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A SENSITIVE LC-MS/MS METHOD FOR QUANTITATIVE ESTIMATION OF DOLUTEGRAVIR IN HUMAN PLASMA

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ABSTRACT

A sensitive Liquid chromatrography tandem mass spectroscopy method for quantitative estimation of Dolutegravir (DTG) in human plasma was developed and successfully validated. Dolutegravir belong to anti-retroviral class which is integrase strand transfer inhibitor (INSTI) which blocks the functioning of HIV (Human Immuno Virus) integrase which is needed for viral replication. Dolutegravir and the Internal Standard Dolutegravir D6 was extracted from K3EDTA based Human Plasma samples by Solid Phase extraction procedure and processed samples were then subjected to analysis by Phenomenex Luna C18 (2), 50×4.6 mm, 5µm column using Acetonitrile: Water (80:20 v/v) containing 0.1 mL of Formic Acid as Mobile Phase at a flow rate of 1.0000 ml/min with 80% flow splitting. The retention time of Dolutegravir and Dolutegravir D6 was found to be 0.60 minute and 0.59 minute respectively. The standard curve was linear (R^2 > 0.99) over the concentration range of 28.44 ng/mL to 7054.37ng/mL. All the bioanlaytical validation parameters were determined as per US FDA guidelines. The developed bioanalytical method was sensitive and reliable as sensitivity, selectivity, matrix effect and other method validation parameters were falls under the acceptance criteria as stated by guidelines. The peaks obtained for the Dolutegravir and Dolutegravir D6 were well resolved from each other without any interferences from human plasma. The presented work provides a validated bioanalytical method for determination of Dolutegravir and it can be successfully applied to clinical research samples as part of phase I/II clinical studies.

KEYWORDS: HIV, Integrase inhibitor, LC-MS/MS, Dolutegravir, Internal standard, etc.

1. INTRODUCTION

Dolutegravir (Fig. 1) is a newly developed anti-viral drug from ViiV Healthcare (Research Triangle Park, NC, USA). Dolutegravir belong to anti-retroviral class which is a integrase strand transfer inhibitor (INSTI) which blocks the functioning of HIV (Human Immuno Virus) integrase which is needed for viral replication. Dolutegravir does not require ritonavir for inhibition of P450 3A4 and block the strand transfer step of viral genome integration into the host cell's DNA.[1] Dolutegravir inhibits the binding of the integrase viral DNA complex to host cell DNA by means of chelating Mg2+ ions at the active site.[2] When the integration is blocked, HIV can no longer replicate and results in interruption of viral replication cycle. In phase II studies, dolutegravir shown to be highly effective at rapid decrease in viral burden with a subsequent increase in CD4 cell count in treatment naïve patients receiving, 10 or 50 mg once daily in combination with a nucleoside reverse transcriptase inhibitor backround. Dolutegravir with dual nucleoside reverse transcriptase inhibitors (NRTIs) has been shown sustained antiviral activity in treatment naïve subjects in phase III studies. Dolutegravir was also shown to be superior as compared to raltegravir as a part of combination regimen in treatment experienced subjects. This translates into clinical Observations indicating dolutegravir activity in patients having resistance to raltegravir.[3] Dolutegravir has been well tolerated in phase III trails with a very low incidence of adverse effects due to discontinuation. Insomnia and headache were the most common adverse effects of moderate to severe intensity in these trails. The primary rote of metabolism of dolutegravir is glucuronidation via UDP-glucoronosyl transferase 1A1 without inhibition or induction of cytochrome P450 enzymes. Due to lack of significant interactions with other antiretroviral agents, dolutegravir will be administered as a part of a multi-drug regimen. Dolutegravir having an excellent safety and tolerability profile and predictable pharmacokinetic profile with low to moderate inter subject variability. Dolutegravir has the potential for treatment experienced patients but also it will become a first line antiretroviral agent.

Therefore, to promote further understanding of dolutegravir plasma pharmacokinetics, we have developed a method for dolutegravir using solid phase extraction and liquid chromatography coupled with tandem mass spectrometry detection in order to measure concentration of drug in human plasma. Presented work describes bioanalytical method for estimation of stated drug in human plasma in presence of concomitant Medication (Tenofovir and Lamivudine), as combination therapy is mostly preferred in the treatment of AIDS

because no single drug is that much effective. On existing literature we didn't found any developed bioanalytical method for estimation of dolutegravir in presence of tenofovir & lamivudine. Both of these drugs are the best option for combination therapy due to their several advantages. This serve as a novelty of the presented research work.

2. EXPERIMENTAL

2.1 Principle of the method

Dolutegravir and the Internal Standard Dolutegravir D6 was extracted from K3EDTA based Human Plasma samples by Solid Phase extraction procedure and processed samples were then subjected to analysis using Mobile Phase **:** Acetonitrile: Water (80**:**20 v/v) Containing 0.1 mL of Formic Acid as Mobile Phase. Quantitation was done by *'Peak area ratio method'.* All regression and values in the validation procedure were generated by **'***Analyst Software'.*

2.2 Metabolite information

Quantification of Dolutegravir from plasma samples was done using LC/MS/MS instrument. In this LC/MS/MS method Multiple Reaction Monitoring (MRM) mode was used for the analysis. This MRM mode of analysis is very specific to molecule and analyzes the compound on the basis of fragmentation pattern i.e., parent ion (Q1) to daughter ion/s (Q3). In our analytical method MRM used was 420.2/277.2 amu in positive polarity mode which is very specific to Dolutegravir.

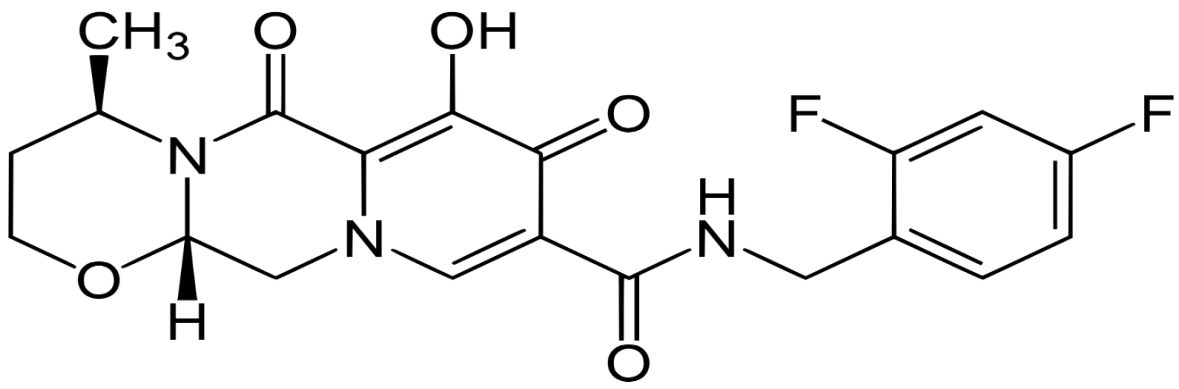

Fig. 1: Dolutegravir (Molecular weight: 419.385 g/mol).

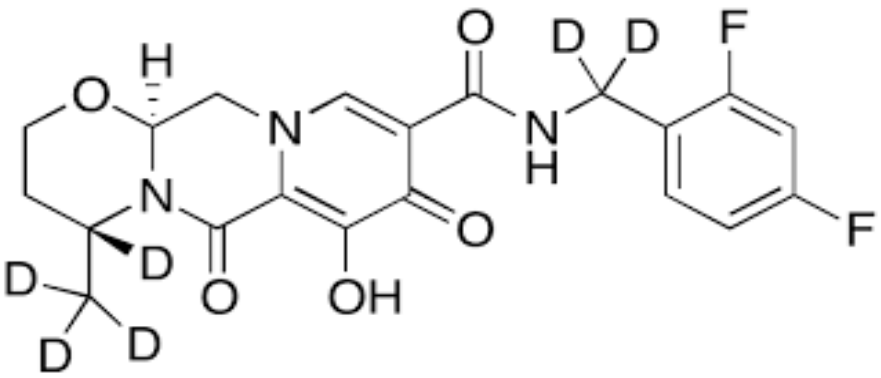

Fig. 2: Dolutegravir D6 (Molecular weight: 425.385 g/mol).

2.3 Reagents and Preparation of solutions

All the solvents, including LC-MS grade methanol and acetonitrile were obtained from Fisher Scientific. Formic acid (92%) was also obtained from Fisher Scientific. Vivan Life science provided both Dolutegravir and dolutegravir D6 which serve as the internal standard (DTG-IS). Multiple lots of K3EDTA plasma were obtained from Biological specialities corporation. Water was purified on-site using Milli-Q water purification system from Millipore Corporation.

