Pharm Methods, 2018; 9(2): 49-55.
A multifaceted peer reviewed journal in the field of Pharm Analysis and Pharmaceutics
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Original Article

Development and Validation of RP-HPLC Method for the Simultaneous Estimation of Lamivudine, Tenofovir Alafenamide and Dolutegravir Bulk and their Combined Dosage Form

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ABSTRACT

Introduction: A simple and Rapid High Performance Liquid Chromatographic method was developed and validated for simultaneous estimation of lamivudine, tenofovir alafenamide and dolutegravir in their tablet dosage form. **Method:** The method was established using Agilent C18 (250 × 4.6 mm, i.d., 5 µm) column, a mobile phase consisting of 0.05M phosphate buffer pH 6.2 (solvent A) and acetonitrile (solvent B) 60:40 v/v at a flow rate of 1 mL/min with isocratic elution, injecting 10 µL sample into the chromatographic system. The eluted compounds were detected by using PDA Detector at a detection wavelength of 260 nm and the temperature was maintained at 30°C. **Result:** Retention times for the three compounds were found to be 3.09 min, 6.19 min and 9.61min for lamivudine, tenofovir alafenamide, and dolutegravir respectively. The linearity range was 10-80 µg/ml for three drugs with values of LOD found to be 0.56, 0.39µg, 1.35µg and LOQ were found to be 1.50µg, 0.99µg and 3.61 µg for lamivudine, tenofovir alafenamide and dolutegravir respectively which were linear enough showing correlation coefficient 0.999 in all the cases. **Conclusion:** The proposed method is therefore, suitable for the purpose in quality-control laboratories for quantitative analysis of the drugs individually and in the combined dosage form. The method was found to be as it is simple and rapid with tremendous precision and accuracy. The method can be used as a routine quality control method for triple combined dosage forms.

Key words: Alafenamide, Dolutegravir, Lamivudine, RP-HPLC, Tenofovir.

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DOI : 10.5530/phm.2018.2.10

INTRODUCTION

Lamivudine, chemically 4-amino-1-[(2R, 5S)-2-(hydroxyl methyl)-1, 3-oxathiolan-5-yl]-1, 2-dihydropyrimidin-2-one. Lamivudine is reverse transcriptase reported to be active against HIV-1, HIV-2 and hepatitis B virus. Lamivudine [Figure 1][1] has been used for treatment of chronic hepatitis B at a lower dose than for the treatment of HIV. It improves the seroconversion of e-antigen positive hepatitis B and also improves histology staging of the liver.

Dolutegravir [Figure 2][2] inhibits integrate strand transfer thereby inhibiting viral multiplication. Chemically, it is known as (4R,12aS)-N-(2,4-difluorobenzyl)-7-hydroxy-4- methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2H-pyrido- [1',2':4,5]pyrazino[2,1-b][1,3]oxazine-9-carboxamide.

Tenofovir alafenamide (TA) [Figure 3][3] is chemically isopropyl (2S)-2-[[[(1R)-2-(6-aminopurin-9-yl)-1-methyl-ethhoxy]methyl-phenoxy-phoshoryl]amino]propanoate. TA is a nucleotide reverse transcriptase inhibitor and a prodrug of tenofovir. It is used in the treatment of HIV infection and chronic hepatitis B. It is closely related to the commonly used reverse-transcriptase inhibitor tenofovir disproxil, TA has greater antiviral activity and better distribution into lymphoid tissues than that tenofovir disproxil.

Literature survey reveals that various HPLC[4-5] LC-MS,[6] HPTLC[7-8] method and have been reported for the estimation of Tenofovir disproxil fumerate and Lamuvidine. The present study illustrates development and validation of a simple, accurate and precise procedure for "Development and validation of RP-HPLC method for the simultaneous estimation of lamivudine, tenofovir alafenamide and dolutegravir bulk and their combined dosage form".

