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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF DOLUTEGRAVIR, EMTRICITABINE AND TENOFOVIR ALAFENAMIDE BY HPLC IN BULK AND PHARMACEUTICAL DOSAGE FORM

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Abstract: **A simple, rapid, economical, precise and accurate HPLC method for simultaneous estimation of Dolutegravir, Emtricitabin And Tenofovir Alafenamide in their combined dosage form has been developed.**

A indicating reverse phase high performance liquid chromatographic method was developed for the simultaneous estimation of Dolutegravir, Emtricitabin And Tenofovir Alafenamide in their combined dosage form. The separation was achieved by C_{18} (250mm x 4.6 mm i.d.,5µm) column and Buffer (pH 5.0): Methanol (50:50) at a flow rate of 1 ml/min. Detection was carried out at 263 nm. Retention time 4.653 min, 5.603 min and 11.083 min for Dolutegravir, Emtricitabine And Tenofovir Alafenamide respectively. The method has been validated forlinearit y, accuracy and precision. Linearity observed for Dolutegravir 5-15 µg/ml, Emtricitabine 20-60 µg/ml and for Tenofovir Alafenamide 2.5-7.5 µg/ml. Developed method was found to be accurate, precise and rapid for simultaneous estimation of Dolutegravir, Emtricitabine And Tenofovir Alafenamide in their combined dosageform. The proposed method was successfully applied for the simultaneous estimation of both the drugs in commercial Combined dosageform.

The drug was subjected to stress condition of hydrolysis, oxidation, photolysis and Thermal degradation, Considerable Degradation was found in Thermal degradation. The proposed method was successfully applied for the simultaneous estimation of both the drugs in commercial Combined dosage form.

Keywords: **Dolutegravir, Emtricitabin And Tenofovir Alafenamide simultaneous estimation, HPLC Method, validation.**

1. INTRODUCTION

Emtricitabine is 5-fluoro-1-(2R,5S)-[2-(hydroxymethyl)-1,3 oxathiolan-5-yl]cytosine. Emtricitabine, a synthetic nucleoside analog of cytidine, is phosphorylated by cellular enzymes to form emtricitabine 5'-triphosphate. Emtricitabine 5'-triphosphate inhibits the activity of the HIV-1 reverse transcriptase by competing with the natural substrate deoxycytidine 5'-triphosphate and by being incorporated into nascent viral DNA which results in chain termination. Emtricitabine 5'-triphosphate is a weak inhibitor of mammalian DNA polymerase α, β, ε, and mitochondrial DNA polymerase γ.

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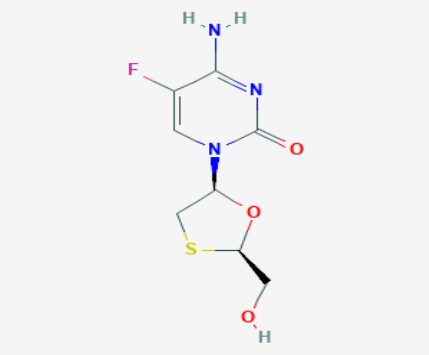

Fig 1: Structure of Emtricitabine

Dolutegravir is a monocarboxylic acid amide obtained by formal condensation of the carboxy group of (4R,12aS)-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-carboxylic acid with the amino group of 2,4-difluorobenzylamine. Used (as its sodiumsalt) for treatment of HIV-1. It has a role as a HIV-1 integrase inhibitor. It is an organofluorine compound, a monocarboxylic acid amide and an organic heterotricyclic compound. It is a conjugate acid of a dolutegravir(1-).