Solution preparation

2.3.1 **0.1% Formic acid solution:** 1mL formic acid + water upto 1000 mL, mixed and sonicated.

2.3.2 **Mobile Phase:** 1600 mL of Acetonitrile + 400 mL of water containing 2 mL of Formic Acid.

2.3.3 **Diluent/Rinsing Solution:** 500 mL of methanol + 500 mL of water, mixed.

2.3.4 **5% Methanol Solution:** 50 mL of methanol and volume made upto 1000 mL with water.

2.4 Biological matrix

Biological matrix was K3EDTA based Human Plasma which was procured from M/s Srinivasa clinical Lab, Hyderabad. Whole Blood used for stability experiment was collected in In-house Clinical Laboratory.

2.5 Precautions

Dolutegravir is known to be light sensitive molecule and hence all the activities were done under Sodium Vapour lamp.

2.6 Instrumentation and software

Shimadzu Integrated UFLC-XR system (consisting of a CMB-20A Lite controller, LC-30AD pump, a DGU-20A_{5R} degasser, a SIL-30AC_{MP} autosampler, and a CTO-20AC column oven) coupled with an AB Sciex Linear Ion Trap Quadrupole 5500 were utilized for the separation and detection of Dolutegravir and Dolutegravir internal standard. Both the UFLC and mass spectrometer were controlled remotely using Analyst software v. 1.6.3. All statistical calculations were performed using Microsoft Excel capabilities.

Table 1: Summary of MS/MS parameters optimized for dolutegravir detection.

Sr. no.	Parameter	Details		
1.	MS transition	Parameters	Dolutegravir	Dolutegravir D6
		Polarity	+ve	+ve
		DP (volts)	110.00	110.00
		EP (volts)	6.32	11.35
		CE (volts)	38.00	37.66
		CXP (volts)	9.59	8.93
		Dwell Time (msec)	200.00	200.00
2.	Source dependent parameters	CUR : 30.00 IS (volts) : 5500.00 TEM (^{0}C) : 550.00 GS1 : 40.00 GS2 : 60.00 CAD : 7.00		

2.7 UPLC separation and MS-MS detection

All samples were subjected to chromatographic separation using a Shimadzu integrated UFLC-XR system with Phenomenex Luna C18(2), 50×4.6 mm, 5µm column. Chromatographic analyses were performed isocratically at 30^{0}C and at a flow rate of 1.0000 mL/min, with an overall run time of 2.00 minute. The mobile phase was composed of Acetonitrile: Water (80**:**20 v/v) Containing 0.1 mL of Formic Acid. Samples are maintained at 5^{0}C in the autosampler with a 5 µL aliquot of each sample being injected onto the column. The needle was washed before sample aspiration with Methanol: Water (50:50 v/v), to minimize carryover between injections. Dolutegravir and Dolutegravir-IS eluted from the column at approximately 0.60 min, and were detected using multi reaction monitoring (MRM). The API 4500 instrument was used in positive TurbolonSpray mode and the following transitions for protonated products $[M+H]^+$ were monitored and acquired: m/z Dolutegravir, 420.000 →277.200; m/z Dolutegravir-IS 426.200 →127.100. Settings for the individual mass spectrometer parameters are listed in Table 1. Traces correlating to the above

transitions were integrated using the MultiQuant software and concentration values were obtained using Dolutegravir to Dolutegravir-IS peak area ratio.

2.8 Stock solutions, working solutions, plasma calibration and control samples

2.8.1 Dolutegravir stock solution (For calibration curve standards)

5.054 mg of Dolutegravir was weighed and dissolved in 5 mL of Methanol. Final concentration of Dolutegravir Stock Solution was 1007767.60ng/mL by considering % Purity as 99.70%.

2.8.2 Dolutegravir stock solution (for quality control)

5.045 mg of Dolutegravir was weighed and dissolved in 5 mL of Methanol. Final concentration of Dolutegravir Stock Solution was 1005973.00ng/mL by considering % Purity as 99.70%.

2.8.3 Dolutegravir intermediate solution (For quality control samples)

2.500 mL of Dolutegravir Stock Solution was taken and volume was made up to 10 mL with diluent. Final concentration of Dolutegravir Intermediate Solution was 251493.25ng/mL.

2.8.4 Lamivudine stock solution

2.017 mg of Lamivudine working standard was weighed and transferred into 2 mL volumetric flask. About 1 mL of Methanol was added and sonicated to dissolve Lamivudine. The volume was made upto 2 mL with Methanol. Final concentration of Lamivudine .Stock Solution was 1003760.05ng/mL by considering % Purity as 99.53%.

2.8.5 Tenofovir stock solution

2.023 mg of Tenofovir working standard was weighed and transferred into 2 mL volumetric flask. About 1 mL of Methanol was added and sonicated to dissolve Tenofovir. The volume was made upto 2 mL with Methanol. Final concentration of Tenofovir. Stock Solution was 1001991.90ng/mL by considering % Purity as 99.06%.

2.8.6 Preparation of spiking solution

Using respective Dolutegravir Stock Solutions and Diluent, the Spiking Solutions of Calibration Curve Standards and Quality Control Samples were prepared as given in Table below.

Table 2: Preparation of spiking solutions for calibration curve standards.

Parent solution ID	Concentration of parent solution (ng/mL)	Volume of parent solution (mL)	Volume of Diluent (mL)	Final Volume (mL)	Concentration of spiking solution (ng/mL)	Spiking solution ID
Dolutegravir stock solution (CC)	1007767.60	1.400	8.600	10.000	141087.46	SS STD I
SS STD I	141087.46	7.000	3.000	10.000	98761.22	SS STD H
SS STD H	98761.22	6.000	4.000	10.000	59256.73	SS STD G
SS STD G	59256.73	6.000	4.000	10.000	35554.04	SS STD F
SS STD F	35554.04	5.000	5.000	10.000	17777.02	SS STD E
SS STD E	17777.02	4.000	6.000	10.000	7110.81	SS STD D
SS STD D	7110.81	4.000	6.000	10.000	2844.32	SS STD C
SS STD C	2844.32	4.000	6.000	10.000	1137.73	SS STD B
SS STD B	1137.73	5.000	5.000	10.000	568.87	SS STD A
SS STD A	0.00	10.000	0.000	10.000	0.00	DILUENT

Table 3: Preparation of spiking solutions for quality control samples of dolutegravir in presence of tenofovir and lamivudine.

Concentration of Dolutegravir Intermediate Solution	Volume of Dolutegravir Intermediate Solution (mL)	Volume of Lamivudine Stock solution (mL)	Volume of Tenofovir Stock Solution (mL)	Volume of Diluent (mL)	Final Volume (mL)	Concentration of Dolutegravir in Spiking Solution (ng/mL)	Spiking Solution ID
251493.25	4.500	0.388	0.078	5.034	10.00	113171.96	SS HQC
	2.805	0.388	0.078	6.729	10.00	70543.86	SS MQC
	0.067	0.388	0.078	9.467	10.00	1685.00	SS LQC
	0.023	0.388	0.078	9.511	10.00	578.43	SS LLOQ

2.8.7 Preparation of Bulk spiked calibration curve standards and quality control samples

Table 4: Preparation of Bulk spiked calibration curve standards.

Spiking solution ID	Concentration of spiking solution of analyte (ng/mL)	Volume of spiking solution of analyte (mL)	Volume of plasma (mL)	Final volume (mL)	Final concentration (ng/mL)
SS STD I	141087.46	0.150	2.850	3.000	7054.37
SS STD H	98761.22	0.150	2.850	3.000	4938.06
SS STD G	59256.73	0.150	2.850	3.000	2962.84
SS STD F	35554.04	0.150	2.850	3.000	1777.70
SS STD E	17777.02	0.150	2.850	3.000	888.85
SS STD D	7110.81	0.150	2.850	3.000	355.54
SS STD C	2844.32	0.150	2.850	3.000	142.22
SS STD B	1137.73	0.150	2.850	3.000	56.89
SS STD A	568.87	0.150	2.850	3.000	28.44
Diluent	0.00	0.300	5.700	6.000	0.00

Table 5: Preparation of Bulk spiked quality control samples.

