTG concentrations than non-pregnant individuals [12]. The distribution and excretion of DTG may be affected by placental transfer in utero and breast milk transfer to the infant [13]. Quantitation of DTG in plasma and breast milk can inform infant drug exposures during lactation.

Several publications describe liquid chromatographic tandem mass spectrometric (LC–MS/MS) methods for quantifying DTG in human plasma, either in isolation [14] or as a component of multiplexed methods for antiretroviral quantitation [15,16]. These previously published methods report average run times of 9 min, with lower limits of quantitation (LLOQ) ranging from 5 to 30 ng/mL. Methods for detecting DTG in breast milk are scarce; one report details the quantitation of DTG in dried breast milk spots with a lower limit of quantitation of 10 ng/mL [17].

To build upon the existing literature, we examined the pharmacokinetic profile of DTG in plasma and breast milk to define clinically relevant analytical measuring ranges (AMR). The differential distribution of DTG into plasma versus breast milk necessitates separate measuring ranges. An early pharmacokinetic study of healthy volunteers found that the average maximum plasma DTG concentration (C_{max}) was 6200 ng/mL following a single 50 mg dose [18]. A subsequent study reported a trough concentration (C_{trough}) of 830 ng/mL in persons living with HIV after 10 days on a 50 mg per day regimen [19]. The DTG plasma assay has an AMR of 100 ng/mL to 10,000 ng/mL, which encompasses both the C_{max} and C_{trough} while also extending to a lower quantitation limit to evaluate elimination parameters.

The DolPHIN-1 trial was the first clinical study to examine both maternal plasma and breast milk DTG concentrations and reported breast milk concentrations of 10–100 ng/mL, which was roughly 3 % of maternal plasma concentrations [13]. Similar concentrations were also observed in a maternal-infant case study [20]. The DTG breast milk assay ranges from 0.500 ng/mL to 1000 ng/mL, covering the range of anticipated physiologic concentrations and detect low levels of DTG transfer into the matrix.

Herein, we describe methodologies to quantify DTG in plasma and whole breast milk via protein precipitation with analytical run times of less than 2.5 min. The methods have been validated in accordance with regulatory recommendations, and assessments included precision, accuracy, stability, and matrix effects.

2. Methods

2.1. Chemicals

DTG was purchased from Toronto Research Chemicals (North York, ON, Canada); the isotopically labeled internal standard, $^{13}C^{2}H_{5}$-DTG, was purchased from Alsachim (Illkirch Graffenstaden, France). Drug-free whole blood, K_2EDTA plasma, hemolyzed K_2EDTA plasma (2 % lysed whole blood), lipemic K_2EDTA plasma (≥300 mg/dL triglyceride content), and whole breast milk were purchased from BioIVT (Westbury, NY, USA). Optima-grade methanol, water, acetonitrile, ammonium hydroxide, and dimethyl sulfoxide were purchased from Fisher Scientific (Fairlawn, NJ, USA). ACS reagent grade formic acid was purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Preparation of standards and quality controls

DTG master stocks were prepared at concentrations of 1 mg/mL in DMSO. Separate working stocks were prepared for calibration standards and quality controls (QC). For the plasma assay, a 1 mg/mL working stock was also prepared. For all other working stocks, master stocks were diluted with 50:50 water:methanol to generate working stock solutions of 0.100 μg/mL, 1.00 μg/mL, 10.0 μg/mL, and 100 μg/mL. Calibration standards were prepared from working stock solutions by spiking the appropriate volume of DTG into drug-free plasma or whole breast milk. Plasma calibrators were prepared as follows: 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL, 2500 ng/mL, 5000 ng/mL, 7500 ng/mL, and 10,000 ng/mL. Plasma QCs were prepared at final concentrations of 100 ng/mL, 300 ng/mL, 1500 ng/mL and 8500 ng/mL, representing the assay LLOQ, and low, mid, and high QC levels. For DTG quantitation in breast milk, standards were prepared at 0.500 ng/mL, 1.00 ng/mL, 5.00 ng/mL, 10.0 ng/mL, 50.0 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL, and 1000 ng/mL; QCs were prepared at 0.500 ng/mL (LLOQ), 1.50 ng/mL (low), 75 ng/mL (mid), and 800 ng/mL (high).

2.3. Sample preparation

Plasma and whole breast milk samples were prepared using protein precipitation on a conditioned 0.45 μm Captiva 96-well filter plate from Agilent Technologies (Wilmington, DE, USA). For the plasma DTG assay, 25 μL of plasma was combined with 50 μL internal standard and 500 μL acetonitrile. Following precipitation under vacuum pressure, 50 μL of eluent was transferred to a new 96 well plate and 250 μL 0.1 % formic acid in water was added to each well and vortexed; 3.0 μL was subjected to downstream LC–MS/MS analysis. To quantify DTG in breast milk, 30 μL of breast milk was combined with 30 μL internal standard and 500 μL acetonitrile. Following protein precipitation, eluted samples were evaporated to dryness and reconstituted in 100 μL of 0.1 % formic acid in water; 3.0 μL of sample was analyzed via LC–MS/MS.

