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A Review On Development And Validation For Simultaneous Estimation And Stability Indicating Studies Of Emtricitabine, Rilpivirine, And Tenofovir Alafenamide In Solid Dosage Form

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ABSTRACT- A stability indicating RP-HPLC method was developed and validated for the estimation of emtricitabine (EMT), rilpivirine (RIL), and tenofovir alafenamide (TAF) in combined dosage forms and its API. Chromatographic separation of these drugs was performed on INERTSIL column, C18 (150x4.6 ID) 5µm as the stationary phase using solvent system consisted of Phosphate buffer: Acetonitrile 40:60. The method was validated according to the International Conference of Harmonization (ICH) guidelines. The method showed accuracy of 100.19%, 101.30% and 99.70% and percentage assay of 100.04%, 99.74% and 102.14% for Emtricitabine, Rilpivirine and Tenofovir alafenamide, respectively. Percentage relative standard deviation (<2%) was found for both precision and robustness study showing that the proposed method was precise, specificity, robust and stable in accordance with ICH guidelines.

KEYWORDS- Emtricitabine (EMT), rilpivirine (RIL), tenofovir, RP-HPLC, validation, stability indicating studies,

I. INTRODUCTION

[1-3]Emtricitabine (EMT) is a nucleoside reverse transcriptase inhibitor (NRTI) for the treatment of HIV infection in adults which works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA, resulting in early chain termination. Its chemical name is 5-fluoro-1-[(2R, 5S)-2-(hydroxymethyl)-1, 3-oxathiolan-5-yl] cytosine and the molecular formula is C8H10FN3O3S.

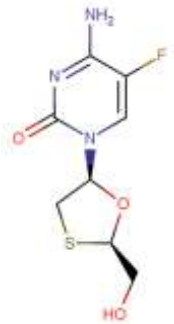

Figure 1. Emtricitabine

Tenofovir alafenamide (TAF) is a NRTI and a novel ester prodrug of the antiretroviral tenofovir. Its chemical name is ({[(2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl] oxy} methyl) phosphoric acid and the molecular formula is C21H29N6O5.

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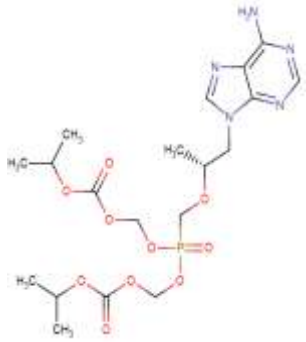

Figure 2. Tenofovir alafenamide

[4]Rilpivirine (RIL) is non-nucleoside reverse transcriptase inhibitor (NNRTI) which is used for the treatment of HIV-1 infections in treatment-naïve patients. It is a diarylpyrimidine derivative, a class of molecules that resemble pyrimidine nucleotides found in DNA 2. Chemically, it is 4-[[4-[(E)-2- cyanoethenyl]-2, 6-dimethyl-anilino] pyrimidin-2-yl] amino] benzonitrile.

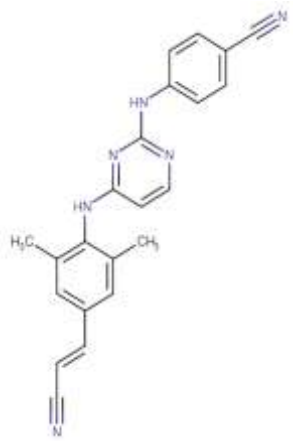

Figure 3. Rilpivirine

[1-3]According to literature survey, there was no official method for the simultaneous estimation of EMT, RIL, and TAF, but only few reverse-phase high-performance liquid chromatography (RP-HPLC) methods have been described in the literature for individual or in combination with other drugs for the estimation which were found to have high retention time (RT) and more total run time for analysis. [5-7]There was no stability indicating analytical methods reported for simultaneous estimation of EMT, RIL, and TAF. [8-13]This study describes the development and validation of high performance liquid chromatographic (HPLC) method for the simultaneous estimation of Emtricitabine (EMT), Rilpivirine (RPV) and Tenofovir alafenamide (TAF) in tablet dosage form and stability indicating study. The developed method was validated with respect to specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity, precision, accuracy and robustness. Forced degradation studieswere performed on the drug product solution to show the stability-indicating nature of the method and to ensure its compliance in accordance with International Conference on Harmonization (ICH) guidelines.

