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Research Article

STABILITY INDICATING UPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF EMTRICITABINE, RILPIVIRINE AND TENOFOVIR IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

A simple, sensitive and precise stability indicating UPLC method has been developed and validated for the simultaneous estimation of Emtricitabine, Rilpivirine and Tenofovir in combined dosage form. The column used was Endoversil C_{18} (2.1 x 50mm, 1.7µm) column. The mobile phase used was Phosphate buffer: Acetonitrile (50:50). Quantification was carried out using PDA Detector at 260 nm. Linearity was found to be 20-100 mcg/ml for Emtricitabine, 25-125 mcg/ml for Rilpivirine and 30-150 mcg/ml for Tenofovir, respectively. The method was validated for system suitability, precision, accuracy, ruggedness, robustness, LOD & LOQ. Emtricitabine, Rilpivirine and Tenofovir were also subjected to acid degradation, alkali degradation, oxidative degradation, thermal degradation and photo degradation. The degradation products obtained were well resolved from the Emtricitabine, Rilpivirine and Tenofovir with different retention times. Since the method can effectively separate Emtricitabine, Rilpivirine and Tenofovir in their combined dosage form, it can be used for the routine determination of Emtricitabine, Rilpivirine and Tenofovir.

Keywords: Emtricitabine, Rilpivirine, Tenofovir, Stability indicating, UPLC.

INTRODUCTION

Emtricitabine is a nucleoside reverse transcriptase inhibitor having the molecular structure given in fig. 1. Chemically, Emtricitabine is 4-amino-5-fluoro- 1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2-one. Rilpivirine is a diaryl pyrimidine derivative and reverse transcriptase inhibitor with antiviral activity against HIV-1 having the molecular structure given in fig. 2. Chemically, Rilpivirine is [4-[4-[(E)-2-cyanoethenyl]-2,6-dimethylanilino] pyrimidin-2-yl]amino]benzonitrile. Tenofovir is an adenine analog reverse transcriptase inhibitor with antiviral activity against HIV-1 having the molecular structure given in fig. 3. Chemically, Tenofovir is [(2R)-1-(6-aminopurin-9-yl)propan-2-yl]oxymethylphosphonic acid.

Literature survey reveals that few analytical methods have been reported for the simultaneous estimation of Emtricitabine, Rilpivirine and Tenofovir in their combined dosage form. In the present investigation a stability indicating UPLC method was described using Endoversil C_{18} (2.1 x 50mm, 1.7µm) column. The mobile phase used was Phosphate buffer: Acetonitrile (50:50), with a flow rate of 0.4 mL/min. Quantification was carried out using PDA Detector at 260 nm. In the proposed method the low values of % RSD, LOD and LOQ indicates

that the developed method is more precise and sensitive than the reported methods. The use of phosphate buffer in the preparation of mobile phase makes the method more economical than the reported methods.

MATERIALS AND METHODS

Instrumentation

Chromatography was carried out using Waters UPLC system, with Empower 2 software, 2695 separation module. Detector used was PDA detector.

Chemicals and solvents

Reference standards Emtricitabine, Rilpivirine and Tenofovir were obtained from Pharma train Laboratory. Solvents used were of UHPLC grade. Other chemicals used were of analytical grade. Commercial tablets (Complera**,** labeled to contain 200 mg Emtricitabine, 25 mg Rilpivirine and 300 mg Tenofovir, respectively**)** were procured from local pharmacy.

Chromatographic conditions

Instrument used was Waters UPLC with auto sampler. The column used was Endoversil C_{18} (2.1 x 50mm, 1.7µm) column. The mobile phase used was Phosphate buffer: Acetonitrile (50:50). Quantification was carried out using PDA Detector at 260 nm.

Preparation of standard solutions

The stock and working standard solutions were prepared with the mobile phase. The standard stock solutions of Emtricitabine (2 mg/ mL), Rilpivirine (0.25 mg/ mL) and Tenofovir (3 mg/ mL) were prepared by transferring accurately weighed amounts (20 mg of Emtricitabine, 2.5 mg of Rilpivirine and 30 mg of Tenofovir) into different 10 mL volumetric flasks. The drugs were dissolved by shaking gently with 5 mL of mobile phase and made upto the mark with the same solvent.