2.4. Chromatography parameters

Chromatographic separation of DTG in plasma and breast milk was performed using an Acquity BEH C8 2.1 × 50 mm UPLC column, with a particle size of 1.7 μm (Waters Corporation, Milford, MA, USA). For the breast milk assay, a pre-column filter was used. The mobile phase system for both methods consisted of 0.1 % formic acid in water (mobile phase A; MPA) and 0.1 % formic acid in acetonitrile (mobile phase B; MPB). For quantitation of DTG in plasma, separation was achieved with a gradient of 25 % MPB to 99 % MPB, a hold at 99 % MPB, and a re-equilibration to starting conditions; the flow rate across the method was 0.550 mL/min. DTG was eluted at 0.81 min with a total analytical run time of 2.0 min. For DTG quantitation in breast milk, separation was achieved with a gradient of 37 % MPB to 99 % MPB, followed by a hold at 99 % MPB and re-equilibration. The flow rate was 0.750 mL/min; DTG was eluted at 0.86 min, and the total analytical run time was 2.2 min.

2.5. Detection parameters

DTG quantitation was performed on an API 5500 triple quadrupole mass analyzer (SCIEX, Redwood City, CA) using an ESI source operated in positive ionization and selective reaction monitoring (SRM) modes; a scheduled SRM was used for DTG quantitation in breast milk. Ion transitions monitored for DTG were m/z 420.2 → 277.1 (quantifier) and m/z 420.2 → 127.1 (qualifier); m/z 426.2 → 133.1 was monitored for $^{13}C^{2}H_{5}$-DTG.

2.6. Data analysis

Data were acquired using Analyst® 1.7 software (SCIEX).

Calculations for validation assessment, which included precision, accuracy, stability, and matrix effects, were performed using Microsoft Office Excel 2019. Outliers were identified by Grubbs' Outlier Test utilizing Prism GraphPad.

2.7. Method validation

The LC–MS/MS methods for DTG quantitation in plasma and breast milk were validated in accordance with FDA Bioanalytical Method Validation Guidance for Industry recommendations [21]. Intra (within) and inter (between) assay precision and accuracy, linearity, selectivity, stability (under several conditions), matrix effects, cross talk, and carryover were evaluated.

2.7.1. Precision and accuracy

Intra-assay precision was evaluated by the analysis of six samples containing DTG at aforementioned LLOQ, low, mid, and high QC concentrations for both plasma and breast milk specimen sources. Inter-assay precision was assessed by testing the QC materials in replicates of six across three independent runs. Observed means, standard deviations (SDs) and coefficients of variation (%CV) were calculated for all QC levels. Intra- and inter-assay accuracy was also evaluated at established QC levels. Accuracy was described by percent deviation (%DEV), which is the difference between the observed and theoretical analyte concentrations divided by the theoretical concentrations and multiplied by 100.

2.7.2. Linearity and dilutional integrity

Standard curves were calculated using the ratio of the peak area of the analyte to isotopically labeled internal standard using quadratic regression with $1/x^2$ weighting. Extended linearity was evaluated by preparing plasma controls containing drug concentrations at three times the upper limit of quantitation (ULOQ; 30,000 ng/mL). Controls were diluted 4-, 8-, and 16-fold with drug-free plasma; precision and accuracy were assessed by comparing theoretical concentrations to observed results. For the breast milk assay, controls containing drug concentrations at ten times the ULOQ (10,000 ng/mL) were evaluated. Controls were diluted 20- and 50-fold with drug-free breast milk; precision and accuracy were assessed as outlined above.

2.7.3. Carryover and cross talk

Carryover was evaluated by running three samples at the ULOQ followed by injection of blank samples (plasma or breast milk). These results were compared to peak areas of samples at the LLOQ. Cross talk was assessed by injecting three blank specimens with internal standard, followed by three samples spiked at the ULOQ without internal standard and three samples at the LLOQ. Cross talk for DTG was assessed by monitoring for the presence of signal in the $^{13}C^{2}H_5$-DTG channel when DTG was evaluated alone at the ULOQ. Internal standard cross talk was assessed by monitoring the presence of signal in the DTG channel when a sample was spiked only with the $^{13}C^{2}H_5$-DTG internal standard.

2.7.4. Stability

Freeze-thaw stability studies were performed using QC materials that were frozen at ≤ -70 °C for at least 72 h, thawed unassisted at room temperature for 4–6 h, and re-frozen under the same conditions for 16–24 h; this was repeated for three cycles. Sample matrix stability was assessed by incubating QC samples at room temperature (21–25 °C) prior to extraction and analysis. Observed values from stability-challenged samples were compared to freshly tested materials. Long-term stability studies were performed by analyzing QC materials at low, mid, and high levels stored at ≤ -70 °C for 865 days for plasma and 195 days for breast milk. These QCs were tested against freshly prepared and analyzed quality controls and calibrators. Whole blood stability was assessed by spiking whole blood with DTG. Unspun samples were held for 24 h at room temperature or were exposed to high heat (40 °C) and humidity (100 %) conditions for 2 h. These test sets were compared to a control set of whole blood that was spiked with DTG and immediately processed and extracted. For the aforementioned temperature and humidity challenges, stability was evaluated by calculating a percent difference (%DIF), which assesses the difference between a stability-challenged sample and a non-challenged QC sample divided by the non-challenged prepared QC sample; this result was then multiplied by 100. For all stability challenges, acceptability was defined as a percent difference of $\leq$15 % between the observed and reference QC values.

