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Development and Validation of Stability Indicating Method for Simultaneous Estimation of Embtricitabine and Tenofovir

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ABSTRACT

A simple rapid, sensitive, selective and reproducible reversed- phase high-performance liquid chromatographic method has been developed and validated for the simultaneous estimation of embtricitabine and tenofovir in pure and Pharmaceutical dosage form. In present work a simple, sensitive and specific method (RP-HPLC assay, stability indicating RP-HPLC) has been developed for the simultaneous estimation of embtricitabine and tenofovir in pure and Pharmaceutical dosage form. A phenomenex BDS C18, column having 5 µm particle size and 150 mm x 4.6 mm in length and gradient mode, with mobile phase containing potassium dihydrogen phosphate (pH3.0, adjusted with O-phosphoric acid) and acetonitrile in the ratio of 96:4. The flow rate was 1.0ml/min and effluents were monitored by UV detector at 254nm. The retention times of emtricitabine and tenofovir is 3.1±0.1 & 6.1±0.1 min was recorded at 254nm. The method is linear and the correlation coefficient was found to be 0.999. The method was validated for linearity, precesion, accuracy, solution stability, ruggedness, and post degradation studies were performed. Recoveries from formulations were between 98.3 and 99.5%. The results of specificity studies indicated no interference from excipients, impurities, and degradation products under various stress conditions and assured that the peak response was due to a single component only. Hence, the present method is cost-effective, faster, and can be used for the routine analysis of these drugs in pure and formulations.

Keywords: RP-HPLC method, Embtricitabine, Tenofovir Validation and Stability studies

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INTRODUCTION

Emtricitabine (4-amino-5-flouro1-[(2S,5R)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one) marketed by Gilead Sciences with the brand name *Emtriva*.[1] Emtricitabine is a nucleoside analogue approved for treatment of human immunodeficiency virus 1 with clinical activity against hepatitis B virus (HBV)[2]. The drug works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA[3]. By interfering with this process, which is central to the replication of HIV, emtricitabine can help to lower the amount of HIV, or "viral load", in a patient's body and can indirectly increase the number of immune system cells (called T cells or CD4+ T-cells). Both of these changes are associated with healthier immune systems and decreased likelihood of serious illness. Like emtricitabine, lamivudine, when used on its own, does not completely suppress viral replication. This allows drug resistant strains to emerge[4]. Emtricitabine is indicated in combination with other antiretroviral agents for the treatment of HIV infection in adults. Emtricitabine is commercially available and is approved by the FDA for treatment of HIV infection.

Tenofovir disoproxil fumarate[5] ({[(2R)-1-(6-amino-9H-purin-9-yl)propan-2-yl]oxy}methyl)phosphoric acid) marketed by Gilead Sciences under the trade name Viread, belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (NRTIs), which block reverse transcriptase, a crucial virus enzyme in human immunodeficiency virus 1 (HIV-1) and hepatitis B virus infections[6]. Tenofovir may be measured in plasma by liquid chromatography. Such testing is useful for monitoring therapy and to prevent drug accumulation and toxicity in patients with renal or hepatic impairment[7-9]. A Cochrane review examined the use of tenofovir as apre-expos ure prophylaxis against HIV infection. It found that both tenofovir alone, as well as the tenofovir/emtricitabine combination, significantly decreased the risk of contracting HIV[10]. The most common side effects associated with tenofovir include nausea, vomiting, diarrhea, and asthenia. Less frequent side effects include hepatotoxicity, abdominal pain, and flatulence. Tenofovir has also been implicated in causing renal toxicity, particularly at elevated concentrations.

Various analytical methods were found to be developed and validated by using various instrumental methods of analysis viz. reverse phase-high performance liquid chromatography (RP-HPLC)[11-13], high performance thin layer chromatography (HPTLC)[14], UV- visible Spectrophotometry[15,16], ultra performance liquid chromatography[17].