MATERIALS AND METHODS

Chemicals and reagents

Working standard of Lamivudine, Dolutegravir and Tenofovir Alafenamide was obtained as gift sample from Hetero Laboratories, Hyderabad, India. Reagents used were KH_2PO_4. AR Grade, Qualizines, India. HPLC graded acetonitrile Qualizines India and Triple distilled water for the entire study.

Instrumentation

The method development and validation were carried out by using HPLC Agilent separation module model, it was equipped with auto-sampler with injection volume 10 µl, column used was Agilent C18 (250 × 4.6 mm, i.d., 5 µm) column and data recorded using EZ chrome elite software.

Chromatographic conditions

Various combinations of mobile phases were screened and finally, the mobile phase consisting of 0.05M phosphate buffer pH 6.2 (solvent A) and acetonitrile (solvent B) 60:40 v/v was set with isocratic programming for 15 min optimized at a flow rate of 1 ml/min, at 260 nm wavelength, the injection volume of 10 µL and ambient temperature was maintained during the entire process to obtain symmetric peaks of Lamivudine,Tenofovir alafenamide and Dolutegravir.

Preparation of Solutions

Diluent: Mix an equal volume of acetonitrile and water in the ratio of 1:1 which was used as a Diluent.

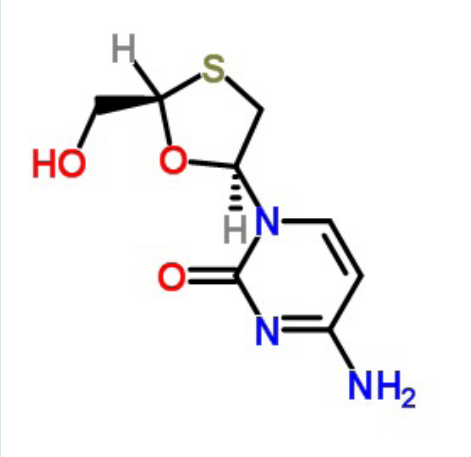

Figure 1: Chemical structure of Valganciclovir

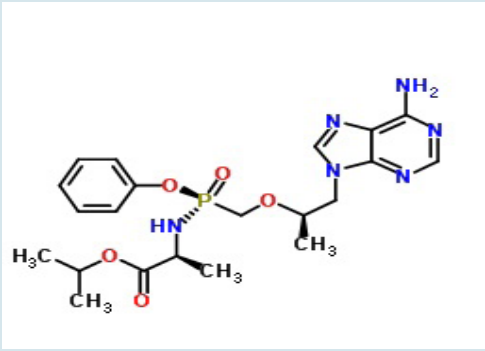

Figure 3: Figure 3(a): Overlain spectra of method B (Zero-order). Figure 3 (b): Overlain spectra of method B (first-order).

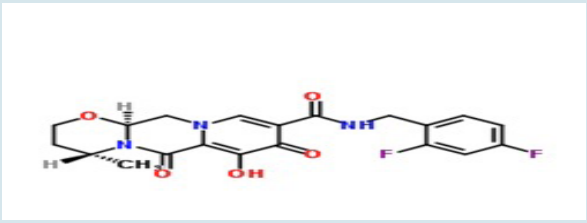

Figure 2: Figure 2 (a): Overlain spectra of method A (Zero-order). Figure 2 (b): Overlain spectra of method A (first-order).

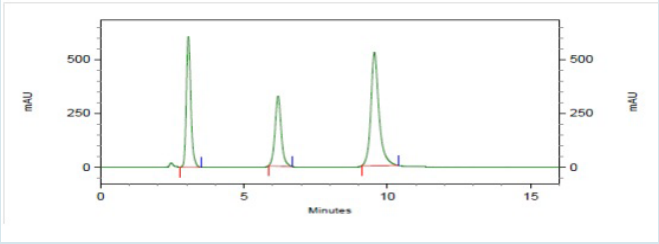

Figure 4: Figure 4. (a): Overlain spectra of method C (Zero-order). Figure 4 (b): Overlain spectra of method C (first-order).

