Original Research Article

Forced Degradation Behaviour with a Developed and Validated RP-UPLC Method for Estimation Lamivudine and Dolutegravir in Combined Pharmaceutical Dosage Form.

ABSTRACT:

A simple, fast, precise, specific, and accurate reversed phase Ultra Performance liquid chromatographic (UPLC) method was developed and validated for the forced degradation studies of the Lamivudine and Dolutegravir in tablet dosage form. Chromatogram was run HSS C_{18} (2.6 x 50mm, 1.6µm). Mobile phases containing 70% 0.01N disodium hydrogen phosphate: 30% Methanol). The elution of analytes was achieved with a flow rate at 0.3 mL/min. Potassium dihydrogen phosphate was used as a buffer in this experiment. Temperature was maintained at 30°C. Optimized wavelength selected at 260nm. The detector response was linear in the concentration range of 25-150µg/mL respectively. Retention time of Lamivudine and Dolutegravir were found to be 1.408min and 1.739min. %RSD of the Lamivudine and Dolutegravir were and found to be 0.8 and 0.8. The % recovery was obtained as 100.39% and 100.37% for Lamivudine and Dolutegravir. LOD, LOQ values obtained from regression equations of Lamivudine and Dolutegravir were 0.41, 1.25 and 0.09, 0.26. Regression equation of Lamivudine is y = 24270x + 12218 and for Dolutegravir was y = 34783x + 1060. Since retention times and run times were reduced, the method established was easy and premium and it could be used in periodic quality control tests in industries.

KEY WORDS: Lamivudine, Dolutegravir, RP-UPLC, Forced degradation.

INTRODUCTION:

Dolutegravir is Chemically (4R,12aS)-N-[(2,4-Difluorophenyl)methyl]-3,4,6,8,12,12a-hexahydro-7-hydroxy-4-methyl-6,8-dioxo-2H-pyrido[1',2':4,5]pyrazino[2,1-b][1,3]oxazine-9-carboxamide (Figure No:1). Its molecular Formula $C_{20}H_{19}F_2N_3O_5$ and Molecular weight 419.38 g/mol. Dolutegravir is an HIV-1 integrase strand transfer inhibitor of the second generation. For the treatment of HIV infection, dolutegravir is now in Phase III clinical studies. Dolutegravir has been proven to inhibit HIV replication in cells infected with a self-inactivating PHIV lentiviral vector, such as peripheral blood mononuclear cells (PBMCs), MT-4 cells, and CIP4 cells. It is slightly soluble in water and Acetonitrile. It binds to the active site of HIV integrase and prevents the strand transfer stage of retroviral DNA integration. This is a critical phase in the HIV replication cycle that will result in viral activity being inhibited. Dolutegravir has a mean EC_{50} value of 0.5nm (0.21 ng/mL) to 2.1nm (0.85 ng/mL) in peripheral blood mononuclear cells (PBMCs) and MT-4 cells. Dolutegravir is authorised for the treatment of HIV-1 infection in adults and children aged 12 years and older in conjunction with other antiretroviral medicines and weighing at least 40 kg[2].

Lamivudine chemically known as 4-amino-1-[(2R, 5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one.Its molecular formula $C_8H_{11}N_3O_3$ (Figure No:2). Lamivudine is a synthetic nucleoside analogue that is phosphorylated intracellularly to produce lamivudine triphosphate, an active 5'-triphosphate metabolite (L-TP). HIV reverse transcriptase and HBV polymerase integrate this nucleoside analogue into viral DNA, resulting in DNA chain termination. Lamivudine is a nucleoside reverse transcriptase inhibitor (NRTI) that inhibits viral DNA synthesis in the HIV-1 and Hepatitis B (HBV). Lamivudine can produce active metabolites when phosphorylated, which compete for inclusion into viral DNA. Lamivudine metabolites function as a chain terminator of DNA synthesis by competitively inhibiting the activity of the HIV reverse transcriptase enzyme via DNA incorporation. Incorporated nucleoside analogues prohibit the development of a 5'-OH group because they lack a 3'-OH group. Phosphodiester linkage that is essential for DNA chain elongation[2-4].

