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Development and Validation of HPLC Method for Simultaneous Estimation of Emtricitabine, Rilpivirine and Tenofovir Disoproxil Fumarate Tablet Dosage form

D.Karunakranth*, Anil Kumar Midha,R.Sridhar Babu and DV.Kishore
OPJS University, Churu, Rajasthan-331303
***Corresponding author**:D.Karunakranth;E-Mail:karun_kranth@rediffmail.com

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ABSTRACT

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 disoproxil fumarate (TFV) in tablet dosage form. 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 calibration curves were linear over the (r2 -0.995) concentrations range from 24-56 µg /ml for Emtricitabine, 3-7 µg /ml for Rilpivirine and 30-70 µg /ml for Tenofovir disoproxil fumarate. 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 disoproxil fumarate, 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.

1. INTRODUCTION

Emtricitabine (EMT), a nucleoside reverse transcriptase inhibitor is chemically known as 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one.
Tenofovir disoproxil fumarate belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors, which block reverse transcriptase, a crucial viral enzyme in HIV-1 and hepatitis B virus infections. It is chemically known as ({[(2R)-1-(6-amino-9H-purin-9-yl) propan-2-yl] oxy} methyl) phosphoric acid. Molecular formula and molecular weight of Emtricitabine, Rilpivarine and TenofovirDisproxilFumerate were C8H10FN3O3S & C9H14N5O4P and 247.248 & 287.213 grams per mole respectively. The molecular structures of Emtricitabine, Rilpivarine and Tenofovir Disproxil Fumerate were presented. As the development of antiviral drugs for the treatment of viral infections has become a very active area, recently the combination of Emtricitabine (EMT) and Tenofovir Disoproxil Fumarate (TDF) has demonstrated significantly greater human immunodeficiency virus (HIV) ribonucleic acid (RNA) suppression compared to the combination of zidovudine and lamivudine. TDF is formulated in binary mixture with the reverse transcriptase inhibitor EMT namely Truvada tablets consisting 200 mg of EMT and 300 mg of TDF to prevent HIV from altering the genetic material of healthy cells. The estimation of Rilpivirine using UV-visible spectroscopy and HPLC has been reported. The study revealed that once daily regimen containing Emtricitabine, Tenofovir Disoproxil Fumarate and Rilpivirine is virologically and immunologically effective, well tolerated and safe with benefits in the lipid profile in the majority of patients. HPLC methods are useful in the determination of drugs in pharmaceutical formulations, especially those containing more than one active component.

Since spectrophotometric methods are lack of sensitivity, though LC/MS/MS technique is highly sensitive but costly and lot of care should be taken during analysis, therefore UPLC or HPLC methods have wide applications in the analysis of pharmaceutical analysis especially in quality assessment.

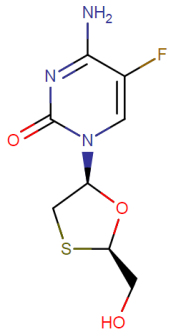

Figure.1.Molecular structure of Emtricitabine

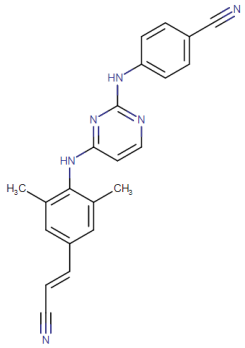

Figure.2.Molecular structure of Rilpivirine

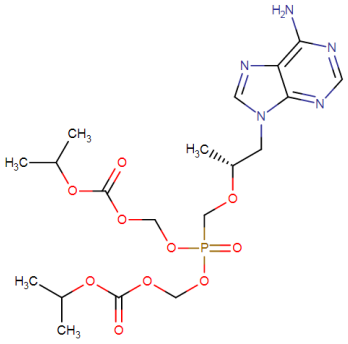

Figure.3.Molecular structure of Tenofovir

2. MATERIALS AND METHODS

Chemicals and Reagents: Active pharmaceutical ingredient (API) of 99.8% potency of Emtricitabine, Rilpivarine and Tenofovir Disproxil Fumerate were obtained from Chandra Labs, Hyderabad, Telangana, India. Pharmaceutical formulations like Complera tablets were procured from the local pharmacy. Analytical grade reagents such as acetonitrile, potassium dihydrogen orthophosphate, tetrahydrofuran, sodium hydroxide and HPLC grade water were procured from Merck India.

Instrumentation: Agilient 1200 series HPLC system equipped with auto sampler and photo diode array detector was used for the present investigation. The data acquisition was obtained from chemstation software.

Preparation of Solutions:

Mobile Phase: 0.68% Potassium dihydrogen orthophosphate buffer solution was prepared by taking 6.8 grams of potassium dihydrogen orthophosphate in a clean 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 sodium hydroxide solution. Then the resulting solu-tion 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. This was used as mobile phase and diluent.