II. MATERIALS AND METHODS

Chemicals And Reagents

[14]Active pharmaceutical ingredient (API) of 99.8% potency of Emtricitabine, Rilpivarine and Tenofovir alafenamide (TAF) were obtained. Analytical grade reagents such as acetonitrile, potassium dihydrogen orthophosphate, tetrahydrofuran, and sodium hydroxide and HPLC grade water were procured.

Instrumentation

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[15]Separation was carried out on Waters Acquity RP-HPLC with PDA detector having with INERTSIL column C18(150x4.6 ID) 5µm, Nicolet Evolution 100 UV/visible, METSAR pH meter, POWERSONIC 405 sonicator, Afcoset er-200a analytical balance and pipettes, beakers, and burettes.

III. PREPARATION OF SOLUTIONS

Mobile Phase

[14]0.68% Potassium dihydrogen orthophosphate buffer solution was prepared by taking 6.8 grams of potassium dihydrogen orthophosphate in 1000 mL volumetric flask and dissolved in water, made up to the mark by adjusting the pH of the solution equal to pH = 6 with 0.1 N NaOH solution. Resulting solution was filtered through 4.5 µ filter under vacuum filtration. Mixture of buffer and acetonitrile in the ratio 40:60 v/v was taken, degassed in ultrasonic water bath for five minutes at room temperature and then filtered through 4.5 µ filter under vacuum filtration.

Standard and sample preparation

[15]Weigh accurately 13 mg of EMT, 1.62 mg of RIL, and 20 mg of TAF in 100 ml of volumetric flask and dissolve in 10 ml of Mobile Phase and make up the volume with Mobile Phase. From that, 13 µg/ml of EMT, 1.62 µg/ml of RIL, and 20 µg/ml of TAF were prepared by diluting 5.3 ml–10 ml with Mobile Phase which was used as stock solution.

5 tablets were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Weight equivalent to 34.62 mg and dissolved. Further dilutions were prepared in five replicates of 13 µg/ml of EMT, 1.62 µg/ml of RIL, and 20 µg/ml of TAF which were made by adding 5.3 ml of stock solution to 10 ml of Mobile Phase which was used as sample solution.

HPLC Method Development

[14]HPLC is a novel technique used in the separation and assay of pharmaceutical especially in combined drugs. The development of liquid chromatographic method was based on physico-chemical properties of selected drugs such as molecular weight, molecular formula, chemical structure, solubility, pKa value, UV absorption maxima and inactive ingredients. The selected drugs were completely soluble in moderately polar and polar solvents such as water, methanol and acetonotrile, hence a reversed phase liquid chromatographic technique was the best method in which a non-polar stationary phase (a nonpolar hydrophobic packing with octyl or octadecyl functional group bonded to silica gel) and a polar mobile phase (potassium dihydrogen ortho-phosphate buffer solution and organic solvents like acetonitrile) were considered.

[16-17]The optimum chromatographic conditions were established by testing different trials by changing one of the chromatographic conditions such as column, mobile phase and its composition, flow rate of the mobile phase, injection volume, run time, column temperature and detection wavelength keeping other constant. Finally the desired separation was achieved by injecting 20 µL of standard solution into the INERTSIL column, C18(150x4.6 ID) 5µm column maintained at ambient temperature; elution was carried out by using mobile phase at a flow rate of 1.2 mL/min, and the detection at wavelength of 275 nm.

Method validation

[18-20]The analytical method was validated with respect to parameters such as linearity, limit of quantification (LOQ), limit of detection (LOD), precision, accuracy, robustness and ruggedness.

