

Research article

Method development and validation for simultaneous estimation of lamivudine, dolutegravir and tenofovir disoproxil fumarate in bulk and pharmaceutical dosage form using RP-HPLC and its application to *in-vitro* dissolution study

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ABSTRACT

A simple, rapid, and economical method has been developed for the simultaneous estimation of the latest FDA approved antiviral drug combination, Dolutegravir, Lamivudine, and tenofovir disoproxil fumarate in tablet dosage form using Shimadzu LC-20 AT HPLC with a Phenomenex Luna column compartment., the method was developed using HPLC graded methanol with o-phosphoric acid as a mobile phase and successfully validated the developed method as per the ICH guidelines. The method was found to be linear, accurate, precise, robust, and rugged. The limit of detection and the limit of quantification was found to be 2.6µg/ml and 8.18µg/ml for Dolutegravir, 14.63 µg/ml and 44.35 µg/ml for Lamivudine and 16.43 µg/ml and 49.81 µg/ml for tenofovir disoproxil fumarate respectively. The retention time was found to be 3.0, 2.3 and 2.7 min for Dolutegravir, Lamivudine and tenofovir disoproxil fumarate respectively. All of assessed parameters complied with the acceptance criteria hence indicated the usefulness of the RP-HPLC method for the determination of assay and in-vitro dissolution study for tablet dosage form which contains lamivudine, tenofovir disoproxil fumarate, and dolutegravir active substances. Hence the method can be applied for routine quality control of the drugs.

Keywords: Dolutegravir, Lamivudine, Tenofovir disoproxil fumarate, RP-HPLC, in-vitro dissolution study, Antiretroviral

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INTRODUCTION

The human immunodeficiency virus (HIV) is a virus that causes the syndrome of acquired immune deficiency (AIDS) and is transmitted by contact with blood and body fluids that are infected HIV infects the body's immune cells, which are important for battling infections, called CD4 positive (CD4+) T cells. HIV turns these cells into factories that create more HIV viruses to infect other healthy cells, thereby killing the CD4+ cells. These three drugs are used as antiretroviral medicines which are used for HIV or AIDS prevention and treatment. The literature survey reveals no study on RP-HPLC with a retention time of less than 4 min and its application to in-vitro dissolution studies for the simultaneous estimation of Dolutegravir, Lamivudine, and Tenofovir disoproxil fumarate in the tablet dosage form.

Lamivudine is an antiretroviral drug that is used for HIV or AIDS prevention and treatment. In the treatment of hepatitis B, it is often used when other options are not possible. The IUPAC: 2',3'-

Dihydroxy-3'thiacytidine 4-Amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidinone. The molecular formula is C₈H₁₁N₃O₃S, molecular weight 229.29 gm/mol. Lamivudine is a synthetic nucleoside analog with anti-hepatitis B (HBV) and HIV activity. Tenofovir disoproxil fumarate is an agent that can be used with other antiretroviral agents, demonstrates its activity as an inhibitor of reverse transcriptase nucleotides, and decreases the virus' ability to replicate. It is used for HIV or AIDS care when there is a high risk before exposure. The Tenofovir disoproxil fumarate IUPAC name is [(2R)-1-(6-aminopurin-9-yl)propan-2-yl]oxymethyl-(propan-2-yloxyphosphoryl)oxymethylpropan-2-ylcarbonate; but-2-enedioic acid having molecular formula C₂₃H₃₄N₅O₁₄P. Tenofovir Disoproxil fumarate molecular weight is 635.5gm/mol. It is a crystalline white powder with a melting point of 229°C. It is an inhibitor of nucleotide reverse transcriptase. Dolutegravir is an antiretroviral agent that is

also used for HIV or AIDS prevention and treatment. DTG is (4*R*,12*aS*)-*N*-(2,4-difluorobenzyl)-7-hydroxy-4-methyl-6,8-dioxo-3,4,6,8,12,12*a*-hexahydro-2*H*-pyrido 9(1',2':4,5) pyrazino [2,1-*b*] [1,3] oxazine-9- carboxamide. The molecular formula of Dolutegravir is: C₂₀H₁₉F₂N₃O₅ with a molecular weight of 419.38gm/mol. It indicates the 190-193oC melting point. Dolutegravir is an orally bioavailable strand-transfer integrase (INSTI) inhibitor. Its activity against infection with the human immunodeficiency virus type 1 (HIV-1).