Spiking Solution ID	Concentration of Dolutegravir Spiking Solution (ng/mL)	Volume of Spiking solution (mL)	Volume of Plasma (mL)	Final Volume (mL)	Final Dolutegravir Concentration of Solution (ng/mL)	Quality control samples ID
SS HQC	113171.96	1.800	34.200	36.00	5658.60	SS HQC
SS MQC	70543.86	1.000	19.00	20.00	3527.19	SS MQC
SS LQC	1685.00	1.800	34.200	36.00	84.25	SS LQC
SS LLOQ	578.43	1.000	19.00	20.00	28.92	SS LLOQ

2.9 Sample preparation

The extraction of dolutegravir was carried out by using solid phase extraction technique. Procedure for extraction of Calibration curve standards and quality control samples described as follows:

200 µL of aliquot + 50 µL Internal standard + 500 µL of 0.1% formic acid subjected to solid phase extraction followed by conditioning with 1 mL of methanol, equilibration with 1 mL of water, followed by washing with 1 mL water and 1 mL 5% methanol subjected to drying for 5 minutes followed by elution with mobile phase (500 µL twice), finally sample was filled in autosampler vials for further analysis.

2.10 Data calculations

Chromatograms were acquired using the Data acquisition software. The concentrations of the samples were calculated using linear regression equation ($y = ax + b$) with $1/x^2$ weighing factor by Analyst software 1.6.3. The final print of the data was taken using Cred-Bio Application, version 1.0.2.215, Scientific Data Management System (SDMS) and these electronically generated printouts were used for further activities. Final concentration values for Analyte were reported in ng/mL. The Precision (% RSD), % Accuracy and % Bias were rounded to the 2nd decimal place.

2.11 Method validation

Method validation is a part of GLP study and it is to ensure the quality, reproducibility and reliability of the method. Developed method was validated as per US FDA guidelines for bioanalytical method validation.

2.11.1 Precision and Accuracy

The precision of the bioanalytical method describes the closeness of repeated individual measures of analyte. Precision is expressed in terms of Coefficient of variation (CV). While the accuracy of an analytical method describes the closeness of the determined value obtained by the method to the true concentration of analyte (expressed in percentage). Precision and Accuracy was performed at LLOQ QC, LQC, LMQC, MQC and HQC levels which were extracted as per extraction procedure. Inter batch and Intra-batch Precision and Accuracy experiments were done as a part of this validation exercise. A single Precision and Accuracy batch consisted of one set of Calibration Curve Standards and six replicates of extracted samples for each concentration of LLOQ QC, LQC, LMQC, MQC and HQC.

% RSD and % Nominal of back calculated concentration of analyte was calculated.

2.11.1.1 Intra-Day Precision and Accuracy

Intra-Day Precision and Accuracy experiment should be evaluated with Precision and Accuracy batches, which were processed separately on same analytical day.

2.11.1.2 Inter-Day Precision and Accuracy

Inter-Day Precision and Accuracy experiment should be evaluated with Precision and Accuracy batches, which were processed on different analytical days.

2.11.2 Sensitivity

Sensitivity is the ability of the method to detect Lower Limit of Quantification (LLOQ). The LLOQ is the lowest concentration of an analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision. Sensitivity was evaluated by injecting extracted samples in K3EDTA based Human Plasma from six different lots. The samples were spiked with spiking solution of STD A (LLOQ) and processed as per extraction procedure along with one Precision and Accuracy batch in K3EDTA based human plasma. Back calculated concentration was obtained from the software.

Signal to Noise ratio (S/N) was calculated for each sensitivity sample. The % RSD and % Nominal of back calculated concentration of Analyte at LLOQ level was calculated.

2.11.3 Calibration curve standards

A Calibration Curve is the relationship between the response of the instrument and known concentrations of the analyte. A sufficient number of concentration level should be used to adequately define the relationship between concentration and the analyte response. The

number of standards used in constructing a calibration curve should depends on the anticipated range of analytical concentration and the nature of the analyte response relationship. Calibration Curve Standards were prepared using pooled K3EDTA based Human Plasma. Calibration Curve Standards consisted of Blank sample (matrix sample processed without analyte and IS), Blank + IS (matrix sample processed with IS) and eight non-zero standards in the concentration range of 28.44ng/mL to 7054.37ng/mL.

Table 6: Range of calibration curve standards.

STD ID	Concentration in Plasma (ng/mL)
Blank	0.00
Blank + IS	0.00
STD A	28.44
STD B	56.89
STD C	142.22
STD D	355.54
STD E	888.85
STD F	1777.70
STD G	2962.84
STD H	4938.06
STD I	7054.37

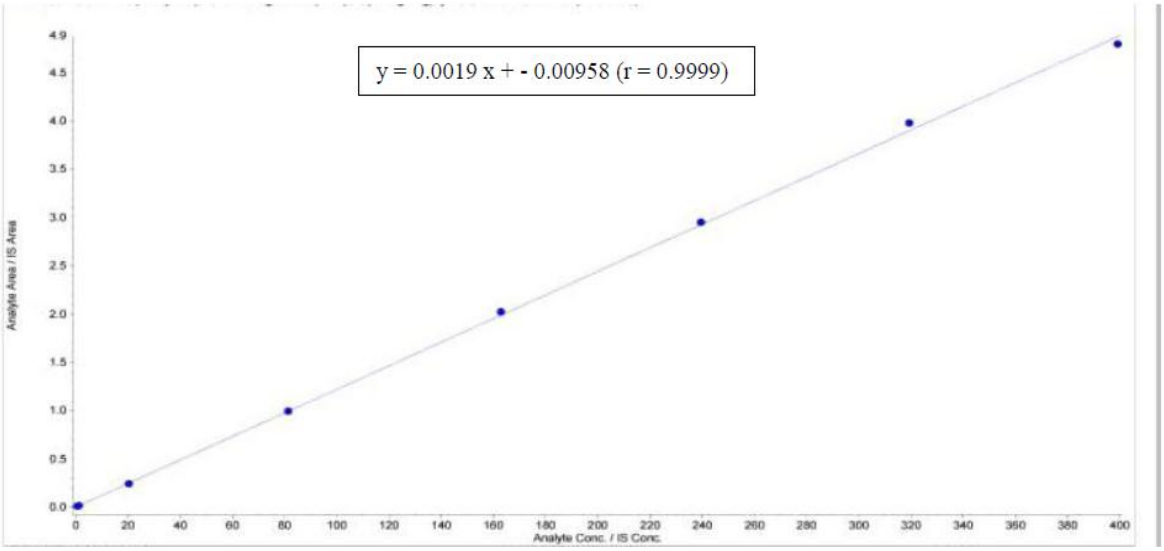

Fig. 3: Calibration curve.

Correlation Coefficient and % Nominal of back calculated concentration of analyte was calculated.

2.11.4 Autosampler carryover test

Autosampler carry over test should be performed to confirm that there is no carryover of analyte from the previous injection. If carry over is unavoidable specific measures should be taken during the validation.

Autosampler Carryover Test was carried out using Aqueous and Extracted samples and injecting in the sequence as given below. Working solutions of analyte were appropriately diluted to the AQ STD I and AQ STD A levels in the appropriate proportion and was subjected to analysis.

Sequence of autosampler carryover test samples

Aqueous: Mobile phase, STD I, Mobile phase, STD A

Extracted: Blank, STD I, Blank, STD A

The Carryover Response indicated by a peak in the Mobile Phase injection as well as in Blank Sample at the retention time of analyte and IS was evaluated.

2.11.5 Selectivity

Selectivity should be assessed to show that the intended analyte is measured and their quantitation is not affected by the presence of the endogenous matrix components such as metabolites, degradation products, etc.

Selectivity was carried out to evaluate the ability of the method to quantify the Analyte and IS from other plasma components after extraction. This was evaluated by injecting extracted Blank samples in K3EDTA based Human Plasma samples from six different lots and comparing the interference in the blank samples with the response of the respective extracted LLOQ sample. In addition, plasma evaluation was also done using one Haemolyzed lot and one Hyperlipidaemic lot. The samples were run at least three times of the run time.

Interferences were checked at the retention time of Analyte and IS.

2.11.6 Reinjection reproducibility

This exercise was done to assess any change occurring due to reacquisition of the same samples in the vials which were kept in the Autosampler at 5ºC. Reinjection Reproducibility was evaluated by preparing and extracting a set of Calibration Curve Standards and six replicates of LQC and HQC samples. These samples were subjected to LC/MS/MS analysis. After analysis, samples from the batch were kept in Autosampler at 5ºC for 16 hours and 45 minutes and then these samples were reanalyzed. After reanalysis, the results for the reanalysed Quality Control Samples were calculated using both the Calibration Curves

derived from initial analysis as well as reanalysed Calibration Curve Standards. The duration of Reinjection Reproducibility of analyte was calculated from the initial injection time of first LQC sample of initial batch to reinjection of the same LQC sample of reinjected batch.

