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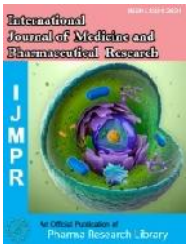

R E S E A R C H A R T I C L E

Analytical Method Validation for Essay of Emitricitabine, Rilpevirene and Tenofovir Alfanamide by RP-HPLC

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A B S T R A C T

A new, simple, precise, accurate and reproducible RP-HPLC method for Simultaneous estimation of Emtricitabine and Tenofovir and Rilpivirine in bulk and pharmaceutical formulations. Separation of Emtricitabine and Tenofovir and Rilpivirine was successfully achieved on a Inertsil ODS C18 (4.6 x 250mm, 5µm) utilizing Phosphate buffer pH 3: Acetonitrile (30:70) at a flow rate of 1.0 mL/min and elute was monitored at 280 nm, with a retention time of 4.4945 and 7.971 and10.513 minutes for Emtricitabine and Tenofovir and Rilpivirine. The assay of Emtricitabine, Tenofovir and Rilpivirine was performed with tablets and the % assay was found to be 99.69 and 100.31 and 99.50 which shows that the method is useful for routine analysis. The linearity of Emtricitabine, Tenofovir and Rilpivirine was found to be linear with a correlation coefficient of 0.999 and 0.999 and 0.999 which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.2 and 0.2 and 0.1 for Emtricitabine, Tenofovir and Rilpivirine which shows that the method is precise. The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.1 and 0.3 and 0.3 for Emtricitabine, Tenofovir and Rilpivirine which shows that the method is repeatable when performed in different days also. The accuracy limit is the percentage recovery should be in the range of 97.0% - 103.0%. The acceptance criteria for LOD and LOQ is 3 and 10.The LOD and LOQ for Emtricitabine was found to be 2.98 and 9.98and LOD and LOQ for Tenofovir was found to be 3.00 and 10.00 and LOD and LOQ for Rilpivirine was found to be 2.94 and 9.97.

Keywords: Emtricitabine, Tenofovir, Rilpivirine, HPLC.

A R T I C L E I N F O

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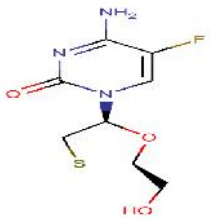
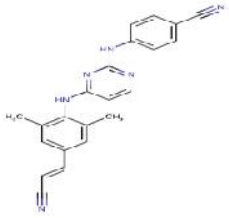
1. Introduction

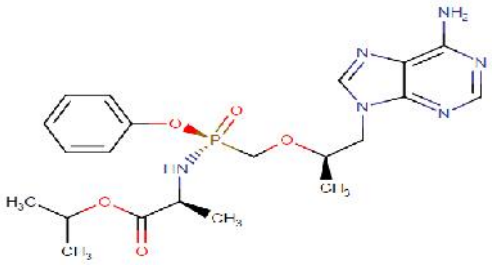
Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are developed to reduce the cost and time for better precision and ruggedness [1]. Trial runs are conducted, method is optimized and validated. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available [2].



Emtricitabin



Rilpivirine



Tenofovir

Description of the Various Analytical Methods

Titrimetric and gravimetric method of analysis is suitable when the sample is present in pure form or when no interference is observed in the mixture with other materials [3]. Ultraviolet and visible spectrometric method is suitable when no Interference is observed in the mixture [4]. HPLC and GC methods are more advantageous than the above due to their capability in separating organic mixtures and quantitative estimations. AAS is used mainly for quantitative estimation in ppm and ppb levels of elements [5]. Infra-red spectroscopy though mainly used for qualitative analysis can be used for quantitative estimation also. Out of all the above methods, thin layer chromatography plays a very important role in analysis due to its adaptability, flexibility, and cost and time. It can be used both for qualitative and quantitative determination. After separation spots can be scanned with the help of a scanner and quantitative measurement can be made [6].