2.7.5. Matrix effect characterization

The effects of ion suppression or enhancement were assessed quantitatively for DTG and $^{13}C^{2}H_5$-DTG in both plasma and breast milk, as previously described [22]. Un-extracted materials were prepared at low, mid, and high QC concentrations without matrix. Post-extracted materials were prepared by spiking post-extracted plasma or breast milk samples with DTG at low, mid and high QC concentrations. Pre- and post-extracted sets were evaluated using independent lots of plasma or breast milk ($n = 6$). Raw peak areas for analyte and internal standard were used to determine overall matrix effects (a comparison of post-extracted samples to un-extracted samples), extraction efficiency (a comparison of pre-extracted samples to post-extracted samples) and processing efficiency (a comparison of pre-extracted samples to un-extracted samples).

2.8. Human sample analysis

Ten remnant human plasma samples were collected from individuals prescribed DTG. This collection was performed in accordance with approval from our local institutional review board (IRB00295353). All samples were blinded and de-identified before delivery to the testing laboratory. The results were not used for clinical decision making.

3. Results

3.1. Method development

Initial sample preparation methods for the extraction of DTG from plasma and breast milk were performed using protein precipitation. Due to the relatively high concentration of DTG in plasma, the eluent obtained after precipitation was diluted 1:5 with 250 μL of 0.1 % formic acid. However, dilution of breast milk samples was not necessary. Instead, breast milk samples were dried and reconstituted in a composition amenable for LC–MS/MS analysis. Several volumes and ratios of mobile phases were examined to optimize the resuspension of breast milk, and eluted material was ultimately resuspended in 100 μL of 0.1 % formic acid in water (MPA).

As DTG is highly hydrophobic (Fig. 1A and B), an octyl column was used, which we have also employed for the separation of other hydrophobic antiretrovirals [23,24]. Gradients were optimized to begin at high organic concentration to facilitate short retention times with minimal carryover. DTG was quantified in both matrices using the same column, mobile phases, and ion transitions. Representative chromatograms of DTG and $^{13}C^{2}H_5$-DTG for the plasma and breast milk assays are shown in Fig. 1C–F.

3.2. Precision and accuracy

Precision and accuracy were calculated using six individual QC samples at four levels (LLOQ, low, mid, and high) prepared in human plasma and whole breast milk. Intra-day precision and accuracy averages for plasma ranged from 1.41 % to 2.82 % and −9.52 % to −6.53 %, respectively. For DTG quantitation in breast milk, average intra-day precision and accuracy ranged from 2.38 % to 7.62 % and −9.80 % to 2.50 %, respectively. Inter-day precision and accuracy for plasma DTG ranged from 2.73 % to 3.41 % and −10.6 % to −5.37 %, respectively.

