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Development and Validation of Stability-Indicating HPLC Method for Simultaneous Determination of Emtricitabine and Tenofovir Disoproxil Fumarate in Bulk and their Tablet Dosage Form

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Abstract:
A reversed-phase high performance liquid chromatographic method is simple, precise, reliable, rapid and reproduciblemethod, developed and validated for the simultaneous estimation of Emtricitabine and Tenofovir disoproxil fumarate. Chromatography is carried out isocratically on Intersil C18 (33mm X 4.6 mm) column with a mobile phase composed of acetonitrile: phosphate buffer (80:20 v/v) at a flow rate of 2.0ml/min. Detected with the help of UV detector at wavelength 260 nm.linearity, precision, accuracy, specificity and ruggedness are the parameters analysed as per the ICH Q2(R1) guidelines. The retention times of Emtricitabine and Tenofovir are 2.1 min and 14.3 min respectively. The l % recoveries of Emtricitabine and Tenofovir are found to be 99.5%, 99.8% respectively. The method developed was found to be accurate, precise, selective and rapid for simultaneous estimation of Emtricitabine and Tenofovir in pharmaceutical dosage forms.

Key words—Reverse phase HPLC, EMITRICITABINE, TENOFOVIR DISOPROXIL FUMERATE, ISOCRATIC, INTERSIL C18, acetonitrile, phosphate buffer.

I. INTRODUCTION:

Emtricitabine: Chemically 4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2one [1]. And with molecular formula of C8H10N3O3S and a molecular weight- 247.24 g/mol. Emtricitabine is a synthetic nucleotide analogue of cytidine. It has solubility in 112mg/ml of distilled water at 25 □ and it is freely soluble in various aqueous solutions, slightly soluble in acetonitrile and very slightly soluble in isopropyl acetate,soluble in methanol. Emtricitabine isUsed in the treatment of HIV.**Emtricitabine** is an analogue of cytidine. The mechanism of action of the drug was by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA.
Side effects likediarrhea, headache, trouble sleeping ,Darkening skin colour on palms of hand sand soles of feet may occur.

TenofovirDisoproxil:Chemically it is 9-[(R)-2-[[(isopropoxcarbonyl)-oxy] methoxy] phosphinyl] methoxy] propyl] adenine fumarate [4e7]. It has molecular formula C9H14N5O4P and a molecular weight 635.52g/mol. It has Solubility in 13.4mg/ml of distilled water at 25°C, it is freely soluble in dimethylformamide,soluble in methanol, acid hydrochloride (0.1N HCl), ethanol, sparingly soluble in acetone,isopropanol, water, slightly soluble in acetonitrile, ethylacetate, insoluble in dichloromethane, hexane.It is also used for the treatment of HIV.**Tenofovir disoproxil Fumarate** :Tenofovir disoproxil Fumarate exhibits activity against HIV-reverse transcriptase.
Sideeffects includes Dizziness, diarrhoea ,headache ,trouble sleeping .

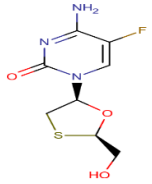


Fig no:1Emtricitabine

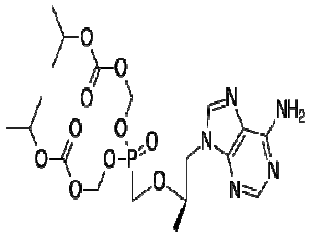


Fig no:2 Tenofovir Disoproxil

The literature survey reveals that there was only one HPLC [3] method was developed in combination of Emtricitabine and Tenofovir disproxil in bulk and dosage forms. There were no reported analytical methods for simultaneous estimation Emtricitabine and Tenofovir disproxil in bulk and their combined dosage forms in presence of their

degradation products. Hence it is an attempt to develop stability indicating specific, sensitive, accurate and precise RP-HPLC method for simultaneous estimation of these drugs. This method was validated as per the guidelines of ICH Q2.