A single run consisted of one set of Calibration Curve Standards and six (6) replicates of LQC and HQC samples. % Nominal and % RSD for LQC and HQC samples were calculated using both the initial and reanalysed Calibration Curve Standards.

2.11.7 Haemolysis effect

Haemolysis effect can be described as he rupture of red blood cells and the release of haemoglobin into the surrounding plasma. Plasma containing more than 2% of lysed blood is considered as Haemolysed plasma. Haemolysis constitute a special cause of matrix effect since certain compounds may behave differently in the presence of RBS's. This exercise should be done to assess the Haemolysis effect throughout the application of developed method. The Biological Matrix for this Bioanalytical method is K3EDTA based Human Plasma. This exercise was done to assess the Haemolysis effect throughout the application of this method. Haemolyzed matrix has a lot of inherent variability. Haemolysis can affect the response of Analyte during the method validation and subsequently in subject analysis. The quantification of Analyte from plasma can be grossly affected by a significant Haemolysis effect. For Haemolysis effect experiment, LQC and HQC concentration levels were spiked in Haemolyzed K3EDTA based Human Plasma (six individual sample preparations). These samples were extracted as per procedure along with one Precision and Accuracy batch in non Haemolyzed K3EDTA based Human Plasma. Back calculated concentrations were obtained from the software.

2.11.8 Lipemic effect

This exercise should be done to assess the Lipemic effect throughout the application of developed method. Matrix containing more than 300 mg/dL of triglycerides is considered as Lipemic matrix. Lipemic matrix has a lot of inherent variability and can affect the response of analyte during the method validation and subsequently in subject analysis. The quantification of analyte from plasma can be grossly affected by a significant Lipid content in sample. The biological matrix for this Bioanalytical method is K3EDTA based Human plasma. This exercise was done to assess the Lipemic effect throughout the application of this method. Lipemic matrix has a lot of inherent variability and can affect the response of Analyte during the Method Validation and subsequently in subject analysis. The quantification of Analyte

from plasma can be grossly affected by a significant Lipid content in sample. For Lipemic Effect experiment, LQC and HQC concentration levels were spiked in Lipemic plasma. These samples were extracted as per procedure along with one Precision and Accuracy batch prepared in non Lipemic plasma. The Back calculated concentrations were obtained from the software.

2.11.9 Matrix effect

Matrix effect results in ion suppression or ion enhancement and it is common in LC-MS bioanalysis. Matrix effect provide incorrect Observations affecting the result and performance of study. Biological matrix contains many components such as salts, phospholipids, proteins, etc. All of these substances are the main source of ion suppression in bioanalysis. The quantitative measure of matrix effect can be termed as matrix factor and defined as a ratio of the analyte peak response in the presence of matrix ion to the analyte peak response in the absence of matrix ion. In Matrix Effect experiment, the spiking solutions of LQC and HQC each were individually spiked in six normal plasma lots and were extracted along with one Precision and Accuracy batch prepared in pooled K3EDTA based Human plasma, as per procedure. Back calculated concentrations were obtained from the Calibration Curve after LC/MS/MS analysis.

2.11.10 Dilution integrity

Dilution of samples will affect the accuracy and precision. Dilution integrity is performed in order to check the validity of method in case the sample needs to be diluted during analysis and also to quantify values which are above the Upper Limit of Quantification (ULOQ). Dilution Integrity was performed to quantify values (concentrations) which are above the Upper Limit of Quantification (ULOQ). These samples cannot be accurately quantified using the Calibration Curve as per the validation Observations used in this Bioanalytical method. It may happen that some of the samples from the study subjects may have values greater than Upper Limit of Quantification (ULOQ). In such cases, the Calibration Curve cannot be extrapolated and hence, there is a need to dilute the samples appropriately so as to fit the concentration within the linear range to enable accurate quantitation. The final concentration is then calculated by considering the appropriate dilution factor, thus leading to an accurate quantitation of Dolutegravir in such plasma samples.

2.11.11 Stability

Drug stability in biological matrix is a function of the storage conditions, the chemical and physical properties of the drug, the matrix and container system. All stability determinations should use a set of comparison samples prepared or processed from a freshly prepared stock solution or stock solutions with proven stability of the analyte and internal standard in the appropriate analyte -free, interference-free biological matrix. Stability procedure should evaluate the stability of the analyte during collection of sample and handling, after short term and long term storage, after going through freeze thaw cycles. Following parameters were evaluated to study the stability aspects for this proposed Bioanalytical method to be used for Quantitation of Dolutegravir from K3EDTA based Human Plasma.

2.11.11.1 Short term stock solution stability

The Stock Solution Stability was evaluated upto 22 hours, 20 minutes for Dolutegravir and 24 hours, 02 minutes for Dolutegravir D6 at ambient temperature. Six aliquots of freshly prepared stock solutions of Analyte and IS were kept on the work bench at ambient temperature (Stability Stock Solutions). After desired stability period, fresh stock solutions of Analyte and IS were prepared (Fresh Stock Solutions). Thereafter, six solutions of concentration equivalent to Spiking Solution of MQC concentration level were prepared individually from six aliquots of Stability Stock Solution of Analyte. Likewise, six fresh solutions of concentration equivalent to Spiking Solution of MQC concentration level samples were prepared from Fresh Stock Solution of Analyte. In case of IS, six samples of concentration equivalent to spiking solution of IS were prepared individually from six aliquots of Stability Stock Solution. In addition, six samples of concentration equivalent to spiking solution of IS were prepared from Fresh Stock Solution. IS from the freshly prepared solution was compared with the area response of Analyte and IS in the Stability Solution. The duration of Short Term Stock Solution was calculated from the time of Stability Stock Solution preparation to Fresh Stock Solution preparation.

The % RSD of the area of Analyte and area of IS was calculated for the six consecutive injections. The % Difference in the area of Analyte and IS of the Stability Samples as compared to Fresh samples was calculated.

2.11.11.2 Long Term Stock Solution Stability at 2 to 8°C.

The Stock Solution Stability of Analyte and Internal Standard were evaluated in refrigerator at 2 to 8°C for 5 Days. Six aliquots of freshly prepared stock solutions of Analyte and IS

were kept in refrigerator at 2 to 8°C (Stability Stock Solutions). After relevant stability period, fresh stock solution of Analyte and IS was prepared (Fresh Stock Solutions). Thereafter, six solutions of concentration equivalent to Spiking Solution of MQC concentration level and spiking solution concentration of IS were prepared individually from six aliquots of Stability Stock Solution of Analyte and IS. Likewise six fresh solutions of concentration equivalent to Spiking Solution of MQC concentration level and spiking solution concentration of IS were prepared from Fresh Stock Solution of Analyte and IS respectively. The area response of the Analyte and IS from the freshly prepared solution was compared with area response of the Analyte and IS in the Stability Solution. The duration of Long Term Stock Solution Stability was calculated from the time of storage of Stability Stock Solution to the retrieval time of Stability Stock Solution from refrigerator.

2.11.11.3 Bench top stability

The Bench Top Stability was evaluated by analysing plasma samples containing Analyte at LQC and HQC concentration levels. These samples were kept on work bench at ambient temperature for 27 hours and 58 minutes (Stability Samples). Freshly prepared plasma samples containing Analyte at LQC and HQC concentration levels were subjected to extraction (Fresh Samples) along with the Stability Samples and one set of freshly prepared Calibration Curve Standards as per the extraction procedure.

The duration of Bench Top Stability of Analyte was calculated from the retrieval time of Stability Plasma Samples from the deep freezer to the start time of sample processing.

A single run consisted of one set of Calibration Curve Standards and six (6) samples each of LQC and HQC levels of Stability and Fresh samples. The % Difference, % RSD and % Nominal of back calculated concentrations of Analyte were calculated for the Stability and Fresh samples.

