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Research Article

Pharmaceutical analysis for Novel Drug Formulation

Simultaneous Determination of Dolutegravir and Lamivudine in Human Plasma by LC-MS/MS

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Abstract: A rapid, simple, sensitive and selective LC-MS/MS method has been developed and validated for quanti? cation of the Dolutegravir and Lamivudine in plasma samples. The analytical procedure involves a liquid–liquid extraction method using Emtricitabine as an internal standard (IS). The precision and accuracy data have to fulfill the requirements for quantification of the analytes in biological matrices to generate data for bioequivalence and bioavailability investigations. The chromatographic separation was achieved on a Hypurity Advance (4.6, 50 mm, 5µ) column using a mobile phase consisting of 0.1% formic acid buffer–acetonitrile (20:80, %v/v) at ? ow rate of 0.8 mL/min. The API-4000 LC-MS/MS was operated in the multiple-reaction monitoring mode using electrospray ionization. The total run time of analysis was 3 min and elution of Dolutegravir, Lamivudine and Emtricitabine (IS) occurred at 1.06, 1.84 and 0.92 min, respectively. A detailed validation of the method was performed as per the US Food and Drug Administration guidelines. The method was validated in terms of linearity, accuracy, precision, specificity, limit of detection and limit of quantitation. The standard curves found to be linear in the range of 0.10–30.0 ng/mL for Dolutegravir and 20.2–6026 ng/mL for Lamivudine, with a coef? cient of correlation of =0.99 for both the compounds. Dolutegravir and Lamivudine were found to be stable in a plasma stability studies, viz. bench-top, autosampler, re-injection, wet-extract and repeated freeze–thaw cycles. The coefficient of variation was =15% for intra- and inter-batch assays. The assay is suitable for pharmacokinetic study samples as demonstrated by its specificity, precision, accuracy, recovery, and stability characteristics.

Keywords: Dolutegravir and Lamivudine; Emtricitabine; plasma; Method validation; LC-MS/MS; Pharmacokinetics

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1. INTRODUCTION

Human immunodeficiency virus (HIV) treatment has improved significantly since from 29 years. Whereas the first therapies emerged in the 1980s. Current drug regimens offer patients durability and improved conveniences so that HIV is now a chronic, rather than a life-threatening, disease. The life expectancy of people living with HIV has dramatically increased, for example, life expectancy at age 20 for individuals with HIV living in California in 2011 has been estimated at 33 - 53 years. As the life expectancy of people living with HIV increases with the age of 34. In addition to carrying HIV, develop age-related comorbidities, such as diabetes[1-3]. In the age of 35, people living with HIV, these comorbidities are more prevalent and can occur at a 36 years age than in the general population. Polypharmacy, in the aging HIV population, leads to an increased risk for drug-drug interactions. The aim of development of new therapies include reducing adverse effects and undesirable drug interactions, and increasing convenience and ease of administration, while maintaining efficacy[4]. Lamivudine, commonly called 3TC, is an antiretroviral medication used to prevent and treat HIV/AIDS. The chemical name of Lamivudine is (2R,cis)-4-amino-1 (2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one. Lamivudine, chemically the (-) enantiomer of a dideoxy analogue of cytidine. Lamivudine has also been referred to as (-)2′,3′-dideoxy, 3′ thiacytidine. It has a molecular formula of C8H11N3O3S and a molecular weight of 229.3 g per mol [5]. Dolutegravir, is an antiretroviral medication used, together with other medication, to treat HIV/AIDS. The chemical 332 name of Dolutegravir sodium is sodium (4R,12aS)-9-{[(2,4-difluorophenyl)methyl]carbamoyl} -3334-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2H-pyrido [1',2':4,5]pyrazino[2,1-b][1,3]oxazin-7-334 olate. The empirical formula is $C_{20}H_{18}F_2N_3NaO_5$ and the molecular weight is 441.36 g/mol[6].