Extensive literature survey was carried out which revealed that there is no work carried out especially on Forced degradation method of the Lamivudine and Dolutegravir in tablet dosage form using UPLC method. The specific aim of the research was to develop a UPLC method and performing forced degradation studies of Lamivudine and Dolutegravir in bulk

and formulated dosage form and to validate the proposed methods in accordance with ICH guidelines for the intended analytical application[5-12]

MATERIALS AND METHODS:

Instruments and Apparatus:

Standard Lamivudine and Dolutegravir pure drugs (API), Combination Lamivudine and Dolutegravir tablet (Dovato), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dehydrogenate ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem Pvt. Ltd, India. The Ultra-High Performance liquid chromatography (WATERS UPLC Auto Sampler TUV Detector with Empower 2 Software). UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2 mm and 10 mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Lamivudine and Dolutegravir solutions.

Preparation of Standard stock solutions:

Accurately weighed 75mg of Lamivudine, 12.5mg of Dolutegravir and transferred to individual 50mL volumetric flasks separately. Both flasks were sonicated for 10 minutes after 3/4 of the diluents were introduced. Standard stock solution 1 and 2 were prepared in flasks with diluents and labelled. (1500µg/mL of Lamivudine and 250µg/mL of Dolutegravir).

Preparation of Standard working solutions (100% solution):

1mL from each stock solution was Pipetted out and taken into a 10 mL volumetric flask and made up with diluent. (150µg/mL Lamivudine of and 25 µg/mLof Dolutegravir).

Preparation of Sample stock solutions:

10 tablets were weighed and was transferred into a 100mL volumetric flask, 50mL of diluents was added and sonicated for 25min, further the volume was made up with diluent and filtered by HPLC filters (3000µg/mL of Lamivudine and 500µg/mL of Dolutegravir).

Preparation of Sample working solutions (100% solution):

0.5 mL of filtered sample stock solution was transferred to 10mL volumetric flask and made up with diluent (150µg/mL of Lamivudine and 25µg/mL of Dolutegravir).

Buffer preparation:

Accurately weighed 1.41gm of sodium dihydrogen Ortho phosphate in a 1000mL of Volumetric flask add about 900mL of milli-Q water added and degas to sonicate and finally make up the volume with water.

Method Development:

Chromatographic analysis was performed on Acquity UPLC HSS C18 (2.6 x 50mm, 1.6µm). The mobile phase consists of 70% 0.01N Na_2HPO_4:30% Methanol was used through the analysis. The flow rate was 0.3mL/min, the injection volume was 1.0mL, column temperature was 10ºC and detection was performed at 260nm using a UV detector.

METHOD VALIDATION:

The analytical method was verified for linearity, accuracy, precision, specificity, robustness, and ruggedness in accordance with ICH recommendations.

Linearity:

Linearity was demonstrated from 1500µg/mL of Lamivudine and 250µg/mL of Dolutegravir of standard concentration using minimum six calibration levels (25%, 50%, 75%, 100%, 125% and 150%) for both the drugs. The data was analysed using the linear regression approach.

Accuracy:

The proximity between the reference value and the obtained value is expressed by the accuracy of an analytical method. The accuracy of the method was evaluated in triplicate at three concentration levels, 50%, 100%, and 150% of standard solutions of Lamivudine and Dolutegravir.

Robustness:

Robustness conditions like Flow mins (0.2mL/min), Flow plus (0.4mL/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature Plus (35°C) was maintained and samples were injected in duplicate manner. The system suitability parameters were relatively unaffected, and all of them were passed. %RSD was within the limit.

Specificity:

Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific and excellent.

Precision:

Preparation of Sample stock solutions:

10 tablets were weighed and was transferred into a 100 mL volumetric flask, 50mL of diluents was added and sonicated for 25min, further the volume was made up with diluent and filtered by HPLC filters (3000µg/mL of Lamivudine and 500 µg/mL of Dolutegravir).