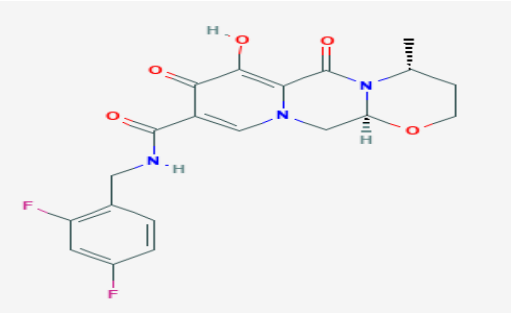

Fig 2: Structure of Dolutegravir

Tenofovir alafenamideisopropyl (2S)-2-[[[(1R)-2-(6-aminopurin-9-yl)-1-methyl-ethoxy]methyl-phenoxy-phosphoryl] amino]propanoate. Tenofovir Alafenamide is being used in a Phase 2 trial (NCT03472326) evaluating an investigational drug called GS-9131. The study is evaluating GS-9131 for HIV treatment in women on a current HIV regimen that is not controlling the virus. In Part 2 of the trial, tenofovir alafenamide as a stand-alone drug is used in a GS-9131-containing HIV regimen.

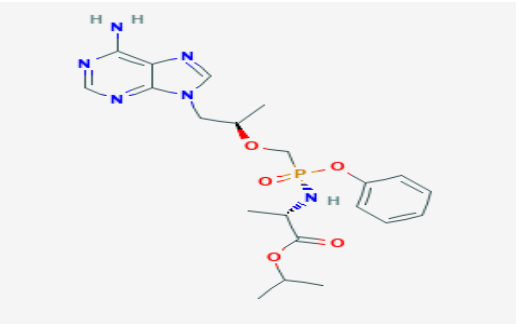

Fig 3: Structure of Tenofovir Alafenamide

2. MATERIALS AND METHODS

Reagents and Chemical

Emtricitabine, Dolutegravir And Tenofovir Alafenamide were procured from Prudence pharmachem AnklEmcure Pharmaceuticals Pvt Ltd ,Pune, Maharashtra 411057, India. The market. HPLC grade reagents methanol, acetonitrile (Merck specialists pvt, Ltd Mumbai) were used for study. All the reagent prepared by carbon dioxide free water and whereas the sample solution prepared in carbon dioxide free water double Distilled water for HPLC Purpose.

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Apparatus and chromatographic conditions

HPLC method development and validation was done on a HPLC instrument(LC 20AT) PDA detector, Stationary Phase used was C_{18} (250mm x 4.6 mm i.d.,5µm) particle size and mobile phase consisting of Buffer (pH 5): Methanol (50:50)was used. The flow rate was 1.0 ml/min and the effluents were monitored at 263 nm. The mobile phase was filtered through nylon 0.45 µm membrane filter (Millipore Pvt., Ltd, Bangalore, India). Injection volume was 20 µL. All weighing were done on analytical balance.

Preparation of mobile phase

The mobile phase was prepared with accurately weighed 5.772g of sodium acetate buffer and transfer to 1000ml volumetric flask, add 800 ml of distilled water after add 1.778g of acetic acid to the solution. Adjust solution to desired pH using 10N HCL (typically pH 5.0).

Taken 50ml of buffer solution and 50ml of methanol and mixed well, filtered with 0.45µ membranes filter paper and sonicate to degas it.

Preparation of Standard solutions

(A) Dolutegravir standard solutions: (500µg/ml)

A 50mg of Dolutegravir was weighed and transferred to a 100 ml volumetric flask. Volume was made up to the mark with methanol And sonicate to degas it.

(B) Emtricitabine standard solutions: (2000µg/ml)

A 200mg of Emtricitabine was weighed and transferred to a 100 ml volumetric flask. Volume was made up to mark with acetonitrile And sonicate to degas it.

(C) Tenofovir Alafenamide standard solution: (250µg/ml)

A 25mg of Tenofovir Alafenamide was weighed and transferred to a 100 ml volumetric flask. Volume was made up to mark with acetonitrile And sonicate to degas it.

Preparation of Sample solutions

Take 1 ml from the dolutegravir stock solution, 1ml form the Emtricitabine stock solution and 1ml form the Tenofovir Alafenamide stock solution and transferred to 10 ml volumetric flask and volume made up to the mark by mobile phase which use in particular trials.