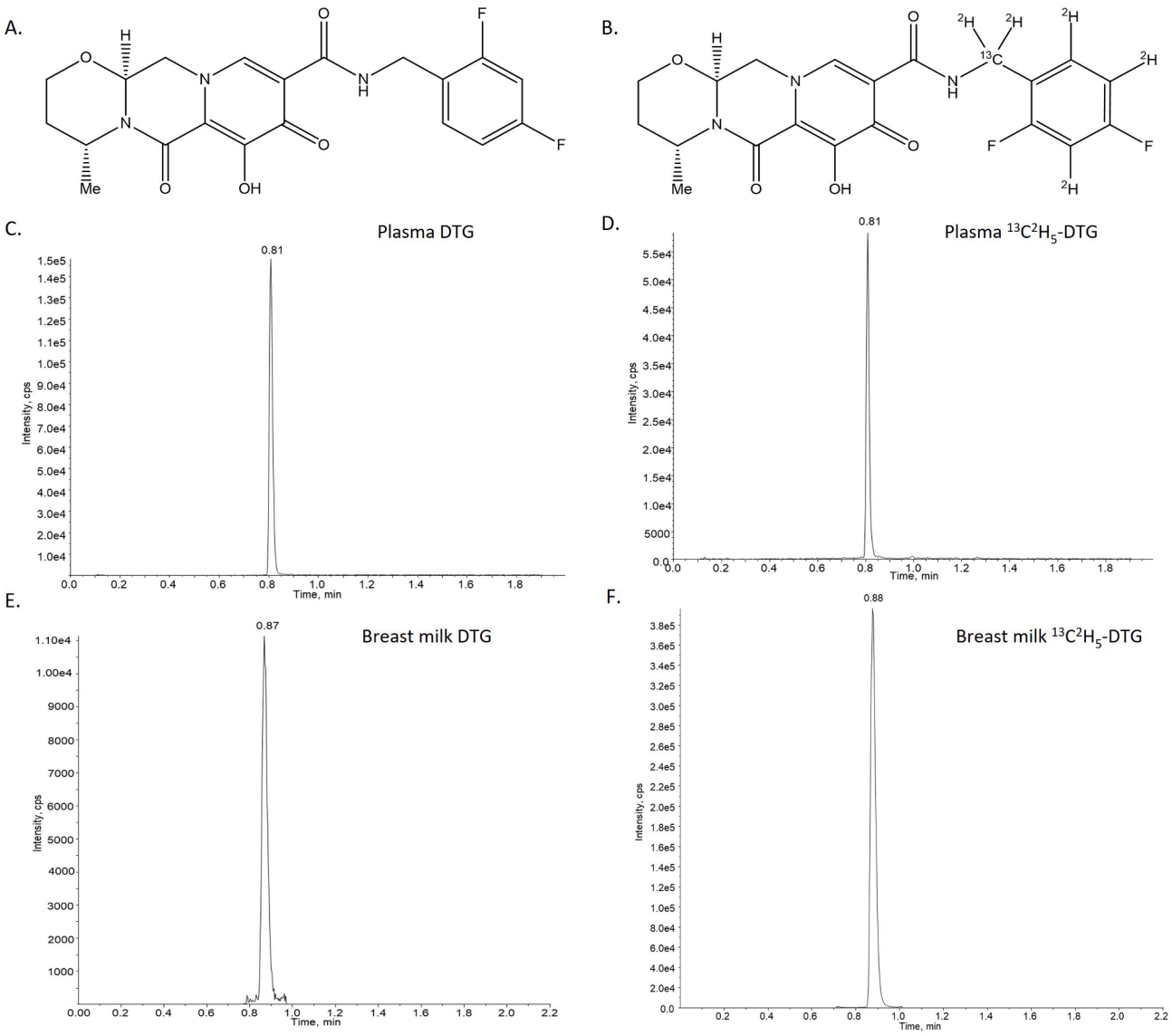

Fig. 1. Chemical structures of (A) Dolutegravir (DTG, $C_{20}H_{19}F_2N_3O_5$) and (B) $^{13}C^{2}H_5$-DTG (DTG-IS, $C_{19}{}^{13}CH_{14}D_5F_2N_3O_5$) are displayed. Representative chromatograms for low QC for plasma DTG (C), plasma DTG-IS (D), breast milk DTG (E), and breast milk DTG-IS (F) are shown. For low QC preparations, DTG was spiked into plasma at a concentration of 300 ng/mL and breast milk at a concentration of 1.5 ng/mL.

Breast milk DTG inter-day precision and accuracy ranged from 4.24 % to 12.4 % and −5.63 % to 7.49 %, respectively. Data are summarized in Table 1. Observed results meet the recommended FDA bioanalytical acceptance criteria.

3.3. Linearity, dilutional integrity, carryover, and cross talk

Given the dynamic range of DTG in both plasma and breast milk, calibration curves were generated using quadratic regression with $1/x^2$ weighting of analyte-to-internal standard peak area ratios. Representative standard curves are shown in Supplemental Fig. 1. For the plasma assay, DTG samples were diluted 1:4, 1:8, and 1:16 to assess dilutional integrity; diluted samples were within 10 % of theoretical concentrations. Similarly, breast milk samples were diluted 1:20 and 1:50 and met acceptability criteria (data not shown). Furthermore, there was no significant carryover or cross talk from analyte or internal standard in either matrix (data not shown).

3.4. Stability

Stability of DTG in K_2EDTA plasma and whole breast milk was evaluated under several conditions, including freeze–thaw, in-matrix, in whole blood (plasma assay), and long-term storage at ≤−70 °C. Data are summarized in Table 2. Under three freeze–thaw cycles at ≤−70 °C, DTG exhibited acceptable stability, with %DIFs ranging from −4.50 % to 6.21 % for plasma and −4.87 % to −0.116 % for breast milk when compared to freshly tested samples, respectively.

Sample matrix stability was assessed through room temperature incubation. Plasma samples were incubated for 72 h, while breast milk was analyzed after 83 h. When compared to freshly thawed and tested materials, the differences ranged from −3.01 % to 1.41 % for plasma and −2.18 % to 0.122 % for breast milk.

QC samples were maintained at ≤−70 °C for prolonged periods—865 days for plasma and 195 days for whole breast milk. Samples were quantified using freshly prepared calibration curves and compared against freshly prepared QCs; %DIFs ranged from −15.0 % to −10.7 % for plasma and −14.9 % to 10.9 % for breast milk. These data illustrate acceptable long-term stability of DTG in K_2EDTA plasma for up to 865

Table 1
Intra and inter assay precision and accuracy for DTG in plasma and breast milk.

Intra-assay Precision and Accuracy (n = 6)					
		Mean	SD	%CV	%Dev
Plasma	LLOQ (100 ng/mL)	92.4	2.61	2.82	−7.64
	Low (300 ng/mL)	280	6.81	2.43	−6.53
	Mid (1500 ng/mL)	1357	25.6	1.89	−9.52
	High (8500 ng/mL)	7937	112	1.41	−6.63
Breast milk	LLOQ (0.500 ng/mL)	0.451	0.0343	7.62	−9.80
	Low (1.50 ng/mL)	1.37	0.0327	2.38	−8.44
	Mid (75 ng/mL)	76.5	2.28	2.98	2.04
	High (800 ng/mL)	820	22.0	2.69	2.50
Inter-Assay Precision (n = 18)					
		Mean	SD	%CV	%Dev
Plasma	LLOQ (100 ng/mL)	89.4	3.05	3.41	−10.6
	Low (300 ng/mL)	284	7.94	2.80	−5.37
	Mid (1500 ng/mL)	1381	39.1	2.83	−7.92
	High (8500 ng/mL)	7958	218	2.73	−6.37
Breast milk	LLOQ (0.500 ng/mL)	0.533	0.0662	12.4	6.57
	Low (1.50 ng/mL)	1.42	0.0600	4.24	−5.63
	Mid (75 ng/mL)	72.7	4.76	6.55	−3.10
	High (800 ng/mL)	860	60.6	7.05	7.49

days and in whole breast milk for 195 days when stored at ≤−70 °C.