Chromatography:

Chromatography is a technique used in analytical chemistry to separate and identify components of mixtures. The name comes from the Greek term for "color writing" because this method was originally used to separate colored samples. The advent of high-performance liquid chromatography (HPLC).in this system pressure is applied to the column, forcing the mobile phase through at much higher rate[7]. The pressure is applied using a pumping system. The action of the pump is critical, since it must not pulsate and mix up the sample being separated in the solvent, causing it to lose resolution [8]. Development of pumps has proceeded quite quickly over the last several years, and now it is possible to achieve good resolution under the conditions required for HPLC [9].

2. Materials and Method

Apparatus: WATERS HPLC, 2695 separation module, Photo diode array detector (PDA), with an automated sample injector. The output signal was monitored and integrated using Empower 2 software [10].

Chemicals:

KH2PO4, Methanol, Water, Orthophosphoric acid, Emtricitabine and Tenofovir and Ralpiverine Preparation of 0.025M Phosphate buffer: 3.4g of potassium dihydrogen ortho phosphate was weighed and taken in a 1000ml volumetric flask and adjust the PH with Diluted NaOH up to 3, finally the solution was filtered by using 0.45 Micron membrane filter, sonicate it for 10 mins [11].

Preparation of mobile phase:

Accurately measured 300 ml (30%) of above buffer and 700 ml of Acetonitrile HPLC (70%) were mixed and degassed in an ultrasonic water bath for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration [12]. Optimization Chromatographic trials for Simultaneous Estimation of Emtricitabine and Tenofovir and Rilpivirine by RP- HPLC.

Optimization chromatographic conditions

Mobile phase: Phosphate buffer pH 3: Acetonitrile (30:70)

Flow Rate: 1.0ml/min

Column Temperature: Ambient

Column: Inertsil ODS C18 (4.6 x 250mm, 5 m)

Injection Volume : 20µl

Detection wave length: 280 nm

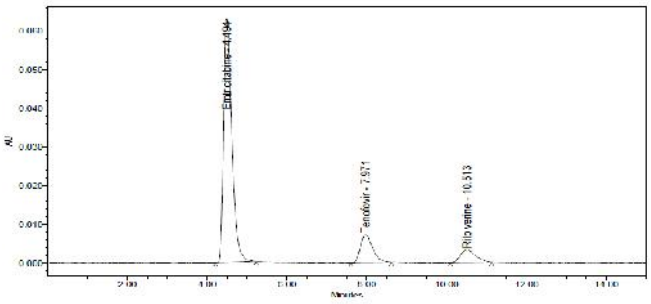


Figure 1: Optimization Chromatogram

Observation: Resolution between three analytes was good. No peak asymmetry was observed. No other impurity interference was seen. All the results were found to be within the acceptance criteria. Hence the method was considered to be optimized.

Method Validation Parameters

Preparation of the emtricitabine, tenofovir and rilpivirine standard & sample solution:

Standard Solution Preparation: Accurately weigh and transfer 100mg of Emtricitabine, 12.5mg of Tenofovir and 12.5mg of Rilpivirine working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and

sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [13].

Sample Solution Preparation:

Accurately weigh 10 tablets crush in mortor and pestle and transfer equivalent to 100mg of Emtricitabine, 12.5mg of Tenofovir and 12.5mg of Rilpivirine in (marketed formulation=126.53 mg of tablet Powder) sample into a 10mL clean dry volumetric flask add about 7 mL of Diluent and sonicate it up to 30 mins to dissolve it completely and make volume up to the mark with the same solvent. Then it is Filtered through 0.45 micron Injection filter. (Stock solution). Further pipette 0.3 ml of Emtricitabine, Tenofovir and Rilpivirine from the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure:

Inject 20 µL of the standard, sample into the chromatographic system and measure the areas for Emtricitabine, Tenofovir and Rilpivirine peaks and calculate the %Assay by using the formulae.

Linearity:

Preparation of stock solution: Accurately weigh and transfer 100mg of Emtricitabine, 12.5mg of Tenofovir and 12.5mg of Rilpivirine working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent [14]. (Stock solution)

Preparation of Level – I: 0.1 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – II: 0.2 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – III: 0.3 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent.