IV. STABILITY INDICATING STUDIES

[21]Forced degradation studies of the fixed dose combination of the drug were carried out by treating the sample under tress conditions like acid and base hydrolysis, oxidation, photolytic and thermal degradation, and resultant degradation products was investigated. These study help to know the stability characteristics of the drug and the possible degradation products.

Preparation of Solution for Degradation Studies: Weight equivalent to 1 tablet, i.e., 200 mg of EMT, 25 mg of RIL, and 25 mg of TAF into 50 ml capacity standard volumetric flask. The contents in the flask were dissolved using methanol and sonicate it and diluted up to the mark with methanol.

Preliminary Study: In the preliminary examination, observations were made about sample stability, including exposure of solid state samples to heat and light and exposure of solutions to various pH and oxidative conditions. The preliminary study can also be used to aid in the development of an analytical method.

Acid Degradation Condition: Pipette 2 ml of the above solution into a 10ml volumetric flask and 3 ml of 0.1N HCl was added. Then, the volumetric flask was kept at 60 ºC for 6 h and then neutralized with 0.1 N NaOH and makeup to 10ml with

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diluent. Filter the solution with 0.22 microns syringe filters and placed in vials. Using mobile phase finally the volume was made up to the mark, and the percentage of degradation was calculated.

Alkali Degradation Condition: Pipette 2 ml of the above solution into a 10 ml volumetric flask into a 10ml volumetric flask and add 3 ml of 0.1N NaOH was added in 10 ml of volumetric flask. Then, the volumetric flask was kept at 60 ºC for 6 h and then neutralized with 0.1N HCl and makeup to 10 ml with Diluent. Filter the solution with 0.22 microns syringe filters and placed in vials. Finally, volume was made up to the mark with the mobile phase, and the percentage of degradation was calculated.

Thermal Induced Degradation Condition: Rilpivirine, Emtricitabine, and Tenofovir alafenamde sample was taken in Petri dish and kept in a hot air oven at 110 ºC for 24 h. Then the sample was taken and diluted with diluents and injected into HPLC and percentage of degradation was calculated.

Photolytic Degradation Condition: A 5 ml aliquot of the above stock solution was exposed to sunlight for about 6 h, and then the sample diluted with 5 ml of mobile phase and the percentage of degradation was calculated.

Oxidative Degradation Condition: Pipette 2 ml above stock solution 2 into a 10 ml volumetric flask solution into a 10ml volumetric flask 1 ml of 3% w/v of hydrogen peroxide added in 10 ml of volumetric flask and the volume was made up to the mark with diluent. The volumetric flask was then kept at room temperature for 15 min. Filter the solution with 0.45 microns syringe filters and place in vials and percentage of degradation was calculated.

V. RESULT AND DISCUSSION

System suitability

[22]The RT of EMT, RIL, and TAF using optimum conditions was 2.517, 3.273, and 6.697 min, respectively. The peak symmetries were <1.5, theoretical plates were >2000, and % relative standard deviation (RSD) was <2 as shown.

Table 1 System Suitability Parameters

Parameter	EMT	RIL	TAF
Peak area	1012865	1105605	1118501
Theoritical Plate	2862.66	6433	6402.16
Retention Time	2.517	3.273	6.697
Tailing Factor	0.96	1.22	1.335