From the literature review it is known that various methods have been reported for the estimation of Emtricitabine and Tenofovir and it was found that stability indicating methods of

Emtricitabine and Tenofovir in Pharmaceutical dosage form by RP-HPLC were not reported. Hence an attempt has been made to develop simple and accurate method for the estimation of Emtricitabine and Tenofovir in tablet dosage form.

MATERIALS AND METHODS

Instrumentation:

Chromatographic separation was performed on Shimazdu HPLC system consist of model 2010 having UV detector. LC solution software was applied for data collecting and processing.

Chemicals and Reagents

All chemicals and reagents used were of analytical grade only. Milli-Q-water was used throughout the process, potassium dihdrogen phosphate, ortho phosphoric acid are of analytical reagent grade of Merck Pharmaceuticals. Acetonitrile of HPLC grade were procured from Merck Chemical Laboratories, Bangalore, India. Pure standard Embtricitabine & Tenofovir are received as gift samples from Cipla Ltd, Bangalore and formulation TRAVUDA (Label claim – 200mg of Embtricitabine & 300mg of Tenofovir) was used for the study.

METHOD DEVELOPMENT

Selection of mobile phase:

Various Mobile Phases were tried in different ratios for selection of Mobile Phase. The drug Emtricitabine and Tenofovir were injected with different mobile phases at different ratios with different flow rates till a sharp peak, without any interference peaks containing spectrum was obtained. The mobile phase selected was phosphate buffer (pH 3), and acetonitrile in the ratio 96:4.

Preparation of mobile phase:

Solution A(Buffer): Buffer solution is prepared by dissolving 4.4gm of potassium dihydrogen phosphate in 1000ml of Millipore water and sonicated to dissolve, then filtered through 0.45µ membrane filter; Solution B: Acetonitrile; Mobile Phase**:** 96 volumes of solution A and 4 volumes of solution B.

Standard stock solution of Emtricitabine and Tenofovir:

8mg of Emtricitabine working standard and 12 mg of Tenofovir working standard was transferred into 100 ml volumetric flask, added about 80 ml of diluents and sonicated to dissolve and volume made with diluent (80 µg/ml Emtricitabine and 120 µg/ml Tenofovir).

Preparation of calibration curve: From this stock solution 0.5-2.5ml were pipetted into 10ml volumetric flasks. After this volume was made upto 10ml with milli pore water to get conc 4-20

µg/ml Emtricitabine and 6-30 µg/ml of tenofovir.

RESULTS AND DISCUSSION

Method Validation:

System Suitability:

To verify that the analytical system is working properly and can give accurate and precise results, the system suitability parameters are to be set. System suitability tests are used to verify the reproducibility of the chromatographic system. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared standard solutions. The chromatographic conditions and results of system suitability are shown in Table 1 and 2 respectively.

Table 1: Chromatographic Conditions

Instrument	HPLC 2010	
Column	C18 phenomenex luna C8 column 250 x4.6	
Wavelength	254nm	
Temperature	30°C	
Flow rate	1.0ml/min	
Detector	UV	
Injection volume	10 µl	
Mobile phase	Phosphate buffer (pH 3.0),and acetonitrile which were of HPLC grade in the ratio 96:(v/v).	
Retention time	Embtricitabine	3.15±0.10
	tenofovir	6.1±0.05

Table 2: Results of System Suitability parameters

Sno	System Suitability Parameters	Proposed Acceptance Criteria	Results	
			Emtricitabine	Tenofovir
1	Tailing factor of Emtricitabine and Tenofovir peaks	NMT 2.0.	1.17	1.24
2	Theoretical plates of Emtricitabine and Tenofovir peaks	NLT 2000	4219	5610
3	%RSD for the peak area of 5 replicate injections of Emtricitabine and Tenofovir peaks	NMT 2.0%	0.1%	0.31%

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Linearity:

The linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range. The range of analytical method is the interval between upper and lower level of analyte including levels that have been demonstrated to be determined with precision

and accuracy using the method. The standard chromatogram of embtricitabine and enofovir are shown in figure. 1. Linearity ranges are 8-40 µg/ml and 12-60 µg/ml for embtricitabine and enofovir respectively. The calibration graph are shown figure 2 and 3. The area responses are reported in Table 3.