Preparation of 0.05M Phosphate buffer of pH 6.2

Weigh accurately about of 1.37g of monobasic potassium dihdro-ortho-phosphate (KH2PO4) and transfer into1000 ml volumetric flask containing 200ml distilled water, shake for 5 mns and final volume was made up with distilled water, sonicate for 15 mns and ph 6.2 was adjusted with orthophosphoric acid.

Mobile Phase

0.05M phosphate buffer pH 6.2 and acetonitrile (60:40)v/v was prepared and filtered through 0.45micron membrane filter to remove the impurities and sonicate using ultrasonicator to remove the un dissolved gases.

Preparation of standard stock solution

A standard stock solution was prepared accurately weigh and transfer 10 mg of lamivudine, tenofovir alafenamide and dolutegravir into a 10 ml clean dry volumetric flask and dissolved in 7 ml of diluent after vigorous shaking the volume was made with up to the mark with diluent to get conc. of 1000µg/ml for each drug .from this 5 ml was transferred to 50 ml volumetric flask and volume made up with the mobile Phase to obtain the concentration of 100 µg/ml of lamivudine, tenofovir alafenamide and dolutegravir respectively.

Chromatographic condition

Agilent C_{18} (250×4.6 mm I.D; 5 µm) column is used for detection at a wavelength of 260nm, using 0.05M phosphate buffer pH 6.2 (solvent A) and acetonitrile (solvent B) 60:40 v/v) in a isocratic elution mode as a mobile phase. The contents of the mobile phase was degassed, with a helium sparge for 15 min and filtered through vaccum filtration pumped from the respective solvent reservoirs to the column at a flow rate of 1 mL/min. The column temperature was maintained at 30°c and run time was 15 mins. The injection volume of sample was 10 µl. Retention times for the three compounds were found to be 3.09 min, 6.19 min and 9.61min for lamivudine, tenofovir alafenamide and dolutegravir respectively. The optimized chromatogram and the conditions are shown [Table 1] and [Figure 4].

Construction of calibration curves for LAM, TAF, DOL

For the construction of calibration curves, a series of aliquots were prepared from the above stock solution by transferring 1 ml, 2 ml, 3 ml, 4 ml, 5 ml, 6 ml, 7 ml, 8 ml into a 10ml volumetric flask and final volume was made with mobile phase to get the conc. of 10-80 µg/ml of each drug. Each concentration was injected 6 times into the column and each time retention time and peak area of LAM, TAF and DOL was noted. Construct the calibration curves separately by taking concentration (µg/ml) on the x-axis and average peak area on the y-axis for the drugs [Figure 5]. From the calibration curves, regression equations were calculated, these regression equations were used to calculate the amount of LAM, TAF and DOL present in the commercial dosage form.

Preparation of sample solution

Laboratory synthetic mixture was prepared by accurately weighed Lamivudine (150mg), Tenofovir alafenamide (25mg), Doltegravir (50 mg) and excipients were mix well in a mortor and pestle from this powder equivalent to 10mg of Tenofovir alfenamide contained 20mg of Doltegravir and

Table 1: Optimized chromatographic conditions

Column	Agilent, C18(250 X 4.6mm, 5µ)
Mobile phase	0.05M phosphate buffer pH 6.2 and acetonitrile (60:40)
Flow rate	1ml/min
Column temperature	30^{0}C
Injection volume	10µl
Detection Wavelength	260nm
Run time	15min
Retention time	Lamivudine: 3.09 min, Tenofovir: 6.19 min, Dolutegravir: 9.61min

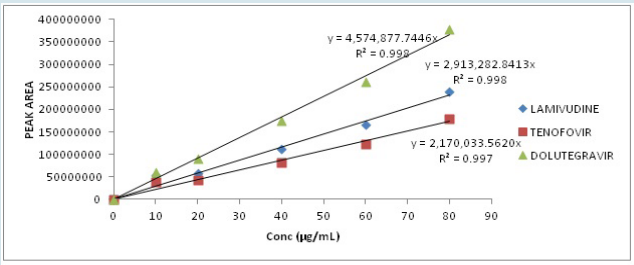

Figure 5: Figure 5.(a):Overlain spectra of method D (Zero-order). Figure 5(b): Overlain spectra of method C (first-order).

