Development of a Rapid and Sensitive LC-MS/MS Assay for Dolutegravir Quantitation in Breast Milk

Ashley R. Rackow, Aashish Pandey, Mark A. Marzinke

Johns Hopkins University Baltimore, MD USA

ABSTRACT

Background:
The use of antiretroviral therapy (ART) in pregnant people living with HIV can prevent peri- and post-natal transmission of the virus. Dolutegravir (DTG) is an integrase strand transfer inhibitor that commonly used in ART regimens. DTG is a lipophilic molecule and may deposit in breast milk during the postpartum period[1,2]. Previous work has shown transplacental and breastfeeding-mediated transfer of DTG, resulting in neonatal and infant drug exposure[3]. However, DTG pharmacokinetics (PK) in breast milk are incompletely understood. Bioanalytical tools are required to understand the multi-compartment pharmacology and efficacy of DTG in preventing mother-to-child HIV transmission.

Methods:
- Breast milk was acquired from BioIVT (Hicksville, NY)
- DTG (TRC, Toronto, ON) and its isotopically labeled internal standard, ^{13}C, $^{2}H_5$-DTG (Alsachim, Illkirch, FR) were spiked into breast milk
- 30 µL drug-spiked breast milk was subjected to protein precipitation with 500 µL of acetonitrile in a 96-well Captiva plate (Waters, Milford, MA)
- Eluted materials were evaporated and reconstituted in 100 µL of 0.1% formic acid in water
- 3 µL of reconstituted material was subjected to LC-MS/MS analysis on a API 5500 (SCIEX, Redwood City, CA) QTRAP interfaced with an LC-40 system (Shimadzu, Kyoto, JN).
- Assay was validated in accordance with FDA Bioanalytical Method Validation, Guidance for Industry, recommendations

Results:
The observed elution time for DPV is 0.87 minutes and the calibration curve was fit using a weighted $1/x^2$ quadratic regression analysis. The analytical measuring range was 0.500-1,000 ng/mL. Quality control (QC) materials prepared at the lower limit of quantitation (LLOQ), low, mid and high QC levels yielded intra- and inter-assay coefficients of variation (%CVs) ranging from 2.08 to 14.6% and 2.89 to 11.6%, respectively. Intra and inter-assay accuracies ranged from -11.1 to 12.7% and -4.06 to 10.3%, respectively. Stability challenges demonstrated <15% difference between control and treated samples. Selectivity and matrix effects analyses[4] were also acceptable. The method was validated in accordance with US FDA Guidance for Industry, Bioanalytical Method Validation recommendations.

Conclusion:
A robust analytical LC-MS/MS method was developed and validated to quantify DTG in human breast milk.

EXPERIMENTAL DESIGN AND WORKFLOW

Sample Preparation

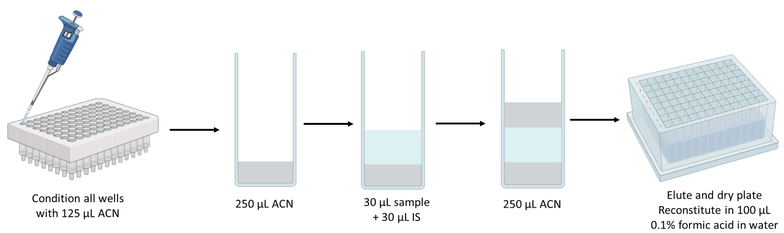

Figure 1. Schematic of Sample Preparation. DTG was extracted from human breast milk by solid-phase extraction. Breast milk was equilibrated at room temperature for 30 minutes. A Captiva plate was conditioned with 150 µL acetonitrile; 250 µL acetonitrile was then pipetted into the filter plate, followed by 30 µL breast milk and 30 µL internal standard followed by 250 µL of acetonitrile. The mixture was incubated for 5 minutes prior to elution into a deep well 96-well plate. The plate was evaporated until completely dry and resuspended in 0.1% formic acid in water; 3 µL was injected for analysis.

Method Parameters

Liquid Chromatography Conditions	
Instrument	Shimadzu LC-40 LC System
Column	Acquity UPLC BEH C8 1.7 µm, 2.1 x 50 mm
Injection Volume	3 µL
Mobile Phase A	0.1% Formic Acid in Water
Mobile Phase B	0.1% Formic Acid in Acetonitrile
Flow Rate	0.550 mL/min
Retention Time	0.860 min

Mass Spectrometry Conditions	
Instrument	SCIEX QTRAP 5500
Scan Type	Scheduled MRM
MRM Detection Window	12 s
Dwell Time	50 ms
Polarity	Positive
Ion Source	Turbo Spray
Analyte transitions	420.2→277.1 (Quant) 420.2→127.1 (Qual)
IS transition	426.2→133.1 (Quant)

***Table 1.* Liquid chromatographic conditions.** Separation was carried out using a Waters BEH-C8 UPLC column (1.7 µm, 50 x 2.1 mm) through a gradient elution (Mobile Phase A: 0.1% Formic Acid in Water; Mobile Phase B: 0.1% Formic Acid in Acetonitrile) using scheduled MRM.