3. RESULT AND DISCUSSION

Method validation

The method was validated according to International Conference on Harmonization guidelines for validation of analytical procedures.

To evaluate the linearity of the method, six different dilutions were made from the standard stock solutions in the working range of 50mg, 200mg and25mg for Dolutegravire, Emtricitabine and Tenofovir Alafenamide.

In order to determine the accuracy of the method, three different concentrations (80%, 100% and 120%) of tablet formulation were used and their recovery was calculated. Regarding the determination of the precision (repeatability) five replicate injections of the working standard Dolutegravir, Emtricitabine and Tenofovir Alafenamide were injected and the relative standard deviation (RSD) of the peak areas were calculated for the replicate injections. To determine the LOD and LOQ, serial dilutions of the combination were made from the standard stock solution the signal from the samples was compared with those of blank samples.

System suitability

suitability tests were carried out on freshly prepared standard stocksolution of Dolutgravir, Emtricitabine and Tenofovire Alafenamide chromatographic condition and parameters were studied to evaluate the suitability of the system.Results are shownTable.1

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Table 1: System suitability testing

Parameters	Data Observed		
	Dolutegravire	**Emtricitabine**	**Tenofovir Alafenamide**
Theoretical Plate Per Column	**5789**	**7328**	**7325**
Symmentry Factor/Tailing Factor	**4.633**	**5.577**	**11.030**
Resolution	**14.054**		

Linearity and Range

The linearity for Emtricitabine Dolutegravir and Tenofovir Alafenamide were assessed by analysis of combined standard solution in range of 20-60µg/ml ,5 to 15µg/ml and 2.5 to 7.5µg/ml respectively. 0.5,0.75,1.0,1.25,1.5 ml solutions were pipette out from the Stock solution of Emtricitabine (200 µg/ml) Dolutegravir(75 µg/ml) and Tenofovir Alafenamide (25 µg/ml) and transfer to 10 ml volumetric flask and make up with mobile phase to obtain 20,30,40,50 and 60µg/ml, 5,705,10,12.5 and 15µg/ml and 2.5,3.75,5,6.25 and 7.5for Emtricitabine Dolutegravir and Tenofovir Alafenamide respectively.

Table 2: Linearity data for Emtricitabine

Sr.no	Concentration (µg/ml)	area
1	20	4633.059
2	30	7003.539
3	40	9079.044
4	50	11424.241
5	60	13463.017

Table 3: Linearity data for Dolutegravir

Sr.no	Concentration (µg/ml)	area
1	5	1706.569
2	7.5	2581.36
3	10	3336.578
4	12.5	4209.745
5	15	4981.00

Table 4: Linearity data for Tenofovir alafenamide

Sr.no	Concentration (µg/ml)	area
1	2.5	495.645
2	3.75	742.735
3	5	977.866
4	6.25	1220.885
5	7.5	1430.128

Chromatography :- The mobile phase buffer-ammo-acetate (ph 5.0) : methanol (60:40v/v) was selected because it was found to ideally resolve the peaks with retention time (RT) 3.393min, 4.170min and 11.497min for Dolutegravir, Emtricitabine and Tenofovir Alafenamide respectively and the same is show in figure.