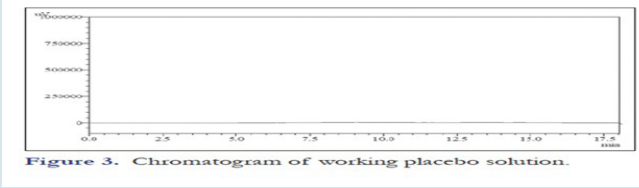

Figure 6: Figure: 6. (a) Calibration curve of method A (Zero-order derivative). Figure: 6 (b) Calibration curve of method A (first-order derivative).

Table 2 : Results for the synthetic mixture

Name of the drug	Labelled amount (mg)	Test conc µg/mL	Estimated conc µg/mL	% of Assay
Lamivudine	150	60	59.1	98.5
Tenofovir alafenamide	25	10	9.94	99.4
Doltegravir	50	20	19.8	99

Table 3 : Optical characteristic of proposed method

Drugs names	Beers limit	Regreession equation	Correlation coefficient(r^2)	Wavelength (nm)
Lamivudine,	10-80 µg/ml	y = 2,913,282.8413x,	0.998	260
Tenofovir alafenamide	10-80 µg/ml	y = 2,170,033.5620x	0.997	260
Dolutegravir	10-80 µg/ml	y = 4,574,877.7446x	0.998	260

60 mg of Lamivudine was taken into a 10 ml volumetric flask and dissolved with diluents to get the conc of 1000µg/mL of tenofovir alafenamide from this test solution was prepared by diluting the above sample stock solution which contains 10µg/mL of TAF, 20 µg/mL of DOL, 60µg/ml LAM, by using a mobile phase. The test solution was injected 6 times into the column, each time peak area and retention time was noted. The % of drug content in the Laboratory synthetic mixture was estimated by using regression equation and the result obtained were noted as shown in the [Table 2]

Method validation

The developed method for simultaneous estimation of Lamivudine, Tenofovir alafenamideb and Dolutegravir has been validated in accordance with the International Conference on Harmonization guidelines.

Linearity

Several aliquots of standard stock solution of Lamivudine,Tenofovir alafenamide and Dolutegravir were taken in different 10 ml volumetric flask and diluted up to the mark with the mobile phase such that their final concentrations were 10-80µg/ml for LAM, 10-80µg/ml for TAF, 10-80µg/ml and respectively. Peak areas were plotted against the corresponding concentrations to obtain the calibration graph for each compound as shown in [Table 3]. A good linear relationship (r=0.998 for LAM and 0.998 TAF, 0,997 DOL) respectively. Linearity equation obtained for Lamivudine, Tenofovir alafenamide, and Dolutegravir were y = 2,913,282.8413x, y = 2,170,033.5620x, and y = 4,574,877.7446x, respectively. Figures 5, shows linearity graphs for Lamivudine,Tenofovir alafenamide and Dolutegravir.

Accuracy

The accuracy of the method for assay determination was achieved at three concentration levels of 50%, 100%, and 150% for Lamivudine,Tenofovir alafenamide, Dolutegravir. Known amount of standard drug concentration was added to the pre analysed sample concentration and mean peak area was determined. The mean percentage recovery values are shown in [Table 4].

Precision: Precision is the degree of repeatability of an analytical method under normal operating condition.

Precision is of 3 types.

1. system precision.
2. method precision.
3. intermediate precision.

a.inter- day precision.

Method precision was achieved by repeating the same procedure of preparation solution six times and injecting.