Standard Stock Solution: Weigh accurately 13mg of Emtricitabine and 1.62mg of Rilpivirine and 20mg of Tenofovir in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase From abovestock solution 13µg/ml of Emtricitabine and 1.62µg/ml of Rilpivirine and 20µg/ml of Tenofovir is prepared by diluting 1ml to 10ml with mobile phase.

Sample Stock Solution: 5tablets (each tablet contains 200mg of Emtricitabine and 25mg of Rilpivirine and 300mg of Tenofovir) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Weight equivalent to 34.62mg of Emtricitabine, Rilpivirine and Tenofovir and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 100ml with mobile phase.

HPLC Method Development: High performance liquid chromatography (HPLC) is a novel technique used in the separation and assay of pharmaceutical formulations 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. 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 262 nm.

3. RESULTS AND DISCUSSION

A precise and accurate stability indicating RP - HPLC method was developed and validated for the determination of Emitricitabine, Rilpivarine and Tenofovir in pure and tablet dosage forms. The

separation of the components was achieved by using Agilent HPLC system equipped with auto sampler. The components were detected at 262 nm and separated by using a mobile phase of potassium dihydrogen orthophosphate buffer and acetonitrile in the ratio 40:60 v/v at a flow rate of 1.2 mL/min through Inertsil C18 (150 mm × 4.6, 5 µm) at ambient tem-perature.

System Suitable Parameters: Triplicate chromatograms of standard solution of concentration 60 µg/mL of EMT and 90 µg/mL of TDF were recorded. System suitable parameters such as plate count, tailing and resolution for EMT and TDF were found to be 2427 & 3685, 1.16 & 1.23 and 3.12 respectively. The chromatographic parameters like retention time, peak area and peak height of EMT and TDF were found to be 0.684 & 0.930, 694200 & 8778000 and 272881 & 3685 respectively.

Specificity: To determine specificity of the proposed method, number of peaks, tailing factor, number of theoretical plates, peak area and peak height of each peak, and resolution were determined. The chromatogram of sample was compared with the chromatogram of standard and found no additional peaks except three peaks at retention time 2.52, 3.27 and 6.65 minutes for Emitricitabine, Rilpivarine and Tenofovir respectively, whereas the blank chromatogram contains no peaks. The results of specificity were represented below as chromatograms.

Method Validation: Validation is a procedure having of documental evidence to demonstrate method is able or not to produce the expected results under the stated experimental conditions.

System Suitability Parameters: Exactly 5.3 mL of standard stock solution was accurately measured, transferred into a 10 mL volumetric flask and diluted up to the mark with diluents. The concentration of the resulting solution was found to be 13 µg/mL of Emtricitabine and 1.62 µg/mL of Rilpivirine and 20 µg/mL of Tenofovir respectively. Then precisely 20 µL of the this solution was injected into the col-umn in five times, 0.68% Potassium dihydrogen orthophosphate buffer solution and acetonitrile in the ration 40:60 v/v pH 6.0 were allowed to flow through the column at a rate of 1.2 mL per min from two separate channels, and the response of the instrument was recorded at 262 nm as a function of time for a run time of 7.0 min. A typical system suitable chromatogram was presented.

Precision: Precision describes the reproducibility of results under a set of experimental conditions. Exactly 1 mL of standard stock solution was accurately measured, transferred into a 10 mL volumetric flask and diluted up to the mark with diluents. The concentration of the resulting solution was found to be 13 µg/mL of Emtricitabine and 1.62 µg/mL of Rilpivirine and 20 µg/mL of Tenofovir respectively. Then precisely 20 µL of this solution was injected into the column six times into column, chromatograms were recorded under the optimized conditions and chromatographic parameters were evaluated. In the study of method precision, 5.3 mL of sample stock solution was accurately transferred into five separate 10 mL volumetric flasks and diluted up to the mark with diluents, exactly 20 µL of each of these solutions was injected into the column, chromatograms were recorded and chromatographic parameters were obtained under similar conditions.

Accuracy: Accuracy describes the correctness of an experimental result expressed as the closeness of the measurement to the true or accepted value. The study of accuracy was carried out at three different levels i.e. 50%, 100% and 150% with respect to target concentration by standard addition method in which known amounts of standards were added to pre-analyzed sample. An amount of tablet fine powder equivalent to 20 mg of EMT and 30 mg of TDP was taken in three different 100 mL volumetric flasks, 32/40/48 mg of Emtricitabine 4/5/6 mg of Rilpivarine & 40/50/60 mg of Tenofovir was prepared, sonicated for ten minutes, filtered through 0.45 µ membrane filter, then chromatograms were obtained in triplicate as per the procedure.