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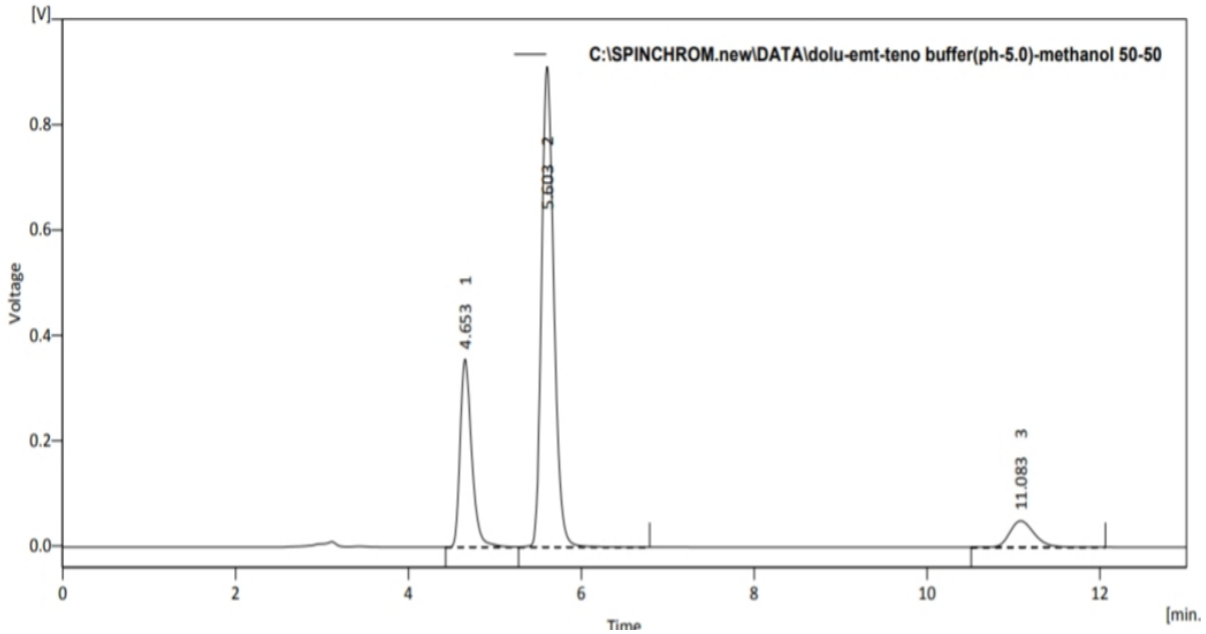

Fig.4: Chromatogram Of Dolutegravir, Emtricitabine And Tenofovir Alafenamide in Buffer(pH-5): Methanol (50:50v/v) (Flow rate-1.0ml/min).

Overlay chromatogram of different concentrations of mixtures of Emtricitabine, Dolutegravir And Tenofovir Alafenamide

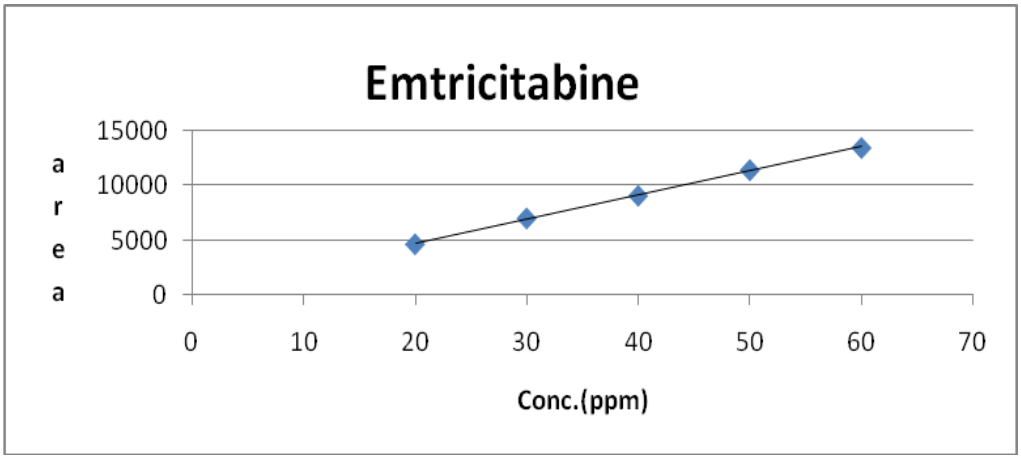

Fig 5: Calibration Curve of Emtricitabine (20-60 µg/ml)