System precision is checked by injecting using a standard chemical

Table 4 : Accuracy results

Recovery Level(n=6)	Sample number	Preanalysed concentration (µg/ml)	µg/ml added	µg/ml found	% Recovery	% Mean
50%	1	20	10	30.12	100.16	99.59
	2	10	5	15.21	100.21	
	3	20	10	30.12	100.16	
100%	1	20	20	40.40	99.40	99.59
	2	10	10	20.38	99.40	
	3	20	20	40.13	99.40	
150%	1	20	30	49.33	100.20	99.59
	2	10	15	25.26	100.16	
	3	20	30	50.33	100.20	

Table 5 : Results of the intra &intermediate precision

Drug	Drug concentration (µg/ml)	Intraday		Interday	
		Mean ± SD	%RSD	Mean ± SD	%RSD
lamivudine	60	180525650±206281	1.142	184864279±5688	0.030
	70	85239708±2486541	1.211	18239708±24865	0.124
	80	18965544±4565433	1.132	18965544±45654	0.412
Taf	10	55031234±1296654	0.030	53031234±12966	0.190
	20	58665525±1308641	0.124	59665525±13086	0.540
	30	64876615±1489982	0.412	63876615±14899	0.412
dolt	20	58665525±1308641	0.190	59665525±13086	0.030
	30	64876615±1489982	0.540	65876615±14899	0.224
	40	71975235±1697365	0.412	72975235±16973	0.312

substance to ensure that the analytical system is working properly. In this peak area and percentage of the drug of six determination is measured and percentage relative standard deviation should be calculated.

In method precision, a homogenous sample of the single batch should be analyzed 6 times. The indicates weather a method is giving a constant result for a single batch. In this analysis the inject the sample six times and calculate the % RSD and the results are shown in [Table 5].

LOD and LOQ

LOD: It is the lowest amount of analyte in a sample that can be detected but not necessarily quantities as an exact value under the stated, experimental conclusions. The detection limit is usually expressed as the concentration of the analyte. The standard deviation and response of the slope.

$$LOD = 3.3*\text{standard deviation (6)}/s$$

LOQ: The quantitation limit of an analytical procedure is the lowest amount of an analyte of a sample which can be quantitatively determined with suitable precision and accuracy. The standard deviation and response of the slope and the results obtained.

$$LOQ= 10^* \text{ standard deviation (6)}/s$$

The results of LOD and LOQ are shown in the [Table 6].

System Suitability

Six replicates of standard stock solution for each drug were injected sample containing Lamivudine, Tenofovir alafenamide, and Dolutegravir was given to evaluate equipment, electronics and analytical operations and samples suitability. Parameters calculated for system suitability were percentage of relative standard deviation of retention time, number of theoretical plates and tailing factor. Results found were given in [Table 7] and within acceptable limits.

Robustness

To evaluate the robustness of the method, the chromatographic conditions were deliberately altered and degree of reproducibility was evaluated. During robustness testing each condition was varied separately, all other conditions being held constant at the optimized values. Robustness of the proposed method was assessed with respect to small alterations in the flow rate (1.0 ± 0.2ml/min), organic composition and the results obtained from as shown the [Table 8].

Selectivity

Selectivity test determines the effect of excipients on the assay result. To determine the selectivity of the method, a standard solution of Lamivudine, Tenofovir alafenamide and Dolutegravir, excipient solution and blank solutions were run in the instrument one after another. The results of the tests proved that the components other than the drug did not produce any detectable signal at the retention time of Lamivudine, Tenofovir alafenamide and Dolutegravir. In [Figure 6] and [Figure

Table 6 : LOD and LOQ of LAM, DOL, TAF

	LAM	DOL	TAF
LOD [µg/ml]			
	0.56038	0.394033	1.353279
LOQ [µg/ml]	1.5038	0.994038	1.6100845

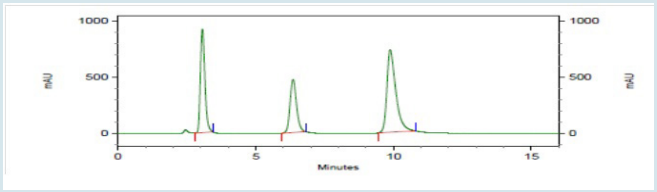

Figure 7: Figure: 7. (a) Calibration curve of method B (Zero-order derivative). Figure: 7 (b) Calibration curve of method B(first-order derivative).