Preparation of Level – IV: 0.4 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent

Preparation of Level – V: 0.5 ml of above stock solutions has taken in different 10ml of volumetric flasks, dilute up to the mark with diluent

Procedure: Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Accuracy:

Preparation of Standard stock solution: Accurately weigh and transfer 100mg of Emtricitabine, 12.5mg of Tenofovir and 12.5mg of Rilpivirine working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [15].

Preparation Sample solutions: For preparation of 50% solution (With respect to target Assay concentration):

Accurately weigh and transfer 500mg of Emtricitabine, 6.25.5mg of Tenofovir and 6.25mg of Rilpivirine working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

For preparation of 100% solution (With respect to target Assay concentration):

Accurately weigh and transfer 100mg of Emtricitabine, 12.5mg of Tenofovir and 12.5mg of Rilpivirine working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [16].

For preparation of 150% solution (With respect to target Assay concentration):

Accurately weigh and transfer 150mg of Emtricitabine, 18.75mg of Tenofovir and 18.75mg of Rilpivirine working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure: Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Emtricitabine, Tenofovir and Rilpivirine and calculate the individual recovery and mean recovery values [17].

Precision:

Preparation of stock solution:

Accurately weigh and transfer 100mg of Emtricitabine, 12.5mg of Tenofovir and 12.5mg of Rilpivirine working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents [18].

Procedure:

The standard solution was injected for six times and measured the area for all six. Injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Intermediate precision/Ruggedness:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day.

Preparation of stock solution:

Accurately weigh and transfer 100mg of Emtricitabine, 12.5mg of Tenofovir and 12.5mg of Rilpivirine working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock

solutions into a 10ml volumetric flask and dilute up to the mark with diluents [19].

Procedure: The standard solutions prepared in the precision was injected on the other day, for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Robustness:

As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

A. The flow rate was varied at 0.9 ml/min to 1.1ml/min. Standard solution 300ppm of Emtricitabine, 37.5ppm of Tenofovir & 37.5ppm of Rilpivirine was prepared and analyzed using the varied flow rates along with method flow rate [20].

Limit of detection: (for Rilpivirine)

Preparation of 37.5 µg/ml solution:

Accurately weigh and transfer 12.5mg of Rilpivirine working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.14 µg/ml solution: Further pipette 1.0ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Further pipette 0.36ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents [21].

Limit of quantification:

Preparation of 37.5 µg/ml solution: Accurately weigh and transfer 12.5mg of Rilpivirine working standard into a 10 ml clean dry volumetric flask add about 7 mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Further pipette 0.3 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent.

Preparation of 0.49µg/ml solution:

Further pipette 1.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents. Further pipette 1.0ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure for LOD and LOQ:

The LOD and LOQ solutions was prepared injected, for three times and measured the area for all three injections in HPLC. The %RSD for the area of three replicate injections was found to be within the specified limits.

3. Results and Discussion

Method Validation Parameters

Assay:

Standard and sample solution injected as described under experimental work. The corresponding chromatograms and results are shown below.

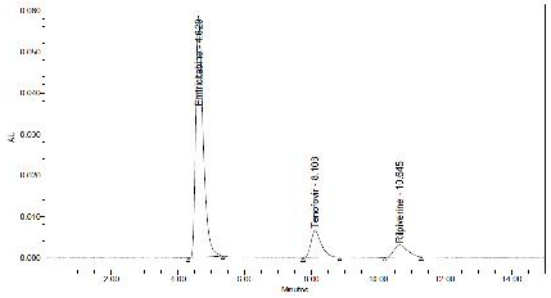


Figure 2a: Chromatogram for Standard

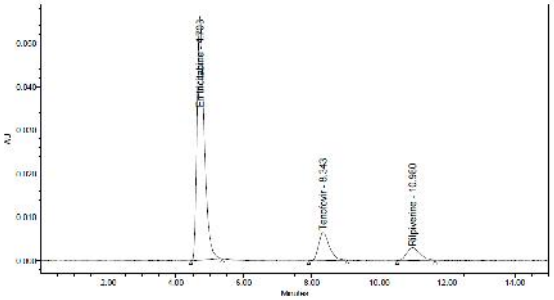


Figure 2b: Chromatogram for Sample

Table 1: Results of Assay for Emtricitabine and Tenofovir and Rilpivirine

	Label Claim (mg)	% Assay
Emtricitabine	200	99.69
Tenofovir	25	100.31
Ralpevirine	25	99.50

Linearity:

The linearity study was performed for the concentration of 100 ppm to 500ppm for Emtricitabine and 12.5 ppm to 62.5ppm for Rilpivirin and12.5 ppm to 62.5ppm for Tenofovir and chromatograms are shown below.