Preparation of Sample working solutions (100% solution):

In a 10 mL volumetric flask, 0.5 mL of filtered sample stock solution was transferred and made up with diluent and made up with diluent. (150µg/mL of Lamivudine and 25µg/mL of Dolutegravir).

Forced degradation studies:

Oxidation:

To 1mL of stock solution of Lamivudine and Dolutegravir, 1mL of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were maintained at 60°C for 30 minutes. For UPLC study, the resultant solution was diluted to obtain 150µg/mL & 25 µg/mL solution and 1.0µL were injected into the system.

Acid Degradation Studies:

To 1 mL of stocks solution Lamivudine and Dolutegravir, 1 mL of 2N Hydrochloric acid was added and refluxed for 30 mins at 60^0c. The resultant solution was diluted to obtain 150 µg/mL & 25 µg/mL solution and 1.0µl solutions were injected into the system.

Alkali Degradation Studies:

To 1 mL of stock solution Lamivudine and Dolutegravir, 1mL of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 150 µg/mL & 25µg/mL solution and 1.0 µL were injected into the system.

Dry Heat Degradation Studies:

To investigate dry heat degradation, the standard medication solution was baked for 6 hours at 105°C. The resulting solution was diluted to 150µg/mL & 25µg/mL for UPLC testing.to and 1.0µL were injected into the system.

Photo Stability studies:

The photochemical stability of the drug was also studied by exposing the 1500µg/mL & 250µg/mL solution to UV Light by keeping the beaker in UV Chamber for 7 days or 200-Watt hours/m^2 in photo stability chamber. For UPLC study, the resultant solution was diluted to obtain 150µg/mL & 25µg/mL solutions and 1.0µL were injected into the system.

Neutral Degradation Studies:

Stress testing under neutral conditions was studied by refluxingthedruginwaterfor6hrs at temperature of 60º.For UPLC study, the resultant solution was diluted to 150µg/mL & 25µg/mL solution and 1.0µL were injected into the system. (Figure 6 to 9 and Table 6)

RESULTS AND DISCUSSION:

Optimized method:

Lamivudine and Dolutegravir were eluted at 1.408 min and 1.739 min respectively with good resolution. Because the plate count and tailing factor were both good, this method was refined and validated. (Figure No. 3)

Linearity:

Six linear concentrations of Lamivudine (37.5-225µg/mL) and Dolutegravir (6.25-37.5µg/mL) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Lamivudine was y = 24270x + 12218 and for Dolutegravir was y = 34783x + 1060. The two drugs have a correlation value of 0.999. (Figure No: 4 &5 and Table 1).

Precision:

From a single volumetric flask of working standard solution six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 0.8% and 0.8% respectively for Lamivudine and Dolutegravir. (Table 5).

Accuracy:

Three levels of Accuracy samples were prepared by standard addition method. Triplicate injections were given for each level of accuracy and mean %Recovery was obtained as 100.39% and 100.37% for Lamivudine and Dolutegravir respectively.(Table 2 and 3)

Robustness:

Robustness conditions like Flow minus (0.2 mL/min), Flow plus (0.4 mL/min), mobile phase minus (75B:25M), mobile phase plus (65B:35M), temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner. The system suitability parameters were largely unaffected, and all of them were passed. %RSD was within the limit. (Table 4).

CONCLUSION

A simple, Accurate, precise method for the simultaneous estimation of the Lamivudine and Dolutegravir in tablet dosage form was developed and validated. Retention time of Lamivudine and Dolutegravir were found to be 1.408min and 1.739min. %RSD of the Lamivudine and Dolutegravir were and found to be 0.8 and 0.8 respectively. %Recovery was obtained as 100.39% and 100.37% for Lamivudine and Dolutegravir respectively. LOD,

LOQ values obtained from regression equations of Lamivudine and Dolutegravir were 0.41, 1.25 and 0.09, 0.26 respectively. Regression equation of Lamivudine is y = 24270x + 12218 and y = 34783x + 1060 of Dolutegravir. The developed UPLC method in the present study for the estimation were found to be simple, rapid, accurate, precise, specific, linear and rugged. They are thus suitable for the simultaneous estimation in raw materials and formulations. The newly developed analytical methods can be used in research institutions, academic institutes, quality control department in industries, approved testing laboratories, biopharmaceutics & bioequivalence studies and clinical & pharmacokinetic studies after suitable modification.

DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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FIGURES:

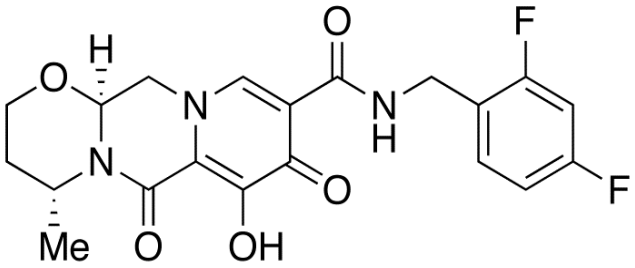

Figure 1.Structure of Dolutegravir

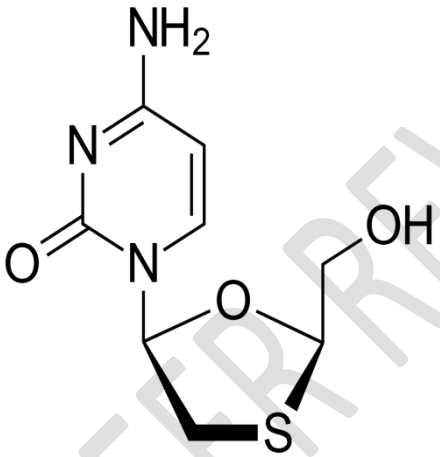

Figure 2.Structure of Lamivudine

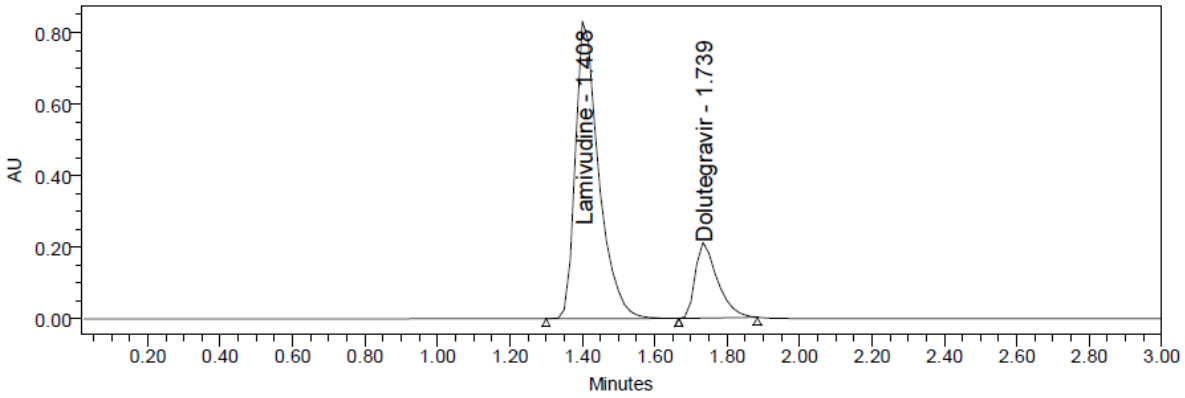

Figure 3.Optimized Chromatogram

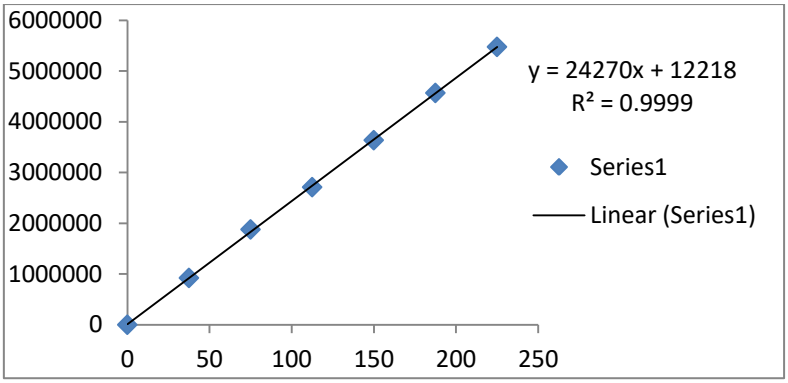

Figure 4.Calibration curve of Lamivudine

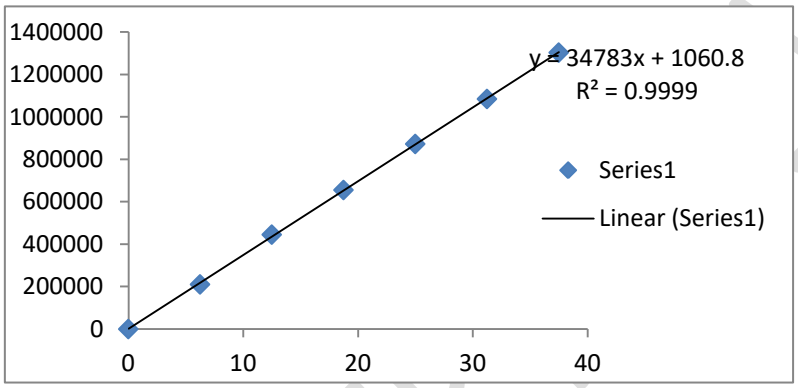

Figure 5. Calibration curve of Dolutegravir

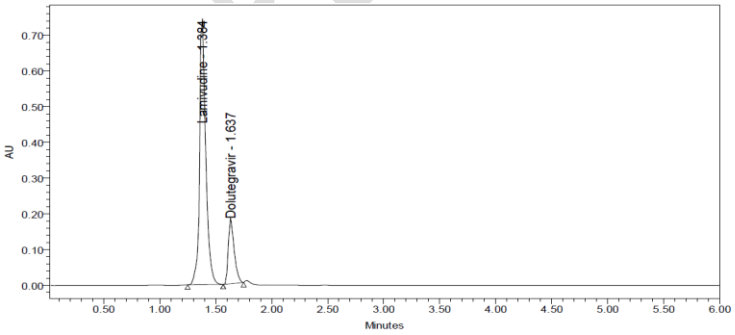

Figure 6.Acid degradation chromatogram

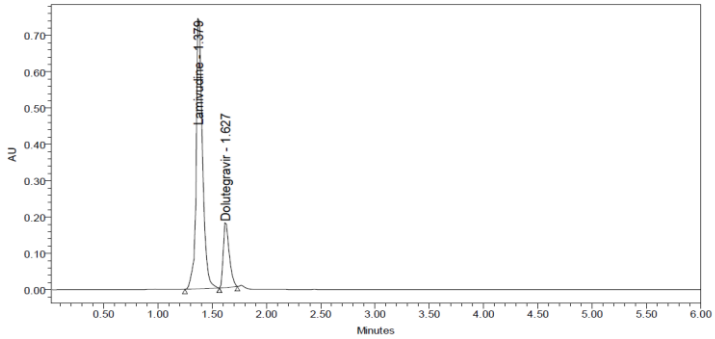

Figure 7.Base degradation chromatogram

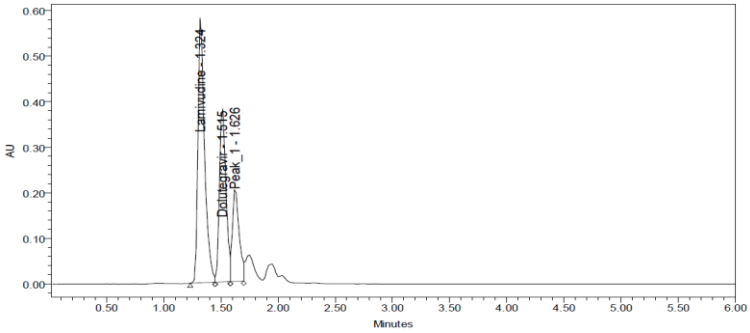

Figure 8.Peroxide degradation chromatogram

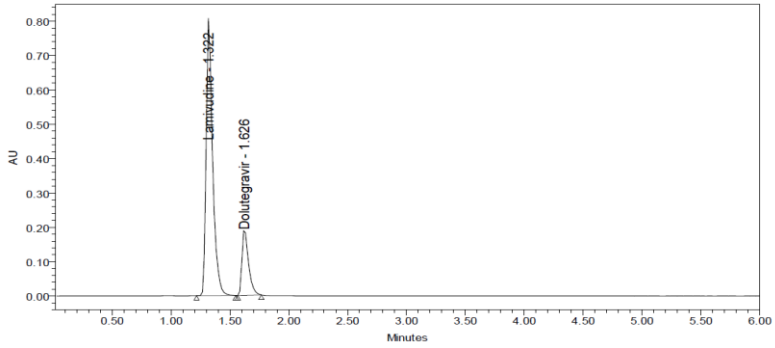

Figure 9.Thermal degradation chromatogram

TABLES:

Table 1. Linearity table for Lamivudine and Dolutegravir