Table 7 : System suitability results for Lamivudine,Tenofovir alafenamide and Dolutegravir

Parameter	Drugs		
	Lam	Taf	Dol
Retention time	3.090	6.193	9.617
Resolution	1.686	3.103	3.424
Theoretical plates	2078	3364	6239
Tailing factor	1.2	1.3	1.14
% RSD	0.37	0.26	0.92

Table 8 : Results of Robustness by variation in flow rate and organic rate

Parameters	Retention time			Peak area			%RECOVERY		
	LAM	TAF	DOLT	LAM	TAF	DOLT	LAM	TAF	DOLT
Flow Minus(0.8)	3.76	7.47	11.0	5330886	3939765	6017721	100.3	100.5	100.2
Flow Plus(1.2)	2.51	5.95	7.12	5830819	4339900	6717544	100.7	100.3	100.3
Organic Minus 35%acetonirile	3.62	7.15	11.8	5330887	4239723	6317758	100.5	100.4	100.2
Organic Plus 45%acetonitrile	2.86	5.52	6.95	5130886	3739716	591775	100.1	100.3	100.3

7] shows the chromatogram of Lamivudine, Tenofovir alafenamide and Dolutegravir mobile phase and placebo respectively. There were no interfering peaks at retention time of Lamivudine, Tenofovir alafenamide and Dolutegravir).

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

A simple, accurate and precise RP-HPLC method was developed and validated for the simultaneous estimation of Lamivudine, Tenofovir alafenamide and Dolutegravir**.** A mobile phase consisting of .05M phosphate buffer (solvent A) pH 6.2 and acetonitrile (solvent B) 60:40v/v was set with isocratic programming for 15 min. Chromatographic conditions were optimized for mobile phase using Agilent C_{18} (250 × 4.6 mm, i.d., 5 µm) column at a flow rate of 1 ml/min. Effluents were detected at 260 nm by variable wavelength PDA detector. Column compartment temperature was in ambient. Chromatogram of Lamivudine,Tenofovir alafenamide, and Dolutegravir at optimized chromatographic condition was shown in [Figure 4].

Selectivity

[Figure 6 and 7] shows the chromatogram of blank, sample. There were no interfering peaks at retention time of LAM, TAF and DOL was observed.

Linearity and range

The linearity regression coefficient (R2) values were found to be 0.998 for LAM and 0.998 TAF, 0,997 DOL. Regreession equation obtained LAM, TAF and DOL were y = 736752x+48197, y = 8E+06x+2487.4 and y = 6E+07x+3787. Figures 5, shows linearity graphs for Lamivudine, Tenofovir alafenamide and Dolutegravir respectively. The method was linear over the range of 10-80 µg/ml of each drug.

System suitability

Six replicates of standard stock solution containing Lamivudine, Tenofovir alafenamide and Dolutegravir were given to evaluate equipment, electronics, and analytical operations and samples suitability. Parameters calculated for system suitability were a number of theoretical plates, tailing factor, resolution, retention time, and area. The results as shown in [Table 7]. Indicates the system is a suitable or proposed method.

LOD and LOQ

The LOD and LOQ were measured, LOD value of LAM, TAF and DOL was found to be 0.5603, 0.3940 and 1.3532µg/mL, respectively. The LOQ values were found to be 1.5038, 0.9940 and 1.6100 for LAM, TAF and DOL respectively. The statistical data were presented in [Table 6].