The study aims to develop simple, rapid, accurate, precise method for the determination of Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate in tablet dosage form and to validate the proposed method in accordance with ICH guidelines for the intended analytical application i.e., to apply the proposed method for the routine quality control analysis of Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate in tablet dosage form. In future the method developed will be useful for the quality control departments into daily operations for assessing the amount recovery and percentage purity of the drug component using RP-HPLC.

MATERIALS AND METHODS

Chemicals and reagents

DTG, 3TC, TDF gift samples were obtained from Emcure pharmaceutical Ltd., Hyderabad, India. HPLC grade methanol and analytical grade 0-Phosphoric acid and Sodium lauryl sulphate reagents were purchased from Thermo fisher Scientific India Pvt. Ltd, Mumbai, India. VIROPIL□ tablets (DTG- 50mg, 3TC-300mg, TDF- 300mg) was purchased from local market.

Equipment

The chromatographic separations were performed on a Shimadzu LC-20 AT HPLC with a Double pump (Prominence LC 20 AT), a DAD detector system (LC 20 AT) and a Phenomenex Luna column compartment. The data acquisition was processed using LC Solution software. The dissolution tests were performed in an Electro lab with USP-II (paddle) dissolution apparatus (Electrolab, India).

Sample preparation

Preparation of Mobile phase

Mobile phase solution consists of HPLC grade Methanol and pH was adjusted to 4.6 using 0- phosphoric acid. It was then sonicated for 15 min

Preparation of Diluent

The Same mobile phase used as diluent

Analysis of Formulation

Twenty tablets were weighed accurately and the average weight was calculated. The tablets were then grounded to a fine powder. An accurately weighed tablet powder equivalent to 10mg of DTG, 60 mg of 3TC and 60mg of TDF was transferred into 100ml standard flask. Dissolved the content in a little amount of methanol. The solution was then sonicated using an ultra-sonicator for 20 minutes and was filtered using a membrane filter. 3ml of the above

prepared solution was transferred into a 10ml standard flask and the volume was made up to 10ml with diluent. Appropriate aliquots contain 30µg/ml DTG, 180µg/ml 3TC and 180µg/ml TDF were analyzed by the proposed method. 20µl of this solution was injected into the RP-HPLC system and the chromatogram was recorded.

$$\text{Amount of sample} = \frac{\text{Peak area of Sample}}{\text{Peak area of Standard}} \times \frac{\text{Weight of Standard}}{\text{Dilution of Standard}} \times \frac{\text{Dilution of Sample}}{\text{Weight of Standard}} \times \text{Avg wt}$$

$$\text{Percentage purity} = \frac{\text{Amount of sample}}{\text{Label claim}} \times 100$$

EXPERIMENTAL DESIGN

Method development and Optimization

The chromatographic Separations were achieved at a temperature of 25°C with an isocratic elution of samples on a Phenomenex Luna 5µ C18 (12) 100A, (250× 4.6mm × i.d 5µ) with Buffered mobile phase containing Methanol with PH 4.6 adjusted by using O-phosphoric acid at flow rate of 0.6 mL/min. The mobile phase was degassed in an ultra sonicator and filtered with a 0.45 µm membrane filter by a Millipore vacuum filter system. Peak identity was confirmed by comparing the spectra obtained from the PDA detector at 260nm. Under the above optimized conditions, all three components were well separated from each other with 4 min. Dissolution samples were collected at different time points using an auto sampler and analyzed using HPLC.