The stability of DTG was also evaluated in whole blood maintained at room temperature and under extended heat and humidity conditions. DTG demonstrated acceptable stability for up to 24 h in whole blood at room temperature and for up to 2 h under heat (40 °C) and 100 % humidity. The percent differences from spiked and immediately processed whole blood were −8.00 % to 0.527 % for whole blood maintained at room temperature for 24 h and −4.31 % to 6.01 % for 2 h under extended heat and humidity conditions (Table 3).

3.5. Matrix effects

Matrix effects were evaluated by examining peak areas of DTG and $^{13}C^{2}H_{5}$-DTG under unextracted, post-extracted, and pre-extracted conditions. Data are summarized in Table 4. In plasma, matrix effects ranged from 101 % to 108 % for DTG and 103 % to 106 % for $^{13}C^{2}H_{5}$-DTG, while the breast milk assay demonstrated matrix effects of 78.2 % to 99.3 % for DTG and 78.4 % to 100 % for $^{13}C^{2}H_{5}$-DTG. When considered individually, there is minor ion suppression in the breast milk assay; however, there is good agreement between DTG and the internal standard, indicating that there would be no significant matrix effects after normalization to $^{13}C^{2}H_{5}$-DTG.

Recovery efficiencies for the plasma assay ranged from 88.5 % to 95.0 % for DTG and 85.5 % to 89.1 % for $^{13}C^{2}H_{5}$-DTG. For the breast milk assay, recovery efficiencies were 84.9 % to 112 % for DTG and 88.2 % to 109 % for $^{13}C^{2}H_{5}$-DTG. This method can successfully isolate the analyte and internal standard from plasma and breast milk for accurate drug quantitation. Plasma assay performance was also assessed in the context of hemolyzed and lipemic samples, demonstrating that DTG can be accurately quantified in the presence of biological interferents (Supplemental Table 1).

3.6. Analysis of remnant samples

Remnant plasma samples were acquired from individuals prescribed DTG. DTG was quantifiable in 90 % of the samples, with concentrations ranging from 466 ng/mL to 2013 ng/mL (Table 5). One sample was unquantifiable. This observation is not attributed to a methodological limitation; rather, the unquantifiable result is expected as the sample was collected 24 days after discontinuation of DTG. These data support the capability of the assay to quantify DTG in plasma.

4. Discussion

To build upon existing tools to advance the study of DTG drug disposition and pharmacokinetics, we developed and validated methods to quantitate DTG in K_2EDTA plasma and whole breast milk. Both methods feature simple sample preparation workflows, dynamic analytical measuring ranges, and rapid run times of under 2.5 min. The assay met all evaluated acceptance criteria. Notably, minimal matrix effects were observed, and DTG recovery from K_2EDTA plasma and whole breast milk exceeded 80 %, demonstrating that protein precipitation was an appropriate sample preparation strategy for DTG isolation from these specimen sources. Furthermore, we established DTG stabilities of at least 865 days in plasma and 195 days in breast milk when stored at ≤−70 °C; these data support the use of stored samples to accurately determine DTG concentrations. The extensive stability studies conducted highlight the robustness of DTG under a variety of conditions and confirm that DTG is stable in both plasma and breast milk under challenged conditions.

Table 3
Whole blood stability of DTG.

Conditions	QC Level	Control Mean PAR[a]	Test Mean PAR	%Diff
24 h	Low QC	2.37	2.39	0.527
21–25 °C	Mid QC	11.3	10.7	−5.32
	High QC	56.3	51.8	−8.00
2 h	Low QC	2.37	2.52	6.01
40 °C	Mid QC	11.3	11.8	4.43
100 % humidity	High QC	56.3	53.9	−4.31

[a] PAR: Peak area ratio for DTG to $^{13}C^{2}H_{5}$-DTG.

Table 2
DTG stability in plasma and breast milk.

		Freeze Thaw[a]			Sample Matrix[b]			Long Term Stability[c]		
		Control Mean	Test Mean	%Diff	Control Mean	Test Mean	%Diff	Control Mean	Test Mean	%Diff
Plasma	Low (300 ng/mL)	289	307	6.21	289	293	1.41	339	288	−15.0
	Mid (1500 ng/mL)	1416	1424	0.572	1416	1423	0.553	1607	1432	−10.9
	High (8500 ng/mL)	7907	7551	−4.50	7907	7669	−3.01	9399	8392	−10.7
Breast milk	Low (1.50 ng/mL)	1.44	1.44	−0.116	1.53	1.50	−2.18	1.54	1.31	−14.9
	Mid (75.0 ng/mL)	74.2	70.6	−4.87	73	72.4	−0.845	77.6	86.0	10.9
	High (800 ng/mL)	860	820	−4.71	820	819	0.122	839	772	−8.05

[a] Freeze-thaw stability was established after three freeze thaw cycles for plasma and breast milk assays.
[b] Sample matrix stability for plasma was assessed after 72 h and 83 h for breast milk.
[c] Long term stability was assessed after 865 days for plasma and 195 days for breast milk.

Table 4
Matrix effects, recovery efficiency and processing efficiency for DTG in plasma and breast milk.