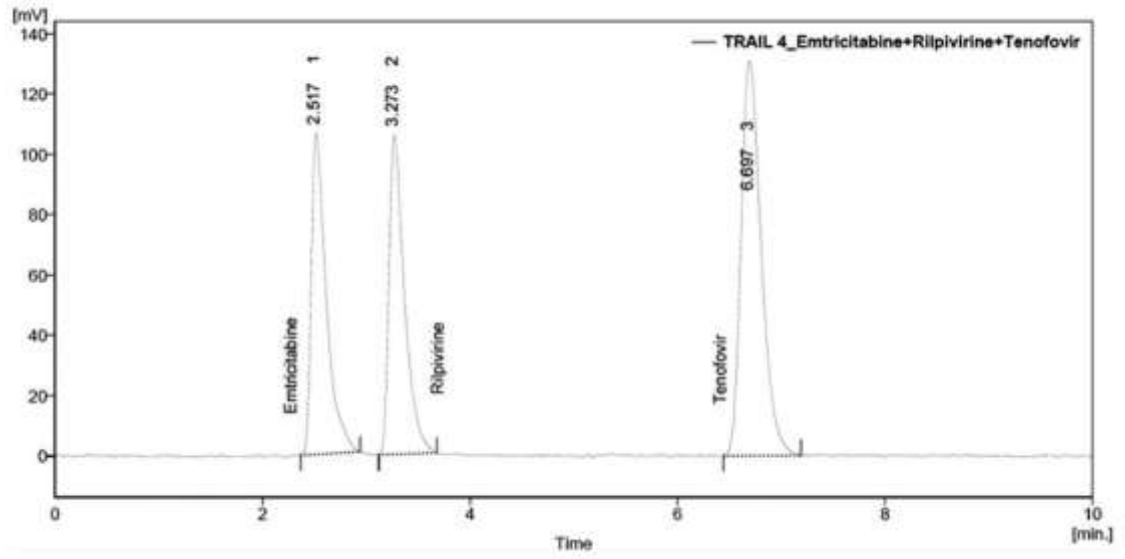

Figure 4. Optimized chromatogram of emtricitabine, rilpivirine, and tenofovir alafenamide

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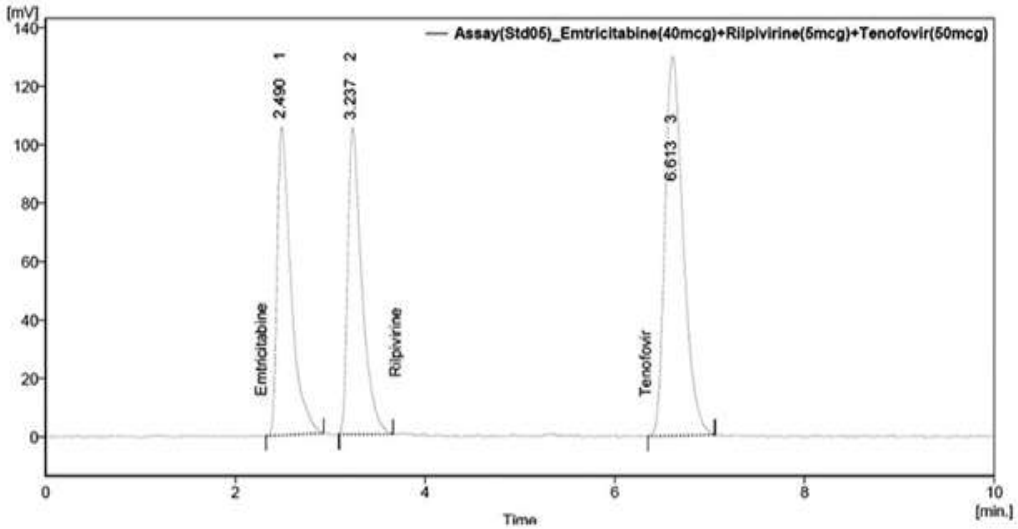
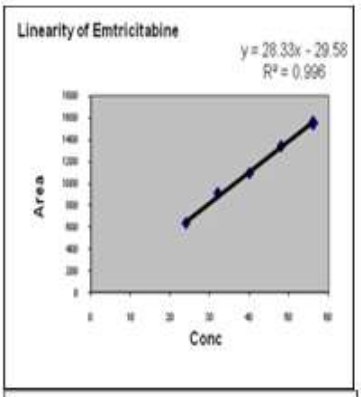
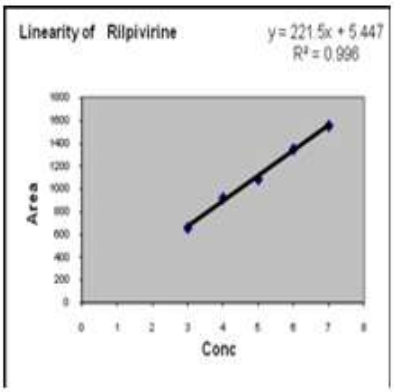
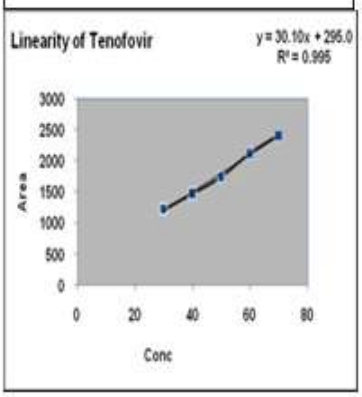