In-vitro dissolution study

After the development of the analytical method for simultaneous analysis of Tablet APIs, then dissolution medium for every single APIs in the tablet was used to test whether specific dissolution media for lamivudine, teno After the development of the analytical method for simultaneous analysis of Tablet APIs, then dissolution medium for every single APIs in the tablet was used to test whether specific dissolution media for lamivudine, tenofovir disoproxil fumarate and dolutegravir were able to simultaneously release all the three APIs in the tablets. Tablet's dissolution study carried out in 0.1 M hydrochloric acid (HCL) medium, in water medium and lastly dissolved in sodium lauryl (SLS) media which contained 1g, 2g, 5g, 10g and 20g equivalent to 0.1%, 0.2%, 0.5%, 1.0% and 2.0% of sodium lauryl sulphate in water. Sodium lauryl sulphate media at 0.1%, 0.2%, 0.5%, 1.0% and 2.0% were used to test the minimum concentration of SLS enough to simultaneously release all APIs in the formulation. Dissolution tests were performed at 37°C by using a paddle method at 50 rpm for 120 min. Dissolution samples were collected at different time points using an autosampler. At each time point, a 1 mL sample was collected from each vessel and transfer to 10 ml standard flask, then made up to 10ml using diluent. It was filtered through a 0.45µm membrane filter and analyzed using HPLC. fover disoproxil fumarate and dolutegravir were able to simultaneously release all the three APIs in the tablets.

Tablet's dissolution study carried out in 0.1 M hydrochloric acid

(HCL) medium, in water medium and lastly dissolved in sodium lauryl (SLS) media which contained 1g, 2g, 5g, 10g and 20g equivalent to 0.1%, 0.2%, 0.5%, 1.0% and 2.0% of sodium lauryl sulphate in water. Sodium lauryl sulphate media at 0.1%, 0.2%, 0.5%, 1.0% and 2.0% were used to test the minimum concentration of SLS enough to simultaneously release all APIs in the formulation. Dissolution tests were performed at 37°C by using a paddle method at 50 rpm for 120 min. Dissolution samples were collected at different time points using an autosampler. At each time point, a 1 mL sample was collected from each vessel and transfer to 10 ml standard flask, then made up to 10ml using diluent. It was filtered through a 0.45µm membrane filter and analyzed using HPLC.

Method validation

The method was validated using ICH guidelines by determining the following parameters: Linearity, Accuracy, Precision, Robustness, Ruggedness, Precision, Detection limit and Quantification limit.

Accuracy

The accuracy of the method was determined using recovery analysis. A known quantity of the mixed pure drug was added to the sample solution at 50%, 75%, and 100% levels.

Precision

To determine the precision of the proposed method sample solution at a particular concentration level (within the working range) was prepared and analyzed in three replicates during single day (intra-day) and on three different days (inter-day). And the percentage relative standard deviation was also calculated.

Linearity

The linearity of the method was ranging from Five different concentrations of standard Dolutegravir (10, 20, 30, 40, 50µg/ml), Lamivudine (60, 120, 180, 240, 300 µg/ml) and tenofovir disoproxil fumarate (60, 120, 180, 240, 300 µg/ml) were prepared. The calibration curve was drawn by plotting peak area against the concentration of drugs.

Robustness

Robustness is the capability of the method to remain unaffected by small deliberate changes in test parameters. The Robustness of the method was checked by analyzing (n = 3) the solutions used for precision studies with small changes ±2 pH and ±1 flow rate. The quantitative influence of the variables was determined by evaluating the value theoretical plate and tailing factor.

Ruggedness

Ruggedness was determined by performing an analysis of the formulation following the recommended procedures by three different analysts.