Table 3: Concentrations and Linearity of Embtricitabine and Tenofovir

Levels	ml. Added	Diluted to	Conc. (ppm) Emtricitabine	Tenofovir	Area Response Emtricitabine	Tenofovir
1	0.50	50	8	12	215524	284322
2	1.0	50	16	24	413256	554879
3	1.5	50	24	36	640125	844442
4	2.0	50	32	48	839584	1114098
5	2.5	50	40	60	105287	1394225

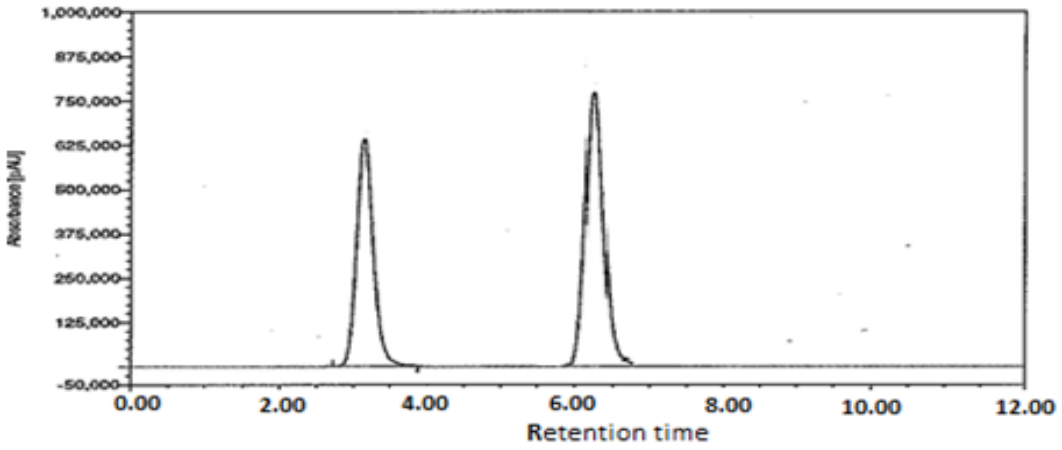

Figure 1: Chromatogram of Standard Embtricitabine and tenofovir

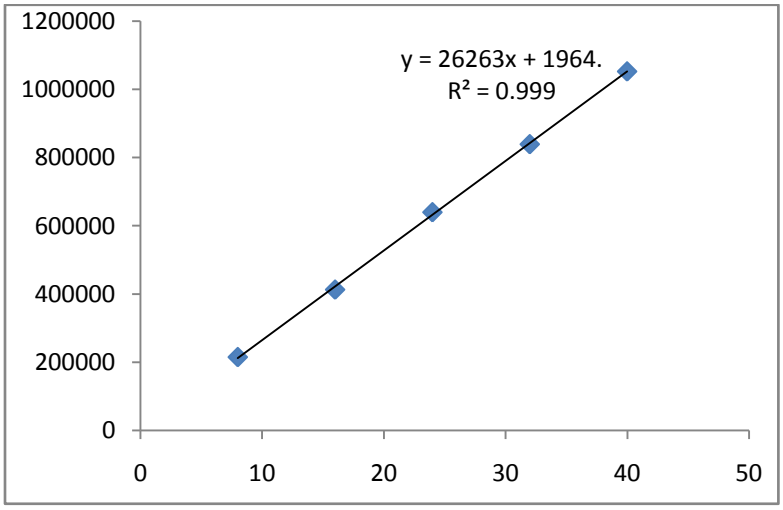

Figure 2: Linearity plot for Embtricitabine

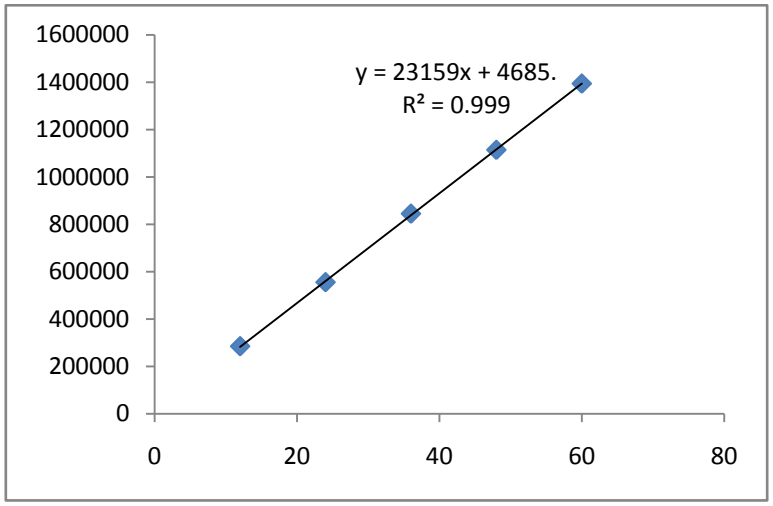

Figure 3: Linearity plot for Tenofovir

Table 4: Peak Results for accuracy of Embtricitabine

Target Conc.	Wt. taken	Mg spiked	Area Inj.1	Area Inj.2	Avg. Area	Mg recov.	% recovery	Avg. recov.	% RSD
50	149.1	148.95	2070631	2084844	2077738	147.62	99.1	99.3	0.2
50	150.1	149.95	2088675	2105466	2097071	148.99	99.4		
100	300.2	299.90	4178760	4151575	4165168	295.92	98.7	98.3	0.6
100	299.6	299.30	4131497	4111538	4121518	292.82	97.8		
150	450.1	449.65	6230375	6239630	6235003	442.98	98.5	98.4	0.2
150	449.9	449.45	6211612	6210415	6211014	441.27	98.2		
Overall Recovery								98.6	0.6

Table 5: Peak Results for accuracy of Tenofovir

Target Conc.	Wt. taken	Mg spiked	Area Inj.1	Area Inj.2	Avg. Area	Mg recov.	% recovery	Avg. recovery	% RSD
50	300.10	299.80	3192269	3213399	3202834	298.00	99.4	99.5	0.1
50	300.90	300.60	3205017	3230119	3217568	299.37	99.6		
100	599.20	598.60	6385922	6343518	6364720	592.19	98.9	98.5	0.6
100	599.00	598.40	6319484	6286416	6302950	586.44	98.0		
150	899.00	898.10	9495283	9505885	9500584	883.96	98.4	98.2	0.3