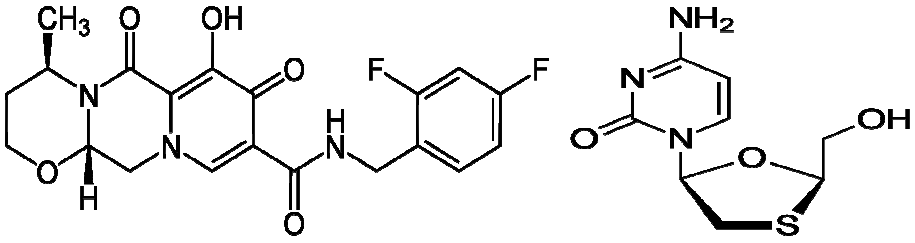


Fig1. Chemical structures of A) Dolutegravir B) Lamivudine

Literature survey reveals that various UV-VIS spectroscopy[7-8], HPTLC[9], HPLC [10-13], LC-MS[14-30] methods have been reported individually for the estimation of Dolutegravir and Lamivudine. None of the methods were reported for simultaneous estimation of Dolutegravir and Lamivudine. The present study illustrates development and validation of a simple, accurate and precise procedure for Development and validation of bio-analytical method for the simultaneous estimation of Dolutegravir and Lamivudine biological matrices by LC-MS/MS.

2. MATERIALS AND METHODS

2.1 HPLC operating conditions

A Shimadzu LC-20 AD Series HPLC system (Shimadzu Corporation, Kyoto, Japan) was used to inject 20 mL aliquots of the processed samples on a Hypurity Advance column (4.6, 50 mm, 5µ), which was kept at ambient temperature. The isocratic mobile phase, a mixture of acetonitrile–0.1%formic acid (80:20, %v/v) was filtered through a 0.45mm membrane filter (Millipore, USA), then degassed ultrasonically for 5 min and delivered at a flow rate of 0.8 mL/min into the mass spectrometer electrospray ionization chamber.

2.2 Mass spectrometry operating conditions

Quantitation was achieved with MS/MS detection in positive ion mode for the analytes and IS using a MDS Sciex API-4000 mass spectrometer at C. The ion spray voltage was set at 5500 V. The source parameters, viz. the nebulizer gas, curtain gas, auxiliary gas and collision gas, were set at 40, 20, 45 and 8 psi, respectively. The compound parameters viz. the declustering potential, collision energy, entrance potential and collision cell exit potential were 60, 30, 10 and 8 V for Dolutegravir, 80, 30, 10 and 10 V for Lamivudine 54, 34, 10 and 12 for Emtricitabine (Internal standard-IS), respectively. MRM ions were identified as m/z 420.20 and 192.20 for Dolutegravir, m/z 287.20 and 130.0 for Emtricitabin.

