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A NOVEL VALIDATED ANALYTICAL METHOD FOR SIMULTANEOUS ESTIMATION OF EMTRICITABINE, TENOFOVIR DISOPROXIL FUMARATE AND RILPIVIRINE – HYDROCHLORIDE BY RP-HPLC

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ABSTRACT

A simple, precise, accurate, efficient and reproducible, isocratic Reverse Phase- High Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride in bulk and tablet dosage form. Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride were separated using an Phenomenex Luna 3µ C8(2) 100A°, LC Column 150cm x 45mmand the mobile phase contained a mixture of 10mM Ammonium acetate (pH adjusted to 5.7 with 0.1% triethylamine), Acetonitrile and Methanol (30:55:15v/v/v). The flow rate was set to 0.8ml/min with the response measured at 250nm. The retention time of Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride was found to be 2.079min, 2.726min, 3.982min respectively with a resolution of 3.283, 5.675. Linearity was established for Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride in the range of 10-50µg/ml for Emtricitabine, 20-100µg/ml for Tenofovir disoproxil fumarate, 5-40µg/ml for Rilpivirine-hydrochloride with correlation coefficient of 0.9998, 0.9993 and 0.9994. The percentage recovery of Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-

hydrochloride was found to be 99.98%, 100.03%, 100.05% respectively. Validation parameters such as specificity, linearity, precision, accuracy, robustness, limit of detection (LOD), limit of quantification (LOQ) was evaluated for the method according to the International Conference on Harmonization (ICH) Q2 R1 guidelines.

Keywords: Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride RP-HPLC, ICH

INTRODUCTION

Emtricitabine (EMT) is a Nucleoside reverse transcriptase inhibitor (NRTI). It is chemically 4-amino-5-fluoro-1- [(2R, 5S)-2-(hydroxyl methyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one. It works by inhibiting reverse transcriptase, the enzyme that copies HIV RNA in to new viral DNA which can indirectly increase the number of immune system cells **[1-4]**. Tenofovir disoproxil fumarate (TDF) is also a Nucleoside reverse transcriptase inhibitor. It is chemically ({[(2R)-1-(6-amino-9H-purin-9yl) propan-2yl] oxy} methyl) phosphonic acid. It works by blocking reverse transcriptase, a crucial viral enzyme in HIV-1 and hepatitis B virus infections **[2-6]**. Rilpivirine hydrochloride (RPV) is a second-generation non-nucleoside reverse transcriptase inhibitor (NNRTI). It is chemically 4-{[4-({4-[(E)-2cyanovinyl]-2,6-dimethylphenyl} amino) pyrimidin-2-yl] benzonitrile**.** It is used for the treatment of HIV-1 infections **[1, 7, 8]**.

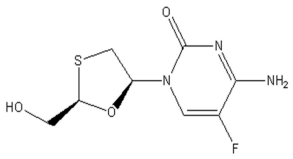

Figure: 1 Emtricitabine

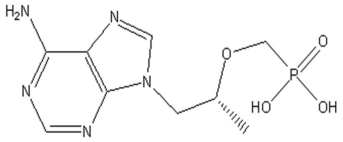

Figure 2: Tenofovir disoproxil fumarate

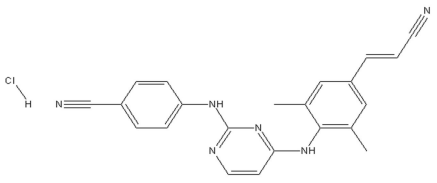

Figure 3: Rilpivirine-HCL

MATERIALS AND METHODS

Chemicals:

Emtricitabine, Tenofovir disoproxil fumarate, Rilpivirine-hydrochloride Pharmaceutical formulations were procured from Merck manufactures and Ammonium acetate, Acetonitrile, Methanol and Triethyl amine procured from National Scientific laboratories.

Instrumentation: Equipment used was manufactured by Shimadzu of 2030 LC Prominencei-series with high detection

capabilities of PDA detector and Autosampler was used for sampling. Also used pH meter manufactured by LABINDIA, Analytical balance manufactured by ESSAE.

Preparation of 10mM Ammonium acetate buffer: 10mM Ammonium acetate buffer was prepared by dissolving 0.77gm of ammonium acetate and 0.3ml of glacial acetic acid in 100ml of HPLC grade water and pH was adjusted to 5.7 with triethyl amine. The buffer was filtered through 0.45µm membrane filter to remove all fine particles and gases.