		% Matrix Effects		% Recovery Efficiency		% Processing Efficiency	
		DTG	$^{13}C^2H_5$-DTG	DTG	$^{13}C^2H_5$-DTG	DTG	$^{13}C^2H_5$-DTG
Plasma	Low (300 ng/mL)	108	104	95.0	89.1	103	92.3
	Mid (1500 ng/mL)	104	106	88.5	86.9	92.0	91.7
	High (8500 ng/mL)	101	103	90.9	85.5	92.1	88.2
Breast milk	Low (1.50 ng/mL)	78.2	78.4	112	109	87.8	85
	Mid (75.0 ng/mL)	99.3	100	84.9	88.2	84.3	88.2
	High (800 ng/mL)	87.8	85.9	99.8	101	87.7	86.8

DTG has a half-life of approximately 15 h, with most individuals achieving steady state after five days of treatment [25], with trough concentrations of 830 ng/mL and peak concentrations of 6200 ng/mL [18,19]. The DolPHIN-1 trial reported DTG breast milk concentrations of approximately 3 % of what is found in plasma [13]. The AMRs for the plasma and breast milk assays were designed to cover therapeutic ranges for individuals on DTG, while also providing a limit of quantitation that allows for examination of multi-compartment elimination parameters for future pharmacokinetic studies.

The DolPHIN trials and subsequent analyses were instrumental in informing maternal-fetal medicine for persons living with HIV. First, these trials demonstrated rapid viral suppression with DTG late in pregnancy, which is critical to preventing vertical transmission [10,12]. In addition, maternal plasma and breast milk were collected to characterize DTG pharmacokinetics during pregnancy and the postpartum period. They found that maternal plasma DTG concentrations were lower during pregnancy compared to the postpartum period and that DTG was distributed into breast milk [13]. Follow-up protein binding studies suggest that the decreased total DTG plasma concentrations may be a result of reduced albumin content during pregnancy; however, the free proportion of the drug is maintained, mitigating concerns regarding the therapeutic efficacy of DTG during pregnancy [26]. Data from the DolPHIN trials also indicate delayed clearance of DTG in infants [13]. DTG is extensively metabolized, primarily via glucuronidation by UGT1A1, although a minor pathway driven by CYP3A4 accounts for approximately 8 % of drug metabolism [25]. The difference in clearance is likely due to decreased expression of UGT1A1 in the neonatal period.

Several factors may contribute to interindividual variability in DTG pharmacokinetics. Genetic polymorphisms can result in altered metabolism; for example, decreased function of UGT1A1 may lead to impaired clearance and elevated DTG plasma levels in children and adults [27]. Other genes affecting DTG plasma concentrations include ABCG2, a member of the ATP-binding cassette family, and NR1I2, which is a steroid and xenobiotic transcription factor [27]. Interestingly, the effects of ABCG2 variants may differ in children compared to adults [28]. As DTG is a highly lipophilic compound, administration with a high-fat diet can increase its maximal plasma concentration by 20–30 % [29,30]. Although less common with INSTIs compared to other antiretrovirals, medication interactions should also be considered in the context of pharmacokinetics and pharmacodynamics. All of these variables contribute to the interindividual variability in DTG pharmacokinetics.

Although this work is limited by lack of assay application to breast milk samples, we have demonstrated performance in remnant plasma samples and yielded quantifiable drug concentrations. Furthermore, based on the partitioning of DTG into breast milk and our assay's AMR, there is high confidence that DTG can be quantified in breast milk from individuals using DTG. This work will enable future pharmacokinetic analyses, including the assessment of intra- and inter-individual variability in DTG distribution.

Herein, we demonstrate data for the development, validation, and applications of assays to quantify DTG in human plasma and breast milk. All experiments met acceptance criteria and were conducted in accordance with regulatory recommendations. These methods may now be applied to future clinical trials examining the pharmacokinetics of antiretroviral regimens, including those involving pregnant populations.

Table 5
Deidentified human K_2EDTA plasma from subjects prescribed DTG.

De-Identified Remnant Sample	Plasma DTG (ng/mL)
A-1	1554
A-2	1704
A-3	<LLOQ[a]
A-4	551
A-5	466
A-6	2013
A-7	655
A-8	786
A-9	1095
A-10	476

[a] Value obtained 24 days after DTG discontinuation.

Ethics statement

Testing of anonymized, remnant human plasma samples was performed in accordance with approval from our local institutional review board.

Funding

This work was supported in part by the HIV Prevention Trials Network Laboratory Center, sponsored by the National Institute of Allergy and Infectious Diseases (NIAID), the National Institute of Mental Health (NIMH), and the National Institute of Drug Abuse (NIDA), Office of AIDS Research, of the National Institutes of Health (NIH), Department of Health and Human Services (DHHS), grant UM1-AI068613. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. This work was also partially supported by the Johns Hopkins University Center for AIDS Research (CFAR; P30AI094189).

CRediT authorship contribution statement

Ashley R. Rackow: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Aashish Pandey:** Writing – review & editing, Validation, Methodology, Formal analysis. **Amelia L. Price:** Writing – review & editing, Validation, Formal analysis. **Mark A. Marzinke:** Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mark Marzinke reports financial support was provided by National Institutes of Health. If there are other authors, they declare that they have

no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2024.09.001.

References

[1] Center for Disease Control (CDC), Estimated HIV incidence and prevalence in the United States 2017–2021, Atlanta, GA, USA, 2023.