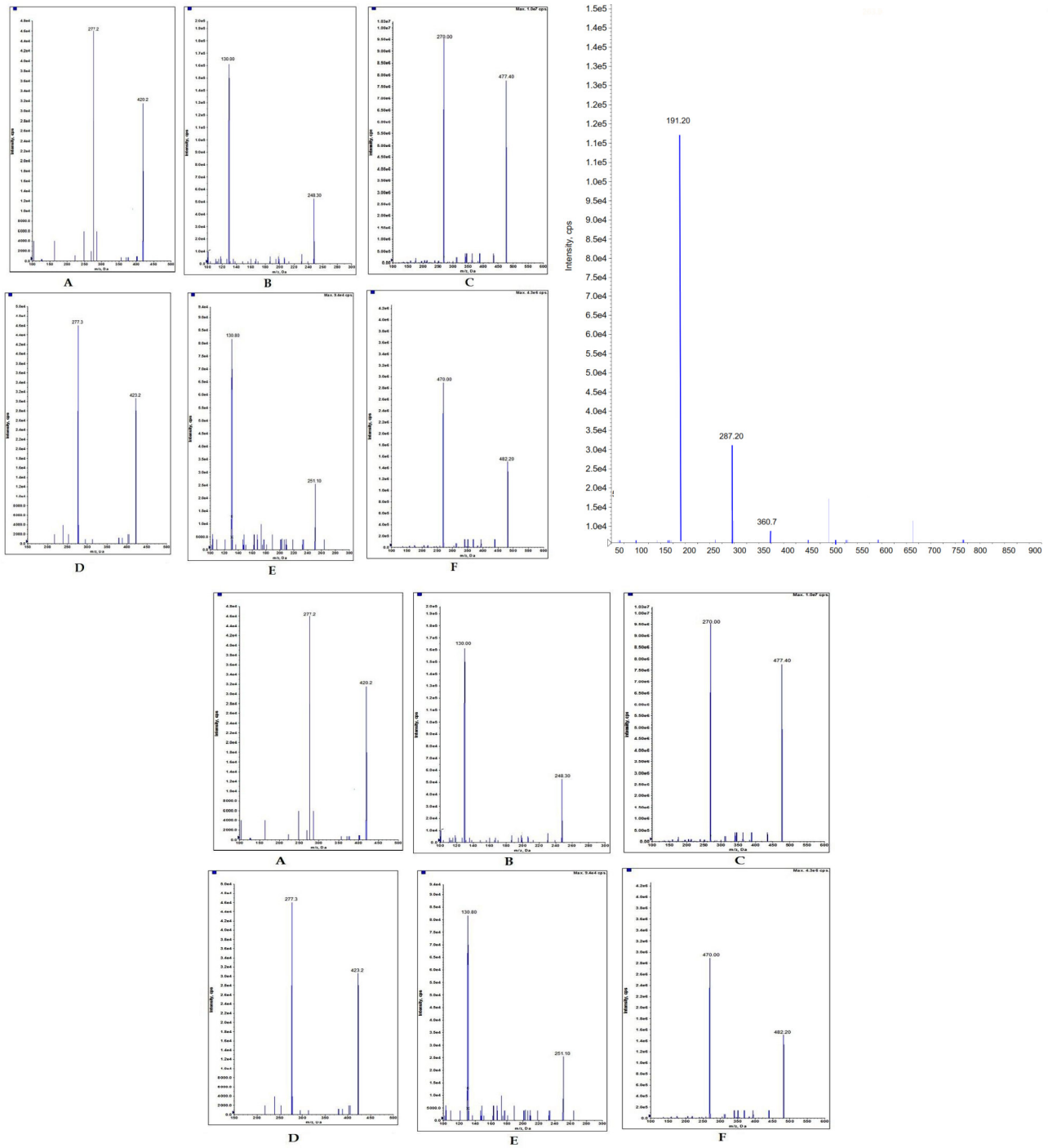


Fig 2. Mass Spectra of Q1→Q3 A) Dolutegravir B) Lamivudine C) Emtricitabine (Internal Standard).

2.3 Experimental

2.3.1 Chemicals and reagents

The reference standards of Dolutegravir (97.9%), Lamivudine (99.5%) and Emtricitabine (Internal standard-IS) (95.7%) were purchased from Neucon Pharma Private Limited, Goa, India. Chemical structures are presented in Fig.1. Water used for the LC-MS/MS analysis was prepared from a Milli-Q water purification system procured from Millipore (Bangalore, India). All chemicals used used in this research were HPLC grade

2.3.2 Preparation of stock solutions of analytes and IS

The primary stock solutions of Dolutegravir and Lamivudine were prepared in methanol and the stock solutions of Dolutegravir and Lamivudine and Emtricitabine (IS) were stored at 2–8° C. They were consecutively diluted with methanol: water (50:50, %v/v) to prepare working solutions for preparation of calibration curve standards. Another set of working stock solutions of Dolutegravir and Lamivudine was made in methanol: water for preparation of QC samples. Working stock solutions were stored at 2–8° C.