Recovery

The percentage recovery was calculated by preparing standard drug concentrations of LAM, TAF and DOL with concentration levels of

50%, 100%, and 150%. A known amount of the standard drug was added to the pre analyzed concentration at each level. Good recovery of the spiked drugs was obtained at each added concentration and the mean percentage recovery of LAM, TAF and DOL was achieved between 99.93–100.08 ± 0.5%. The results are given in [Table 4].

Precision

Precision is expressed as the closeness of agreement between a series of measurements obtaining from multiple sampling of the same homogeneous sample. Six replicate injections of a known concentration of LAM 60,70,80 (µg/ml), TAF 10,20,30 (µg/ml) and DOL 20,30,40 (µg/ml) have been analyzed by injecting them into a HPLC column on the same day and on consecutive days. From the results obtained, % RSD was calculated and was found to be within the limits <2. The results are given in [Table 5].

Robustness

Robustness of the proposed analytical method is a measure of its capacity to remain unaffected, and it reflects the reliability of the analysis with respect to deliberate changes in the parameters such as flow rate (1.0 ± 0.2ml/min), column temperature (ambient), mobile phase ratio and pH of the mobile phase. The parameters chosen for the study of robustness is the flow rate and mobile phase composition. From the results obtained, there were no significant changes observed at the end of the study. The results are given in [Table 8].

CONCLUSION

In this study, a simple, fast and reliable HPLC method was developed for the simultaneous estimation of Lamivudine, Tenofovir Alafenamide and Dolutegravir. The simultaneous estimation of Lamivudine, Tenofovir Alafenamide and Dolutegravir could be achieved successfully, although there was a great difference in polarity between the compounds. it shows that the validated method was specific.

From the results, it can be concluded that the method has been successfully applied for the analysis of marketed tablets and can be used for the routine analysis of formulations containing any one of the selected drugs or their combinations, without any alteration in the assay. Since the method was successfully applied for the estimation of selected drugs in bulk as well; therefore, this method can also be adopted for the study of pharmaceutical release patterns of the drugs while designing new dosage forms. This method can be used as a routine quality control method for triple combined dosage forms. The method also shows a good performance with respect to specificity, sensitivity, linearity, accuracy and precision.

ACKNOWLEDGEMENT

I am very thankful to the Principal, University College of Pharmaceutical Sciences, Acharya Nagarjuna University, Guntur for providing the laboratory facilities chemicals to carry out entire study. I am also thankful to Hetero Laboratories, Hyderabad, India for providing working standard as a gift sample.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

LAM: Lamivudine; **DOL:** Doltigravir; **TAF:** Tenofovir alafenamide; **TDF:** Tenofovir Disoproxil Fumerate; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **PDA:** Photo Diode Array.

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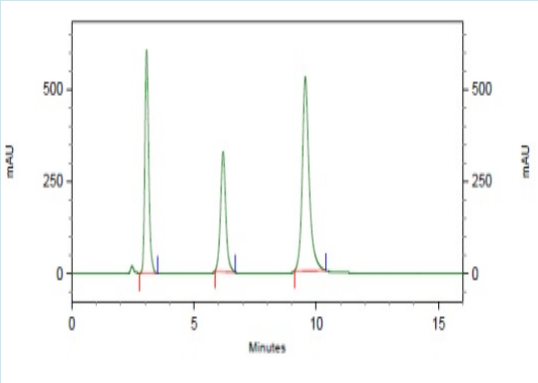
PICTORIAL ABSTRACT



SUMMARY

- Lamivudine, tenofovir alafenamide and dolutegravir are used for HIV infection.it is a new combination.instead of tenofovir disoproxil fumerate (TDF) we use tenofovir alafenamide (TAF). TAF containing regimens can improve bone and renal safety compared with TDF containing regimens. this new combination useful work for researchers to develop formulation. If anyone develop this formulation the proposed method used for the estimation of drug content in the combined dosage form during routine analysis.
- Stability reviewls that the proposed analytical method has been employed to separate and identify possible degradation products.

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