2.11.11.4 Freeze and Thaw stability

Six aliquots at each concentration level of LQC and HQC samples were exposed to five Freeze and Thaw cycles (Stability Samples) at -70^{0}C. Freshly prepared plasma samples (six preparations each) containing Analyte at LQC and HQC concentration levels (Fresh Samples) were subjected to extraction along with the Stability Samples and one set of freshly prepared Calibration Curve Standards as per the extraction procedure. The concentration of Fresh samples and Freeze and Thaw Stability Samples were determined from the Calibration Curve Observations. A single run consisted of one set of freshly prepared Calibration Curve

Standards and six (6) samples each of LQC and HQC levels of Stability and Fresh Samples. Analyte stability was determined after the fifth Freeze and Thaw cycle.

2.11.11.5 Autosampler stability

The Autosampler Stability was evaluated by keeping samples of LQC and HQC concentration levels (six preparations each) in the Autosampler for 29 hours and 17 minutes at 5ºC (Stability Samples). Freshly prepared plasma samples (six preparations each) containing Analyte at LQC and HQC concentration levels (Fresh Samples) were subjected to extraction along with one set of freshly prepared Calibration Curve Standards as per the extraction procedure. The Stability samples were then analyzed and compared with Fresh samples for stability evaluation. A single run consisted of one set of freshly prepared Calibration Curve Standards and six (6) samples each of LQC and HQC levels of Stability and Fresh Samples. The duration of Autosampler stability of Analyte was calculated from the Autosampler loading time of samples to the injection time of the First Stability sample.

2.11.11.6 Wet extract stability

The Wet Extract Stability was evaluated by extracting plasma samples containing analyte at LQC and HQC concentration levels (six preparations each) as per the procedure. These extracted samples were kept in the refrigerator for 27 hours and 48 minutes at 2 to 8ºC (Stability Samples). Freshly spiked plasma samples (six preparations each) containing Analyte at LQC and HQC concentration levels (Fresh Samples) were subjected to extraction along with one set of freshly prepared Calibration Curve Standards as per the extraction procedure. The Stability samples were then analyzed and compared with Fresh samples for stability evaluation.

2.11.11.7 Whole blood stability

Whole Blood Stability was evaluated by analyzing plasma samples containing Analyte at low (LQC) and high (HQC) concentration level samples (six preparations each). These samples were prepared by spiking 120 µL of SS LQC/ SS HQC in respective poly propylene tubes containing 2280 µL of Whole Blood and kept on work bench at ambient temperature for 02 hours and 28 minutes (Stability Samples). After 02 hours and 28 minutes, the Fresh samples were prepared by Spiking 120 µL of SS LQC/ SS HQC in respective pp tubes containing 2280 µL of whole blood. Fresh and stability samples were centrifuged at 4500 rpm at 4ºC for 10 minutes; plasma was separated and transferred in pre-labeled pp tubes (six aliquots each). These Plasma samples were then subjected to extraction as per the extraction procedure. The

area ratio of Analyte to Internal Standard from the freshly prepared samples was compared with area ratio of Analyte to Internal Standard of Stability Samples. The duration of Whole Blood Stability was calculated from the time of spiking of Stability Samples to the spiking of fresh Samples.

2.11.12 Ruggedness test

The ruggedness test was evaluated for different Analyst, different Column and different equipment. The Calibration Curve was generated using linear regression y = ax + b with weighting ($1/x^2$). A Precision and Accuracy batch was processed by different analyst, by using different column and different equipment.

2.11.13 Cross specificity

Cross specificity is carried out to evaluate the ability of the method to specifically quantify the analyte and IS in presence of other drugs. Cross Specificity was evaluated by injecting three replicates of pooled Blank plasma samples spiked with ULOQ concentration of Tenofovir and Lamivudine, along with one sample at LLOQ concentration for Dolutegravir. Interference was checked at the retention time of Analyte and IS.

3. RESULT AND DISCUSSION

3.1 Chromatography, detection and quantitation of dolutegravir

Initial optimization of the triple quadrupole mass spectrometer for the detection of an dolutegravir begins with optimizing for the parent compound, in this case dolutegravir, in the single-quadrupole mode. This step of optimization revealed a large peak at $[M+H]^+$ for DTG at m/z 420.00, which correlated with the expected molecular ion mass of dolutegravir. Five main product ions were found for DTG at m/z 277.2, 125.0, 138.0, 104.0 and 109.0 and mass spectrometer conditions were initially optimized for detection of dolutegravir (Table 1) and its three strongest product ions. Plasma samples were then spiked with DTG, extracted and reconstitution volume and product ions were optimized further. Ultimately, only a single channel each for DTG and DTG-IS (m/z 420.00→277.20) and (m/z 426.20→127.10) was chosen and used for quantitation (Fig. 3).

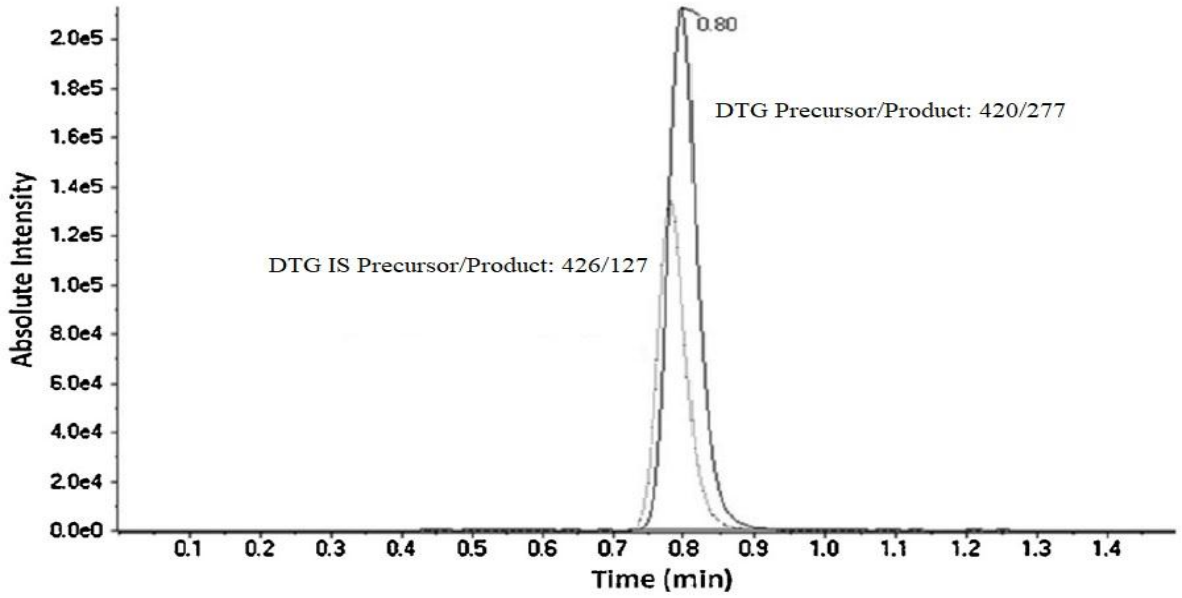

Figure 3: LC-MS/MS chromatogram of DTG and isotopic internal standard from plasma extract.

3.2 Method validation

Validation parameters were performed on different days. Each validation run contained Blank and Blank + IS samples, a full 9-point curve, plus six replicates each of LLOQ, LQC, MQC and HQC respectively.

3.2.1 Precision and Accuracy

Table 7: Observations of Inter-batch Precision and Accuracy.

QC ID	LLOQ QC	LQC	MQC	HQC
Nominal concentration (ng/mL)	28.92	84.25	3527.19	5658.60
Acceptance limit (ng/mL)	23.14 to 34.70	71.61 to 96.89	2998.11 to 4056.27	4809.81 to 6507.39
Mean (Back calculated concentration ng/mL)	27.99	77.62	3538.98	5598.77
SD	0.8347	1.7385	82.9300	163.1956
%RSD	2.98	2.24	2.34	2.91
%Nominal	96.78	92.13	100.33	98.94
%Bias	-3.22	-7.87	0.33	-1.06

Acceptance criteria: % RSD for LLOQ QC level should NMT 20.00%, % RSD for samples other than LLOQ QC should NMT 15.00%, % Bias for LLOQ QC level should ± 20.00%, % Bias for samples other than LLOQ QC should ± 15.00%, % Nominal for LLOQ QC level should 80.00% to 120.00%, % Nominal for samples other than LLOQ QC should 85.00% to 115.00%. At least 67% of total QC samples and 50% at each concentration level must comply with above mentioned criteria of % Nominal.

Result: The acceptance criteria were met.

Table 8: Intra-batch Precision and Accuracy.