150	899.40	898.50	9465773	9465752	9465763	880.72	98.0		
Overall Recovery								98.7	0.7

Accuracy:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy of an analytical method should be established across its range. Accuracy is performed in three different levels for Embtricitabine and Tenofovir . Spiked known quantity of Embtricitabine and Tenofovir at 50%, 100% and 150% levels. Analysed samples in duplicate for each level. From the results, % recovery was calculated. % Recovery at each spike level shall be not less than 98.0 and not more than 102.0. % RSD for the duplicate observations

shall be not more than 2.0. Overall % RSD for the % Recovery shall be not more than 2.0. Peak results for Embtricitabine and Tenofovir are shown in Table 4 & 5 respectively.

Procedure:

Accuracy at 50%:

Accurately 149.1 mg of embtricitabine and 300.1 mg of tenofovir transferred into 200 ml volumetric flask, added 100 ml of diluent and sonicated it for 10 minutes and sonicated it for 25 minutes with intermittent shaking and allowed to cool at room temperature, made upto mark with diluent and mixed well. Filter this through 0.45µ nylon filter. Further 5ml was diluted to 50 ml with diluents and mixed well.

Accuracy at 100%:

Accurately weighed 300.2 mg of embtricitabine and 599.5 mg of tenofovir transferred into 200 ml volumetric flask, added 100 ml of diluent and sonicated it for 10 minutes and sonicated it for 25 minutes with intermittent shaking and allowed to cool at room temperature, made upto mark with diluent and mixed well. Filter this through 0.45µ nylon filter. Further 5ml was diluted to 50 ml with diluents and mixed well.