Detection and Quantification Limit

The limit of detection (LOD) and the limit of quantification (LOQ) of the developed method were determined by injecting progressively low concentrations of the standard solutions using the

developed RP-HPLC method and calculated based on the intercept standard deviation and the curve slope.

$$\text{LOD} = \frac{3.3\sigma}{S} \quad \text{LOQ} = \frac{10\sigma}{S}$$

RESULTS AND DISCUSSION

Analysis of Formulation

Simultaneous estimation of DTG, 3TC, TDF in formulation by HPLC was carried out using optimized chromatographic conditions. The pure sample solutions were prepared and chromatograms were recorded. The recorded for 3TC, TDF, DTG standard and formulation chromatograms are given in Figures 1 to 5. The assay procedure was repeated for three times. The percentages of individual drugs found in formulations, mean and relative standard deviation in formulations were calculated and presented in Table 1.

Figure.1 Chromatogram of standard Lamivudine drug

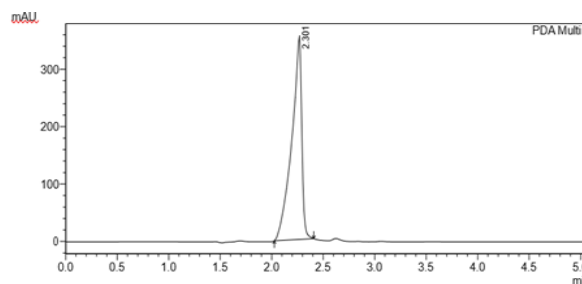


Figure. 2 Chromatogram of standard Tenofovir disoproxil fumarate drug

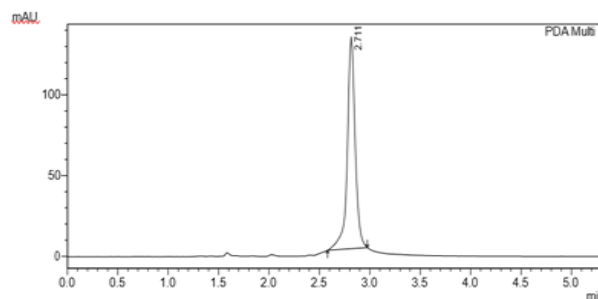


Figure 3 Chromatogram of standard Dolutegravir drug

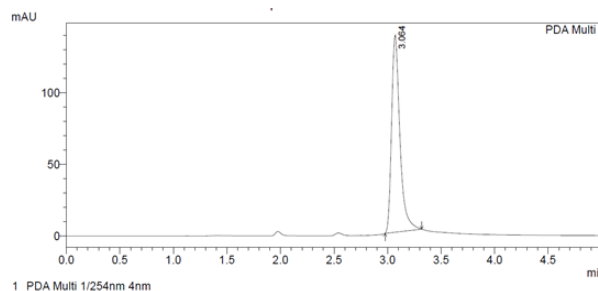


Figure.4 Chromatogram of standard drug in combination

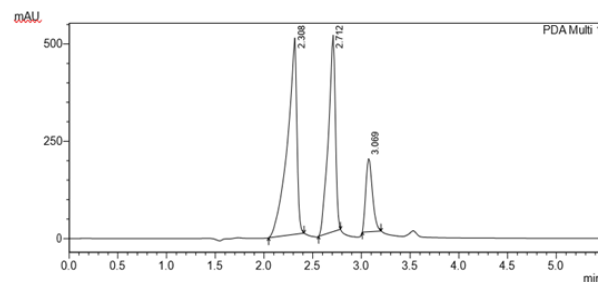
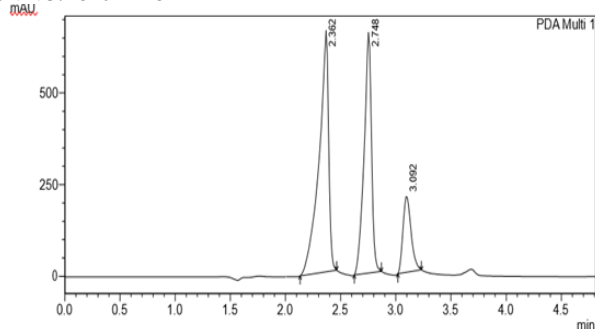