The working standard solutions (40 mcg/ml of Emtricitabine, 5 mcg/ml of Rilpivirine and 60 mcg/ml of Tenofovir) were prepared by transferring 2 mL of stock standard solution into 100 mL volumetric flask and the volume was made upto the mark with the mobile phase. All the solutions were filtered through 0.1 µm membrane filters before use.

Calibration curves

Standard calibration curves were prepared with six calibrators over a concentration range of 20-100 µg/mL for Emtricitabine, 25-125 µg/mL for Rilpivirine and 30-150 µg/mL for Tenofovir. 2 µL of solutions were injected in triplicate and chromatographed under the optimized conditions as described above. The peak areas measured were plotted against the concentration of the corresponding drug and the regression equation was derived.

Preparation of tablet sample solution

Ten tablets were weighed and their average weight was determined. The tablets were crushed to a homogenous powder and an amount equivalent to 200 mg of Emtricitabine, 25 mg of Rilpivirine and 300 mg of Tenofovir was accurately weighed and transferred into a 100 mL volumetric flask to which 30 mL of mobile phase was added. After sonication for 15 min, the mixture in the flask was diluted to the mark with mobile phase and mixed. An aliquot of 2 mL was transferred to a 100 mL flask and filled to the mark with mobile phase. The solution was filtered through 0.1 µm membrane filter before use. 2 µL of solution was injected under the optimized conditions as described above. The contents of the analytes were obtained from the corresponding regression equation/corresponding calibration curve.

METHOD VALIDATION

After development, the method was subjected to validation as per ICH guidelines

System suitability

The system suitability parameters were evaluated by injecting standard solution of 40 µg/mL Emtricitabine, 5 µg/mL Rilpivirine and 60 µg/mL Tenofovir. The results are presented in Table 1. The system was found to be suitable, as the parameters are within the acceptable limits (fig. 4).

Linearity

The linearity of the method was evaluated by analyzing a series of solutions containing Emtricitabine, Rilpivirine and Tenofovir in the concentration range of 20-100 µg/mL, 25-125 µg/mL and 30-150 µg/ mL, respectively. The calibration curves were constructed. The regression coefficients of the curves were found to be ≥ 0.9990 for the three drugs, enabling the linear behavior of the method in the established concentration range. Emtricitabine, Rilpivirine and Tenofovir showed linearity in the range of 20-100 µg/mL, 25-125 µg/mL and 30-150 µg/ mL,

respectively (fig. 5). Linear regression equations and correlation coefficient are presented in Table 2.

Precision

The precision of the method was evaluated by analyzing standard solutions of Emtricitabine, Rilpivirine and Tenofovir with a concentration of 40 µg/mL, 5 µg/mL and 60 µg/mL, respectively. Six replicates were analyzed to determine the precision. The % RSD of peak areas was calculated and was found to be below 2.0 %. This indicates the precision of the method for the simultaneous estimation of Emtricitabine, Rilpivirine and Tenofovir (fig. 6). The results are shown in Table 3.

Accuracy

To determine the accuracy of the method, recovery studies were carried out by application of the standard addition method. Known amounts of the Emtricitabine, Rilpivirine and Tenofovir at three different concentration levels (50 %, 100 % and 150 %) were added to a pre-analyzed tablet sample; the prepared samples were then analyzed by the proposed method and the percentage recoveries were then calculated. Good percentage recoveries were obtained, confirming the accuracy of the proposed method (fig. 7-9). The results are shown in Table 4.

Ruggedness

To evaluate the intermediate precision of the method, analysis was carried out using a different analyst. The precision of the method was evaluated by analyzing standard solutions of Emtricitabine, Rilpivirine and Tenofovir with a concentration of 40 µg/mL, 5 µg/mL and 60 µg/mL, respectively. Six replicates were analyzed to determine the precision. The % RSD of peak areas was calculated and was found to be below 2.0 %. This indicates the precision of the method for the simultaneous estimation of Emtricitabine, Rilpivirine and Tenofovir (fig. 10).