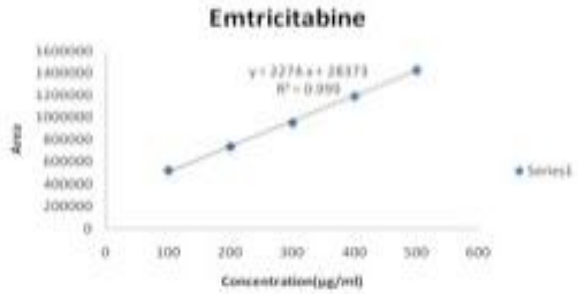


Figure 3a: Calibration graph of Emtricitabine

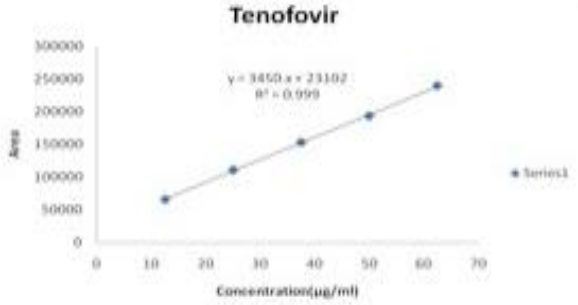


Figure 3b: Calibration graph of Tenofovir

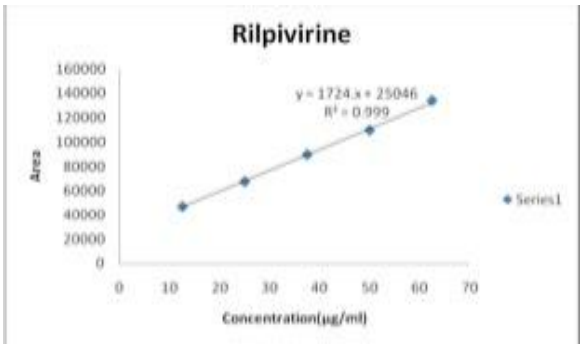


Figure 3c: Calibration graph of Rilpivirine

Robustness: The standard and samples of Emtricitabine and Tenofovir were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Table 2: Linearity Results: (for Emtricitabine)

S. No	Linearity Level	Concentration	Area
1	I	100	522088
2	II	200	734633
3	III	300	950658
4	IV	400	1192066
5	V	500	1430452
Correlation Coefficient			0.999

Table 3: Linearity Results: (for Rilpivirine)

S. No	Linearity Level	Concentration	Area
1	I	12.5	47257
2	II	25	67723
3	III	37.5	89884
4	IV	50	109712
5	V	62.5	134068
Correlation Coefficient			0.999

Table 4: Linearity Results: (for Tenofovir)

S. No	Linearity Level	Concentration	Area
1	I	12.5	65477
2	II	25	110790
3	III	37.5	153097
4	IV	50	193120
5	V	62.5	239955
Correlation Coefficient			0.999

Table 5: Accuracy (recovery) data for Emtricitabine

% Concentration (at specification Level)	50%	100%	150%
Area	476290	957024	1448027
Amount Added (mg)	50	100	150
Amount Found (mg)	49.69	99.85	151.07
% Recovery	99.38	99.85	100.72
Mean Recovery	99.98		

Table 6: Accuracy (recovery) data for Tenofovir

% Concentration (at specification Level)	50%	100%	150%
Area	77719	154381	231466
Amount Added (mg)	6.25	12.5	18.75
Amount Found (mg)	6.27	12.45	18.66
% Recovery	100.25	99.57	99.52
Mean Recovery	99.99		