II. MATERIALS AND METHODS:

Instruments used:

s.no	Name of instrument	Make	Model	Software
1	HPLC	AGILENT	UV Detector	Empower3
2	Weighing Balance	SARTORIUS	BSA224S-CW	N/A
3	P^H meter	LABINDIA	AD102U	N/A
4	Sonicator,Centrifuge	ENERTECH	SE6OUS	N/A

Table no :1 Instruments used in the method

Chemicals used:

S.No.	Chemicals/Reagents	Make/Grade
1	Methanol	Merck (HPLC-Grade)
2	Acetonitrile	Merck (HPLC-Grade)
4	Potassium dihydrogen Orth phosphate	Merck (GR-Grade)
5	O-Phosphoric acid	Merck (GR-Grade)

Table no:2 Chemicals used in the method

Working reference standards used:

S.No	Name	Batch number	%Potency (As is basis)
1	Emtricitabine	VJR(681)187	99.2%
2	Tenofovir Disoproxil fumarate	KSC(708)164	98.9%
3	placebo	AS(895)166P	99.1%
4	Tablets	AS(895)	98.6%

Table no:3 Standards used for the reference

Selection of Buffer and Mobile phase:

Initially development was started with a buffer which was used for Emtricitabine, Tenofovir Disoproxil Fumarate method (0.01M KH2PO4+1g-1-Octane Sulfonic Acid pH3.0). But to separate impuritiesfrom analytes buffer 0.01M KH2PO4+1g-1-Octane Sulfonic Acid pH3.0 with Acetonitrile andMethanol with different gradient programmers were tried. Out of this Buffer pH 3.0 with Acetonitrile: Methanol (80:20) was finalized Based on peak parameters like resolution power, retention time, good peak shape, peak tailing and no blank interference.

Selection of HPLC column:

Various HPLC columns polar packing material like Hypersil BDS C18 (100 x 4.6mm, 5 µm), Hypersil BDS C18 (250 x 4.6mm, 5 µm), µ Bond pack column (300x4.6mm, 10 µm)used for this study. Out of these Inertsil C8 33*4.6mm, 3µm was finalized based on peak parameters like retention time, peak shape, resolution power and peak tailing.

OPTIMIZED HPLC METHOD

Preparation of Buffer:

Transfer about 2.72g of Potassium dihydrogen Phosphate into a beaker containing 1000 mLof Milli-Q water Adjust pH of the solution to 4.0±0.05 with diluted Ortho phosphoric acid solution. Filter the solution through 0.45µm membrane filter.

Transfer about 3.4g of Potassium dihydrogen Phosphate into a beaker containing 1000 mLof Milli-Q water Adjust pH of the solution to 3.0±0.05 with diluted Ortho phosphoric acid solution. Filter the solution through 0.45µm membrane filter

Preparation of Mobile phase-A: Use Buffer (pH 4.0)

Preparation of Mobile Phase-B: Prepare a degassed mixture of buffer (pH 4.0) and Acetonitrile in the ratio of 20:80 (%v/v)

Preparation of diluent: Prepare a degassed mixture of buffer (pH 3.0) and Methanol in the ratio of 50:50 (%v/v)

Preparation of Emtricitabine and Tenofovir disoproxil fumarate standard stock solution:

Accurately weigh and transfer about 40mg of Emtricitabine and 60mg Tenofovir disoproxil fumarate working standard into a 50mL volumetric flask. Add about 30mL of Methanol and sonicate to dissolve. Dilute to volume with Methanol and mix.

Transfer 10mL of above solution into a 50mL volumetric flask, dilute to volume with diluent and mix. Filter the solution through 0.45µm membrane filter.

Preparation of sample solution:

For 200/300mg Tablets

Transfer 5tablets in to a 500mL volumetric flask, add about 200mL of buffer (pH 3.0) and mechanical shaker for 1hour 150rpm. Add Methanol up to 1cm bellow the mark and sonicate for not less than 30minutes with occasional shaking {maintain the sonicator bath temperature between 20 to 25°C). Dilute to volume with Methanol and mix. Centrifuge a portion of the solution at 5000rpm for about 5minutes.

Transfer 4mL of above solution into a 50mL volumetric flask, dilute to volume with diluent and mix. Filter the solution through 0.45µm membrane filter and discard first few ml of the filtrate.

Procedure

Equilibrate the column for not less than 60 minutes with initial mobile phase composition at a flow rate of 1.0mL/minute.

Separately inject 10uL of Blank (diluent), Standard solution (five injections) and Sample solution into the chromatographic system. Record the chromatograms and measure the peak responses.