Figure 5 Chromatogram of Formulation



Analysis of formulation

Table 1: Assay result of Formulation

Sample	Drug	Peak Area	*Estimated amount (mg) (mean \pm SD)	% purity	%RSD
Formulation (Viropil)	DTG (label claim 50 mg)	651542	49.73 \pm 0.34	99.71	0.68
		646214	50.13 \pm 0.27		
		649821	49.60 \pm 0.31		
	3TC (label claim 300mg)	2245715	297.82 \pm 2.01	99.97	1.09
		2254814	299.02 \pm 3.59		
		2289112	303.57 \pm 2.32		
TDF (label claim 300mg)	1648185	301.23 \pm 3.15	99.20	1.19	
	1604956	293.67 \pm 1.96			
	1630415	297.98 \pm 2.03			

*Triplicate performance

In-vitro dissolution study

The dissolution profile of tablets was performed to determine the optimal release of the APIs in tablets. Lamivudine, tenofovir and Dolutegravir tablets were dissolved in different dissolution media; 0.1M hydrochloric acid, water and different concentrations of sodium dodecyl sulphate (SLS). Its result was shown in fig.6, 7.

Figure 6. Dissolution profile of lamivudine, Tenofovir disoproxil fumarate and dolutegravir in tablets in Water and 0.1 N HCl dissolution media.

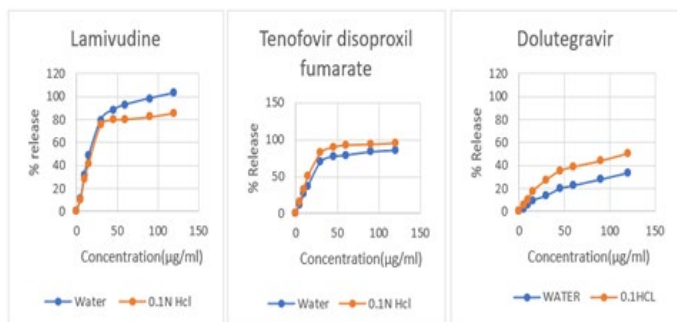
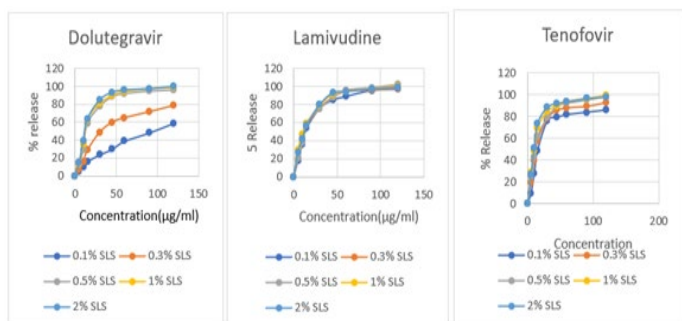


Figure 7. Dissolution profile of dolutegravir, lamivudine, and Tenofovir disoproxil fumarate in tablets in different concentration of SLS dissolution media.



Dolutegravir is poorly soluble in acid and water, but its solubility was improved with SLS. The release of all the three APIs in

tablets was more than 75% at 45 min in 0.3%, 0.5%, 1.0%, and 2.0% concentrations of SLS (Figures 7). In 0.1% SLS dissolution medium, lamivudine and tenofovir disoproxil fumarate release was more than 75%, but for Dolutegravir the release was only 23.64% at 45 min and 58.87% at 120 min in 0.1% SLS. Dolutegravir release was poor at 0.1% SLS dissolution medium because of poor solubility in water and also the concentration of SLS was below CMC. As the concentration of SLS increased, dolutegravir solubility improved therefore its release increased up to more than 75% at 30 min.

Method validation

Accuracy

The recovery studies were carried out three times and the percentage recovery and percentage relative standard deviation was calculated and presented in table 2.