Table 7: Accuracy (recovery) data for Rilpivirine

% Concentration (at specification Level)	50%	100%	150%
Area	45290	8972	134564
Amount Added (mg)	6.25	12.5	18.75
Amount Found (mg)	6.29	12.46	18.69
% Recovery	100.63	99.67	99.66
Mean Recovery	99.99		

Table 8: Results of Precision for Emtricitabine, Tenofovir and Rilpivirine

Injection	Emtricitabine	Tenofovir	Rilpivirine
Injection-1	957498.0	158363.0	89485.0
Injection-2	958373.0	158376.0	89474.0
Injection-3	958377.0	158237.0	89648.0
Injection-4	958374.0	158373.0	89467.0
Injection-5	959484.0	158932.0	89364.0
Injection-6	954484.0	158383.0	89464.0
Average	957765.0	158444.0	89483.7
Standard Deviation	1726.6	245.3	91.7
%RSD	0.2	0.2	0.1

Table 9: Results of Intermediate precision for Emtricitabine, Tenofovir and Rilpivirine

Injection	Emtricitabine	Tenofovir	Rilpivirine
Injection-1	959473.0	158387.0	87983.0
Injection-2	958474.0	158327.0	87838.0
Injection-3	958373.0	158363.0	87537.0
Injection-4	958363.0	158736.0	87538.0
Injection-5	959373.0	158373.0	87373.0

Injection-6	958363.0	157368.0	87293.0
Average	958736.5	158259.0	87593.7
Standard Deviation	534.3	461.8	267.1
%RSD	0.1	0.3	0.3

Table 10: System suitability results for Emtricitabine

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	2263.65	1.40
2	1.0	2112	1.45
3	1.1	2151.29	1.44

Table 11: System suitability results for Tenofovir

S. No	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	0.9	3331.30	1.29	7.38
2	1.0	3186.09	1.33	7.31
3	1.1	2971.64	1.41	7.11

Table 12: System suitability results for Rilpivirine

S. No	Flow Rate (ml/min)	System Suitability Results		
		USP Plate Count	USP Tailing	USP Resolution
1	0.9	3035.38	1.40	3.87
2	1.0	3353.63	1.27	3.90
3	1.1	3465.98	1.41	3.89

Table 13: Results of LOD for Emtricitabine and Tenofovir and Rilpivirine

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
Emtricitabine	66	197	2.98
Tenofovir	66	198	3.00
Rilpivirine	66	194	2.94

Table 14: LOQ Results for Emtricitabine and Tenofovir and Rilpivirine

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio
Emtricitabine	66	659	9.98
Tenofovir	66	660	10
Rilpivirine	66	658	9.97

4. Conclusion

The estimation of Emtricitabine, Tenofovir and Rilpivirine was done by RP-HPLC. The assay of Emtricitabine, Tenofovir and Rilpivirine was performed with tablets and the % assay was found to be 99.69 and 100.31 and 99.50 which shows that the method is useful for routine analysis. The linearity of Emtricitabine, Tenofovir and Rilpivirine was found to be linear with a correlation coefficient of 0.999 and 0.999 and 0.999 which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.2 and 0.2 and 0.1 for Emtricitabine, Tenofovir and Rilpivirine which shows that the method is precise. The acceptance criteria of intermediate precision is RSD should be not more than 2.0% and the method show precision 0.1 and 0.3 and 0.3 for Emtricitabine, Tenofovir and Rilpivirine which shows that the method is repeatable when performed in different days also. The accuracy limit is the percentage recovery should be in the range of 97.0% - 103.0%. The total recovery was found to be 99.98% and 99.78% and 99.97 for Emtricitabine, Tenofovir and Rilpivirine. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility. The acceptance criteria for LOD and LOQ is 3 and 10.The LOD and LOQ for Emtricitabine was found to be 2.98 and 9.98and LOD and LOQ for Tenofovir was found to be 3.00 and 10.00 and LOD and LOQ for Rilpivirine was found to be 2.94 and 9.97. The robustness limit for mobile phase variation and flow rate variation are well within the limit, which shows that the method is having good system suitability and precision under given set of condition.

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