Figure 5. Assay chromatogram of emtricitabine, rilpivirine, and tenofovir alafenamide

Linearity: Linearity between peak area and concentration of Emtricitabine, Rilpivirine and Tenofovir in the proposed method was determined by drawing plots taking mean peak area on y-axis against concentration on x-axis. From the plots it was evident that linearity for Emtricitabine, Rilpivirine and Tenofovir was found to be 24 - 56 µg/mL, 3 - 7 µg/mL and 30 - 70 µg/mL respectively. Slope, intercept and correlation coefficient of the data was determined and given as below.







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LOD and LOQ: LOD and LOQ of the developed method was determined from noise-to-signal ratio method, the average baseline noise for blank and average peak area for LOD/LOQ concentration with was determined and calculated signal to noise ration and found to be more than 22.19/30.31,3.85/17.12 and 17.23/50.91 for Emtricitabine, Rilpivirine and Tenofovir respectively.

Precision: Precision of finite replicate measurements either in system precision or method precision is expressed as percent of relative standard deviation (%RSD) in statistical analysis, and the acceptability should be %RSD $\leq$ 2.0. In both cases chromatographic parameters such as peak area and retention time between three peaks were determined for six measurements. Mean peak area (M), standard deviation (SD) and percent of relative standard deviation (%RSD) of peak area were determined using Microsoft Excel Sheet. The results of system precision and method precision were presented below respectively.

Table.2.Precision data of Emtricitabine, Rilpivirine and Tenofovir

	Emtricitabine		**Rilpivirine**		**Tenofovir**	
S.No.	Rt	Area	Rt	Area	Rt	Area
1	2.52	1087.80	3.28	1094.37	6.71	1753.45
2	2.52	1089.67	3.27	1101.76	6.69	1754.74
3	2.52	1067.84	3.28	1073.60	6.71	1729.09
4	2.52	1097.28	3.27	1108.43	6.71	1754.49
5	2.52	1059.01	3.27	1079.24	6.69	1729.17
6	2.52	1071.85	3.27	1075.58	6.69	1727.32
AVG	2.52	1088.73	3.27	1088.83	6.70	1741.38
STD	0.002	1.32	0.003	14.71	0.008	14.10
%RSD	99.88	102.76	0.10	1.35	0.12	0.81

Accuracy: To determine accuracy of the proposed method, chromatograms were obtained at three different concentration levels (32, 40 and 48 mg of Ematricitabin 4,5 and 6 mg of Rilpivarine and 40, 50 and 60 mg of Tenofovir) and the percent of recovery was evaluated at each spike level from the peak area, and then mean recovery was calculated and found to be 100.19, 101.30 and 99.70 respectively. According to ICH guidelines, the mean percent of recovery should be 98% - 102%, and hence the percent of recovery was within the acceptable limits. The results of accuracy were presented below.