Robustness

The robustness of the method was studied by varying the chromatographic conditions with respect to the flow rate of the mobile phase and mobile phase combination. The study was conducted at three different flow rates (0.3 mL/min, 0.4 mL/min and 0.5 mL/min) and at three different mobile phase combinations. The effect of these changes on the different chromatographic parameters was studied. The results are summarized in Table 3. Negligible difference was found in system suitability parameters for Emtricitabine, Rilpivirine and Tenofovir such as USP plate count, resolution and the USP tailing factor, therefore the method found to be robust (fig. 11-14).

Limit of detection (LOD) and Limit of quantification (LOQ)

The limits of detection and quantification were evaluated based on residual standard deviation of the response and the slope. The LOD and LOQ values for Emtricitabine, Rilpivirine and Tenofovir are presented in Table 2. The values indicate the adequate sensitivity of the method (fig. 15 &16).

Specificity

The chromatograms of mobile phase blank, placebo blank, test sample (40 µg/mL Emtricitabine, 5 µg/mL Rilpivirine and 60 µg/mL Tenofovir) and standard (40 µg/mL Emtricitabine, 5 µg/mL Rilpivirine and 60 µg/mL Tenofovir) were compared to give reason for the specificity of the method. The method was specific & selective since excipients in the formulation and components of the mobile phase did not interfere in the simultaneous analysis of Emtricitabine, Rilpivirine and Tenofovir (fig. 17&18).

Forced degradation

Forced degradation studies were performed on tablet sample using different stress conditions such as acidic, basic, oxidative, thermal and photolytic stresses and then the samples are filtered through 0.1 µm membrane filter and subjected to UPLC analysis. When Emtricitabine, Rilpivirine and Tenofovir was subjected to different forced degradation conditions (acid, base, oxidative, thermal, and photolytic), significant degradation was observed. The percentage of degradation and percent relative standard deviation values are summarized in Table 5. The degradants produced in all the forced degradations were well separated from Emtricitabine, Rilpivirine and Tenofovir. The method therefore proved to be stability-indicating.

Acidic degradation

Acidic degradation was carried out using 0.1 N HCl. For this, tablet powder equivalent to 200 mg of Emtricitabine, 25 mg of Rilpivirine and 300 mg of Tenofovir was taken in 100 mL volumetric flask. 10 mL of 0.1 N HCl was added and sonicated for 30 min. After completion of the stress, the solution was neutralized using 0.1N

NaOH and filled up to the mark with mobile phase. The sample was injected into UPLC and analysed (fig. 19).

Alkali degradation

Alkali degradation study was carried out using 0.1 N NaOH. For this, tablet powder equivalent to 200 mg of Emtricitabine, 25 mg of Rilpivirine and 300 mg of Tenofovir was taken in 100 mL volumetric flask. 10 mL of 0.1 N NaOH was added and sonicated for 30 min. After completion of the stress, the solution was neutralized by using 0.1 N HCl and filled upto the mark with mobile phase. The sample was injected into UPLC and analysed (fig. 20).

Oxidative degradation

Oxidative degradation was carried out using 30 % H_2O_2. To perform this, tablet powder equivalent to 200 mg of Emtricitabine, 25 mg of Rilpivirine and 300 mg of Tenofovir was taken in 100 mL volumetric flask. 10 mL of 30 % H_2O_2 was added to it. The contents of the flask were sonicated for 30 min. After completion of the stress, the volume of the flask was made up to the mark with mobile phase. The sample was injected into UPLC and analysed (fig. 21).

Thermal degradation

Thermal degradation was performed in hot air oven at 110°C. For this study, tablet powder equivalent to 200 mg of Emtricitabine, 25 mg of Rilpivirine and 300 mg of Tenofovir was taken in glass petri dish and placed in oven at 110 °C for 30 min. After specified time, the sample was cooled, transferred into a 100 mL volumetric flask and dissolved in 30 mL of mobile phase and the volume was made upto mark with mobile phase. The sample was injected into UPLC and analysed (fig. 22).

Photolytic degradation

For photolytic degradation study, 200 mg of Emtricitabine, 25 mg of Rilpivirine and 300 mg of Tenofovir tablet powder was taken in glass petri dish and placed in the direct sunlight for 24 h. After completion of the stress, the drug sample was cooled, transferred into a 100 mL volumetric flask and dissolved in 30 mL of mobile phase and the volume was made upto mark with mobile phase. The sample was injected into UPLC and analysed (fig. 23).