VALIDATION OF OPTIMIZED METHOD:

The proposed method for the assay of Emtricitabine, Tenofovir disoproxil fumarate and tablet dosage form was subjected to validation to check its suitability for routine analysis**.**

A. SPECIFICITY:

Blank interference

Diluent was prepared as per the test method and injected into the chromatographic system and the chromatograms were recorded.

Placebo Interference:

Diluent was injected into Chromatographic system and found no interference. Placebo sample was prepared by taking the placebo equivalent to about the weight portion of test preparation and injected into the HPLC system.

Impurity Interference:

All the related compounds are injected into the HPLC system and recorded the chromatograms.

B. LINEARITY:

The linearity of the method was demonstrated over the concentration range of 50% - 150% of the target concentration. Aliquots of 50%, 70%,80%,90% 100%,110% ,130% and 150% were prepared from standard stock solution.

Procedure

Standard solutions of 50 – 150% concentration were injected separately into the chromatographic system and the chromatograms were recorded. Peak areas were recorded for each injected concentration of drugs and the calibration curves, concentration vs. peak area were constructed for the drugs.

C. PRECISION:

The precision of the method was determined by system precision and method precision using 100% standard and sample solutions.

System precision

The system precision was established by injecting six replicate injections of standard solution in to the chromatographic system and the chromatograms were recorded.

Method precision

Six assay samples of drug product at 100% of the sample concentration were prepared and injected into the chromatographic system and the chromatograms were recorded.

The system and method precision were calculated by using formulae:

-

Standard deviation, $$S.D = \sqrt{\frac{\sum_{i=1}^{i=n}(x_i - \bar{x})}{N-1}}$$

$$\% \ RSD = \frac{S.D}{\bar{x}} \times 100$$

Where, $\overline{X}$ = Mean

N = No. of samples

S. D=Standard deviation

%RSD = Percent relative S.D

D. **ACCURACY:**

The solutions were prepared in replicate at levels 50%,100% and 150% of test concentration using Emtricitabine and Tenofovir disoproxil fumarate drug substance and Emtricitabine and Tenofovir disoproxil fumarate 200/300 tablets placebos per the test method and injected each solution into HPLC as per methodology.

Acceptance criteria: Recovery should be between 98.0% to 102.0%

E. ROBUSTNESS:

As part of evaluation of robustness, deliberate changes were made in the flow rate, Organic phase modifications and wavelength to evaluate the impact on the method.

Procedure

Effect of variation of flow rate

Standard solution prepared as per the test method was injected into the chromatographicsystem

maintaining flow rates, less flow(1.8mL/min), more flow(2.2mL/min) and actual flow (2.0mL/min).

Effect of variation of Organic phase

Standard solution prepared as per the test method was injected into the chromatographic system maintaining flow rates, 2% less organic phase ,2% more organic phase and actual organic phase.

Effect of variation of wavelength

Standard solution prepared as per the test method was injected into the chromatographic system maintaining flow rates, less wavelength (255nm), more wavelength(265nm) and actual wavelength(260nm).

Stress degradation studies

Stress degradation study was conducted in acid, base, peroxide and homogeneity of the peak was assessed in terms of peak purity.

Filter validation

Filter validation was performed by filtering standard and sample solutions with different filters.

Solution stability

Solution stability was performed by injecting standard and sample solution after 12Hrs,24Hrs at 6˚c.

III. RESULTS AND DISCUSSION:

Optimised Chromatographic Conditions:

A Gradient Rapid and simple RP-HPLCmethod was developed and validated for the simultaneous estimation of drugs. Injection volume is setted to 10µl. Mobile phase consisting of degassed mixture of phosphate dihydrogen buffer (pH 4.0) and Acetonitrile in the ratio of 20:80 (%v/v) was set with gradient programming for 25 minutes. chromatographic conditions were optimised for mobile phase using inertsilC8(33mm×4.6mm,3µm) column at a flow rate of 2.0ml/min. effluents was detected at 260nm by variable wavelength UV detector. column compartment temperature was 30˚C.

Validation parameters:

a) Specificity:

The chromatograms of **Emtricitabine, Tenofovir** DF were taken and shown in Figure-3, The peak areas were observed.