2.3.3 Preparation of calibration curve and quality control samples

Calibration curve samples were prepared by spiking 200 mL of control human plasma with the appropriate working solutions of the Dolutegravir and Lamivudine was prepared in combination (50 mL). Calibration curve standards consisting of a set of 10 non-zero concentrations at 0.10–30.0 ng/mL for Dolutegravir and 20.2-6026 ng/mL for Lamivudine were prepared. Portion of 250 mL plasma samples transferred to freshly PP (poly propylene) tubes. The

QCs prepared for each analyte were: for Dolutegravir, 0.10 (lower limit of quantization, LLOQ), 0.30 (low quality control, LQC), 4.96 (medium quality control, MQC1), 17.7 (MQC2) and 24.6 ng/mL (high quality control, HQC); and for Lamivudine, 21.0 (LLOQ), 60.8 (LQC), 1014 (MQC1), 3621 (MQC2) and 5030 ng/mL (HQC). All the samples were stored at -70 degree. 0.10–30.0 ng/mL for Dolutegravir and 20.2–60.26 ng/mL for Lamivudine.

2.3.4 *Sample preparation*

A simple liquid–liquid extraction method was followed for extraction of Dolutegravir and Lamivudine from human plasma. To an aliquot of 250 mL plasma, working solution of Emtricitabine (IS) (25 mL of 5000 ng/mL) and 25 mL of 100 % formic acid were added and mixed for 15 s on a cyclomixer (Remi Instruments, Mumbai, India). After the addition of 5 mL of ethyl acetate, the samples were placed on a reciprocating shaker for 15 min at 200 rpm, followed by centrifugation for 10 min at 4000 rpm on a Multifuge 3SR at 4 deg (Heraus, Germany). The supernatant was dried and reconstituted in mobile phase.

2.4 *Method validation*

In selectivity, the endogenous matrices interference was less than 20%. The precision of the method acceptable in range less than 20%. The Linearity curve shows regression >0.998 for Dolutegravir and Lamivudine in the concentration ranges 0.10–30.0 and 20.2–6026 ng/mL. In stability experiments, QC shows %CV less than 15% indicates analytes were stable in biological samples.