Table.3. Accuracy data of Emtricitabine

Recovery level	**Accuracy of Emtricitabine**			**Average % Recovery**
	Amount taken(mcg/ml)	**Area**	**% of mean Recovery**	
50%	32	950.13	102.87	100.19
	32	930.06		
	32	921		
100%	40	1166.96	99.6	
	40	1050.71		
	40	1043.14		
150%	48	1350.73	98.1	
	48	1300.25		
	48	1293.06		

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Table.4.Accuracy data of Rilpivirine

Recovery level	Accuracy of Rilpivirine			Average % Recovery
	Amount taken(mcg/ml)	Area	% of mean Recovery	
50%	4	976.81	102.52	101.3
	4	930.02		
	4	920.92		
100%	5	1091.76	99.08	
	5	1085.16		
	5	1051.3		
150%	6	1400.54	102.55	
	6	1380.54		
	6	1367.54		

Table.5.Accuracy data of Tenofovir

Recovery level	Accuracy of Tenofovir			Average % Recovery
	Amount taken(mcg/ml)	Area	% of mean Recovery	
50%	40	1494.81	99.217	99.7
	40	1464.75		
	40	1453.16		
100%	50	2115.12	101.28	
	50	2005.19		
	50	1197.74		
150%	60	2212.62	98.1338	
	60	2029.82		
	60	2012.5		

Robustness: In the study of robustness, chromatograms were recorded for flow rate and mobile phase composition variation, and chromatographic parameters were evaluated. It was found that there was no considerable variation in retention time, wavelength for these variations. In the present investigation ruggedness of the proposed method was demonstrated between different flow rates and different wavelengths.

Table.6.Robustness of the method

Parameter	Emtricitabine		Rilpivirine		Tenofovir	
	Retention time(min)	Tailing factor	Retention time(min)	Tailing factor	Retention time(min)	Tailing factor
Flow						
1.0ml/min	2.99	1.34	3.88	1.68	7.89	1.53
1.4ml/min	2.17	1.76	2.81	1.35	5.70	1.55
Wavelength						
260nm	2.51	1.70	3.26	1.31	6.62	1.60
264nm	2.49	1.77	3.24	1.31	6.63	1.57

Ruggedness: In the study of ruggedness, the reproducible results were obtained by the analysis of the same samples by two different analysts. The results of study of ruggedness were shown below.

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Table.7.Ruggedness of the method

Emtricitabine	%Assay	Rilpivirine	%Assay	Tenofovir	%Assay
Analyst 01	100.86	Analyst 01	100.48	Analyst 01	100.72
Analyst 02	99.98	Analyst 02	100.51	Analyst 02	99.10

Stability Indicating Studies: In the present study Forced degradation studies were carried out to develop stability profile for the fixed-dose combination of Emtricitabine, Rilpivirine and Tenofovir alafenamide and ensure the effective separation of drugs from degradation products. Degradation was observed by the generation of different peaks with different retention time with respective original peaks of the drug. The percentage assay of degradation was calculated from the peak area obtained in degradation conditions, and it was compared with assay of non degraded conditions. The stability of an analytical method was determined by forced degradation studies, in which the stability of the method was carried out by performing Acid stress study, Base stress study, Peroxide stress study, Water stress study, UV light exposure study, and Dry heat stress study. The net degradation was found to be within limits. The results and chromatograms were summarized.

TABLE 8. Stability Indicating Studies

Condition	EMT		RIL		TAF	
	Area	% Degraded	Area	% Degraded	Area	% Degraded
Control	1087.9	-	1088.83	-	1748.04	6.7
Acid	988.1	6.25	1638.98	6.04	1229.21	5.76
Base	985.1	5.08	1649.68	5.19	1224.89	7.55
Peroxide	985.53	7.05	1641.59	6.99	1227.22	3.59
Thermal	985.1	3.42	1649.64	2.54	1224.89	3.59
Photo	985.02	1.85	1649.68	1.16	1224.55	2.32

VI. CONCLUSION

A simple, rapid, accurate, and precise stability-indicating HPLC analytical method had been developed and validated for the routine simultaneous estimation of EMT, RIL, and TAF in API and tablet dosage forms. The RT of EMT, RIL, and TAF using optimum conditions was 2.517, 3.273, and 6.697 min, respectively. The simplicity of the HPLC procedure, the short runtime, and the low volume of injection make this method suitable for quick and routine analysis. The stability indication nature of the analytical method provides confidence to use the developed method in a regulatory environment of the pharmaceutical industry without any further modification.

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