QC ID	LLOQ QC	LQC	MQC	HQC
Nominal concentration (ng/mL)	28.92	84.25	3527.19	5658.60
Acceptance limit (ng/mL)	23.14 to 34.70	71.61 to 96.89	2998.11 to 4056.27	4809.81 to 6507.39
Mean (Back calculated concentration ng/mL)	28.08	79.24	3618.85	5475.35
SD	0.7371	1.1573	81.4967	88.9671
%RSD	2.63	1.46	2.25	1.62
%Nominal	97.10	94.05	102.60	96.76
%Bias	-2.90	-5.95	2.60	-3.24

Result: The acceptance criteria were met.

3.2.2 Sensitivity

Table 9: Observations of sensitivity.

Sample ID	LLOQ	
Nominal concentration (ng/mL)	28.44	
Acceptance limit (ng/mL)	22.75 to 34.13	
Sr. No.	Back calculated concentration (ng/mL)	Signal to noise ratio
1.	27.05	1088.07
2.	26.26	879.63
3.	28.05	1169.48
4.	27.69	1813.80
5.	26.86	3473.36
6.	27.96	4314.68
Mean	27.31	
SD	0.7053	
%RSD	2.58	
%Nominal	96.03	

Acceptance criteria: Precision and Accuracy batch acceptance criteria must be met.

Result: The acceptance criteria were met.

3.2.3 Calibration curve standards.

Table 10: Details of freshly spiked calibration curve standards.

STD ID	STD A	STD B	STD C	STD D	STD E	STD F	STD G	STD H	STD I
Nominal concentration (ng/mL)	28.47	56.83	142.08	355.19	887.97	1755.94	2959.91	4933.18	7047.39
Acceptance Limit (ng/mL)	22.78 to 34.16	48.31 to 65.35	120.77 to 163.39	301.91 to 408.47	754.77 to 1021.17	1509.55 to 2042.33	2515.92 to 3403.90	4193.20 to 5673.16	5990.28 to 8104.50
Back calculated concentration (ng/mL)	28.47	57.13	140.29	53.20	894.88	1796.97	2976.20	4996.72	6871.65
%Nominal	99.99	100.53	98.74	99.44	100.78	101.18	100.55	101.29	97.51
Correlation Coefficient	0.9999								

Acceptance Criteria: % Nominal at STD A level should 80.00% to 120.00%, % Nominal at standards other than STD A should 85.00% to 115.00%, Correlation Coefficient should ≥ 0.99, At least seven out of nine non-zero standards (at least 75%) must meet the above criteria, including STD A and STD I.

Result: Calibration Curve Standards were found within the acceptance criteria.

3.2.4 Autosampler carryover test.

Table 11: Observations of autosampler carryover test.

Sr. No.	Autosampler Carry Over Test Samples		Area observed at RT of Analyte	Area observed at RT of IS	% Carryover of IS	% Carryover of Analyte
1.	Aqueous (AQ)	Mobile phase	584	0	0.00	0.00
2.		AQ STD	23184813	2018400		
3.		Mobile phase	1093	0		
4.		AQ STD A	117354	2158259		
5.	Extracted	Blank	0	0	0.00	0.00
6.		STD I	18379645	1853715		
7.		Blank	486	0		
8.		STD A	84842	1951359		

Acceptance criteria

If any peak is present at the retention time of the Analyte in Mobile Phase injection or Blank sample, its area response must be < 20.00% of Analyte area response of Aqueous STD A or Extracted STD A respectively.

If any peak is present at the retention time of the IS in Mobile Phase injection or Blank sample, its area response must be < 5.00% of IS area response of an Aqueous STD A or Extracted STD A respectively.

Result: Carryover observed in Blank at RT of Dolutegravir was within acceptance criteria and No Carryover observed at retention time of Dolutegravir D6.

3.2.5 Selectivity

Table 12: Observations of selectivity.

Sr. No.	Sample ID	Type of Biological matrix	Area at RT of analyte	% Interference of analyte	Area at RT of IS	% Interference of IS
1.	Blank	Normal plasma	0	0.00	0	0.00
2.	LLOQ		105730		2491104	
3.	Blank		0	0.00	0	0.00
4.	LLOQ		117121		2615868	
5.	HE blank	Hyperlipidemic plasma	0	0.00	0	0.00
6.	HE LLOQ		75977		1822505	
7.	LP blank	Haemolyzed plasma	0	0.00	0	0.00
8.	LP LLOQ		95089		2247863	

Acceptance criteria

Response of an interfering peak at the retention time of Analyte must be < 20.00 % of respective LLOQ and response of interfering peak at the retention time of IS must be < 5.00 % of the response of IS in LLOQ Sample.

Result: No interference was observed in blank samples at the retention time of Analyte and IS.

3.2.6 Reinjection reproducibility

Table 13: Observations of Reinjection Reproducibility analysed with initial calibration curve standards.

Sample ID	LQC	HQC
Nominal concentration (ng/mL)	84.25	5658.60
Acceptance limit (ng/mL)	71.61 to 96.89	4809.81 to 6507.39
Sr. No.	Back Calculated Concentration (ng/mL)	
1.	75.31	5397.91
2.	75.27	5459.69
3.	77.51	5633.11
4.	77.72	5496.73
5.	75.99	5499.56
6	75.90	5630.12
Mean	76.28	5519.52
SD	1.0748	94.2565
%RSD	1.41	1.71
%Nominal	90.54	97.54

Table 14: Observations of Reinjection Reproducibility analysed with Reinjected calibration curve standards.

Sample ID	LQC	HQC
Nominal concentration (ng/mL)	84.25	5658.60
Acceptance limit (ng/mL)	71.61 to 96.89	4809.81 to 6507.39
Sr. No.	Back Calculated Concentration (ng/mL)	
1.	75.07	5463.55
2.	75.03	5526.10
3.	77.29	5701.66
4.	77.51	5563.59
5.	75.75	5566.46
6	75.66	5698.64
Mean	76.05	5586.67
SD	1.0874	95.4237
%RSD	1.43	1.71
%Nominal	90.27	98.73

Acceptance criteria

% RSD should not more than 15.00%, % Nominal should in between 85.00 to 115.00%

Result: The acceptance criteria were met.

3.2.7 Haemolysis effect

Table 15: Observations of haemolysis effect.

Sample ID	LQC	HQC
Nominal concentration (ng/mL)	84.25	5658.60
Acceptance limit (ng/mL)	71.61 to 96.89	4809.81 to 6507.39
Sr. No.	Back calculated concentration (ng/mL)	
1.	77.26	5577.30
2.	78.29	5405.51
3.	78.34	5690.28
4.	79.39	5760.92
5.	77.16	5922.33
6.	80.14	5316.58
Mean	78.43	5612.15
SD	1.1709	226.1525
% RSD	1.49	4.03
% Nominal	93.09	99.18

Acceptance criteria

% RSD should not more than 15.00%, % Nominal should in between 85.00 to 115.00%

Result: The acceptance criteria were met.

Conclusion: Quantitation of Dolutegravir was not affected by Haemolysis of samples.

3.2.8 Lipemic effect

Table 16: Observations of lipemic effect.

Sample ID	LQC	HQC
Nominal concentration (ng/mL)	84.25	5658.60
Acceptance limit (ng/mL)	71.61 to 96.89	4809.81 to 6507.39
Sr. No.	Back calculated concentration (ng/mL)	
1.	78.66	5487.76
2.	78.02	5476.58
3.	76.94	5607.17
4.	76.09	5551.77
5.	79.52	5852.82
6.	79.14	4967.14
Mean	78.06	5490.54
SD	1.3263	290.7744
% RSD	1.70	5.30
% Nominal	92.65	97.03

Acceptance criteria

% RSD should not more than 15.00%, % Nominal should in between 85.00 to 115.00%

Result: The acceptance criteria were met.

Conclusion: Quantitation of Dolutegravir was not affected by Lipid content of samples.

3.2.9 Matrix effect

Table 17: Observations of matrix effect.

Sample ID	LQC	HQC
Nominal concentration (ng/mL)	84.25	5658.60
Acceptance limit (ng/mL)	71.61 to 96.89	4809.81 to 6507.39
Sr. No.	**Back calculated concentration (ng/mL)**	
1.	76.53	5599.23
2.	80.24	5412.58
3.	81.65	5597.72
4.	82.52	5916.78
5.	78.15	5740.07
6.	80.37	5360.98
Mean	79.91	5604.56
SD	2.2218	205.9646
% RSD	2.78	3.67
% Nominal	94.85	99.04

Acceptance criteria: % Nominal for LQC & HQC samples should in between 85.00 to 115.00% and % RSD of back calculated concentration of six samples should not more than 15.00%.