Mobile phase: The above prepared ammonium acetate buffer, Acetonitrile and Methanol of HPLC grade were used in the proportion of 30:55:15v/v/v as mobile phase.

Diluent 1: Acetonitrile and Methanol were used in the ratio of 80:20v/v.

Diluent 2: Acetonitrile, Methanol and 0.1% Ortho phosphoric acid was used as a diluents in the ratio of 50:25:25v/v/v.

Method development: Emtricitabine, Tenofovir disoproxil fumarate, Rilpivirine-hydrochloride was analyzed in an Phenomenex Luna 3µ C8(2) 100A°, LC Column 150 x 45 for the chromatographic separation. The mobile phase was composed of 10mM Ammonium acetate (pH adjusted to 5.7 with 0.1% triethylamine), Acetonitrile and Methanol (30:55:15, v/v/v).The buffer was filtered through 0.45µm membrane filter under vaccum filtration and pumped at ambient temperature, at a flow rate of 0.8ml/min with UV detection wavelength at 250nm. Injection volume was 20µl. The run time was 7 min and the retention time of Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride was found to be 2.079min, 2.726min, 3.982min respectively with a resolution of 3.283, 5.675

Preparation of standard stock solutions of Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride: Standard stock solutions of Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride were prepared by dissolving 100mg of Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride in 100ml of acetonitrile and methanol (80:20v/v) solution into a clean dry volumetric flask to get the concentration of 1000µg/ml of Emtricitabine,Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride. **Preparation of sample solution:** 20 tablets (each contains 300mg of Emtricitabine, 200mg of Tenofovir disoproxil fumarate and 600mg of Rilpivirine-hydrochloride) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Weight equivalent to one tablet powder of

Emtricitabine, Tenofovir disoproxil fumarate, Rilpivirine-hydrochloride was dissolved in sufficient mobile phase. After that filtered the solution using 0.45µm membrane filter under vacuum filtration and sonicated for 5min and dilute to 100ml with mobile phase.

RESULTS AND DISCUSSION

The proposed method was validated according to ICH guidelines.

System suitability test: System suitability test parameters were checked by repetitively injecting the drug solution at the concentration level of 30µg/ml, 20µg/ml, 60µg/ml for Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride to check the reproducibility of the system. System suitability parameters like number of theoretical plates (N), Tailing factor, Resolution of repetitive injections were studied. The theoretical plate count is above 2000, Tailing factor is less than 2.0 and Resolution is above 2.0. The results were shown in **Table 1**.

Linearity: A series of standards of EMT, TDF and Rilpivirine-hydrochloride were prepared over a range of 10-500µg/ml, 20-100µg/ml and 5-40µg/ml respectively from the stock solution and calibration graph was obtained by plotting peak area versus concentration of Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride. The chromatograms were shown in **Figures 4-6** and the results are given in **Table 2**.

Precision:

Method precision: Precision is the measure of closeness of the data values to each other for a number of measurements under the same analytical conditions. Precision of the test method was determined by six replicates (n=6) solutions were prepared and each solution was injected in duplicate under the same conditions and mean value of peak area response for each solution were considered. The results are given in **Table 3**.

Acceptance criteria: The % RSD for the peak area of six standard injections should not be more than 2.0%.

Intermediate precision/Ruggedness: The intermediate precision of the method was evaluated by performing precision on different lab by different analyst and different days. The standard preparation concentrations of 30µg/ml of Emtricitabine, 20µg/ml Tenofovir disoproxil fumarate, 60µg/ml of Rilpivirine hydrochloride was injected six times in to the HPLC and the %RSD for the area of 6 replicate injections was calculated. The results are given in **Table 4**.

Acceptance criteria: The % RSD for the peak area of six standard injections should not be more than 2.0%.

Accuracy: The accuracy of the method was determined by calculating recovery of Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride at 50%, 100%, 150% was added to a pre quantified sample solution and injected in to the HPLC system. The mean percentage recovery of Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride at each level was calculated and given in **Table 5, 6, 7**.

