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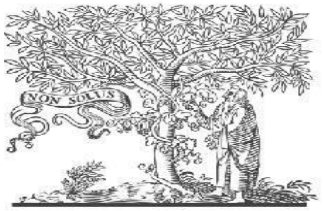

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Validated Method for Separation and Quantification of Dolutegravir and Lamivudine in Bulk and Pharmaceutical Dosage Forms by RP-UPLC

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Abstract
A simple, specific and accurate reverse phase ultra-performance liquid chromatographic method was developed for the simultaneous determination of Dolutegravir and Lamivudine in pharmaceutical dosage form. The column used was ACQUITY UPLC BEH C18 column (2.1 mm x 50 mm, 1.7 µm) in isocratic mode, with mobile phase containing Acetonitrile: Phosphate buffer pH (3.0) (50:50). The flow rate was 1.2 ml/min and effluents were monitored at 260 nm. The retention times of Dolutegravir and Lamivudine were found to be 1.430 min and 2.347 min, respectively. The linearity for Dolutegravir and Lamivudine were in the range of 100-300 µg/ml and 10–50 µg/ml respectively. The recoveries of Dolutegravir and Lamivudine were found to be 98.13% to 98.89% and 99.6 to 101% respectively. The proposed method was validated and successfully applied to the estimation of Dolutegravir and Lamivudine in combined tablet dosage forms. Therefore, a sensitive, robust, accurate method with high degree of sensitivity was developed for practical utility.

Keywords: Dolutegravir, Lamivudine, Validation, Buffer and ICH Q2 (R1) guidelines.

Introduction
Dolutegravir
Dolutegravir Fig.1 is an antiviral agent used for the treatment of HIV-1 infections in combination with other antiretroviral agents. The strand transfer step is essential in the HIV replication cycle and results in the inhibition of viral activity. Dolutegravir has a mean EC50 value of 0.5 nm (0.21 ng/mL) to 2.1 nM (0.85 ng/mL) in peripheral blood mononuclear cells (PBMCs) and MT-4 cells. IUPAC name is sodium; (3S,7R)-13-[(2,4-difluorophenyl) methylcarbamoyl]-7-methyl-9,12-dioxo-4-

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oxa-1,8-diazatricyclo [8.4.0.0^{3,8}] tetradeca-10,13-dien-11-olate. Having the molecular formula $C_{20}H_{18}F_2N_3NaO_5$ with molecular weight 441.4.

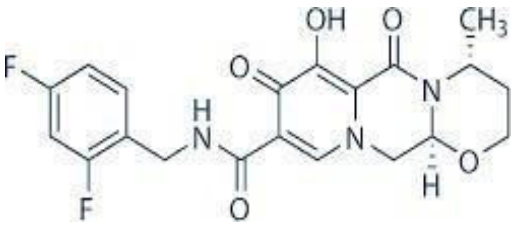

Fig 1: Chemical Structure of Dolutegravir

Lamivudine

Lamivudine Fig. 2 is a nucleoside analog reverse transcriptase inhibitor (NRTI) used to treat AIDS and chronic Hepatitis B. It is a synthetic nucleoside analogue and is phosphorylated intracellularly to its active 5'-triphosphate metabolite lamivudine triphosphate (L-TP). This nucleoside analogue is incorporated into viral DNA by HIV reverse transcriptase and HBV polymerase resulting in DNA chain termination. IUPAC name is ((2R-cis)-4-Amino-1-[2-(hydroxymethyl-1,3-oxathiolan -5- yl)cytosine. Having the molecular formula C8H11N303S with molecular weight 229.3.

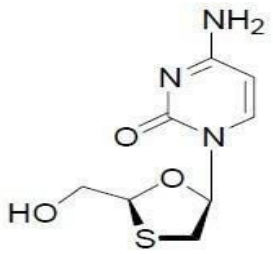

Fig 2: Chemical Structure of Lamivudine

Combination of these two drugs (Dolutegravir -50mg and Lamivudine -300mg:is available in local pharmacy under the brand name of DOVATO Hetero drugs Pvt Ltd. The mechanism of action involves that Dolutegravir inhibits the HIV integrase by binding to the active site and blocking the strand transfer step of retroviral DNA integration in the host cell. The strand transfer step is essential in the HIV replication cycle and results in the inhibition of viral activity. Lamivudine must be converted intracellularly to its triphosphate form, which then competes with cytosine triphosphate for incorporation into the developing viral strand. The present study aimed to develop a simple, sensitive, short retention time and accurate RP-UPLC method for the simultaneous determination of both Dolutegravir and Lamivudine together in pure and tablet dosage forms with high sensitivity, selectivity that can be used for the routine analysis of production samples.