[2] M. Mahy, K. Marsh, K. Sabin, I. Wanyeki, J. Daher, P.D. Ghys, HIV estimates through 2018: data for decision-making, AIDS 33 (Suppl 3) (2019) S203–S211.

[3] R.M.S.M. Domingues, C.L. Szwarcwald, P.R.B. Souza, M. do C. Leal, Prenatal testing and prevalence of HIV infection during pregnancy: data from the "Birth in Brazil" study, a national hospital-based study, BMC Infect. Dis 15 (2015) 100.

[4] C.O. Ozim, R. Mahendran, M. Amalan, S. Puthussery, Prevalence of human immunodeficiency virus (HIV) among pregnant women in Nigeria: a systematic review and meta-analysis, BMJ Open 13 (2023) e050164.

[5] N. Wolter, S. Walaza, C. von Mollendorf, A. von Gottberg, S. Tempia, M. L. McMorrow, J. Moyes, F. Treurnicht, O. Hellferscee, M. Moleleki, M. Makhasi, N. Baute, C. Cohen, Association of HIV exposure and HIV infection with in-hospital mortality among hospitalized infants <1 year of age, South Africa, 2016–2018, J. Pediatric Infect. Dis. Soc. 12 (2023) 646–651.

[6] N. Yeganeh, D.H. Watts, J. Xu, T. Kerin, E.C. Joao, J.H. Pilotto, G. Theron, G. Gray, B. Santos, R. Fonseca, R. Kreitchmann, J. Pinto, M.M. Mussi-Pinhata, V. Veloso, M. Camarca, L. Mofenson, J. Moye, K. Nielsen-Saines, Infectious morbidity, mortality and nutrition in HIV-exposed, uninfected, formula-fed infants: results from the HPTN 040/PACTG 1043 trial, Pediatr. Infect. Dis. J. 37 (2018) 1271–1278.

[7] R.R. Lilian, E. Kalk, K.-G. Technau, G.G. Sherman, Birth diagnosis of HIV infection in infants to reduce infant mortality and monitor for elimination of mother-to-child transmission, Pediatr. Infect. Dis. J. 32 (2013) 1080–1085.

[8] A.L. Slogrove, A. Bovu, S. de Beer, F. Phelanyane, P.L. Williams, A. Heekes, E. Kalk, U. Mehta, G. Theron, E.J. Abrams, M.F. Cotton, L. Myer, M.-A. Davies, A. Boulle, Maternal and birth outcomes in pregnant people with and without HIV in the Western Cape, South Africa, AIDS 38 (2024) 59–67.

[9] P. Cahn, A.L. Pozniak, H. Mingrone, A. Shuldyakov, C. Brites, J.F. Andrade-Villanueva, G. Richmond, C.B. Buendia, J. Fourie, M. Ramgopal, D. Hagins, F. Felizarta, J. Madruga, T. Reuter, T. Newman, C.B. Small, J. Lombaard, B. Grinsztejn, D. Dorey, M. Underwood, S. Griffith, S. Min, extended SAILING Study Team, Dolutegravir versus raltegravir in antiretroviral-experienced, integrase-inhibitor-naive adults with HIV: week 48 results from the randomised, double-blind, non-inferiority SAILING study, Lancet 382 (2013) 700–708.

[10] K. Kintu, T.R. Malaba, J. Nakibuka, C. Papamichael, A. Colbers, K. Byrne, K. Seden, E.M. Hodel, T. Chen, A. Twimukye, J. Byamugisha, H. Reynolds, V. Watson, D. Burger, D. Wang, C. Waitt, M. Taegtmeyer, C. Orrell, M. Lamorde, L. Myer, S. Khoo, DolPHIN-2 Study Group, Dolutegravir versus efavirenz in women starting HIV therapy in late pregnancy (DolPHIN-2): an open-label, randomised controlled trial, Lancet HIV 7 (2020) e332–e339.

[11] Consolidated Guidelines on HIV Prevention, Testing, Treatment, Service Delivery and Monitoring: Recommendations for a Public Health Approach, World Health Organization, Geneva, 2021. http://www.ncbi.nlm.nih.gov/books/NBK572729/ (accessed June 3, 2024).

[12] C. Waitt, C. Orrell, S. Walimbwa, Y. Singh, K. Kintu, B. Simmons, J. Kaboggoza, M. Sihlangu, J.-A. Coombs, T. Malaba, J. Byamugisha, A. Amara, J. Gini, L. Else, C. Heiburg, E.M. Hodel, H. Reynolds, U. Mehta, P. Byakika-Kibwika, A. Hill, L. Myer, M. Lamorde, S. Khoo, Safety and pharmacokinetics of dolutegravir in pregnant mothers with HIV infection and their neonates: a randomised trial (DolPHIN-1 study), PLoS Med. 16 (2019) e1002895.

[13] L. Dickinson, S. Walimbwa, Y. Singh, J. Kaboggoza, K. Kintu, M. Sihlangu, J.-A. Coombs, T.R. Malaba, J. Byamugisha, H. Pertinez, A. Amara, J. Gini, L. Else, C. Heiberg, E.M. Hodel, H. Reynolds, L. Myer, C. Waitt, S. Khoo, M. Lamorde, C. Orrell, DolPHIN-1 Study Group, Infant exposure to dolutegravir through placental and breast milk transfer: a population pharmacokinetic analysis of DolPHIN-1, Clin. Infect. Dis. 73 (2021) e1200–e1207.