Table 2: Results of accuracy studies

Drug	Theoretical % target level	*Amount recovered(mg) (mean \pm SD)	% Recovery	% RSD
DTG (Label claim 50 mg)	50	48.96 \pm 0.67	97.93	1.71
	75	49.43 \pm 0.04	98.86	0.12
	100	51.5 \pm 0.40	103.07	0.91
3TC (Label claim 300 mg)	50	296.43 \pm 2.95	98.78	1.2
	75	295.6 \pm 3.67	98.44	1.57
	100	295.67 \pm 3.96	97.70	0.86
TDF (Label claim 300 mg)	50	296.46 \pm 2.93	98.78	1.20
	75	296.67 \pm 3.75	98.89	1.57
	100	296.8 \pm 2.98	97.70	0.86

*Triplicate performance

Precision

In the intraday studies, three repeated injections of standard were made with in same day and amount recovered from drug peaks and percentage RSD were calculated and presented in Table 3. In the Interday variation studies, six repeated injections of standard were made for three consecutive days and amount recovered from drugs peak and percentage RSD were calculated and presented in Table 4.

Table 3: Results of Interday Precision

Drug	Day	Peak area	*Amount recovered (mg) (mean \pm SD)	% RSD
DTG	Day 1	645749	49.29 \pm 0.18	0.32
	Day 2	646372	49.34 \pm 0.69	
	Day 3	647828	49.45 \pm 0.82	
3TC	Day 1	2250328	298.43 \pm 3.02	1.45
	Day 2	2240555	297.13 \pm 3.44	
	Day 3	2201647	291.90 \pm 2.06	
TDF	Day 1	1625732	297.09 \pm 3.68	1.34
	Day 2	1647392	301.02 \pm 4.04	
	Day 3	1604718	293.28 \pm 2.65	

Table 4: Results of Intraday Precision

Drug	Time	Peak area	*Amount recovered(mg) (mean \pm SD)	% RSD
DTG	0 Hour	651542	49.65 \pm 0.18	0.51
	3 Hour	656214	49.72 \pm 0.23	
	6 Hour	649821	49.58 \pm 0.21	
3TC	0 Hour	2245715	297.77 \pm 2.36	1.02
	3 Hour	2254814	299.90 \pm 2.47	
	6 Hour	2289112	303.57 \pm 1.65	
TDF	0 Hour	1628185	297.57 \pm 2.68	1.11
	3 Hour	1654956	302.40 \pm 2.61	
	6 Hour	1660415	303.34 \pm 1.58	

Linearity

Calibration plots were linear over the tested. Correlation coefficient >0.995 for all the three components shows the good correlation between concentration and peak area. The linearity was evaluated using linear regression analysis and are presented in fig .8, 9 and 10.

