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Stability Indicating Method Development and Validation Foe the Simultaneous Estimation of Dolutegravir, Lamivudine and Tenofovir Disoproxil Fumarate by Rp-Hplc

Dr.K. Mangamma, P. Rohini*, G. Dimple Manvitha, P. Ujwala rama Chandra, Pushpala Rohini

School of pharmaceutical sciences, jntuk Andhra Pradesh,Pin code: 535003
**Corresponding author : Pushpala Rohini*

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ABSTRACT: A simple, Accurate, precise method was developed for the simultaneous estimation of the Dolutegravir, Lamivudine, Tenofovir Disoproxil Fumarate in Tablet dosage form. Good chromatographic separation was achieved with Acetonitrile & methanol in (80:20v/v) with gradient mode usingKinetex Biphenyl 250*4.6mm, 5µm or equivalent column as stationary phase with flow rate of 1.0 mL/min and column temperature of 30°C at a wavelength of 260nm. The optimized mobile phase produced sharp peak, well defined peak with retention times (11.449 min for lamivudine, 26.789 min for tenofovir and 30.281 min for Dolutegravir). The % RSD was less than 1 which was indicated high degree of precision. The linearity was found to be at the concentration range 120.27-362.16 µg/mL with a correlation coefficient (r^2) of 0.9995Lamivudine,120.27-362.16 µg/mL with a correlation coefficient (r^2) of 0.9998 for Tenofovir and 20.28- 63.07 µg/mL with a correlation coefficient (r^2) of 0.9994 for Dolutegravir. The overall recovery rates were 99.6 ,99.6 and 99.6 for lamivudine, tenofovir and Dolutegravir accuracy of the method and good recovery of the analytes. The results of the robustness study indicate that the method was unaffected by small variations in the chromatographic conditions. The forced degradation study showed that there is no interference from degradants and peak purity of analytes has been passed.

Keywords: Dolutegravir, Lamivudine, Tenofovir and HPLC

I. INTRODUCTION

Dolutegravir[19](DTG, is a newly developed human immunodeficiency virus (HIV) integrase inhibitor from ViiV Healthcare (Research Triangle Park, NC, USA). DTG is an integrase strand transfer inhibitor (INSTI) that does not require ritonavir for cytochrome P450 3A4 inhibition, and preferentially blocks the strand transfer step of integration of the viral genome into the host cell's DNA, which is a two-step process mediated by the viral integrase enzyme. Once integration is blocked, HIV-1 can no longer replicate, and the viral replication cycle is interrupted.

Lamivudine[20] is a nucleoside reverse transcriptase inhibitor (NRTI)reported to be active against HIV-1, and hepatitis B virus. Lamivudine used for treatment of hepatitis B (chronic) at a lower dose than for treatment of HIV. It improves the seroconversion of e-antigen positive hepatitis B and also improves histology staging of the liver.

Tenofovir disoproxil Fumarate[21] is fumaric acid salt of the bis iso-propoxy carbonyl oxy methyl ester derivative of Tenofovir Chemically it is 9-[(R)-2- [[(isopropoxcarbonyl)-oxy] methoxy] phosphinyl] methoxy] propyl] adenine fumarate [4e7]. Tenofovir exhibits activity against HIV-reverse transcriptase.

II. METHODS

Instrumentation

The chromatography separation was performed on Agilent water system which is equipped with a quaternary pump and photodiode array detector. Empower-3.0 is used as chromatography software for data integration, data collection.

Chemicals and reagents

Acetonitrile (HPLC grade), Methanol (HPLC grade) both are purchased from merk and Water was purified by milli Q purification system. Dolutegravir, lamivudine and tenofovir disoproxil fumarate reference standards were received from Hetero drugs pvt.Ltd.

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Chromatographic condition

The HPLC analysis was performed on reverse phase chromatography by using the column Kinetex Biphenyl 250*4.6mm, 5µm or equivalent. The mobile phase composition (80:20) ratio of Acetonitrile and OPA with a flow rate of 1.0 ml/min at 260 nm by using PDA detector. Column temperature maintained at 30°C with injection volume 10µl, followed by run time 45min.chromatographic analysis was done using Gradient elution.