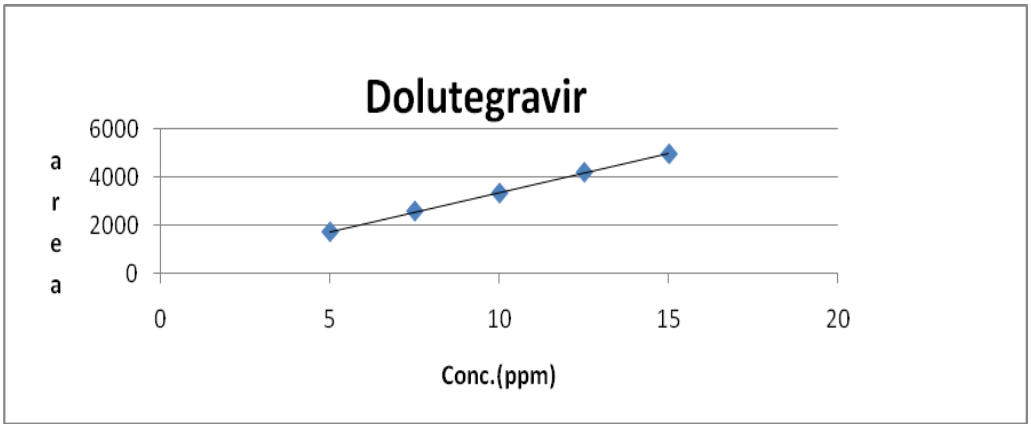

Fig 6: Calibration Curve of Dolutegravir (5-15 µg/ml)

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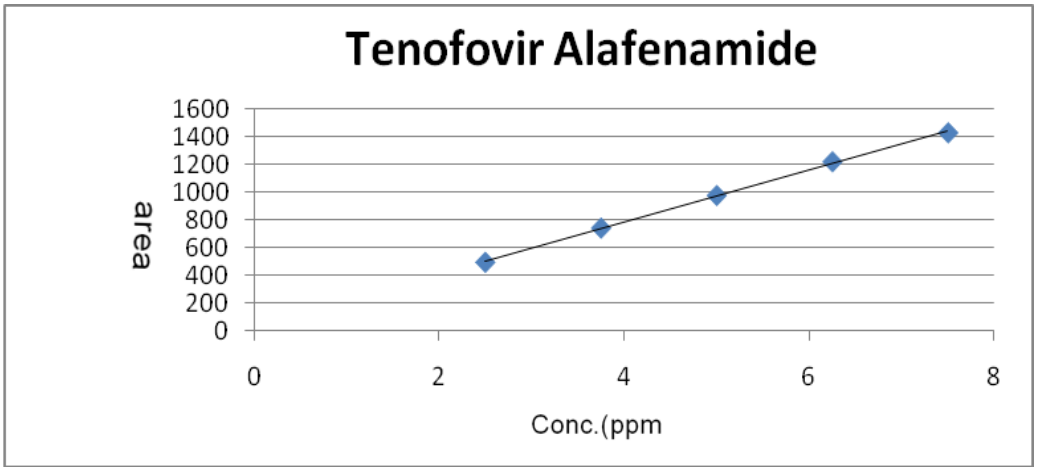

Fig 7: Calibration Curve of Tenofovir Alafenamide (2.5-7.5 µg/ml)

Accuracy

Accuracy was determined by recovery studies of Dolutegravir, Emtricitabine and Tenofovir Alafenamide, known amount of standard was added to the preanalysed sample and subjected to the proposed HPLC analysis. Results of recovery study are shown in Table 5,6 & 7. The study was done at three different concentration levels.