Table 1: Results of system suitability

Parameter	Emtricitabine	Rilpivirine	Tenofovir	Recommened Limits
Retention Time	0.329	0.8	0.483	----
% RSD	0.159	0.357	0.412	RSD ≤2
Tailing factor	1.15	1.10	1.16	≤ 2
Theoretical plates	3350	2747	2776	> 2000

Table 2: Results of Linearity, LOD, LOQ and precision

Parameter	Emtricitabine	Rilpivirine	Tenofovir
Linearity (µg/mL)	20-100	25-125	30-150
Regression equation	y = 15683x + 60060	y = 4942.x + 22134	y = 24599x + 17186
Regression Coefficient	0.999	0.999	0.999
LOD (µg/mL)	0.169	0.124	0.176
LOQ (µg/mL)	0.465	0.482	0.373
RSD (%)	0.245	0.307	0.431

Table 3. Results of Robustness

3.1: System suitability results for Emtricitabine

S. No.	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.3	3347.4	1.16
2	0.4	3576	1.14
3	0.5	3353	1.18

3.2: System suitability results for Rilpivirine

S. No.	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	0.3	2745.7	1.15	2.41
2	0.4	2795	1.13	2.36
3	0.5	2442.59	1.35	2.37

3.3: System suitability results for Tenofovir

S. No.	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	0.3	2763.8	1.19	9.61
2	0.4	2786	1.19	9.26
3	0.5	4101.72	1.21	9.41

3.4: System suitability results for Emtricitabine

S. No.	Organic Phase Ratio	System Suitability Results	
		USP Plate Count	USP Tailing
1	Less Organic	2696.70	1.41
2	**Actual**	2968.97	1.39
3	More Organic	2711.61	1.34

3.5: System suitability results for Rilpivirine

S. No.	Organic Phase Ratio	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	Less Organic	2566	1.24	2.37
2	**Actual**	2542	1.34	2.35
3	More Organic	2583	1.45	2.39

3.6: System suitability results for Tenofovir

S. No.	Organic Phase Ratio	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	Less Organic	4243.88	1.21	9.46
2	**Actual**	4076.31	1.20	9.25
3	More Organic	4296.02	1.23	9.51

Table 4: Results of Accuracy studies

4.1: The accuracy results for Emtricitabine

%Concentration (at specification Level)	Area	Amount Added (mcg)	Amount Found (mcg)	% Recovery	Mean Recovery
50%	498339	20	20.08	100.4	100.04
100%	990504	40	39.94	99.85	
150%	1482568	60	59.93	99.88	

4.2: The accuracy results for Rilpivirine

%Concentration (at specification Level)	Area	Amount Added (mcg)	Amount Found (mcg)	% Recovery	Mean Recovery
50%	201168	25	25.08	100.32	99.99
100%	399477	50	49.98	99.96	
150%	601078	75	74.78	99.70	

4.3: The accuracy results for Tenofovir

%Concentration (at specification Level)	Area	Amount Added (mcg)	Amount Found (mcg)	% Recovery	Mean Recovery
50%	1176673	30	30.06	100.2	99.93
100%	2377073	60	59.93	99.88	
150%	3494692	90	89.74	99.71	

Table 5: Results of Degradation studies

	Emtricitabine		Rilpivirine		Tenofovir	
	Area	% Degradation	Area	% Degradation	Area	% Degradation
Standard	993051		399362		2359335	
Acid	920116	7.34	385764	3.40	2241008	5.02
Base	927681	6.58	386313	3.27	2242549	4.95
Peroxide	922023	7.15	387109	3.07	2244556	4.86
Thermal	921805	7.17	386720	3.17	2245584	4.82
Photo	921845	7.17	386646	3.18	2245686	4.82