Result: The acceptance criteria were met.

Conclusion: Quantitation of Dolutegravir was not affected by Matrix Effect.

3.2.10 Dilution integrity

Table 18: Observations of dilution integrity.

Sample ID	DI 1:2	DI 1:5
Nominal concentration (ng/mL)	11317.20	28267.84
Acceptance limit (ng/mL)	9619.62 to 13014.78	24027.66 to 32508.02
Sr. No.	**Back calculated concentration (ng/mL)**	
1.	11445.87	28483.73
2.	11778.57	29296.61
3.	11865.18	29636.09
4.	10932.24	27398.26
5.	11782.24	28518.27
6.	11651.96	28958.69
Mean	11576.01	28715.28
SD	347.6565	783.8436
% RSD	3.00	2.73
% Nominal	102.29	101.58

Acceptance criteria: % RSD should not more than 15.00% and % Nominal should in between 85.00 to 115.00%

Result: The % RSD and % Nominal of back calculated concentration of Dilution Integrity samples were within the acceptance criteria.

Conclusion: Samples having concentration above ULOQ can be analyzed by diluting the sample with maximum concentration of 28267.84ng/mL.

3.2.11 Stability

3.2.11.1 Short term stock solution stability.

Table 19: Observations of Short term stock solution stability of Dolutegravir.

Sr. no.	Fresh	Stability
	Nominal concentration (ng/mL)	
	1017937.00	1005973.00
	Peak area of analyte	
1.	12330276	11917931
2.	11551435	12347004
3.	11844845	12260892
4.	12761000	13231610
5.	12516658	12167830
6.	12849218	12844133
Mean	12308905.33	12844133
SD	515569.3048	12461566.67
% RSD	4.19	484673.0090
% Mean stability	102.44	
% Difference	2.44	

Table 20: Observations of Short term stock solution stability of dolutegravir D6.

Sr. no.	Fresh	Stability
	Nominal concentration (ng/mL)	
	1017937.00	1005973.00
	Peak area of Internal Standard	
1.	1999964	2187175
2.	1738077	1767988
3.	1970027	1910443
4.	2025992	1747480
5.	1623921	2040948
6.	2048728	1909978
Mean	1901118.17	1924335.33
SD	176250.8181	166583.6918
% RSD	9.27	8.64
% Mean stability	100.09	
% Difference	0.09	

Acceptance criteria: % RSD should not more than 15.00% and % Difference should be ±15.00%

Result: The acceptance criteria were met.

Conclusion: The Stock Solution was found to be stable upto 22 hours, 20 minutes for Dolutegravir at ambient temperature.

3.2.11.2 Long Term Stock Solution Stability at 2 to 8°C.

Table 21: Observations of long term stock solution stability.

Sr. No.	Fresh	Stability	Fresh	Stability
	Drug		Internal standard	
	Nominal concentration (ng/mL)			
	1016940.00	1005973.00	1006940.00	1019377.50
	Peak area of analyte		Peak area of IS	
1.	3246873	3101259	463664	458834
2.	3421131	3380410	483104	483286
3.	3303420	3306451	476925	471740
4.	3363562	3223073	483331	476804
5.	3491826	3505274	470106	486563
6.	3319547	3302758	484756	463372
Mean	3357726.50	3303204.17	476981.00	473433.17
SD	88013.8946	137117.8715	8519.1021	10936.7538
% RSD	2.62	4.15	1.79	2.31
% Mean stability	99.45		98.05	
% Difference	-0.55		-1.95	

Acceptance criteria: % RSD should not more than 15.00% and % Difference should be ±15.00%

Result: The acceptance criteria were met.

Conclusion: Stock Solution of DTG & Dolutegravir D6 were found to be stable upto 5 Days at 2 to 8°C.

3.2.11.3 Bench Top Stability

Table 22: Observations of bench top stability.

QC ID	LQC		HQC	
	Fresh	Stability	Fresh	Stability
Nominal concentration (ng/mL)	85.34	84.25	5647.38	5658.60
Acceptance limit (ng/mL)	72.54 to 98.14	71.61 to 96.89	4800.27 to 6494.49	4809.81 to 65.7.39
Sr. No.	Back calculated concentration (ng/mL)			
1.	78.03	77.49	5643.58	5466.92
2.	81.09	78.53	5685.84	5555.73
3.	76.91	78.42	5569.83	5375.94
4.	76.93	79.63	5499.50	5534.54
5.	77.31	79.07	5430.32	5550.17
6.	76.42	79.04	5367.51	5209.53
Mean	77.78	78.70	5532.76	5448.81
SD	1.7070	0.7333	123.2854	135.6085
% RSD	2.19	0.93	2.23	2.49
% Mean stability	91.14	93.41	97.97	96.29
% Difference	2.49		-1.71	

Acceptance criteria: % RSD should not more than 15.00% and % Difference should be ±15.00% and % Nominal should in between 85.00 to 115.00%.

Result: The acceptance criteria were met.

Conclusion: Dolutegravir was found to be stable in plasma upto 27 hours and 58 minutes at ambient temperature.

3.2.11.4 Freeze and Thaw stability

Table 23: Observations of Freeze and Thaw stability.

QC ID	LQC		HQC	
	Fresh	Stability	Fresh	Stability
Nominal concentration (ng/mL)	85.34	84.25	5647.38	5658.60
Acceptance limit (ng/mL)	72.54 to 98.14	71.61 to 96.89	4800.27 to 6494.49	4809.81 to 65.7.39
Sr. No.	Back calculated concentration (ng/mL)			
1.	78.03	77.37	5643.58	5422.56
2.	81.09	78.16	5685.84	5553.38
3.	76.91	80.72	5569.83	5578.51
4.	76.93	78.89	5499.50	5101.04
5.	77.31	77.34	5430.32	5519.69
6.	76.42	77.79	5367.51	5354.06
Mean	77.78	78.38	5532.76	5421.54
SD	1.7070	1.2833	123.2854	178.2861
% RSD	2.19	1.64	2.23	3.29
% Mean stability	91.14	93.03	97.97	95.81
% Difference	2.08		-2.20	

Acceptance criteria: % RSD should not more than 15.00% and % Difference should be ±15.00% and % Nominal should in between 85.00 to 115.00%.

Result: The acceptance criteria were met.

Conclusion: Dolutegravir was found to be stable upto five Freeze and Thaw cycles at -70^{0}C.

3.2.11.5 Autosampler stability

Table 24: Observations of autosampler stability.

QC ID	LQC		HQC	
	Fresh	Stability	Fresh	Stability
Nominal concentration (ng/mL)	85.34	84.25	5647.38	5658.60
Acceptance limit (ng/mL)	72.54 to 98.14	71.61 to 96.89	4800.27 to 6494.49	4809.81 to 65.7.39
Sr. No.	Back calculated concentration (ng/mL)			
1.	78.03	75.79	5643.58	5511.27
2.	81.09	79.20	5685.84	5647.21
3.	76.91	79.80	5569.83	5440.12
4.	76.93	77.40	5499.50	5465.73
5.	77.31	77.23	5430.32	5417.73
6.	76.42	78.82	5367.51	5733.44
Mean	77.78	77.99	5532.76	5535.92
SD	1.7070	1.5878	123.2854	126.5546
% RSD	2.19	2.04	2.23	2.29
% Mean stability	91.14	92.57	97.97	97.83
% Difference	1.57		-0.14	

Acceptance criteria: % RSD should not more than 15.00% and % Difference should be ±15.00% and % Nominal should in between 85.00 to 115.00%.

Result: The acceptance criteria were met.

Conclusion: The processed samples of Dolutegravir were found to be stable upto 29 hours and 17 minutes in Autosampler at 5ºC.

3.2.11.6 Wet extract stability

Table 25: Observations of wet extract stability.

QC ID	LQC		HQC	
	Fresh	Stability	Fresh	Stability
Nominal concentration (ng/mL)	85.34	84.25	5647.38	5658.60
Acceptance limit (ng/mL)	72.54 to 98.14	71.61 to 96.89	4800.27 to 6494.49	4809.81 to 65.7.39
Sr. No.	Back calculated concentration (ng/mL)			
1.	78.03	80.86	5643.58	5716.83
2.	81.09	80.86	5685.84	5485.95
3.	76.91	78.66	5569.83	5365.19
4.	76.93	77.38	5499.50	5241.93
5.	77.31	77.90	5430.32	5547.14
6.	76.42	76.35	5367.51	5643.51
Mean	77.78	78.67	5532.76	5500.09
SD	1.7070	1.8564	123.2854	175.9567
% RSD	2.19	2.36	2.23	3.20
% Mean stability	91.14	93.38	97.97	97.20
% Difference	2.45		-0.79	

Acceptance criteria: % RSD should not more than 15.00% and % Difference should be ±15.00% and % Nominal should in between 85.00 to 115.00%.