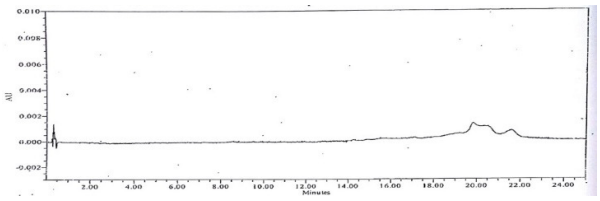


Fig no :3 HPLC Chromatogram of Placebo

Observation: No interference was observed

Acceptance: No peak should not be found at the retention time of main peak

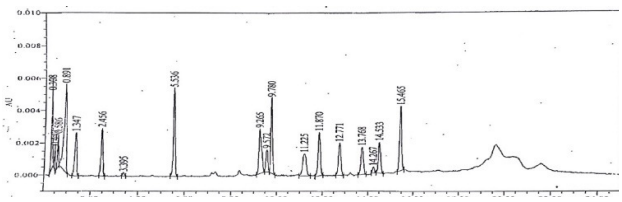


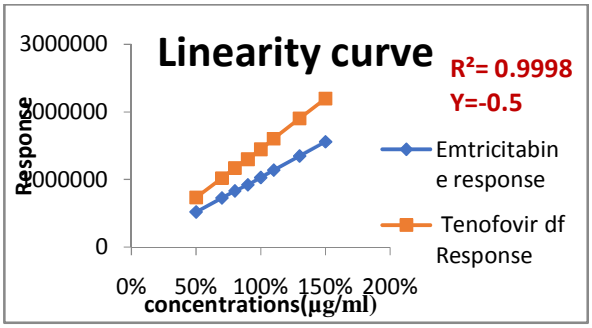
Fig no:4 Impurity Interference Chromatogram of Emtricitabine, Tenofovir Disoproxil Fumarate

Observation : No interference was observed

Acceptance: No peak should be found at the retention time of main peak

b) Linearity

The chromatograms of Emtricitabine, Tenofovir Disoproxil Fumarate were taken and shown in Figures7.16, 7.17, 7.18, 7.19, 7.20, 7.21, 7.22, 7.23. The peak areas were observed. The correlation coefficient® 0.9998 and %y intercept - 0.3for emtricitabine, .and for tenofovir disoproxil fumarate was 0.9998 and %y intercept -0.5



Graph no: 1 Linearity curve of Emtricitabine and Tenofovir disproxil responses

c) Precision

Six sample solutions were prepared. The chromatograms were observed and shown in Figures

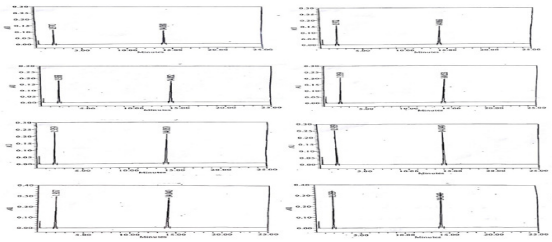


Fig no: 5 HPLC chromatographs for linearity 50%,70%,80%,90%,100%,110%,130%,150% respectively

Table no: 5 Linearitydata of Emtricitabine and Tenofovir disproxil

S.NO	Linearity level	Emtricitabine response	Tenofovir df Response
1	50%	521048	734215
2	70%	727336	1021828
3	80%	831900	1170230
4	90%	922284	1299237
5	100%	1028697	1446794
6	110%	1139566	1604068
7	130%	1348370	1902641
8	150%	1558431	2197338
	Correlation coefficient	0.9998	0.9998
	%y-intercept	-0.3	-0.5

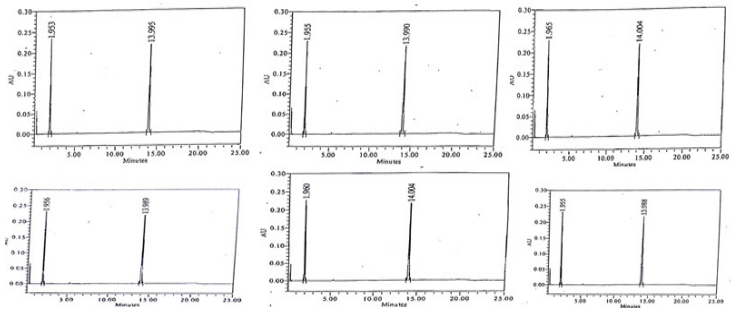


Fig no: 6 HPLC chromatogram of precision

S.NO	Sample Name	Emtricitabine	Tenofovir DF
1	Precision sample-1	1029888	1447111
2	Precision sample-2	1027490	1447505
3	Precision sample-3	1033097	1452702
4	Precision sample-4	1034906	1455942
5	Precision sample-5	1036324	1457164
6	Precision sample-6	1037325	1453083
Mean		1033172	1453083
Std. Dev.		3828	4830
%RSD		0.4	0.3