Figure 8. Linearity graph of Dolutegravir

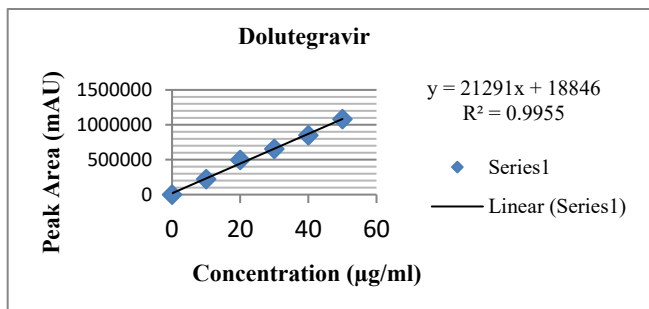


Figure 9 Linearity graph of Lamivudine

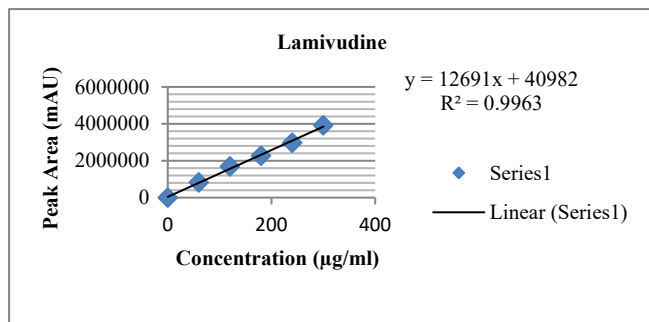
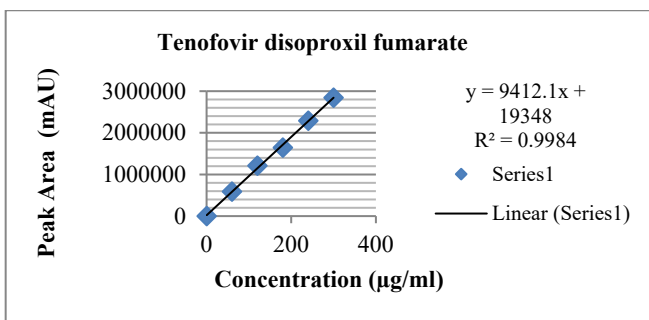


Figure 10 Linearity graph of tenofovir disoproxil fumarate



Robustness

The results (Table 5) show that minor changes PH and flow rate of the given values in chromatographic conditions do not influence the results for DTG, 3TC and TDF. The value of RSD for replicates (n = 3) at particular concentration.

Table 5: Results of robustness studies

Drug	Parameter		Theoretical plate	Tailing factor
DTG	flow rate (±0.1 ml/min)	Decreasing (0.5 ml/min)	6627.850	1.318
		At 0.6ml/min	6882.894	1.014
		Increasing (0.7ml/min)	6454.485	1.265
	pH (±0.2 pH)	Decreasing (4.4)	6342.613	1.494
		pH 4.6	6648.547	0.987
		Increasing (4.8)	6427.845	1.224
3TC	flow rate (±0.1 ml/min)	Decreasing (0.5 ml/min)	2283.273	0.606
		At 0.6ml/min	2379.687	0.578
		Increasing (0.7ml/min)	2140.635	0.653
	pH (±0.2 pH)	Decreasing (4.4)	2892.527	0.718
		pH 4.6	2978.554	0.608
		Increasing (4.8)	2671.529	0.648
TDF	flow rate (±0.1 ml/min)	Decreasing (0.5 ml/min)	4824.984	0.834
		At 0.6ml/min	4967.148	0.638
		Increasing (0.7ml/min)	4722.400	0.799
	pH (±0.2 pH)	Decreasing (4.4)	4887.268	0.887
		pH 4.6	5086.594	0.687
		Increasing (4.8)	4961.274	0.745

Ruggedness

Ruggedness was determined by performing analysis of the sample solution following the recommended procedures by three different analysts and are presented in table 6.

Table 6: Results of ruggedness studies

Drug	Analyst	Amount taken (µg/ml)	Amount recovered (µg/ml)	% Content	% RSD
DTG	Analyst I	50	49.8±0.15	99.6	0.31
	Analyst II		49.9±0.09	99.8	
	Analyst III		50.1±0.13	100.2	
3TC	Analyst I	300	299.21±0.94	99.73	0.45
	Analyst II		298.65±1.06	99.55	
	Analyst III		301.24±1.28	100.41	
TDF	Analyst I	300	298.48±2.01	99.49	0.43
	Analyst II		297.14±1.54	99.04	
	Analyst III		302.52±1.09	100.84	

*Triplicate performance

LOD and LOQ

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the intercept standard deviation and curve slope and the results were shown in Table 7.

Table 7: Results of LOD and LOQ

Drug	LOD (µg/ml)	LOQ (µg/ml)
DTG	2.6	8.18
3TC	14.63	44.35
TDF	16.43	49.81

CONCLUSION

The proposed developed method was simple, rapid, accurate, precise robust and sensitive for the simultaneous determination of lamivudine, Dolutegravir and Tenofovir disoproxil fumarate from bulk, tablet dosage form using an HPLC method. The process was validated by ICH standards, and all validation acceptance requirements were met. From the findings, the method is suitable for routine quality control tests for assay and dissolution in pharmaceutical products which contain lamivudine, tenofovir disoproxil fumarate and dolutegravir active substances in resource-poor settings laboratories.

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