Acceptance criteria: The % Mean recovery should be within 99.00-102.00%

Assay: 20 tablets (each contains 300mg of Emtricitabine, 200mg of Tenofovir disoproxil fumarate and 600mg of Rilpivirine-hydrochloride) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Weight equivalent to one tablet powder of Emtricitabine, Tenofovir disoproxil fumarate, Rilpivirine-hydrochloride was dissolved in sufficient mobile phase. After that filtered the solution using 0.45µm membrane filter under vaccum filtration and sonicated for 5min and dilute to 100ml with 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. The percent of assay was calculated by using the following formula. The results are shown in the **Table 8**.

$$\% \text{ Assay} = \frac{\text{Samplearea}}{\text{Standardarea}} \text{x} \frac{\text{Standard dilution factor}}{\text{Standard weight}} \text{x} \frac{\text{Sample weight}}{\text{Sample dilution factor}} \text{x} \frac{\text{Average weight}}{\text{Labledclaim}} \text{x} \frac{\text{Potency}}{100} \text{x } 100$$

Acceptance criteria: The % Assay should be within 99.00-102.00%

LOD and LOQ: LOD and LOQ were calculated as 3.3XSD/S and 10XSD/S respectively as per ICH guidelines, Where SD is the standard deviation of the response (Y-intercept) and S is the slope of the calibration curve. The LOD is the smallest concentration of analyte that gives a measurable response (Signal to noise ratio of 3). The LOD of Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride was found to be 1µg/ml, 0.5µg/ml and 4µg/ml. The LOQ is the smallest concentration of analyte that gives a response that can be accurately quantified (Signalto noise ratio of 10). The LOQ of Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride was found to be 3µg/ml, 1.5µg/ml, 12µg/ml.

Robustness: As part of robustness, deliberate change in the flow rate and the mobile phase proportion was made to evaluate the impact on the method. The theoretical plates for Emtricitabine, Tenofovir disoproxil fumarate and

Rilpivirine-hydrochloride were found to be 2982, 2786, 4424 respectively. The tailing factor for Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride were found to be 1.561, 1.544, 1.499. The results reveal that the method is robust. The results were given in **Table 9, 10**.

Acceptance criteria: The % RSD for the peak area by changing flow rate and mobile phase proportion should not be more than 2.0%.

Table 1: System suitability test

Parameter	Emtricitabine	Tenofovir disoproxil fumarate	Rilpivirine-hydrochloride
Retention time	2.075	2.721	3.981
Theoretical plates	2982	2786	4424
Tailing factor	1.561	1.544	1.499
Resolution	NA	3.283	5.675

Table 2: Linearity

Linearity of Emtricitabine		Linearity of Tenofovir disoproxil fumarate		Linearity of Rilpivirine-hydochloride	
Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area
10	281456	20	111050	5	81050
20	548967	40	212742	10	150742
30	805898	60	329255	20	290255
40	1098401	80	441912	30	421912
50	1371532	100	535142	40	558142