Linearity: The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample. The range of an analytical procedure is the interval between the upper and lower concentration of analyte for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. Weigh accurately 13mg of Emtricitabine and 1.62mg of Rilpivirine and 20mg of Tenofovir in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. This stock solution contains 13µg/ml of Emtricitabine and 1.62µg/ml of Rilpivirine and 20µg/ml of Tenofovir. This solution is used for recording chromatogram. To determine linearity, different aliquots of standard stock solution (0.03 – 0.56 mL) were taken a series of 10 mL standard flasks, made up to the mark, exactly 20 µL of each of these solutions was injected , and chromatograms were obtained under the identical chromatographic conditions. Linearity plots were drawn between mean peak area of drug Emtricitabine, Rilpivirine and Tenofovir.

Ruggedness: Ruggedness is a study of repeatability of results between two analysts, laboratories, different days and different instruments. In the present investigation the author made investigations to find the repeatability of the results between two different

analysts. Exactly 5.3 mL of sample stock solution was accurately transferred into a 10 mL volumetric flask and diluted up to the mark with diluents, then precisely 20 µL of the this solution was injected twice into column, chromatograms were recorded under the optimized conditions and chromatographic parameters were evaluated.

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. To demonstrate method specificity, exactly 20 µL of blank and sample solutions were injected separately into the column and chromatograms were recorded under the optimized chromatographic conditions.

Assay Studies: Standard and sample stock solutions of concentration 13µg/ml of Emtricitabine and 1.62µg/ml of Rilpivirine and 20µg/ml of Tenofovir was made by adding 5.3ml of stock solution to 10 ml of mobile phase freshly prepared as per the procedure given in section preparation of solutions. Exactly 5.3 mL of standard and sample.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The LOD of an individual analytical procedure is the lowest amount of components in a sample which can bedetected but not necessarily quantitated as an exact value. The LOQ is a parameter of quantitative assay for low levels of compounds in sample, and is used particularly for the determination of impurities and/or degradation products. The LOD for this method was found to be 22.19 µg/ml for Emtricitabine, 3.85 µg/ml for Rilpivirine and 17.23 µg/ml for Tenofovir.

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in pH of buffer, mobile phase composition, columns temperature and flow rate, and provides an indication of its reliability during normal usage. The study of robustness in the present investigation was demonstrated by carrying out deliberate variations in flow rate 1.2 ± 0.2 mL and wave length variation.Accurately 5.3 mL of sample stock solution was transferred into a 10 mL volumetric flask and diluted up to the mark with diluents, then exactly 20 µL of the this solution was injected twice into column, chromatograms were recorded under variable conditions. Solutions were accurately transferred into two separate 10 mL volumetric flasks, diluted up to the mark with diluents. Precisely 20 µL of each solution was injected in five times into column; chromatograms were obtained under the optimized chromatographic conditions.

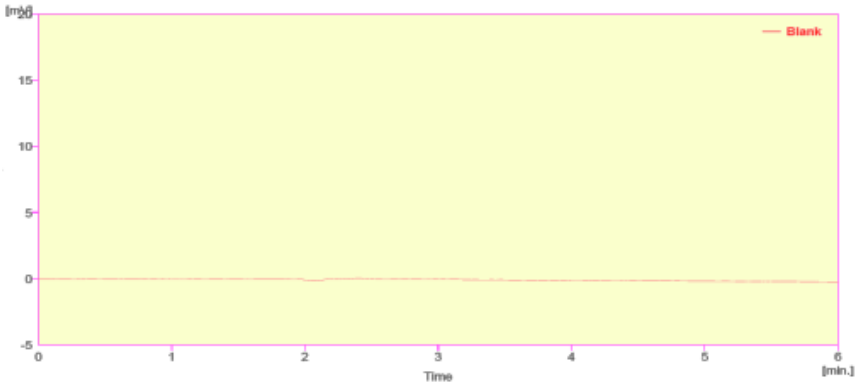

Figure.4.Blank chromatogram

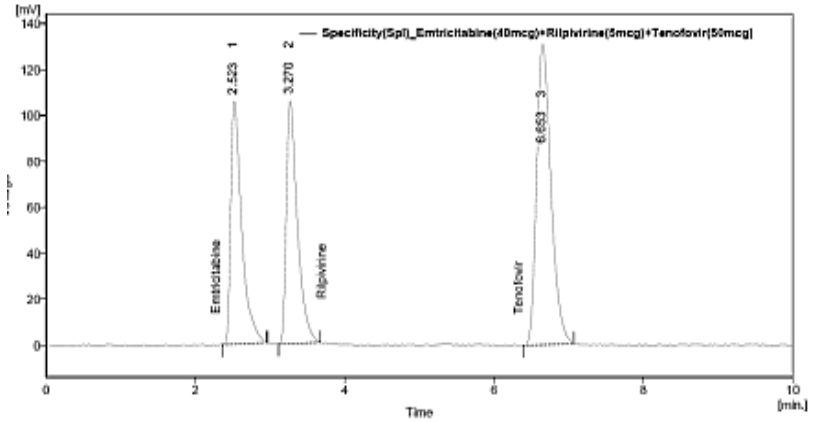

Figure.5.Chromatogram for specificity of Emtricitabine, Rilpivirine and Tenofovir sample

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 ≤ 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.1.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.2. 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		

Table.3.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.4.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		

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.