Preparation of Buffer

Transfer about 1.36g of Potassium Dihydrogen Phosphate and 1g 1-Octane sulfonic acid sodium salt monohydrate into a beaker containing 1000 mL of Milli-Q water and Sonicated to dissolve. Adjust pH of the solution to 3.0±0.05 with Trifluoracetic acid solution. Filter the solution through 0.45µm membrane filter.

Preparation of Mobile phase

Use Buffer as mobile phase -A and acetonitrile and methanol in the ratio of 80:20 (v/v%).

Preparation of diluent

Prepare a degassed mixture of 0.1%Orthophosphoric acid buffer and Methanol in the ratio of 30:70 (%v/v).

Preparation of Dolutegravir standard stock solution

Accurately weigh and transfer about 53mg of Dolutegravir sodium working standard into a 100mL volumetric flask, add about 60mL of Methanol and sonicate to dissolve. Dilute to volume with Methanol and mix.

Preparation of Lamivudine and Tenofovir disoproxil fumarate standard stock solution

Accurately weigh and transfer about 60mg of Lamivudine and Tenofovir disoproxil fumarate working standard into a 50mL volumetric flask. Add about 30mL of Methanol and sonicate to dissolve. Dilute to volume with Methanol and mix. Transfer each 5mL of the Lamivudine and Tenofovir disoproxil fumarate standard stock solution and 2mL of Dolutegravir standard stock solution into a 25mL volumetric flask, dilute to volume with diluent and mix.

Preparation of sample solution

Transfer 5tablets (5/25/25mg) in to a 500mL volumetric flask, add about 50mL of 0.1%OPA Buffer and sonicate for not less than 45minutes with Intermediate shaking. Add about 300mL of Methanol sonicate for not less than 30minutes with occasional shaking {maintain the Sonicator bath temperature between 20 to 25°C). Dilute to volume with Methanol and mix. Centrifuge a portion of the solution at 5000rpm for about 10minutes.Filter the solution through 0.45µm membrane filter and discard first few ml of the filtrate. Transfer 4mL of above solution into a 50mL volumetric flask, dilute to volume with diluent and mix.

III. METHOD VALIDATION:

System suitability

Five sample solutions were prepared for Lamivudine, Tenofovir DF and Dolutegravir. The solutions were injected were into the HPLC system as per test procedure. The Resolution, theoretical plates, tailing factor and %RSD were calculated.

Specificity

Specificity was determined by identification of Lamivudine, Tenofovir disoproxil fumarate and Dolutegravir in sample, standard and Blank injected into HPLC system. Placebo sample was prepared by taking the placebo equivalent to about the weight portion of test preparation and injected into the HPLC system.

Linearity

The linearity of the method was demonstrated over the concentration range of 50% - 150% of the target concentration. Aliquots of 50%, 75%, 100%, 125% and 150% were prepared from standard stock solution. Standard solutions of 50 – 150% concentration were injected separately into the HPLC system. Concentration vs. peak area were constructed for the drugs.

Precision

The precision of the method was determined by system precision and method precision using 100% standard and sample solutions. The system precision was established by injecting six replicate injections of standard solution in to the HPLC system.Six assay samples of drug product at 100% of the sample concentration were prepared and injected into the chromatographic system and the chromatograms were recorded.

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Accuracy

The accuracy of the method was determined by analyzing three solutions containing Fluvoxamine maleate at approximately 50%,100% and 150%. Three samples were injected three times each into the HPLC system.

Robustness

As part of evaluation of robustness, deliberate changes were made in the flow rate, Organic phase modifications and wavelength to evaluate the impact on the method.

Effect of variation of flow rate

Standard solution prepared as per the test method was injected into the chromatographic system maintaining flow rates, less flow (0.8mL/min), more flow (1.2mL/min) and actual flow (1.0mL/min).

Effect of variation of Organic phase

Standard solution prepared as per the test method was injected into the chromatographic system maintaining flow rates, 5% less organic phase ,5% more organic phase and actual organic phase.