Accuracy at 150%:

Accurately weighed 450.1 mg of embtricitabine and 899 mg of tenofovir transferred into 200 ml volumetric flask, added 100 ml of diluent and sonicated it for 10 minutes and sonicated it for 25 minutes with intermittent shaking and allowed to cool at room temperature, made up to mark with diluent and mixed well. Filter this through 0.45µ nylon filter. Further 5ml was diluted to 50 ml with diluents and mixed well.

Precision:

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogenous sample. The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of series of measurement.

System Precision:

The system precision is checked by using standard Embtricitabine and Tenofovir to ensure that the analytical system is precise. The retention time and area of ten determinations was measured and RSD was calculated. % RSD of the assay value for ten determinations shall not be more than 2.0%.

Method Precision:

In method precision, a homogenous sample of a single batch should be analysed six times. This

indicates whether a method is giving consistent results for a single batch. The method precision was performed on Embtricitabine and Tenofovir formulation. The % RSD of the assay value for six determinations shall not be more than 2.0%. Results are shown in Table 6 & 7. It was observed from the data tabulated, that the retention time and area responses are consistent as evidenced by the values of relative standard deviation. Hence, it can be concluded that the system precision parameter meets the requirement of method validation.

Table 6: Method Precision Results for Embtricitabine

Test No.	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6
Area (Inj.1)	4142174	4322107	4289898	4282623	4279268	4308847
Area (Inj.2)	4125529	4346892	4116130	4287899	4335660	4300865
Avg. Area	4133852	4334500	4203014	4285261	4307464	4304856
% RSD	0.3	0.4	2.9	0.1	0.9	0.1
Assay(mg/tab)	29.37	30.78	29.85	30.43	30.59	30.57
Assay (%)	97.9	102.6	99.5	101.4	102.0	101.9
Average Assay		In mg	30.27	In %	100.9	

Table 7: Method Precision Results for Tenofovir

Test No.	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6
Area (Inj.1)	6329842	6604999	6555377	6543307	6539264	6585057
Area (Inj.2)	6306134	6641369	6290971	6551951	6624120	6571480
Avg. Area	6317988	6623184	6423174	6547629	6581692	6578269
% RSD	0.3	0.4	2.9	0.1	0.9	0.1
Assay(mg/tab)	58.78	61.60	59.74	60.89	61.21	61.17
Assay (%)	98.0	102.7	99.6	101.5	102.0	102.0
Average Assay		In mg	60.57	In %	101.0	

Forced Degradation Studies:

Forced degradation studies were carried out on the sample preparations of Emtricitabine and Tenofovir dispersible tablets 30/60/50 mg and the degradation was evaluated by calculating the % degradation of Emtricitabine and Tenofovir in comparison with unstressed sample preparation. The degradation between 10 % and 30% was tried by following stress conditions to prove that the method is has stability indicating characteristics.

The following are the stress conditions which were followed for forced degradation studies:

1. Acid Degradation
2. Alkali Degradation
3. Peroxide Degradation
4. Thermal Degradation

Acid Stress degradation:

Test preparation was subjected to acid stress degradation by treating the sample with hydrochloric acid. The % degradation was evaluated by calculating the % assay and by

comparing the assay results with the assay of unstressed sample. The resultant chromatogram shown in figure. 4.

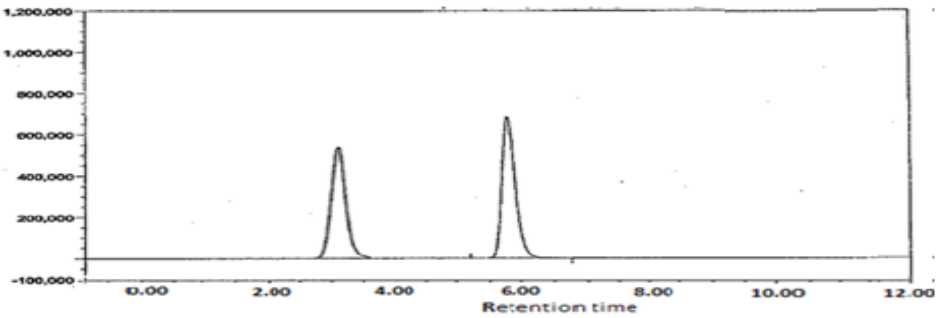

Figure 4: Chromatogram of Acid stressed sample

Test preparation:

Weighed and transferred 553.70 mg of tablet powder into 200 ml volumetric flask, added 5 ml 5N Hydrochloric acid, heated for 1 hour on a water bath at 80°C. Cooled and neutralized with 5 N sodium hydroxide , added 10 ml water, sonicated for 10 minutes, added 130 ml diluent, sonicated for 25 minutes with intermittent shaking, made upto mark with diluent, mixed well, filtered through 0.45µ nylon filter, further diluted 5 ml to 50 ml (filtrate) with diluents, mixed well. Results are shown in Table 8.

Table 8: Peak results for Acid degradation

% Assay Peak				
Name of the analyte	**Unstressed Sample**	**Stressed Sample**	**Purity Results Purity Angle**	**Purity Threshold**
Embtricitabne	98.4	91.1	0.040	0.219
Tenofovir	98.6	91.4	0.096	0.243

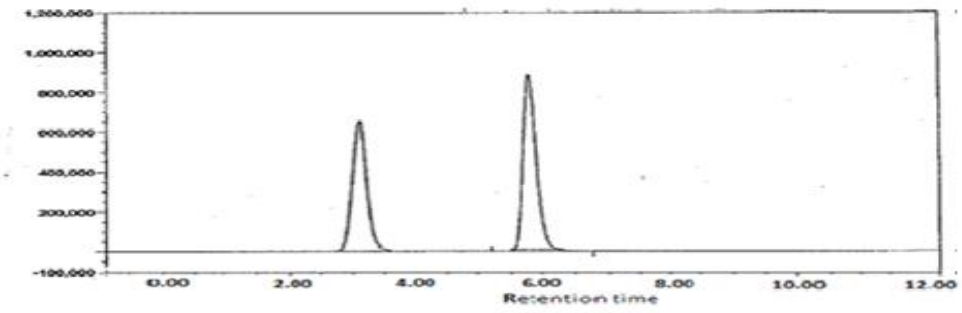

Figure 5: Chromatogram of Alkali stressed sample

Alkali stress degradation:

Test preparation was subjected to alkali stress degradation by treating the sample with sodium hydroxide. The % degradation was evaluated by calculating the % assay and by comparing the assay results with the assay of unstressed sample. Minimum 10 to 30% degradation shall be

achieved. The peak purity for analyte peak shall pass. The resultant chromatogram shown in figure. 5.

Test preparation:

Weighed and transferred 553.90 mg of tablet powder into 200 ml volumetric flask, added 5 ml 5N sodium hydroxide, heated for 1 hour on a water bath at 80°C. Cooled and neutralized with 5 N hydrochloric acid , added 10 ml water, sonicated for 10 minutes, added 130 ml diluent, sonicated for 25 minutes with intermittent shaking, made upto mark with diluent, mixed well, filtered through 0.45µ nylon filter, further diluted 5 ml to 50 ml (filtrate) with diluents, mixed well. Results are shown in Table 9.

Table 9: Peak results for Alkali degradation

% Assay				
Name of the analyte	**Unstressed Sample**	**Stressed Sample**	**Peak Purity Results**	
			Purity Angle	**Purity Threshold**
Embtricitabne	98.4	70.7	0.040	0.214
Tenofovir	98.6	70.5	0.084	0.234

Peroxide Stress degradation:

Test preparation was subjected to peroxide stress degradation by treating the sample with peroxide. The % degradation was evaluated by calculating the % assay and by comparing the assay results with the assay of unstressed sample. Minimum 10 to 30% degradation shall be achieved. The peak purity for analyte peak shall pass (Figure. 6).