Table no: 6 System precision data of Emtricitabine, Tenofovir df

S.NO	Sample Name	Emtricitabine	Tenofovir DF
1	Precision sample-1	101.1	101.3
2	Precision sample-2	99.85	102.6
3	Precision sample-3	101.3	101.7
4	Precision sample-4	100	100.6

5	Precision sample-5	101.2	101.5
6	Precision sample-6	100.4	100.8
Mean		100.6	101
%RSD		0.6	0.5

Table no :6 Method precision data of Emtricitabine and Tenofovir df

Observation: The % RSD and % Assay of Emtricitabine, Tenofovir DF are found to be0.6,0.5, and 100.6,101 respectively

Acceptance criteria: The % RSD should be NMT 2.0% and Average % Assay should be between 95-105% of labelled amount.

d) Accuracy

To determine the accuracy of the method recovery was performed by standard addition method. To pre-analysed sampled, known amount of standards. Emtricitabine, Tenofovir DF were spiked in different concentrations. The recoveries of Emtricitabine, Tenofovir DF at three levels 50%, 100% and 150%.

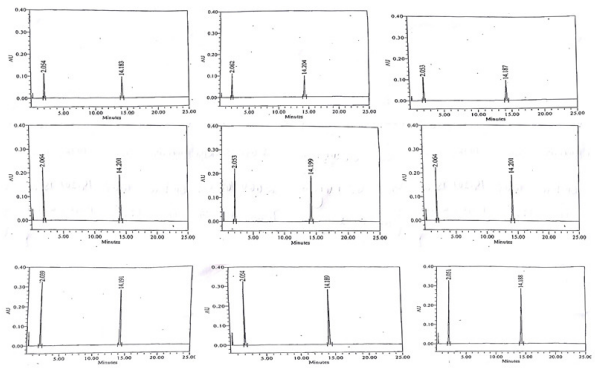


Fig no: 7 Accuracy data of Emtricitabine and Tenofovir df

S.NO	Accuracy Level	% Recovery	Mean recovery	Overall mean recovery
1	Emtricitabine 50%	99.8, 99.2, 100.5	99.8	99.5
2	100%	99.4, 99.4, 99.1	99.3	
3	150%	99.4, 98.8, 99.8	99.3	
4	Tenofovir DF-50%	100.1, 99.4, 101.1	100.2	
5	100%	99.6, 99.6, 99.4	99.5	99.8
6	150%	99.8, 99.3, 100.2	99.8	

Table no: 7 Accuracy Data

Observation: Accuracy was found to be 99.5% and 99.8

e) Robustness:

Robustness conditions

To prove the Robustness of the method, the following parameters were also verified.

Flow,pH,Wavelength, % of organic in mobile phase

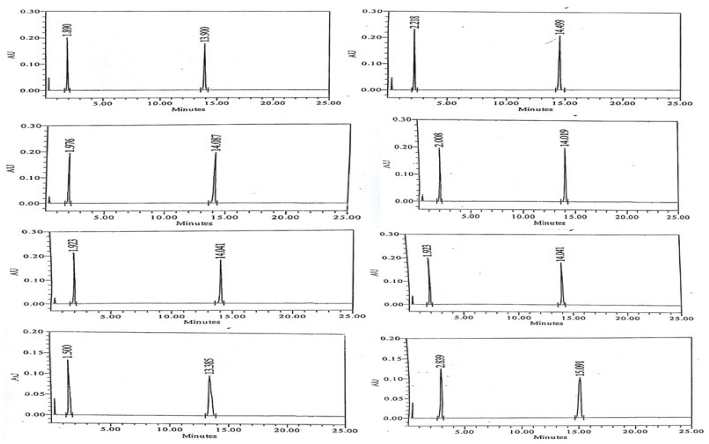


Fig no: 8 Chromatograms of Flow rate (+), Flow rate (-), PH (+), PH (-). Wave length(+)and Wave length(-) respectively.