Effect of variation of wavelength

Standard solution prepared as per the test method was injected into the chromatographic system maintaining flow rates, less wavelength (190nm), more wavelength(292nm) and actual wavelength(260nm).

Stress degradation studies

Stress degradation study was conducted in acid, base, peroxide and homogeny of the peak was assessed in terms of peak purity.

Filter validation

Filter validation was performed by filtering standard and sample solutions with different filters.

Acid degradation

Transfer each 5mL of the Lamivudine and Tenofovir disoproxil fumarate standard stock solution and 2mL of Dolutegravir standard stock solution into a 25mL volumetric flask, add 1 ml 1N HCL solution and place in water bath at 80°C for 15 mins after that take out, cool for some time and add 1ml 1 N NaOH and make up the volume with diluent.

Base degradation

Transfer each 5mL of the Lamivudine and Tenofovir disoproxil fumarate standard stock solution and 2mL of Dolutegravir standard stock solution into a 25mL volumetric flask, add 1 ml 1 N NaOH solution and place in water bath at 80°C for 15 mins after that take out, cool for some time and add 1ml 1 N HCL and make up the volume with diluent.

Peroxide degradation

Transfer each 5mL of the Lamivudine and Tenofovir disoproxil fumarate standard stock solution and 2mL of Dolutegravir standard stock solution into a 25mL volumetric flask, add 1 ml 3% Hydrogen peroxide solution and place in water bath at 80°C for 15 mins after that take out, cool for some time and make up the volume with diluent.

IV. RESULTS AND DISCUSSION:

System suitability

USP plate count was 89766, 291452 and 296504 for lamivudine, Tenofovir DF and Dolutegravir respectively and Tailing factor was 1.1,1.1 and 1.3 for lamivudine, Tenofovir DF and Dolutegravir respectively.

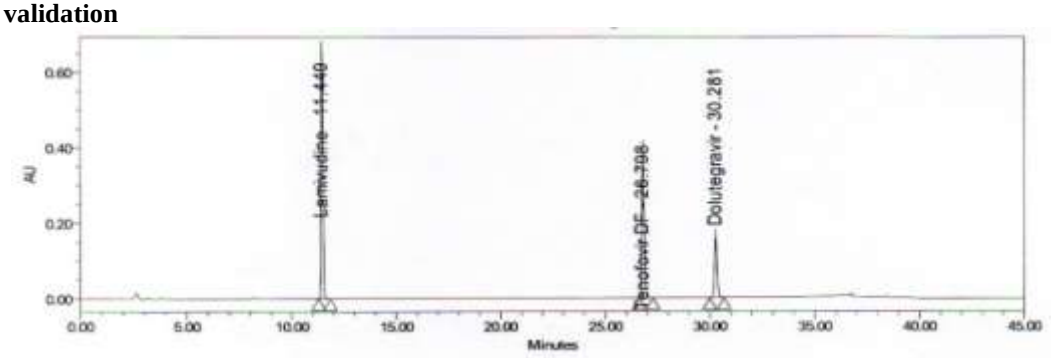

Fig.1: Chromatogram for standards

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Precision

The percentage RSD of precision during assay should be less than 2. The percentage RSDS of system precision of lamivudine, Tenofovir DF and Dolutegravir was found to be 0.3 for all three drugs and the results shown in Table 1.

Table1: Results for system precision

	Sample-1	Sample -2	Sample-3	Sample-4	Sample-5	Sample-6
Name	**Area**					
Lamivudine	3698305	3698305	3698305	3698305	3698305	3698305
Tenofovir DF	3040039	3040039	3040039	3040039	3040039	3040039
Dolutegravir	1351772	1351772	1351772	1351772	1351772	1351772
				Mean	**±S. D**	**%RSD**
lamivudine				3717027	12879	0.3
Tenofovir DF				3048653	12879	0.3
Dolutegravir				1356763	4678	0.3

Linearity

Calibration curve for this assay method was plotted and obtained calibration ranges are calculated and correlation coefficient obtained from curve was greater than 0.999. the obtained curve was linear so the method is said to be linear (Fig 2,3& 4) and the results shown in Table 2.