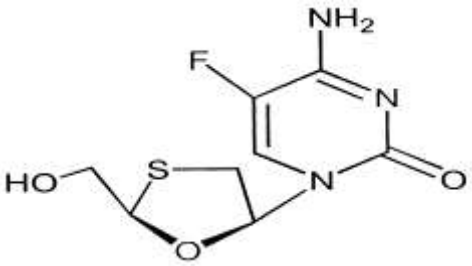

Fig. 1: Chemical structure of Emtricitabine

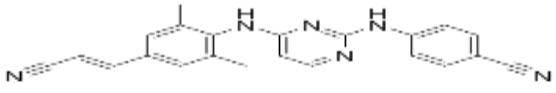

Fig. 2: Chemical structure of Rilpivirine

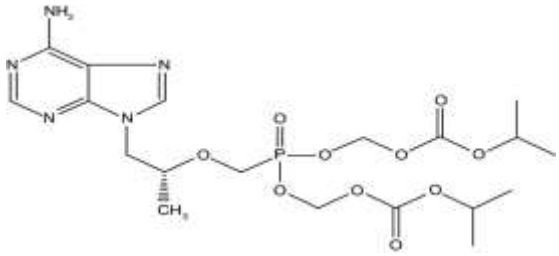

Fig. 3: Chemical structure of Tenofovir

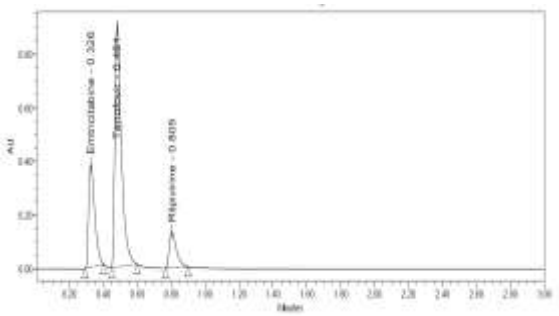

Fig. 4: Chromatogram of Emtricitabine, Rilpivirine and Tenofovir under optimized chromatographic conditions