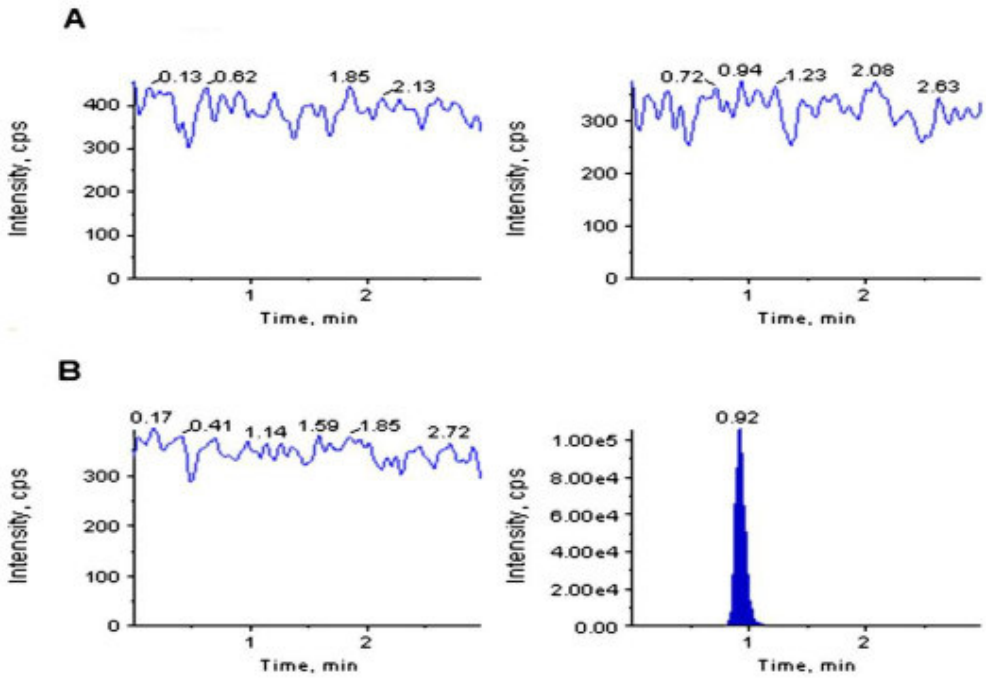
3. RESULTS

3.1 *Method development*

Mass parameters were optimized in both positive and negative ionization modes for the analytes and Emtricitabine (IS). Good response was found in positive ionization mode. Data from multiple reaction monitoring was considered to obtain better selectivity. Use of a buffer with formic acid helped to achieve a good response for MS detection in the positive ionization mode. The chromatographic separation was achieved with 0.1% Formic acid and Acetonitrile (20:80%v/v) using analytical column Hypurity Advance, 50, 4.6mm, 5 mm at flow of 0.8ml/min. The analytes were eluted at 1.06, 1.84 and 0.92 min for Dolutegravir, Lamivudine and Emtricitabine (IS). During extraction, among the different solvents checked alone and in combination for their suitability, ethyl acetate was found to be optimal. It can produce a clean chromatogram for a blank sample and yields the highest recovery for the analytes from the plasma. Isotope-labelled analyte was not available to serve as IS, so in the initial stages of this work, several compounds were investigated to find a suitable IS and finally Emtricitabine was found to be best for the present purpose. Extraction recovery of the IS was almost the same as that of the analytes.

3.2 *Selectivity and chromatography*

As shown in Fig. 2 and 3, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free plasma at the retention times of the analytes and Emtricitabine (IS).



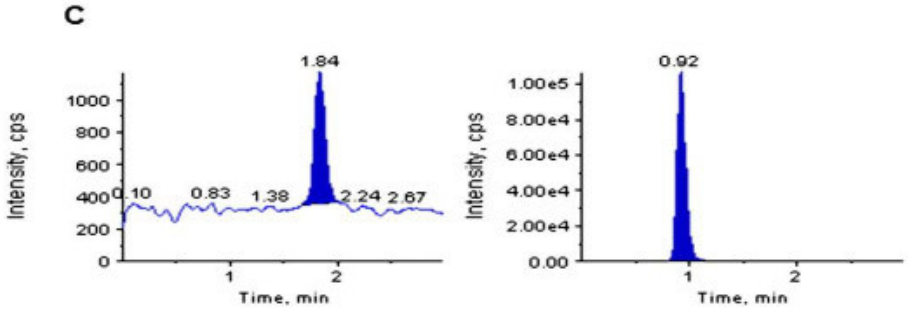


Fig 3. Typical MRM chromatograms of Lamivudine (left panel) and Emtricitabine (IS) (right panel) in (A) human blank plasma, (B) human plasma spiked with IS and (C) an LLOQ sample along with Emtricitabine (IS).

3.3 Sensitivity

The lowest limit of reliable quantification for the analytes was set at the concentration of the LLOQ. The precision and accuracy at LLOQ concentration were found to be 3.10 and 100 %, and 4.07 and 102 % for Dolutegravir and Lamivudine, respectively.

3.4 Extraction efficiency

The method shows good recovery using LLE method and mean overall recovery of Dolutegravir, Lamivudine was 77.85 % and 80.96 %, respectively.

3.5 Matrix effect

No significant matrix effect was observed in all the six batches of human plasma for the analytes at LQC and HQC concentrations respectively. Similarly, the precision and accuracy for Dolutegravir & Lamivudine at HQC concentration were found to be 1.11 & 98.2 %, and 1.36 & 100 %, respectively.