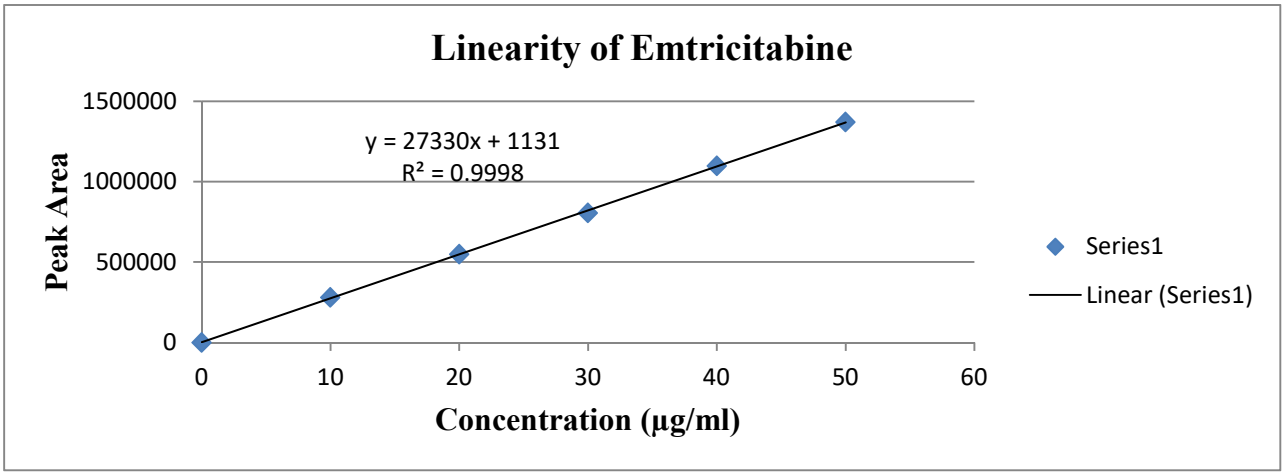

Figure: 4 Linearity of Emtricitabine

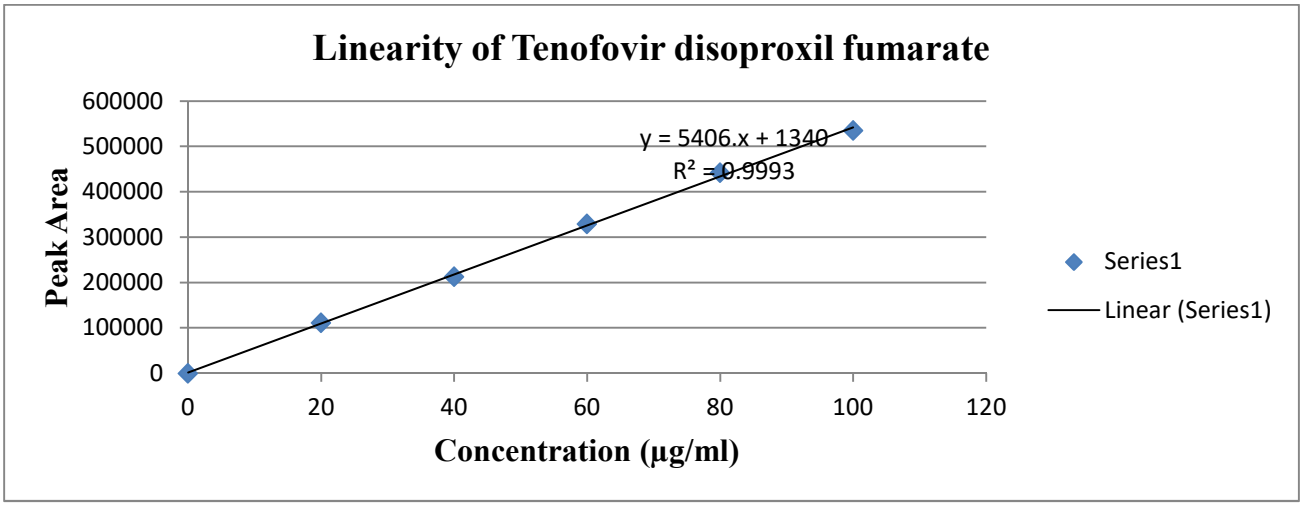

Figure 5: Linearity of Tenofovir disoproxil fumarate

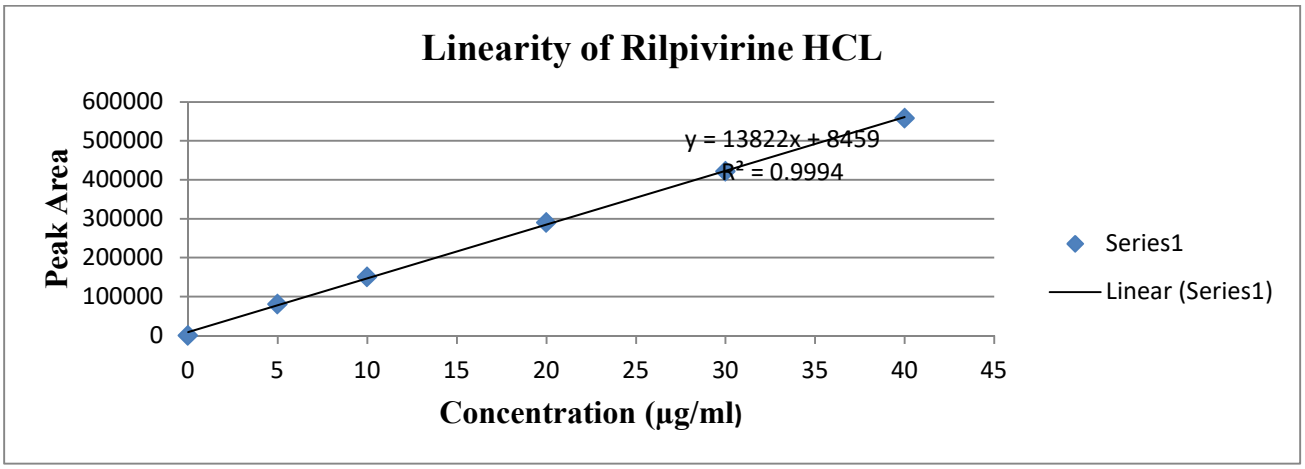

Figure 6: Linearity of Rilpivirine hydrochloride

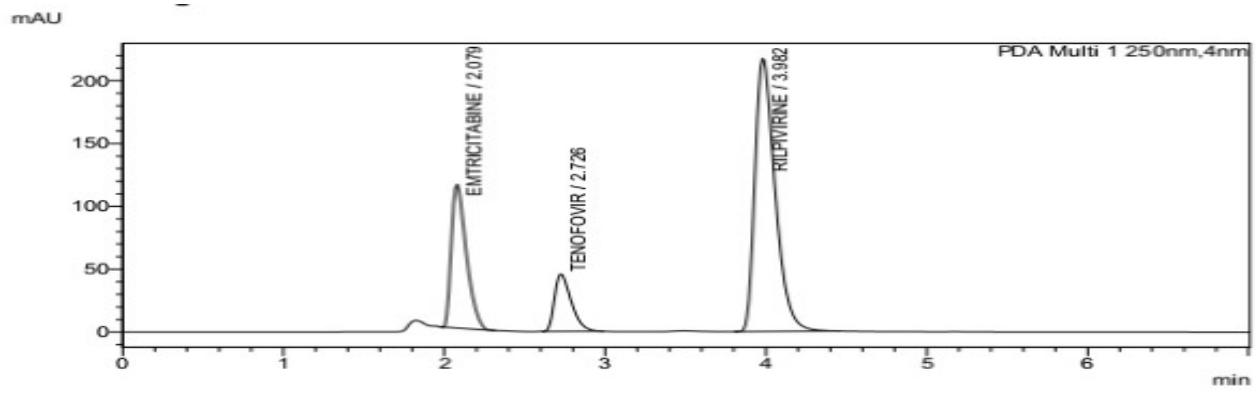

Figure 7: Standard chromatogram

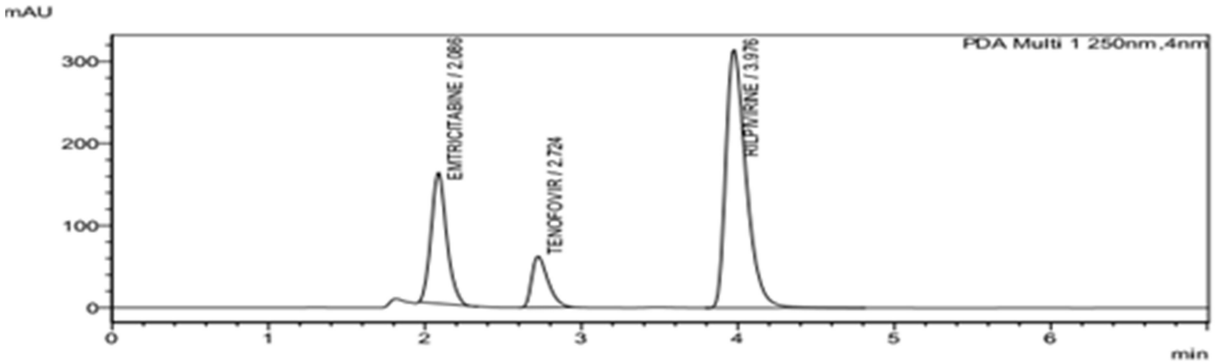

Figure 8: Sample chromatogram

Table 3: Method precision

	Emtricitabine		Tenofovir disoproxil fumarate		Rilpivirine-hydrochloride	
S.NO	Rt	Area	Rt	Area	Rt	Area
1	2.079	703729	2.724	326372	3.990	1899658
2	2.078	704040	2.724	324557	3.987	1865651
3	2.078	705511	2.723	322803	3.984	1874364
4	2.078	706667	2.724	322009	3.984	1884136
5	2.069	707706	2.715	328080	3.972	1898191
6	2.070	709935	2.716	322240	3.974	1898294
AVG	2.075	706265	2.721	324344	3.981	1886716
SDV	0.0045	2351.91	0.00429	2464.531	0.007223	14395.72
%RSD	0.219	0.333007	0.158	0.759852	0.181	0.763004