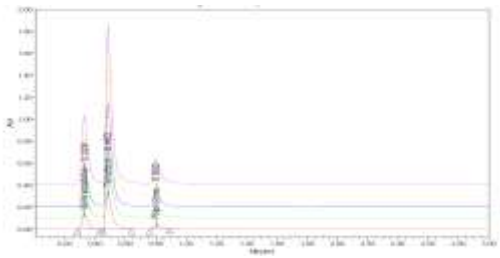

Fig. 5: Chromatogram of Linearity studies

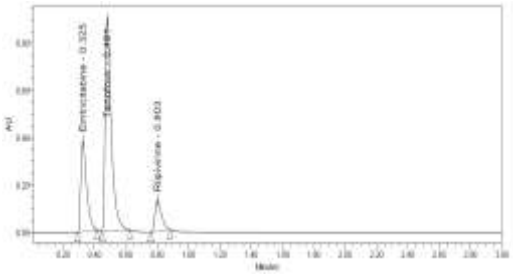

Fig. 6: Chromatogram of Precision studies

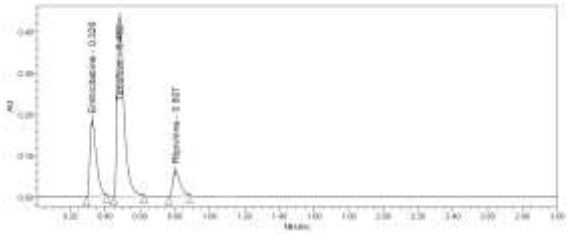

Fig. 7: Chromatogram of Accuracy studies – 50% spiked level

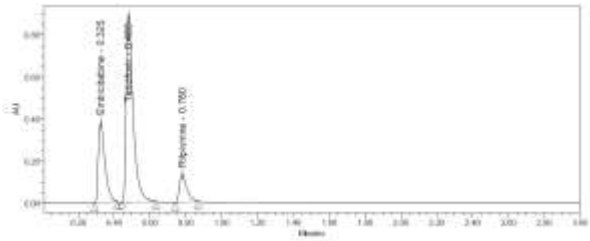

Fig. 8: Chromatogram of Accuracy studies – 100% spiked level

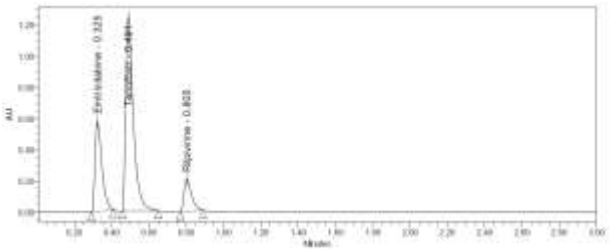

Fig. 9: Chromatogram of Accuracy studies – 150% spiked level

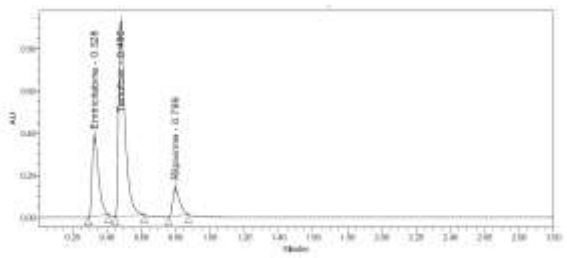

Fig. 10: Chromatogram of Ruggedness studies

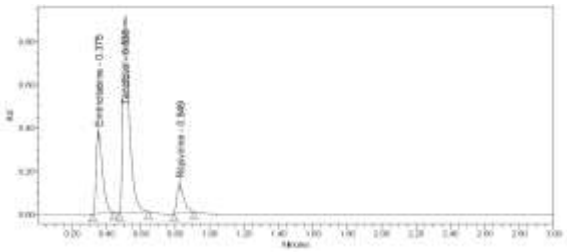

Fig. 11: Chromatogram of Robustness studies – Flow rate 0.3 mL/min

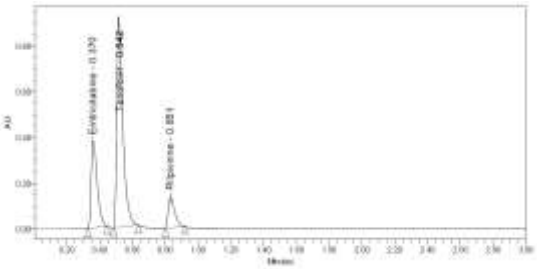

Fig. 12: Chromatogram of Robustness studies – Less organic phase

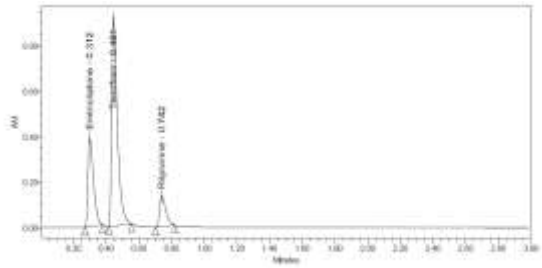

Fig. 13: Chromatogram of Robustness studies – Flow rate 0.5 mL/min

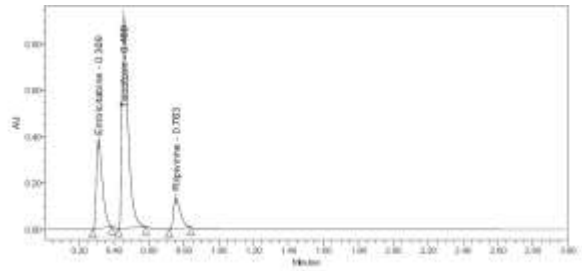

Fig. 14: Chromatogram of Robustness studies – More organic phase

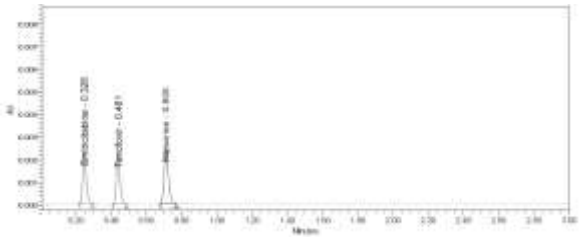

Fig. 15: Chromatogram of LOD

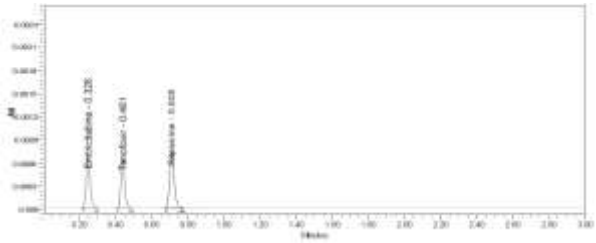

Fig. 16: Chromatogram of LOQ

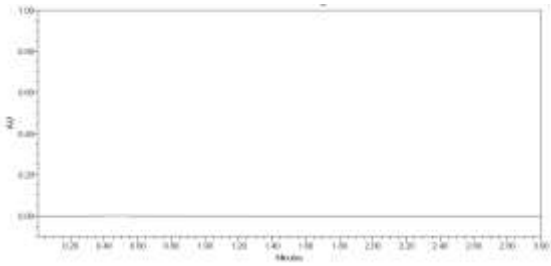