3.6 Linearity

The 10-point calibration curve 0.10, 0.20, 0.50, 1.01, 2.01, 4.02, 8.04, 16.0, 24.0 and 30.0 ng/mL for Dolutegravir; 20.2, 40.4, 101, 202, 404, 807, 1615, 3230, 4821 and 6026 ng/mL for Lamivudine) was constructed by plotting the peak area ratio of analyte–IS against the nominal concentration of calibration standards in human plasma. Following the evaluation of different weighting factors, the results were fitted to linear regression analysis with the use of a 1/x2 (where x is the concentration) weighting factor. The mean correlation coefficient of the weighted calibration curves generated during the validation was ≥0.998.

3.7 Precision and accuracy

As shown in Table 1 and 2, the precision and accuracy of each analyte in the intra-day and inter-day runs were within 15 % at LQC, MQC-1, MQC-2 and HQC concentrations and within 20 % at LLOQ QCs.

Table1. Intraday P&A for Dolutegravir and Lamivudine

Intraday P&A	Dolutegravir				
	Nominal Conc.(ng/mL)				
	LLOQ QC	LQC	MQC1	MQC2	HQC
	0.103	0.297	4.955	17.698	24.581
Mean	0.1057	0.2965	4.9763	18.0650	25.0481
SD	0.00561	0.00967	0.22456	0.61788	0.75883
% CV	5.31	3.26	4.51	3.42	3.03
% Nominal	102.57	99.84	100.43	102.07	101.90
Intraday P&A	**Lamivudine**				
	Nominal Conc.(ng/mL)				
	LLOQ QC	**LQC**	**MQC1**	**MQC2**	**HQC**
	21.017	60.840	1014.000	3621.428	5029.761
Mean	20.3415	59.1983	981.3520	3550.3803	5094.5079
SD	1.53393	3.96304	71.54628	169.76598	161.50765
% CV	7.54	6.69	7.29	4.78	3.17
% Nominal	96.79	97.30	96.78	98.04	101.29

Table 2. Interday P&A for Dolutegravir and Lamivudine

Between Batch/ Intraday P&A	Dolutegravir				
	Nominal Conc.(ng/mL)				
	LLOQ QC	LQC	MQC1	MQC2	HQC
	0.103	0.297	4.955	17.698	24.581
Mean	0.1035	0.2983	4.9575	18.0619	25.1560
SD	0.00474	0.00858	0.16602	0.39970	0.57117

% CV	4.58	2.88	3.35	2.21	2.27
% Nominal	100.49	100.43	100.05	102.06	102.34
Between Batch/ Intraday P&A	**Lamivudine**				
	Nominal Conc.(ng/mL)				
	LLOQ QC	**LQC**	**MQC1**	**MQC2**	**HQC**
	21.017	60.840	1014.000	3621.428	5029.761
Mean	20.7383	59.8393	1016.5092	3518.9283	5078.8632
SD	1.18367	2.72295	86.61183	127.61869	123.48108
% CV	5.71	4.55	8.52	3.63	2.43
% Nominal	98.67	98.36	100.25	97.17	100.98

3.8 Dilution integrity

The upper concentration limits can be extended to 48.0 ng/mL for Dolutegravir and 9642 ng/mL for Lamivudine by 1:2 and 1:4 dilutions with order to depict the plot with clarity having mean SD values of screened human blank plasma. The mean back-calculated for 1:2 and 1:4 dilution samples were within 85–115% of their nominal value. The coefficients of variation (%CV) for 1:2 and 1:4 dilution samples were less than 10% for both the analytes.

3.9 *Stability studies*

In the different stability experiments carried out, viz. bench-top stability (12 h), autosampler stability (50 h), repeated freeze-thaw cycles (five cycles), re-injection stability (30 h), wet-extract stability (48 h at 2–8 Deg) and long-term stability at 70 deg for 60 days, the mean percentage nominal values of the analytes of the biological matrices were found to be within 15% of the predicted concentrations for the deter- for the analytes at their LQC and HQC levels (Table-3). Thus, determination of Dolutegravir and Lamivudine concentrations in human plasma for the results were found to be within the acceptable limits during entire validation.