Materials and Methods

Dolutegravir and Lamivudine were kindly supplied by Hetero drugs Pvt Ltd. Acetonitrile, water (UPLC grade, Merck) and all the other reagents of AR grade were purchased from M R Enterprisers. A tablet DOVATO Hetero drugs Pvt Ltd containing Dolutegravir -50mg and Lamivudine -300mg were used. Acetonitrile, water and orthophosphoric acid are UPLC-grade/ analytical grade chemicals were purchased from Merck (Darmstadt, Germany). Centrifuge tubes and 0.45 µm membrane filters were obtained from Millipore Pvt. Ltd. Bangalore, India. Millipore water from MilliQ water purification system was used. The instrument, Waters photodiode array (PDA) detector was used and the electronic analytical balance (Thermo Fisher Scientific, Hyderabad, India) was used for weighing purposes.

Chromatographic conditions

Waters RP-UPLC instrument with PDA detector was used for UPLC method development and validation of the samples.

The technique was developed using a ACQUITY UPLC BEH C18 column (2.1 mm x 50 mm, 1.7 µm) as a stationary phase and using mobile phase containing sodium dihydrogen orthophosphate (pH 3.0): acetonitrile. The mobile phase was degassed and thoroughly mixed before use. The flow rate of mobile phase was maintained at 1.2 mL/min and the eluted Dolutegravir and Lamivudine was monitored at 260 nm. The injection volumes (10 µL) were set for both standards and samples.

Method Development and Validation

Preparation of standard solution:

Accurately weighed and transferred about 1mg of Dolutegravir and 10 mg of Lamivudine into 10 ml volumetric flasks separately and add 5ml of mobile phase to each flask. Then sonicated for 10 min to dissolve the drug and diluted up to the mark with mobile phase.

Preparation of sample stock solution:

Phosphate buffer pH 3 was prepared by dissolving 0.504gm of disodium hydrogen phosphate and 0.301gm of Potassium dihydrogen phosphate in UPLC grade water and adjusts the pH to 3 with glacial acetic acid and sufficient water was added to produce 100 ml then filtered through membrane filter (0.45µ) and sonicated for 10 min.

Preparation of Pharmaceutical Dosage Sample

As per Indian Pharmacopoeias specifications, marketed tablets were weighed and crushed. Tablet powder equivalent to 50 mg of DOLT and 300 mg of LAM was weighed accurately and transferred to a 25ml volumetric flask. The content was dissolved with 10ml of mobile phase and then sonicated for 15 min. The volume was made up with the mobile phase and filtered through 0.45µ membrane filter and sonicated for 20 min. From the above stock solution working stock solution was prepared.

Results and Discussions

Optimized Chromatographic Conditions

Column	: AQUITY UPLC BEH C18 column (150 mm x 2.1 mm, 2 µm particle size)
Run Time	: 8min
Wavelength	: PDA- 260 nm
Flow rate	:1.0 mL/min
Injection volume	:10 µL
Column temp	: Ambient
Pump mode	: Isocratic
Mobile phase	: Acetonitrile: Phosphate buffer pH(3.0) (50:50)

Inference:

Peak obtained for both Dolutegravir and Lamivudine was good with excellent peak characteristics and it was eluted at 2.430 min and 4.360 min for Dolutegravir and Lamivudine respectively. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated

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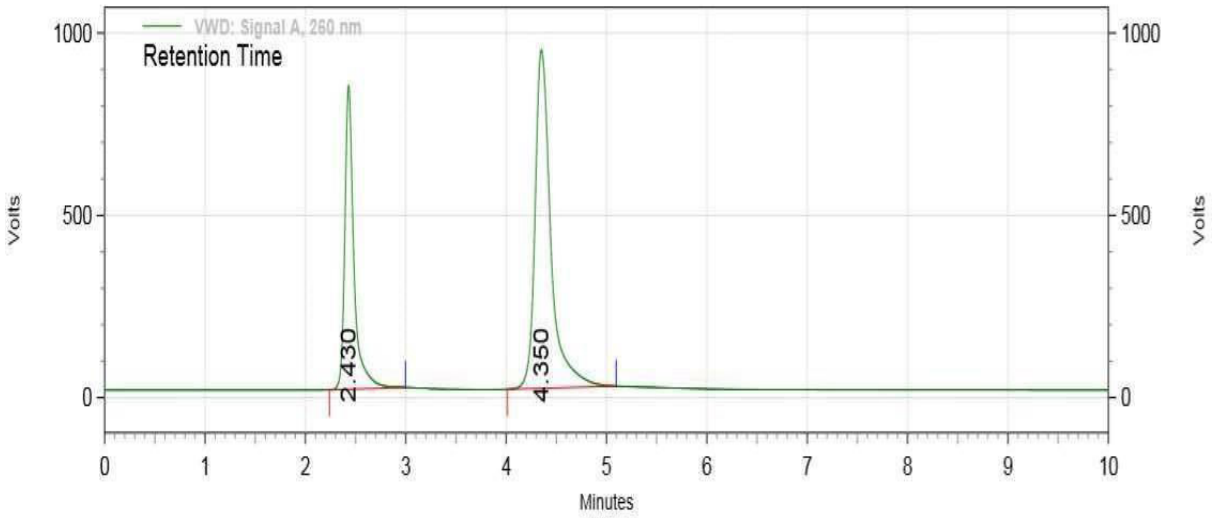

Figure 3: System suitability chromatogram of Dolutegravir and Lamivudine

Method Validation

The optimized method which is derived from the trials can be validated and all the parameters should be checked. The following parameters can be validated in UPLC method.

They are:3,4,5

1) System suitability

2} Specificity

3) Linearity

4) Precision

5) Accuracy

6) Sensitivity

7) Robustness

1. System Suitability

All the system suitability parameters were within the range and satisfactory as per ICH guidelines Q1 R2. The results are reported in table 1.

Table 1: System suitability parameters of Dolutegravir and Lamivudine

Parameters	Dolutegravir	Lamivudine
Retention time (min)	2.430	4.370
Theoretical plates (N)	22.16	22.16
Tailing factor (T)	1.69	1
Resolution (Rs)		1.090

Inference

According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2. All the system suitable parameters were passed and were within the limits.

1. Specificity

Specificity studies were carried for both pure drugs and drug product by comparing with blank and placebo Table 2. These blank and

placebo were compared with standard and sample shows that the analyte chromatographic peak is not attributable to more than one component as the impurities are not available.[8]

Forced Degradation Studies

In the present investigation, as there was no interference of impurities with the analyte peaks, forced degradation studies were conducted with the same LC conditions developed to separate drug peaks of interest from their degradants which proves the stability indicating power of the method.[9] Intentional degradation was attempted to various stress conditions such as acid hydrolysis (using 1.0NHCl), base hydrolysis (using 1.0 N NaOH), oxidative hydrolysis (using 3.0%v/v H2O2), thermal degradation (heated at 70°C for 14 days) and photolytic degradation (to overall illumination of ≥210Wh/m2 at room temperature with UV light for 14 days), to evaluate the ability of the proposed method to separate Dolutegravir and Lamivudine from their degradation products.[10,11]

Acidic hydrolysis:

Forced degradation in acidic media was performed by taking accurately weighed samples of 50 mg of Dolutegravir and 300 mg of Lamivudine each in separate 5mL volumetric flask then .[12]2mL of 1N HCl was added, made to dissolve and final volume was made up to the mark with 1N HCl to get mg/mL solutions and these were kept at 70ºC for 2 days and analyzed after suitable dilution.