Lamivudine		Dolutegravir	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**
0	0	0	0
25	922806	25	210556
50	1878662	50	445258
75	2712498	75	655630
100	3638097	100	872691
125	4569129	125	1084994
150	5477284	150	1303577

Table 2. Accuracy of Lamivudine

% Level	**Amount Spiked (µg/mL)**	**Amount recovered (µg/mL)**	**% Recovery**	Mean %Recovery
50%	75	74.63	99.50	100.39%
	75	74.93	99.91	
	75	75.55	100.74	
100%	150	147.86	98.58	
	150	151.98	101.32	
	150	150.99	100.66	
150%	225	228.58	101.59	
	225	226.97	100.88	
	225	225.78	100.34	

Table 3. Accuracy of Dolutegravir

% Level	**Amount Spiked (µg/mL)**	**Amount recovered (µg/mL)**	**% Recovery**	Mean %Recovery
50%	12.5	12.49	99.94	100.37%
	12.5	12.48	99.83	
	12.5	12.54	100.34	
100%	25	25.34	101.36	
	25	25.28	101.12	

	25	25.25	101.00
150%	37.5	37.32	99.51
	37.5	37.69	100.50
	37.5	37.38	99.68

Table 4. Robustness data for Lamivudine and Dolutegravir

S.No	Condition	%RSD of Lamivudine	%RSD of Dolutegravir