Table 5: Recovery data for Emtricitabine

SR. NO.	Conc. Level (%)	Sample amount (µg/ml)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	80	20	16	16.16475	101.029	100.578±0.52185
2		20	16	16.11161	100.697	
3		20	16	16.00108	100.006	
4	100	20	16	20.04090	100.204	99.2061±1.05354
5		20	16	19.86178	99.3038	
6		20	16	19.62099	98.1049	
7	120	20	16	24.03634	100.151	100.1364±0.26409
8		20	16	23.96765	99.8652	
9		20	16	24.09427	100.392	

Table 6: Recovery data for Dolutegravir

SR. NO.	Conc. Level (%)	Sample amount (µg/ml)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	80	5	4	4.04054	101.0137	99.9432±0.97105
2		5	4	3.98787	99.69696	
3		5	4	3.96476	99.11906	
4	100	5	4	5.00980	100.1960	98.9231±1.26785
5		5	4	4.94565	98.91301	
6		5	4	4.88302	97.66043	
7	120	5	4	6.00816	100.1361	99.8937±0.43934
8		5	4	5.96319	99.38655	
9		5	4	6.00950	100.1584	

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Table 7: Recovery data for Tenofovir Alafenamide

SR. NO.	Conc. Level (%)	Sample amount (µg/ml)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S.D
1	80	0.25	4	4.04651	101.16275	100.2884±1.01308
2		0.25	4	4.02099	100.52450	
3		0.25	4	3.96712	99.17823	
4	100	0.25	5	5.00830	100.16600	99.7265±0.05356
5		0.25	5	4.99183	99.83663	
6		0.25	5	4.95885	99.17707	
7	120	0.25	6	6.02750	100.45837	100.3081±0.26520
8		0.25	6	6.02784	100.46412	
9		0.25	6	6.0001	100.00192	

Repeatability

The data for repeatability of peak area measurement for Emtricitabine (10 µg/ml) Dolutegravir (40µg/ml) and Tenofovir Alafenamide (5 µg/ml) based on six measurements of same solution of Emtricitabine (20 µg/ml) and Tenofovir Alafenamide (2.5 µg/ml). The % RSD for Emtricitabine and Tenofovir Alafenamide was found to be 0.777 ,0.868 and 0.924 respectively in tab.8, 9 And 10.

Table 9: Repeatability data for Emtricitabine

DOLUTEGRAVIR				
Sr.no	Conc.(µg/ml)	Area	Mean±S.D	%R.S.D
1	10	3359.548	3317.325±28.81	0.868503807
		3335.379		
		3321.314		
		3309.954		
		3302.161		
		3275.594		

Table 10: Repeatability data for Dolutegravir

EMTRICITAVIR				
Sr.no	Conc.(µg/ml)	Area	Mean±S.D	%R.S.D
1	40	9122.124	9022.582±70.156	0.777563
		9076.541		
		9017.96		
		9000.116		
		8999.83		
		8918.921		

Table 11: Repeatability data for Tenofovir Alafenamide

TENOFOVIR ALAFENAMIDE				
Sr.no	Conc.(µg/ml)	Area	Mean±S.D	%R.S.D
1	5	978.816	967.5242±8.940	0.924075
		975.895		
		967.66		
		967.635		
		956.177		
		958.962		

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Intraday Precision:

Standred solution containing (20,40,60 µg/ml) of Emtricitabine,(5,10,15 µg/ml) of Dolutegravir and (2.5,5,7.5 µg/ml) of Tenofovir Alafenamide were analyzed three time on the same day and %R.S.D was calculated Tab 12, 13 And 14.