Parameter	Variation	%RSD	
		Emtricitabine	Tenofovir DF
STP	-	0.5	0.5
Flow Rate	-10%	0.4	0.4
	+10%	0.3	0.3
Wavelength	-5nm	0.5	0.5
	+5nm	0.5	0.5
%Organic in mobile phase	-2% absolute	0.2	0.2
	+2% absolute	0.4	0.4

pH	**-0.2 units**	0.6	0.5
	+0.2 units	0.2	0.2

Table no: 8 Robustness data of Emtricitabine and Tenofovir DF

i. DEGRADATION STUDY:

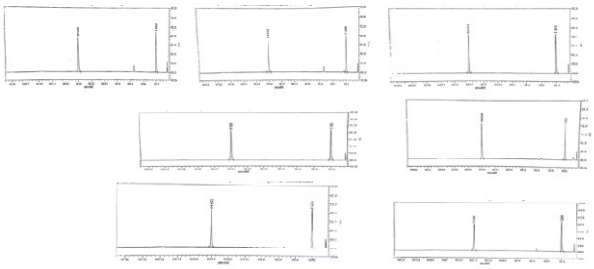


Fig no :9 Chromatograms for Un degradation, Acid degradation, Basedegradation, Peroxidedegradation, Photolyticdegradation, Thermaldegradation, Humidity degradation.

Sample Name	Condition	Purity angle	Purity Threshold
Emtricitabine	Acid Degradation	0.032	0.252
	Base Degradation	0.036	0.243
	Peroxide Degradation	0.036	0.245
	Photolytic Degradation	0.038	0.250
	Thermal Degradation	0.035	0.253
	Humidity Degradation	0.037	0.244
Tenofovir DF	Acid Degradation	0.045	0.260
	Base Degradation	0.038	0.244
	Peroxide Degradation	0.034	0.242
	Photolytic Degradation	0.031	0.244
	Thermal Degradation	0.056	0.262
	Humidity Degradation	0.039	0.242

Table no: 9 Data of forced degradation

Observation: Purity angle is less than purity threshold in all the stress conditions.

Acceptance Criteria: Purity angle should be less than purity threshold.

ii. SOLUTION STABILITY

Solution stability was performed by injecting standard and sample solutionafter 3 Hrs at room temperature 25°C.

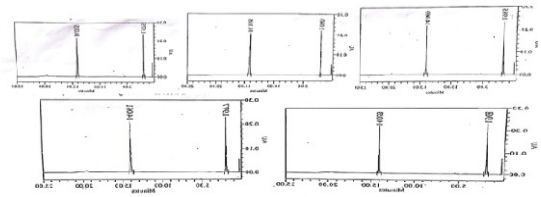


Fig no:10 Solution stability chromatographs at 25°C

Solution stability was performed by injecting standard and sample solution after 12Hrs at refrigerator temperature at 6°C.

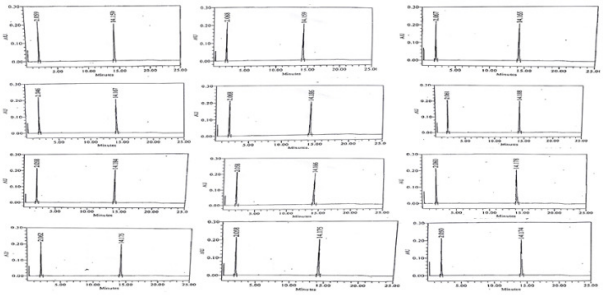


Fig no:11 Solution stability chromatographs at 6°C.

Condition	%Difference	
	Emtricitabine	Tenofovir DF
Room temperature up to 3 Hours (~25°C)	0.5	0.1
Refrigerator condition up to 12 Hours (~6°C)	0.7	0.3

Table no: 10 Solution stability data of Emtricitabine, Tenofovir DF

Observation: %Difference to be found 0.5,0.1and 0.7,0.3 for Emtricitabine, Tenofovir DF in Sample Respectively.

Acceptance criteria: %RSD of Assay difference to the initial value should not be more than 2.0%

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16. MN- Trinath, BM Gurupadayya* Shiva Prasad, Shilpa Kache
17. *Bhushan P. Badgujar*, Moreshwar P.Mahajan, Sanjay D. Sawant.
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