1	Flow rate (-) 0.2mL/min	0.7	1.0
2	Flow rate (+) 0.4mL/min	0.3	0.5
3	Mobile phase (-) 75B:25M	0.2	0.4
4	Mobile phase (+) 65B:35M	0.7	1.1
5	Temperature (-) 25°C	0.1	0.1
6	Temperature (+) 35°C	0.2	0.7

Table 5. System precision table of Lamivudine and Dolutegravir

S. No	Area of Lamivudine	Area of Dolutegravir
1.	3640080	882110
2.	3662158	882532
3.	3623764	870302
4.	3646966	872703
5.	3650353	878651
6.	3710141	889046
Mean	3655577	879224
S.D	29577.7	6902.1
%RSD	0.8	0.8

Table 6. Degradation data for Lamivudine and Dolutegravir

Type of degradation	Lamivudine			Dolutegravir		
	Area	Percentage	Percentage Degraded	Area	Percentage Recovered	Percentage Degraded

		Recovered				
Acid	3408992	93.16	6.84	832130	94.55	5.45
Base	3496767	95.56	4.44	832826	94.63	5.37
Peroxide	3520360	96.20	3.80	814105	92.50	7.50
Thermal	3623820	99.03	0.97	846632	96.20	3.80
UV	3617941	98.87	1.13	849805	96.56	3.44
Water	3651379	99.79	0.21	875607	99.49	0.51