[14] C. Bennetto-Hood, G. Tabolt, P. Savina, E.P. Acosta, A sensitive HPLC-MS/MS method for the determination of dolutegravir in human plasma, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 945–946 (2014) 225–232.

[15] M. Grégoire, G. Deslandes, C. Renaud, R. Bouquié, C. Allavena, F. Raffi, P. Jolliet, E. Dailly, A liquid chromatography-tandem mass spectrometry assay for quantification of rilpivirine and dolutegravir in human plasma, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 971 (2014) 1–9.

[16] S.D. Penchala, S. Fawcett, L. Else, D. Egan, A. Amara, E. Elliot, E. Challenger, D. Back, M. Boffito, S. Khoo, The development and application of a novel LC-MS/MS method for the measurement of Dolutegravir, Elvitegravir and Cobicistat in human plasma, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1027 (2016) 174–180.

[17] J. Gini, S.D. Penchala, A. Amara, E. Challenger, D. Egan, C. Waitt, M. Lamorde, C. Orrell, L. Myer, S. Khoo, L.J. Else, Validation and clinical application of a novel LC-MS method for quantification of dolutegravir in breast milk, Bioanalysis 10 (2018) 1933–1945.

[18] S. Min, I. Song, J. Borland, S. Chen, Y. Lou, T. Fujiwara, S.C. Piscitelli, Pharmacokinetics and safety of S/GSK1349572, a next-generation HIV integrase inhibitor, in healthy volunteers, Antimicrob. Agents Chemother. 54 (2010) 254–258.

[19] S. Min, L. Sloan, E. DeJesus, T. Hawkins, L. McCurdy, I. Song, R. Stroder, S. Chen, M. Underwood, T. Fujiwara, S. Piscitelli, J. Lalezari, Antiviral activity, safety, and pharmacokinetics/pharmacodynamics of dolutegravir as 10-day monotherapy in HIV-1-infected adults, AIDS 25 (2011) 1737–1745.

[20] R. Kobbe, S. Schalkwijk, G. Dunay, J.M. Eberhard, U. Schulze-Sturm, B. Hollwitz, O. Degen, M. Teulen, A. Colbers, D. Burger, Dolutegravir in breast milk and maternal and infant plasma during breastfeeding, AIDS 30 (2016) 2731–2733.

[21] U.S. Department of Health and Human Services and FDA, Bioanalytical Method Validation Guidance for Industry, FDA, United States, 2022.

[22] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019–3030.

[23] L.A. Seserko, J.F. Emory, C.W. Hendrix, M.A. Marzinke, The development and validation of an UHPLC-MS/MS method for the rapid quantification of the antiretroviral agent dapivirine in human plasma, Bioanalysis 5 (2013) 2771–2783.

[24] M. Manohar, M.A. Marzinke, Validation and implementation of an ultrasensitive liquid chromatographic-tandem mass spectrometric (LC-MS/MS) assay for dapivirine quantitation in breast milk, Clin. Biochem. 82 (2020) 66–72.

[25] S. Castellino, L. Moss, D. Wagner, J. Borland, I. Song, S. Chen, Y. Lou, S.S. Min, I. Goljer, A. Culp, S.C. Piscitelli, P.M. Savina, Metabolism, excretion, and mass balance of the HIV-1 integrase inhibitor dolutegravir in humans, Antimicrob. Agents Chemother. 57 (2013) 3536–3546.

[26] J.D. Momper, M. Nikanjam, B.M. Best, M. Mirochnick, E.V. Capparelli, T. R. Cressey, Brief report: dolutegravir plasma protein binding and unbound concentrations during pregnancy and postpartum, J. Acquir. Immune Defic. Syndr. 94 (2023) 332–336.

[27] E.R. Elliot, M. Neary, L. Else, S. Khoo, G. Moyle, D.F. Carr, X. Wang, M. Mcclure, M. Boffito, A. Owen, Genetic influence of ABCG2, UGT1A1 and NR1I2 on dolutegravir plasma pharmacokinetics, J. Antimicrob. Chemother. 75 (2020) 1259–1266.

[28] S.A. Spector, S.S. Brummel, A. Chang, A. Wiznia, T.D. Ruel, E.P. Acosta, for IMPAACT P1093 team, impact of genetic variants in ABCG2, NR1I2, and UGT1A1 on the pharmacokinetics of dolutegravir in children, J. Acquir. Immune Defic. Syndr. 95 (2024) 297–303.

[29] T.P. Dumitrescu, K. Peddiraju, C. Fu, K. Bakshi, S. Yu, Z. Zhang, A.R. Tenorio, C. Spancake, S. Joshi, A. Wolstenholme, K. Adkison, Bioequivalence and food effect assessment of 2 fixed-dose combination formulations of dolutegravir and lamivudine, Clin. Pharmacol. Drug Dev. 9 (2020) 189–202.

[30] S. Weller, S. Chen, J. Borland, P. Savina, B. Wynne, S.C. Piscitelli, Bioequivalence of a dolutegravir, abacavir, and lamivudine fixed-dose combination tablet and the effect of food, J. Acquir. Immune Defic. Syndr. 66 (2014) 393–398.