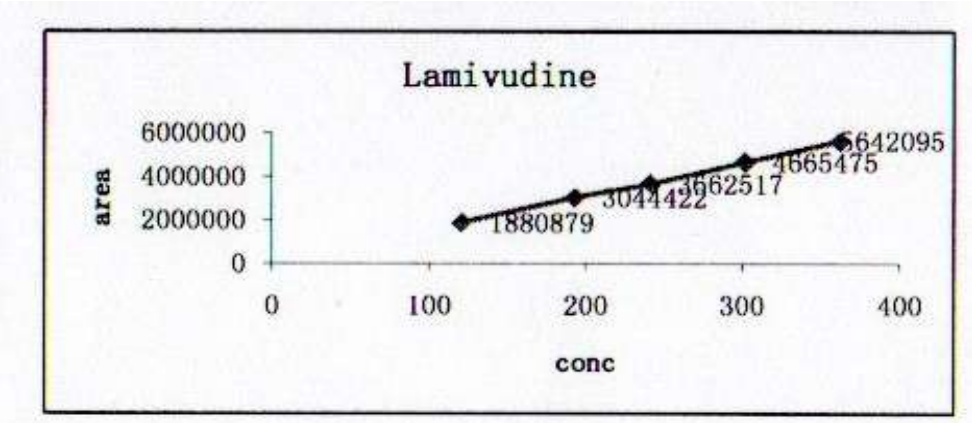

Figure 2: Linearity of Lamivudine

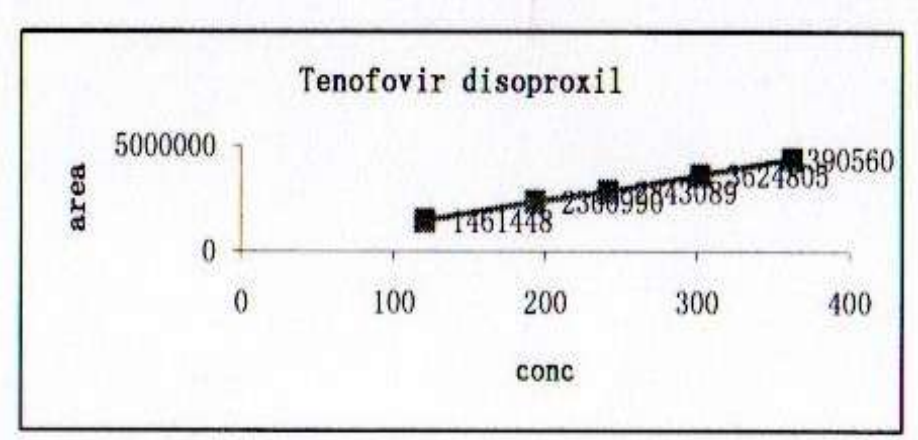

Figure 3: Linearity data of Tenofovir DF

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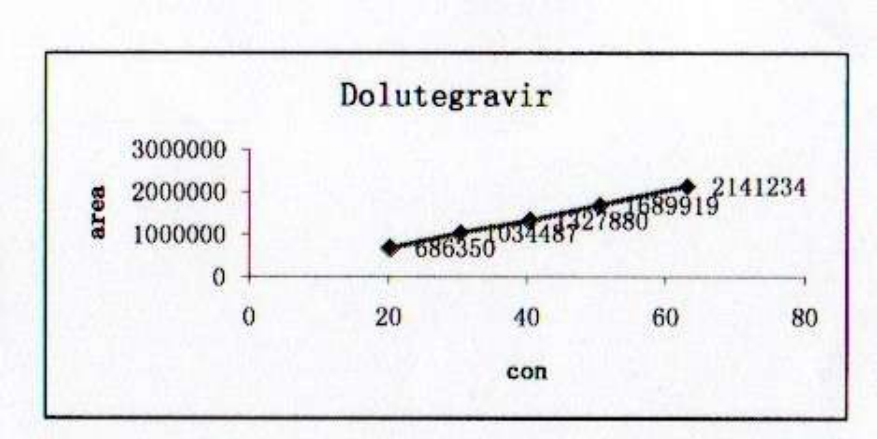