Result: The acceptance criteria were met.

Conclusion: Wet extract samples were found to be stable upto 27 hours and 48 minutes at 2 to 8ºC.

3.2.11.7 Whole blood stability

Table 26: Observations of whole blood stability.

QC ID	LQC		HQC	
	Fresh	Stability	Fresh	Stability
Nominal concentration (ng/mL)	85.34	85.34	5647.38	5647.38
Sr. No.	Peak area ratio of analyte to IS			
QC	WBC LQC	WBC LQC	WBC HQC	WBC HQC
1.	0.1485	0.1469	10.4310	10.3336
2.	0.1411	0.1513	10.3041	9.9911
3.	0.1442	0.1484	10.8334	10.2562
4.	0.1439	0.1475	10.4570	10.4843
5.	0.1441	0.1427	10.1157	10.2342
6.	0.1456	0.1410	10.2288	10.4700
Mean	0.1446	0.1463	10.3950	10.2944
SD	0.0024	0.0038	0.2495	0.1813
% RSD	1.66	2.60	2.40	1.76
% Difference	1.18		-0.97	

Acceptance criteria: % RSD should not more than 15.00% and % Difference should be ±15.00%

Result: The acceptance criteria were met.

Conclusion: Dolutegravir was found to be stable in Whole Blood upto 02 hours and 28 minutes at ambient temperature.

3.2.12 Ruggedness Test

3.2.12.1 Different Analyst

Table 27: Observations of ruggedness test by different analyst.

QC ID	LLOQ QC	LQC	LMQC	MQC	HQC
Nominal concentration (ng/mL)	28.92	84.25	710.47	3527.19	5658.60
Acceptance limit (ng/mL)	23.14 to 34.70	71.61 to 96.89	603.90 to 817.04	2998.11 to 4056.27	4809.81 to 65.7.39
Batch ID	**Back Calculated Concentration (ng/mL)**				
1.	28.48	74.71	703.05	3524.75	5523.39
2.	28.63	79.12	699.27	3504.36	5522.55
3.	26.68	77.54	706.96	3477.21	5639.85
4.	27.41	77.77	708.55	3608.33	5537.00
5.	27.89	76.17	706.09	3489.88	5726.62
6.	28.91	77.52	692.43	3654.58	5639.85
Mean	28.00	77.14	702.73	3528.19	5598.21
SD	0.8436	1.5144	6.0203	49.7855	83.6971
% RSD	3.01	1.96	0.86	1.41	1.50
% Nominal	96.82	91.56	98.91	100.03	98.93
% Bias	-3.18	-8.44	-1.09	0.03	-1.07

Acceptance criteria: Precision and Accuracy batch acceptance criteria must be met.

Result: The acceptance criteria were met.

3.2.12.2 Different column

Table 28: Observations of ruggedness test by different column.

QC ID	LLOQ QC	LQC	LMQC	MQC	HQC
Nominal concentration (ng/mL)	28.92	84.25	710.47	3527.19	5658.60
Acceptance limit (ng/mL)	23.14 to 34.70	71.61 to 96.89	603.90 to 817.04	2998.11 to 4056.27	4809.81 to 65.7.39
Batch ID	**Back Calculated Concentration (ng/mL)**				
1.	27.90	74.25	697.69	3489.29	5622.56
2.	27.37	76.14	703.12	3494.84	5628.55
3.	26.83	76.56	710.19	3514.43	5658.63
4.	26.69	75.11	716.79	3479.25	5640.81
5.	27.38	74.90	704.32	3613.65	5666.81
6.	27.30	77.76	715.50	3479.74	5809.90
Mean	27.25	75.79	707.94	3511.87	5671.21
SD	0.4350	1.2824	7.5092	51.5015	70.0334
% RSD	1.60	1.69	1.06	1.47	1.23
% Nominal	94.23	89.96	99.64	99.57	100.22
% Bias	-5.77	-10.04	-0.36	-0.43	0.22

Result: The acceptance criteria were met.

3.2.12.3 Different Equipment

Table 29: Observations of ruggedness test by different equipment.

QC ID	LLOQ QC	LQC	LMQC	MQC	HQC
Nominal concentration (ng/mL)	28.92	84.25	710.47	3527.19	5658.60
Acceptance limit (ng/mL)	23.14 to 34.70	71.61 to 96.89	603.90 to 817.04	2998.11 to 4056.27	4809.81 to 65.7.39
Batch ID	**Back Calculated Concentration (ng/mL)**				
1.	28.45	74.93	701.92	3635.99	5681.22
2.	28.87	78.64	713.04	3617.29	5796.78
3.	28.14	78.00	713.19	3473.96	5981.69
4.	28.84	78.69	705.11	3560.96	5475.03
5.	27.08	74.25	724.83	3609.00	5922.36
6.	28.45	78.84	723.00	3551.19	5867.88
Mean	28.31	77.23	713.52	3574.73	5787.49
SD	0.6596	2.0724	9.2023	59.3918	185.1362
% RSD	2.33	2368	1.23	1.66	3.20
% Nominal	97.89	91.67701.92	100.43	101.35	102.28
% Bias	-2.11	-8.33713.04	0.43	1.35	2.28

Result: The acceptance criteria were met.

3.2.13 Cross specificity

Table 30: Observations of cross specificity for dolutegravir.

Sr. No.	Sample ID	Type of Biological Matrix	Area at RT of Dolutegravir	% Interference at RT of Dolutegravir	Area at RT of Dolutegravir D6	% Interference at RT of Dolutegravir D6
1.	LLOQ (Dolutegravir)	Normal Plasma	27059	-	517093	-
2.	Blank (ULOQ of Lamivudine + Tenofovir)		0	0.00	0	0.00
3.	Blank (ULOQ of Lamivudine + Tenofovir)		0	0.00	0	0.00
4.	Blank (ULOQ of Lamivudine + Tenofovir)		0	0.00	0	0.00

Acceptance criteria: Response of interfering peak at the retention time of Analyte must be < 20.00 % of LLOQ and response of interfering peak at the retention time of IS must be < 5.00 % of the response of IS in LLOQ.

Result: No interference was observed in blank samples at retention time of DTG and Dolutegravir D6.

4. CONCLUSION

A Sensitive, rapid liquid chromatography – tandem mass spectrometry (LC-MS/MS) method was developed and validated for quantitative estimation of Dolutegravir in human plasma with investigated concentration range 28.44 ng/mL to 7054.37ng/mL. Furthermore, reliability of the method was established by successfully performing full validation notably in compliance with the most recent regulations on bioanalytical method validation and found in the limit as per US FDA guidelines. There were no significant matrix effects were observed by analysing the plasma samples on LC-MS/MS. Further good results were obtained in plasma calibration curve. Stability Observations of Dolutegravir was found to be satisfactory. The lower limit of quantification was found to be in nanograms that means method is capable to detect the stated drug in traces amount. Hence it can be concluded that the Sensitive, rapid LC-MS/MS method was developed and validated for determination of Dolutegravir in Human plasma and can be successfully applied for bioequivalence studies in human subjects. The presented method can serve as a key to unlock the entry of generic drug product into the market by proving bioequivalence of two different formulation of stated drug.

5. Abbreviations

DTG, dolutegravir; DP, declustering potential; EP, entrance potential; CXP, collision cell exit potential; CAD, collisionally activated dissociation; QC, quality control; LQC, lower quality control; LMQC, lower middle quality control; MQC, middle quality control; HQC, higher quality control; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; SD, standard deviation; RSD, relative standard deviation; STD, standard; CC, calibration curve; IS, internal standard; DI, dilution integrity.

6. Declarations

6.1 Availability of observations and materials: All Observations generated or analysed during this study are obtained from Raptim Research limited New Mumbai and included in this article.

6.2 Competing interest: No competing interests.

6.3 Funding: Not applicable.

6.4 Author's contributions: Bioanalytical method for Dolutegravir was developed and successfully validated by Mr Vinayak Raju Bodhankar under the guidance of Dr Milind Bagul and Dr Sarita Pawar. All authors read and approved the final manuscript for publication.

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