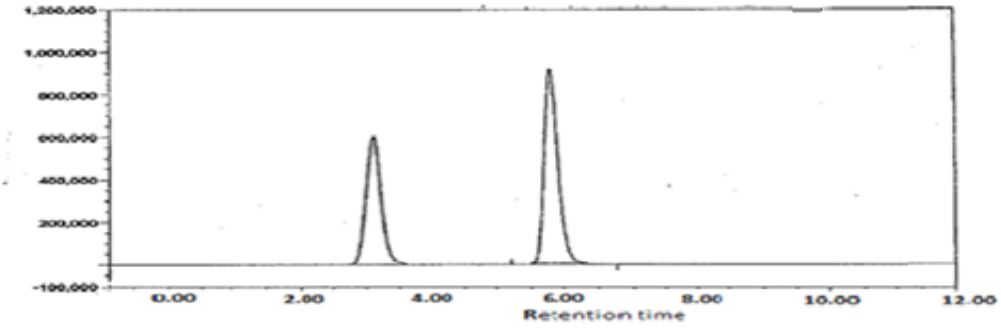

Figure 6: Chromatogram of Peroxide stressed sample

Test preparation:

Weighed and transferred 553.70 mg of tablet powder into 200 ml volumetric flask, added 5 ml 6% H_2O_2, heated for 1 hour on a water bath at 80°C, added 10 ml water, sonicated for 10 minutes, added 130 ml diluent, sonicated for 25 minutes with intermittent shaking, made upto mark with diluent, mixed well, filtered through 0.45µ nylon filter, further diluted 5 ml to 50 ml (filtrate) with diluents, mixed we The resultant chromatogram shown in figure. 7 and results are shown in Table 10.

Table 10: Peak results for Peroxide Stress Degradation

% Assay				
Name of the analyte	**Unstressed Sample**	**Stressed Sample**	**Peak Purity Results**	
			Purity Angle	**Purity Threshold**
Embtricitabne	98.4	86.4	0.035	0.218
Tenofovir	98.6	95.4	0.058	0.245

Thermal stress degradation:

Test preparation was subjected to thermal treatment (80°C) for sufficient time. The % degradation was evaluated by calculating the % assay and by comparing the assay results with the assay of unstressed sample. Minimum 10 to 30% degradation shall be achieved.

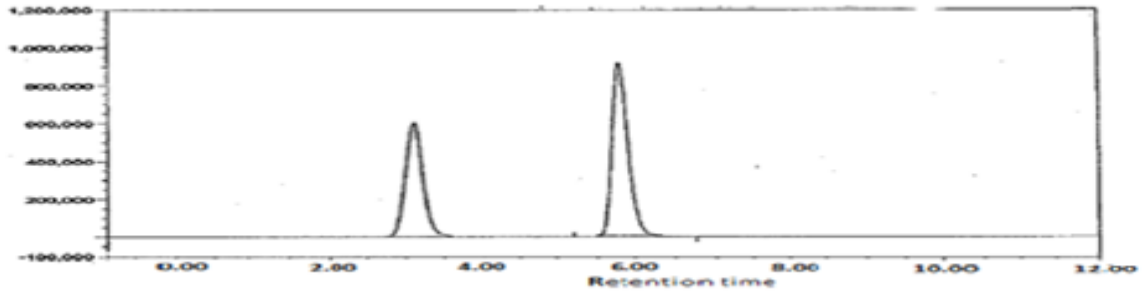

Figure 7: Chromatogram of Thermal stressed sample

Test preparation:

Weighed and transferred 553.60 mg of tablet powder into 200 ml volumetric flask, added 10 ml water, sonicated for 10 minutes, added 130 ml diluent, sonicated for 25 minutes with intermittent shaking, made upto mark with diluent, mixed well, filtered through 0.45µ nylon filter, further diluted 5 ml to 50 ml (filtrate) with diluents, mixed well. Results are shown in Table 11. From results, it can be concluded that purity angle of emtricitabine and tenofovir in stressed sample chromatogram was lesser than the purity threshold. It can be concluded that the method is specific and the peak purity of emtricitabine and tenofovir passed.