Figure 4: Linearity of Dolutegravir

Table 2: Results for linearity

Name	Linearity concentration range(µg/ml)	y-intercept	Correlation coefficient
lamivudine	120.27-362.16	1-6952.543222	0.99953
Tenofovir DF	120.27-362.16	-7737.004314	0.99948
Dolutegravir	20.28- 63.07	-7084.209455	0.99948

Accuracy

The method accuracy was performed by recovery studies which are carried out by three different concentrations levels (50%,100% and 150%). The percentage recovery of lamivudine, tenofovir DF and dolutegravir was found to be 99.6 and results shown in (Table 3).

Table 3: Results for recovery

% of analyte conc. (mg)	lamivudine (% mean Recovery)	Tenofovir DF (% mean Recovery)	Dolutegravir (% mean Recovery)
50	100.5	100.3	99.4
100	99	99	99.9
150	99.4	99.4	99.6

Robustness

The method robustness was done by small changes in optimized conditions like flow rate, sample cooler temperature and mobile phase composition. The % RSD should be less than 2 and results shown in (Table4).

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Table 5: Results for Robustness

S.NO	Robustness Condition	Lamivudine (% RSD)	Tenofovir DF (% RSD)	Dolutegravir (% RSD)
1	Flow minus	0.1	0.0	0.2
2	Flow plus	0.0	0.1	0.1
3	Mobile phase minus	0.6	0.3	0.9
4	Mobile phase plus	0.2	0.3	0.4
5	Temperature minus	0.1	0.3	0.1
6	Temperature plus	0.9	1.6	1.3

Filter variability

The % difference found for PVDF filter 0.0,0.3 and 0, Nylon filters 0.1,0,0.3 for Lamivudine, Tenofovir DF and Dolutegravir Respectively. The % difference of individual % drug release results of centrifuged verses filtered samples should be not more than 2.0% and results shown in (Table 5).

Table 5: Data of Filter Variability

%ASSAY	Centrifuged	PVDF	%Difference	Nylon	%Difference
Lamivudine	99.9	99.9	0	100	-0.1
Tenofovir Df	98.8	98.5	0.3	98.8	0
Dolutegravir	102.6	102.6	0.0	102.9	-0.3

Forced degradation

These studies are done in different stress conditions like acid, base, and peroxide as per ICH guidelines and results are shown in (Table6).

Table 6: Results for forced degradation

Sample Name	Condition	Purity angle	Purity Threshold
Lamivudine	Control Sample	0.122	0.293
	0.1N HCL	0.123	0.316
	0.1N NaOH	0.124	0.303
	3% H_2O_2	0.146	0.302
Tenofovir DF	Control Sample	0.106	0.239
	0.1N HCL	0.090	0.240
	0.1N NaOH	0.088	0.235
	3% H_2O_2	0.122	0.243
Dolutegravir	Control Sample	0.086	0.213

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	0.1N HCL	0.016	0.209
	0.1N NaOH	0.016	0.209
	3% H_2O_2	0.016	0.211

V. CONCLUSION

A simple, Accurate, precise method was developed for the simultaneous estimation of the Dolutegravir, Lamivudine, Tenofovir Disoproxil Fumarate in Tablet dosage form. Retention time of Dolutegravir, Lamivudine, Tenofovir Disoproxil Fumarate were found to be 30min ,11.5min and 26.5min. %RSD of the Dolutegravir, Lamivudine, Tenofovir Disoproxil Fumarate were and found to be 0.3,0.4 and 0.7 respectively. %Recovery was Obtained as 99.6%, 99% and 99.2% for Dolutegravir, Lamivudine, Tenofovir Disoproxil Fumarate. LOD, LOQ values were obtained from regression equations of Dolutegravir, Lamivudine, Tenofovir Disoproxil Fumarate. Retention times and run time areHigh(45min)because ten impurities separated and out of this one impurity of Tenofovir was eluted after 37minSo the method useful for Related substances separation also.The developed method was simple and economical that can be adopted in regular Quality control test in Industries.

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