Table 4: Intermediate precision

	Emtricitabine		Tenofovir disoproxil fumarate		Rilpivirine-hydrochloride	
S.NO	Rt	Area	Rt	Area	Rt	Area
1	2.079	702458	2.724	326562	3.990	1866758
2	2.078	706589	2.724	324357	3.987	1845561
3	2.078	703256	2.723	327083	3.984	1832634
4	2.078	704598	2.724	325609	3.984	1824216
5	2.069	705698	2.715	321180	3.972	1845111
6	2.070	708429	2.716	325140	3.974	1838724
AVG	2.075	705171	2.721	324989	3.981	1842167
SDV	0.0045	2202.677	0.00429	2105.909	0.007223	14490.51
%RSD	0.219	0.312361	0.158	0.647995	0.181	0.786601

Table: 5 Accuracy of Emtricitabine

Recovery level	Accuracy of Emtricitabine				Average percent recovery
	Standard area	Sample Peak Area	%Recovery	%Mean recovery	
50	706198	356894	101.07	100.42	99.98
	706198	352487	99.83		
	706198	354457	100.36		
100	706198	705849	99.96	99.92	
	706198	704568	99.77		
	706198	706458	100.04		
150	706198	1052021	99.32	99.62	
	706198	1057891	99.87		
	706198	1055894	99.69		

Table: 6 Accuracy of Tenofovir disoproxil fumarate

Recovery level	Accuracy of Tenofovir disoproxil fumarate				Average percent recovery
	Standard area	Sample Peak Area	% Recovery	%Mean recovery	
50	322940	160258	99.24	99.91	100.03
	322940	161254	99.87		
	322940	162548	100.64		
100	322940	321548	99.58	99.99	
	322940	322584	99.89		
	322940	324567	100.51		
150	322940	485464	100.23	100.2	
	322940	486521	100.44		
	322940	483987	99.93		

Table: 7 Accuracy of Rilpivirine hydrochloride

Recovery level	Accuracy of Rilpivirine hydrochloride				Average percent recovery
	Standard area	Sample Peak Area	%Recovery	%Mean recovery	
50	1887017	945215	100.17	100.11	100.05
	1887017	944968	100.16		
	1887017	943965	100.02		
100	1887017	1882541	99.77	99.81	
	1887017	1883571	99.82		
	1887017	1884215	99.86		
150	1887017	2854121	100.84	100.24	
	1887017	2832546	100.08		
	1887017	2824587	99.81		

Table 8: Assay

Drug name	Labelled claim (mg)	%Assay
Emtricitabine	300	99.68
Tenofovir disoproxil fumarate	200	100.84
Rilpivirine-hydrochloride	600	99.85

Table 9: Robustness Change in flow rate

Drug	Change in flow (ml/min)	Retention time	Robustness		
			Average peak area	SDV	%RSD
Emtricitabine	0.6	2.747	939191	9821.713	1.05
	1	1.680	584091	1195.01	0.20
Tenofovir disoproxil fumarate	0.6	3.592	425960.5	713.4707	0.17
	1	2.201	266631	1569.777	0.59
Rilpivirine-hydrochloride	0.6	5.234	2424583	7000.357	0.29
	1	3.214	1521606	7281.079	0.48

Table 10: Robustness Change in mobile phase

Drug	Change in mobile phase	Retention time	Robustness		
			Average peak area	SDV	%RSD
Emtricitabine	35:50:15v/v	2.089	753790	777.8175	0.10
	25:60:15v/v	2.089	705105	1131.371	0.16
Tenofovir disoproxil fumarate	35:50:15v/v	2.891	334783	869.7413	0.26
	25:60:15v/v	2.775	323122	719.8347	0.22
Rilpivirine-hydrochloride	35:50:15v/v	4.828	1895022	933.381	0.05
	25:60:15v/v	3.694	1872246	8061.017	0.43

CONCLUSION

The present developed isocratic RP-HPLC method was found to be specific, simple, accurate and rapid for the determination of Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloridein bulk and tablet dosage form. A simple isocratic mode of development was done and there were no interactions with standard and sample with mobile phase. Retention times of the Emtricitabine, Tenofovir disoproxil fumarate and Rilpivirine-hydrochloride were 2.079min, 2.726min, 3.982 min. Accuracy was achieved for the three drugs with 99.98%, 100.03% and 100.05% and correlation coefficient of 0.9998, 0.9993 and 0.9994. Hence the proposed method can be adopted for the routine analysis for quality control in any quality control and testing laboratory.

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