RESULTS

Chromatographic Separation

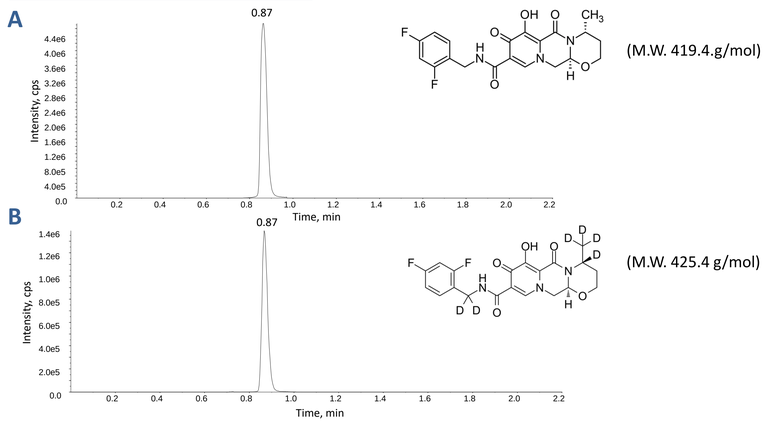

Figure 2. Chromatographic separation of DTG and Internal Standard. Chromatograms of (A) DTG (B) $^{2}H_6$-DTG. Ion transitions monitored for DTG and DTG-IS are *m/z* 420.2→277.1 and *m/z* 426.2→133.1, respectively.

Calibration Curve

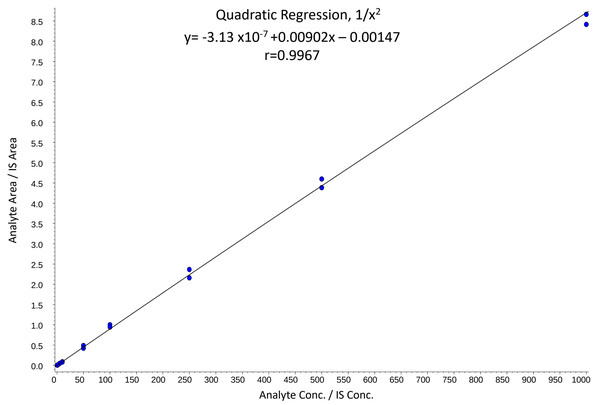

Figure 3. Representative calibration curve of DTG from human breast milk. The analytical measuring range of the assay is 0.500 pg/ml to 1000 pg/ml for DTG. The curve is fit using 9 calibrators which span the analytical measuring range using a quadratic regression with $1/x^2$ weighting.

Precision and Accuracy

A

QC Level (ng/mL)	Mean (ng/mL)	Standard Deviation (ng/mL)	Precision: %CV	Accuracy: % Deviation
LLOQ (0.500)	0.451	0.0343	7.62	-9.80
Low (1.50)	1.37	0.0327	2.38	-8.44
Mid (75.0)	76.5	2.28	2.98	2.04
High (800)	820	22.0	2.69	2.50

B

QC Level (ng/mL)	Mean (ng/mL)	Standard Deviation (ng/mL)	Precision: %CV	Accuracy: % Deviation
LLOQ (0.500)	0.533	0.066	12.4	6.57
Low (1.50)	1.42	0.060	4.24	-5.63
Mid (75.0)	72.7	4.76	6.55	-3.10
High (800)	860	60.6	7.05	7.49

Table 2. Intra- and inter-assay precision and accuracy studies for DTG in breast milk.
Panel A is the intra-assay study, based on n=6 replicates on each analytical run.
Panel B is the inter-assay study, based on n=18 replicates across 3 separate runs.

Stability Studies

	Freeze Thaw Stability			Sample Matrix Stability			Injection Matrix Stability		
QC Levels	Control Mean (ng/mL)	Treated Mean (ng/mL)	% Difference	Control Mean (ng/mL)	Treated Mean (ng/mL)	% Difference	Control Mean (ng/mL)	Treated Mean (ng/mL)	% Difference
Low (1.50)	1.44	1.44	-0.116	1.53	1.50	-2.18	1.37	1.44	5.61
Mid (75.0)	74.2	70.6	-4.87	73.0	72.4	-0.845	68.5	70.6	3.07
High (800)	860	820	-4.71	820	819	0.122	792	830	4.78

Table 3. Stability Studies. Stability challenges for DTG based on n=6 replicates on each analytical run; % Difference = Difference between control and treatment mean.
- Freeze Thaw Stability: 3 cycles
- Sample Matrix Stability: 83 hours at room temperature
- Injection Matrix Stability: 6 days at 4°C after initial injection

Challenge samples are within ±15% of freshly prepared and analyzed specimens.

Matrix Effects

	Matrix Effects (%)[a]		Recovery Efficiency (%)[b]		Processing Efficiency (%)[c]	
QC Levels	DTG	DTG-IS	DTG	DTG-IS	DTG	DTG-IS
Low	78.2	78.4	112	109	87.8	85.0
Mid	99.3	100	84.9	88.2	84.3	88.2
High	87.8	85.9	99.8	101	87.7	86.8

*[a] % M% = Peak area of (post-extracted samples/un-extracted samples) * 100*
*[b] % RE = Peak area of (pre-extracted samples/post-extracted samples) * 100*
*[c] % PE = Peak area of (pre-extracted samples/un-extracted samples) * 100*

Table 4. Matrix Effects. Matrix effects, as well as extraction efficiency and processing efficiency were determined by the approach of Matuszewski and colleagues[4]. Ion suppression is observed for both analyte and internal standard, particularly at the lower end of the analytical measuring range. While absolute matrix effects are exhibited, the relative matrix effects are negligible, thereby having minimal impact on the accurate quantification of DTG in breast milk.

CONCLUSION

A robust analytical LC-MS/MS method was developed and validated to quantify DTG in human breast milk. The developed method will be useful in supporting clinical trials to better understand multicompartment pharmacokinetics in nursing mothers.

REFERENCES

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Contact: Ashley Rackow (arackow1@jhu.edu)