Table 12: Intraday precision data for estimation of Emtricitabine

ENTRICITABINE			
Sr.no	**Conc. (µg/ml)**	**Mean±S.D**	**%R.S.D**
1	20	4622.152±18.77900735	0.406282774
2	40	9036.431±11.29073279	0.124946816
3	60	13604.604±11.2907327	0.124946816

Table 13: Intraday precision data for estimation of Dolutegravir

DOLUTEGRAVIR			
Sr.no	**Conc. (µg/ml)**	**Mean±S.D**	**%R.S.D**
1	5	1703.313±5.603223715	0.328960309
2	10	3324.171±5.540702753	0.166679234
3	15	5001.37633±21.641427	0.432709444

Table 14: Intraday precision data for estimation of Tenofovir alafenamide

TENOFOVIR ALAFENAMIDE			
Sr.no	**Conc.(µg/ml)**	**Mean±S.D**	**%R.S.D**
1	2.5	489.0326±7.539650279	1.541747779
2	5.0	967.3936±6.60386079	0.682644617
3	7.5	1456.5316±11.3892815	0.781945344

Interday Precision:

Standred solution containing (20,40,60 µg/ml) of Emtricitabine,(5,10,15 µg/ml) of Dolutegravir and (2.5,5,7.5 µg/ml) of Tenofovir Alafenamide were analyzed three time on the different day and %R.S.D was calculatedin Tab 15, 16 And 17.

Table 15: Interday precision data for estimation of Emtricitabine

ENTRICITABINE			
Sr.no	**Conc. (µg/ml)**	**Mean±S.D**	**%R.S.D**
1	20	4528.097±38.44494	0.849031
2	40	9008.891±52.19386	0.579359
3	60	13594.07±140.6065	1.034322

Table 16: Interday precision data for estimation of Dolutegravir

DOLUTEGRAVIR			
Sr.no	**Conc. (µg/ml)**	**Mean±S.D**	**%R.S.D**
1	5	1662.431±21.9685	1.321468
2	10	3312.439±20.09945	0.600787
3	15	4994.392±57.14332	1.14415

Table 17: Interday precision data for estimation of Tenofovir alafenamide

TENOFOVIR ALAFENAMIDE			
Sr.no	**Conc.(µg/ml)**	**Mean±S.D**	**%R.S.D**
1	2.5	485.3953333±3.6840	0.758973
2	5.0	969.7383333±5.3800	0.554790
3	7.5	1461.311±13.1881023	0.902484

Limit of detection and Limit of Quantification

The detectionlimitor LOD is the lowest amoun to fanaly teina sample that can be detecte may be expressed as a concentration that gives a signal to noise ratio of approximately 3:1. While the Quantification limit or LOQ is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy with a signal to noise ratio

of approximately 10:1 can be taken as LOQ of the.Our method showed the(LOD)for Emtricitabine, Dolutegravir And Tenofovir Alafenamide were foundto be 3.716 µg/ml, 0.174 µg/ml and 0.210 µg/ml respectively and The LOQ values for Emtricitabine, Dolutegravir And Tenofovir Alafenamide were found 11.26 µg/ml, 0.53 µg/ml and 0.64 µg/ml respectively.

Robustness

The robustness of the proposed method was evaluated by slight modification in the mobile phase compostion, Flow rate and pH values of the mobile phase . During these studies it was found that there was not much change retention time, area and symmetry of peak.The developed method was use fo rth eassay of commercially availabletablets and six replicate determinations were performed. The interference of excipients was studied by comparing the chromatography of standards and formulations. The same shape and retention times of peaks showed that there was no interference from excipients.

4. CONCLUSION

A simple, specific, accurate and precise Stability indicating RP-HPLC method has been developed and validated as per ICH guideline for Simultaneous Estimation of Emtricitabine, Dolutegravir And Tenofovir Alafenamide in their combined dosage form.Validation parameters like Linearity, Accuracy, Precision, Robustness, Systemsuitability, Specificity were tested. Observation fall these parameters leads to the point that developed Stability indicating RP-HPLC method is linear, accurate, precise, specific and robust. It can be successfully adopted for routine quality control analysis of Emtricitabine, Dolutegravir And Tenofovir Alafenamide in Combined dosage formwithout any interference from common excipients and impurity.This method can now transfer to utilize for routine laboratory analysis and assay of Emtricitabine, Dolutegravir And Tenofovir Alafenamide in their combined dosageform.

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