Table 11: Peak results for Thermal stress degradation

% Assay				
Name of the analyte	**Unstressed Sample**	**Stressed Sample**	**Peak Purity Results**	
			Purity Angle	**Purity Threshold**
Embtricitabine	98.4	98.3	0.031	0.219
Tenofovir	98.6	98.5	0.101	0.245

From the forced degradation studies, it can be observed that the proposed acceptance criteria meet the requirements for acid and alkali degradation and it is stable even when more stress conditions like peroxide and thermal stress is applied. Based on the forced degradation studies (Table 12 and 13), the proposed analytical method can be considered as stability indicating method and can be used for release and stability studies for effective evaluations.

Table 12: Forced degradation studies of Embtricitbine

Sr. No	Condition	% degradation Achieved	Assay	Purity Angle	Purity Threshold	Purity pass/ fail
1.	Unstressed Sample	N.A.	98.4	0.036	0.219	Pass
2.	Acid degradation	7.4	91.1	0.040	0.219	Pass
3.	Alkali degradation	28.2	70.7	0.040	0.214	Pass
4.	Peroxide degradation	12.2	86.4	0.035	0.218	Pass
5.	Heat degradation	0.1	98.3	0.031	0.219	Pass

Table 13: Forced degradation studies of Tenofovir

Sr. No	Condition	% degradation Achieved	Assay	Purity Angle	Purity Threshold	Purity pass/ fail
1.	Unstressed Sample	N.A.	98.6	0.054	0.245	Pass
2.	Acid degradation	7.3	91.4	0.096	0.243	Pass
3.	Alkali degradation	28.5	70.5	0.084	0.234	Pass
4.	Peroxide degradation	3.2	95.4	0.058	0.245	Pass
5.	Heat degradation	0.1	98.5	0.101	0.245	Pass

Ruggedness

Ruggedness is a measure of reproducibility of test results under the variation in conditions normally expected from laboratory to laboratory and from analyst to analyst. The results are shown in Table 14.

Table 14: Peak results for Ruggedness

Sr. No.	Embtricitabine in %				Tenofovir in %			
	SET I	SET II	SET III	SET IV	SET I	SET II	SET III	SET IV
1	97.9	99.5	101.6	102.6	98.0	102.7	99.6	101.5
2	102.6	101.9	101.4	97.6	102.7	101.1	102.0	102.1
3	99.5	97.6	99.5	101.9	99.6	98.0	101.0	102.0
4	101.4	102.0	102.6	101.4	101.5	99.6	98.0	99.6
5	102.0	101.4	97.9	101.6	102.0	101.5	101.0	102.0
6	101.9	102.6	105.0	99.5	102.0	102.7	102.0	98.0
Avg	100.9	101.0	100.8	100.5	101.0	100.8	100.0	101.6
SD	1.8060	1.814	1.835	1.833	1.7941	1.7821	1.7800	1.7965
% RSD	1.8	1.8	1.8	1.8	1.8	1.79	1.8	1.76
Overall Avg	101.1				100.8			
Overall % RSD	1.8				1.8			

SET – I: Method precision; SET – II: Variability due to HPLC system; SET – III Variability due to HPLC column; SET – IV: Variability due to analyst

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness was done by changing the column temperature (±5°C), flow rate (±10%), changing the wavelength (±5°nm), pH of buffer solution (±0.2 units). All the system suitability parameters must be met as per the method.

CONCLUSION

Although various methods have been reported for the simultaneous estimation of emtricitabine and tenofovir and it was found that stability indicating methods of emtricitabine and tenofovir in Pharmaceutical dosage form by RP-HPLC were not reported. Hence an attempt has been made to develop simple and accurate method for the estimation of emtricitabine and tenofovir in tablet dosage form Results of analysis of the samples revealed that the proposed method is suitable for their analysis with no interference from the impurities and degradation products and recovery is found to be acceptable. The methods were found to be linear, precise, accurate, specific and all proved to be sensitive, convenient and effective for the simultaneous estimation of emtricitabine and tenofovir. A system suitability test is established and related parameters are recorded. Hence, this method stands validated and can be used for routine and stability sample analysis.

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