Basic hydrolysis:

Forced degradation in basic media was performed by dissolving separately an accurately weighed quantities 25 mg of Dolutegravir and 150 mg of Lamivudine in 1N NaOH in 5mL volumetric flasks and final volume was made up to 5mL with the same to get mg/mL solutions and these solutions dilution.[13]

Oxidative degradation:

Were kept at 70ºC for 2 days and analyzed after suitable Oxidative degradation studies were carried out in 3% (v/v) H2O2. Stock solutions of 25mg of Dolutegravir and 150 mg of Lamivudine were prepared and kept at 70ºC for 2 days and analyzed after suitable dilution. Photodegradation: For photolytic stress, samples of drug substances in solid state were irradiated with UV radiation (overall illumination of ≥210Wh/m2 at room temperature with UV radiation), for 14 days. Stock solutions of 1mg/mL were prepared in methanol from the exposed drug substances individually.

Thermal Degradation:

For thermal stress, 25mg of Dolutegravir and 150 mg of lamivudine of drug substances in solid state were packed in glass 1mg/mL were prepared in methanol from the exposed drug substances individually. For UPLC analysis, all the stressed sample solutions were diluted with mobile phase to obtain final concentration of 60µg/mL of Dolutegravir and lamivudine analysis by UPLC.
Besides, solutions containing 60µg/mL of Dolutegravir and lamivudine for each drug separately were also prepared without performing the degradation of both the drugs. Then 20µL of above solutions were injected into the UPLC system and analyzed. Solution of standard, sample, blank and placebo were prepared as per test procedure and injected into the UPLC system.

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Table 7.9: Results of forced degradation study for Dolutegravir

Type of stress	Degradation products/ Drug (D)	Retention time	% Area	Peak purity	Result
Acidic Hydrolysis (mg/mL in 1N HCl)at 70°C for 2 days	-	2.477	4456894	0.999	Passed
Basic Hydrolysis (mg/mL in 1N NaOH) at 70°C for 2 days	-	2.479	4457894	0.999	Passed
Oxidative Hydrolysis (mg/mL in 3% v/v H2O2) at 70 °C for 2 days	-	2.515	4456712	0.999	Passed
Photo Degradation (to UV light) for 14 days	-	2.476	4568741	0.999	Passed
Thermal Degradation at 70°C for 14 days	-	2.462	4458412	0.999	Passed

Table 7.9: Results of forced degradation study for Lamivudine

Type of stress	Degradation products/ Drug (D)	Retention time	% Area	Peak purity	Result
Acidic Hydrolysis (mg/mL in 1N HCl)at 70°C for 2 days	-	4.321	6541254	0.999	Passed
Basic Hydrolysis (mg/mL in 1N NaOH) at 70°C for 2 days	-	4.312	6541278	0.999	Passed
Oxidative Hydrolysis (mg/mL in 3% v/v) at 70 °c for 2 days	-	4.329	6542151	0.999	Passed
Photo Degradation (to UV light) for 14 days	-	4.328	6585241	0.999	Passed
Thermal Degradation at 70°C for 14 Days	-	4.38	6558712	0.999	Passed

3. Linearity

Linearity is the property of a mathematical relationship or function which means that it can be graphically represented as a straight line.8 Linearity was studied by analyzing five standard solutions covering the range of standard concentrations of sample solutions.

Table:7.3 Results for Linearity (n=3)

Parameters	Lamivudine	Dolutegravir
Slope	782071	396499
y intercept	387706	593776
Correlation coefficientr2	0.9995	0.9999
Regression Equation	y = 782071x + 387706	y =396499x –593776
Linearity range	50-250 µg/ml	50-250 µg/ml
LOD	0.060	0.042
LOQ	0.198	0.132

Inference

Retention times of Lamivudine and Dolutegravir were found to be 3.296 min and 7.257. We did not find any interfering peaks in blank. So this method was said to be specific respectively

The linearity of the method was demonstrated over the concentration range of 10 – 50 µg/ml and 100-300 µg/ml of Lamivudine and Dolutegravir respectively.