Fig. 17: Mobile phase Blank

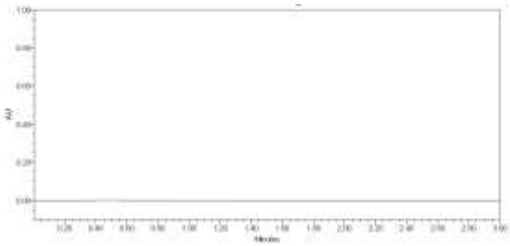

Fig. 18: Placebo

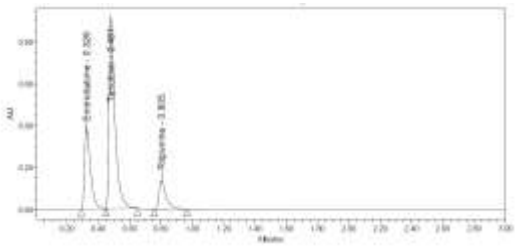

Fig. 19: Acidic degradation

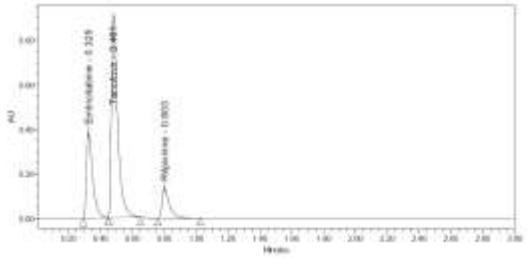

Fig. 20: Alkali degradation

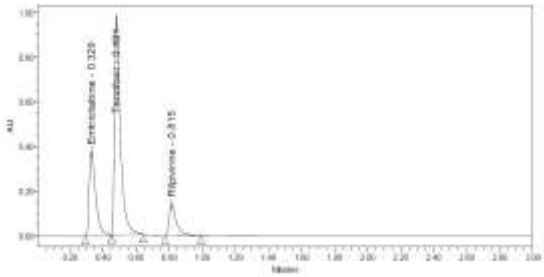

Fig. 21: Oxidative degradation

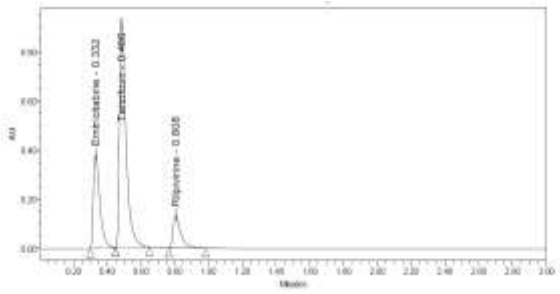

Fig. 22: Thermal degradation

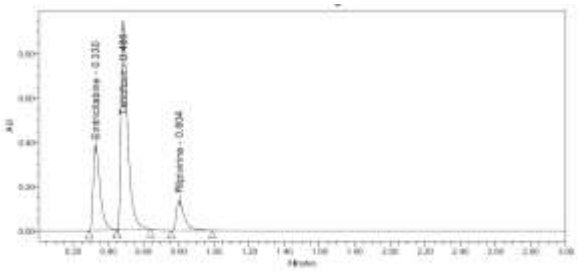

Fig. 23: Photolytic degradation

CONCLUSION

The developed stability indicating UPLC method has been successfully applied for the simultaneous determination of Emtricitabine, Rilpivirine and Tenofovir in their combined dosage form. The method was found to be rapid, simple and accurate. When the developed method was completely validated, the results showed satisfactory data for all the method validation parameters. From the values percentage RSD, LOD and LOQ, it was found that the developed method is more precise and sensitive than the previously reported methods. So the proposed method can be easily and conveniently adopted for routine quality control analysis of Emtricitabine, Rilpivirine and Tenofovir.

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