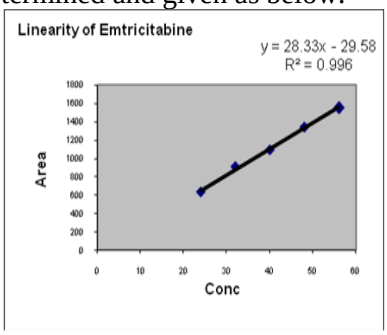

Figure.6.Linearity graph of Emtricitabine

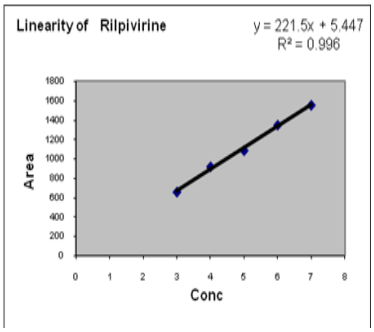

Figure.7.Linearity graph of Rilpivirine

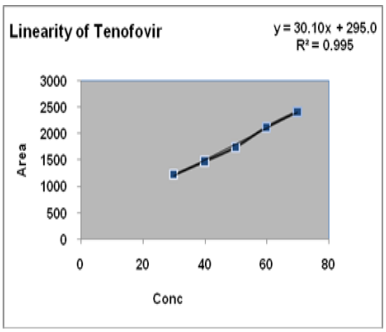

Figure.8.Linearity graph of Tenofovir

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.

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 reten-tion time, wavelength for these variations. In the present investigation ruggedness of the proposed method was demonstrated between different flow rates and different wavelengths.

Table.5.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.

Table.6.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

Analysis of Formulations: 5tablets (each tablet contains 200mg of Emtricitabine and 25mg of Rilpivirine and 300mg of Tenofovir) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Weight equivalent to 34.62mg of Emtricitabine,Rilpivirineand Tenofovir and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 100ml with mobile phase. Further dilutions are prepared in 5 replicates of 13µg/ml of Emtricitabine and 1.62µg/ml of Rilpivirine and 20µg/ml of Tenofovir was made by adding 5.3ml of stock solution to 10 ml of mobile phase. Peak area of both standard and test was determined. The percent of assay was calculated from the peak area of standard and sample, and then mean percent of assay was determined and found to be in good agreement with label claimed. The percent of assay was calculated by using the following formula.

$$\text{Assay} = \frac{\text{Response of test}}{\text{Response of standard}} \times \frac{\text{Weight of standard}}{\text{Dilutionof standard}} \times \frac{\text{Dilutionof test}}{\text{weight of test}} \times \frac{\text{Potency of API}}{100} \times \frac{\text{Average weight of formulation}}{\text{Label claim}} \times 100$$

The mean percent of assay of Emtricitabine, Rilpivirine and Tenofovir was found to be 100.04%, 99.74% and 102.41% respectively and the results were presented below.

Table.7. The mean percent of assay of Emtricitabine, Rilpivirine and Tenofovir

Emtricitabine Area			Rilpivirine Area		Tenofovir Area	
	Standard	Sample	Standard	Sample	Standard	Sample
Injection-1	1077.43	1077.35	1094.98	1073.39	1775.28	1739.8
Injection-2	1082.31	1087.42	1094.09	1095.61	1760.26	1735.75
Injection-3	1079.33	1068.45	1085.07	1069.42	1748.74	1741.1
Injection-4	1088.74	1084.87	1090.77	1105.55	1732.93	1762.02
Injection-5	1075.05	1079.33	1081.97	1092.08	1741.89	1746.09
Average	1080.57	1079.49	1089.37	1087.21	1751.82	1744.95
Assay(%purity)	100.04	1079.485	99.74	1087.21	102.41	1744.95

4. CONCLUSION

The present developed isocratic RP- UPLC method was found to be simple, rapid, accurate and specific for the determination of Emtricitabine, Rilpivirine and Tenofovir desoproxil fumerate in tablet dosages. Hence the proposed method can be adopted for the analysis for quality control in any quality control and testing laboratory.

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