Table 3. Stability Data of Dolutegravir and Lamivudine

Bench top Stability	**Dolutegravir**		**Lamivudine**	
	Nominal Conc.(ng/mL)			
	LQC	**HQC**	**LQC**	**HQC**
	0.297	**24.581**	**60.840**	**5029.761**
Mean	0.2992	25.0048	61.8172	5010.4187
SD	0.00853	0.14577	1.77125	112.53717
% CV	2.85	0.58	2.87	2.25
% Stability	100.74	101.72	101.61	99.62
AutoSampler Stability	**Dolutegravir**		**Lamivudine**	
	Nominal Conc.(ng/mL)			
	LQC	**HQC**	**LQC**	**HQC**
	0.297	24.581	60.840	5029.761
Mean	0.2957	25.4532	60.1567	5086.3437
SD	0.01206	0.37314	1.17684	97.60966
% CV	4.08	1.47	1.96	1.92
% Stability	99.55	103.55	98.88	101.12
Freeze-Thaw Stability	**Dolutegravir**		**Lamivudine**	
	Nominal Conc.(ng/mL)			
	LQC	**HQC**	**LQC**	**HQC**
	0.297	24.581	60.840	5029.761
Mean	0.2952	25.3370	59.8078	5119.6210
SD	0.01115	0.64439	1.40302	227.89192
% CV	3.78	2.54	2.35	4.45
% Stability	99.39	103.08	98.30	101.79
Long term Stability	**Dolutegravir**			
	Nominal Conc.(ng/mL)			
	LQC	**HQC**	**LQC**	**HQC**
	0 Day (PA BATCH-I)		60 Days	
	0.297	24.581	0.297	24.581
Mean	0.2944	25.1164	0.2908	24.9747
SD	0.01177	1.11180	0.01246	0.25357
% CV	4.00	4.43	4.28	1.02
% Stability	99.11	102.18	97.90	101.60
Long term Stability	**Lamivudine**			
	Nominal Conc.(ng/mL)			
	LQC	**HQC**	**LQC**	**HQC**

	0 Day (PA BATCH-I)		60 Days	
	60.840	5029.761	60.840	5029.761
Mean	58.4965	5031.1562	59.2140	5012.1845
SD	4.88222	185.63470	1.83798	166.72356
% CV	8.35	3.69	3.10	3.33
% Stability	96.15	100.03	97.33	99.65

4. DISCUSSION

There are as yet no published methods available for the simultaneous quantification of Dolutegravir and Lamivudine in any of the biological matrices. Validated methods are essential for the determination of Dolutegravir and Lamivudine concentrations in human plasma for bioequivalence studies. To the best of our knowledge and from previous earlier studies, this entire validation is the first report on the simultaneous analysis of Dolutegravir and Lamivudine in human plasma. The projected method is simple, rugged and rapid with a short run time of 3 min for each sample analysis. The method for the determination of Dolutegravir and Lamivudine in plasma has good sensitivity (LLOQ 0.10 ng/mL for Dolutegravir and a 20.2 ng/mL Lamivudine) and uses a single IS with a simple sample preparation.

5. CONCLUSION

The LC-MS/MS assay presented in this paper is rapid, simple, specific and sensitive for quantification of Dolutegravir and Lamivudine in plasma. It is fully validated according to commonly accepted FDA guidelines. The extraction method gave consistent and reproducible recoveries for the analytes and IS from liquid extraction and sample turnover rate of less than 3 min per bioanalysis of Dolutegravir and Lamivudine. From the results it is evident that, the developed method is selective, precise and robust and stable and it can be applicable in pharmacokinetic studies for estimation of drug levels in various biological matrices.

6. AUTHORS CONTRIBUTION STATEMENT

Dr. Venkata Rao.V conceived and designed the study; Mr.Bonthu badru performed the experiment and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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