4. Precision

Precision is a description of random errors, a measure of statistical variability.7 The Precision of the instrument was checked by repeated injection and measurement of peak areas and retention time of solution. Types are:

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Table 4 : Precision data for Lamivudine and Dolutegravir

S. No	Lamivudine			Dolutegravir		
	RT	Area	%Assay	RT	Area	%Assay
Injection1	3.320	4465231	99	7.458	7360011	100
Injection2	3.321	4462350	99	7.471	7368755	100
Injection3	3.316	4464645	100	7.451	7364800	100
Injection4	3.312	4462083	99	7.419	7365230	100
Injection5	3.313	4468154	100	7.398	7361573	100
Injection6	3.312	4466897	99	7.370	7361600	100
Mean			99			100
Std. Dev.			0.18			0.12
% RSD			0.18			0.12

Inference:
The %RSD of precision were found to be 0.18 for Lamivudine and 0.12 for Dolutegravir respectively

5. Accuracy:
Accuracy is the degree of closeness of measurements of a quantity to that quantity's true value. Accuracy of the method was determined by calculating the recoveries of Lamivudine and Dolutegravir by the standard addition method.

Table:7.6 Results for Accuracy (n=3)

Recovery level	Dolutegravir				Lamivudine			
	Amount Added (µg/ml)		Amount Found (µg/ml)	% Recovery	Amount Added (µg/ml)		Amount Found (µg/ml)	% Recovery
	Std	Test			Std	Test		
50%	100	100	197.5	98.75	100	100	199.2	99.6
100%	100	150	245.3	98.13	100	150	257.7	103
150%	100	200	296.6	98.89	100	200	299.3	99.76
Mean Recovery	98.59				100.7			

Inference
The average percentage recovery was between 98-102% and Relative standard deviation of these recovery concentrations was less than 2%.

6. Sensitivity

a) LOD: It is the lowest quantity of a substance that can be distinguished from the absence of that substance (a blank value) within a stated confidence limit.
LOD = (3.3 x S.D.)/ slope

b) LOQ: It is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met.
LOQ = (10 x S.D.)/ slope

Table 7: LOD and LOQ data for Lamivudine and Dolutegravir

S.No	Sample name	LOD data		LOQ data	
		RT	Area	RT	Area
1	**Lamivudine**	3.294	1277996	3.298	1523173
2	**Dolutegravir**	7.248	2276040	7.247	2717390

Inference
The LOD value was found to be 0.090 µg/mL for Lamivudine and 0.090 µg/mL for Dolutegravir at signal to noise ratio 3:1. LOQ value was found to be 0.301 µg/mL for Lamivudine and 2.2063 µg/mL for Dolutegravir at signal to noise ratio 10:1.

7. Robustness

It is the measure of a method remain unaffected by small deliberate changes in method parameters like flow rate and mobile phase composition ratio

Table:7.8 Results for Robustness

Parameters(n=3)	%RSD	
	Dolutegravir	Lamivudine
Detection wavelength at258nm	0.68	0.67
Detection wavelength at262nm	0.16	0.86
Flow rate 1.4ml/min	0.28	0.97
Flow rate 1ml/min	0.51	0.39
Analyst I	0.69	0.72
Analyst II	0.95	0.85

Inference

The %RSD for flow rate was found to be 0.28 and 0.51 for Dolutegravir and 0.97 and 0.39 for Lamivudine, %RSD for wavelength was found to be 0.68 and 0.16 for Dolutegravir and 0.67 and 0.86 for Lamivudine and %RSD for Analyst I & II was found to be 0.69 and 0.95 for Dolutegravir and 0.72 and 0.85 for Lamivudine respectively.

CONCLUSION

The Dolutegravir and Lamivudine showed linearity in the range of 100-300 µg/mL and 10–50 µg/mL respectively. The slope and correlation coefficient values for Dolutegravir were found to be 0.9986 and 0.9998 respectively for Lamivudine which indicates excellent correlation between response factor Vs concentration of standard solutions. Precision of the developed method was studied under system precision and method precision. The %RSD values for precision was found to be within the acceptable limit, which revealed that the developed method was precise. The developed method was found to be robust. The %RSD value for percentage recovery Dolutegravir and Lamivudine was found to be within the acceptance criteria. The results indicate satisfactory accuracy of method for simultaneous estimation of the Dolutegravir and Lamivudine. The forced degradation study showed the method was highly specific.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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