ISSN NO. 2320–7418 DOI: 10.22270/jmpas.V1015.1184

International peer reviewed open access journal

Journal of Medical Pharmaceutical and Allied Sciences

Journal homepage: www.jmpas.com CODEN: JMPACO

Research article

Analytical method development and validation of related substances by rp-hplc of emtricitabine and tenofovir disoproxil fumarate tablets

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ABSTRACT

The developed method was a simple, accurate, precise, specific and robust method for the validation of Emtricitabine and Tenofovir Disoproxil Fumarate Tablets by reverse phase high pressure liquid chromatography. For Emtricitabine and Tenofovir Disoproxil Fumarate Chromatography was performed on Agilent 1200 series, UV and PDA Detector, Waters X-bridge C18 (250 mm x 4.6 mm, 5 µm) by preparing Buffer solution: Dissolve 0.63 g of ammonium formate in 1000 mL of purified water and mix. Adjust to pH of 3.90 +0.05 with diluted formic acid. And used it as mobile phase A. Mobile Phase B: mixture of buffer solution and methanol in the ratio of (20 : 80) % v/v at a flow rate of 1.0 mL/min and at 254 nm wavelength. The retention times of Emtricitabine and Tenofovir Disoproxil Fumarate are approx. 29 min and 70 min. respectively. 5-Fluorocytosinc, Sulfoxide Impurity Isomer 1, Sulfoxide Impurity isomer 2, 5-Fluorouracil analogue, Tenofovir (PMPA) Impurity, Monoester Impurity and Dimer Impurity found linear over the range of LOQ - 150 % of target concentration. Method also found precise by spiking impurities at specification level. Accuracy was demonstrate at LOQ - 150 % level by preparing sample in triplicate for each level and found accurate. Hence, the method could be successfully used for the analysis Impurities in Emtricitabine and Tenofovir Disoproxil Fumarate Tablets.

Keywords: Emtricitabine, Tenofovir Disoproxil Fumarate, HPLC, UV and PDA Detector, Related Substances.

Received - 04-06-2021, Reviewed - 07/07/2021, Revised/ Accepted- 03/09/2021

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INTRODUCTION

In the Era of Sciences and medical, Pharmaceutical industries are playing the vital role worldwide. Now a day's humans are suffering from to many critical diseases however to overcome this pharmaceutical industries are producing innovating new chemical entities. Regulatory bodies like USFDA, TGA, MHRA. WHO etc. have certain guidelines to make qualitative and effective medicines. To fulfill requirement and produce qualitative medicine analytical part also play a vital role and now a day's industries are highly focusing on it [1].

Several reasons are available for the development of a new method of analysis, they are

- There may not a suitable method for a particular analyte in the sample matrix.
- Existing may be too erroneous.
- Existing method may not provide adequate sensitivity.
- Existing methods are too expensive and time consuming [2].

Antiviral drugs are a class of medication used specifically for treating viral infections rather than bacterial ones. Most antiviral are used for specific viral infections, while a broad-spectrum antiviral is effective against a wide range of viruses. Unlike most antibiotics, antiviral drugs do not destroy their target pathogen; instead they inhibit their development [3].

MATERIAL AND METHOD DEVELOPMENT

Instruments used

Table 1: Instrument Used during Development

Name	Make/Model
HPLC	Agilent
Series	1200,1260
Software	Chromeleon
Pump	Isocratic
Column	Waters X-bridge C18 (250 mm x 4.6 mm, 5 µm)
Detector	UV Detector PDA Detector

Reagents used

Ammonium formate (LCMS grade), Formic acid 99% (HPLC grade), Water (Milli Q grade) and Methanol (Gradient grade).

PREPARATION OF SOLUTIONS

Preparation of Diluent

Prepare a mixture of purified water and methanol in the ratio of (80:20) % v/v.

Standard preparation

Transfer an accurately weighed quantity about 40 mg of Emtricitabine working standard and 60 mg of Tenofovir Disoproxil Fumarate working standard in to a 200 mL volumetric flask. Add about 150 mL of diluent and sonicate to dissolve. Make volume up to the mark with diluent and mix. Dilute 4.0 mL of this solution to 100.0 rnL with diluent and mix. (Concentration 8 µg/mL of Emtricitabine and 12 µg/mL of Tenofovir Disoproxil Fumarate)

Proper selection of the method involves certain criteria depends upon the nature of the sample, molecular weight and Solubility. The selected drug for the present study was polar in nature. Polar compounds can be separated by Reverse phase chromatography. Reverse phase chromatographic technique was selected for Initial separations from the knowledge of properties of the compound.

For stationary phase C18 column was chosen and different mobile phases were checked and the most suitable condition was optimized. The objective of this experiment was to optimize the Related Substances method for Emtricitabine and Tenofovir Disoproxil based on the literature survey. So here the trials mentioned describes how the optimization was done.

Selection of wavelength for detection by scanning in uv

The working standard solution of Emtricitabine and Tenofovir Disoproxil Fumarate was scanned in the UV region and spectrum was recorded. Solutions were scanned on spectrophotometer in the UV range of 200-400nm. It was seen that at 280 nm maximum absorbance was found for Emtricitabine and 260 nm for Tenofovir Disoproxil Fumarate. But for common response 254 nm wavelength is choosen. In HPLC, proper peak response was observed using 254 nm. Hence, 254 nm was selected as the wavelength for estimation in HPLC [4].

Trial – I: Chromatographic system

HPLC system is equipped with a UV- Visible detector.

Mobile phase

Buffer solution

In 1000 mL of purified water dissolve 0.63 g of ammonium formate and mix. Adjust to pH of 3.90 ± 0.05 with diluted formic acid. Filter through 0.45µm membrane filter.

Mobile Phase A

Use buffer solution.

Mobile Phase B

Use Methanol and Buffer solution (50 : 50) % v/v.

Chromatographic conditions

Flow rate : 1.0 ml/min

Column : X-bridge C18 (250 mm x 4.6 mm), 5 µm

Detector wavelength : 254 nm

Injection volume : 20 µl

Column temperature : 25°C

Auto sampler temperature : 25°C

Run time : 60 minutes

Diluent : Water : Methanol (20:80) % v/v

Table 2: Gradient program of Trial – I

Time (minutes)	% Mobile phase A	% Mobile phase B
0	100	0
50	0	100
50.1	0	100
60	100	0

Trial – I Observation

In this trail the peak of Degradent Impurities i.e Sulfoxide Impurity Isomer 1, Sulfoxide Impurity Isomer 2 and Tenofovir (PMPA) impurities eluted closely hence required more separation. Also Tenofovir peak didn't eluted hence required run for longer run time.

Trial - II

Mobile phase

Buffer solution

In 1000 mL of purified water dissolve 0.63 g of ammonium formate and mix. Adjust to pH of 3.90 ± 0.05 with diluted formic acid. Filter through 0.45µm membrane filter.

Mobile Phase A

Use buffer solution.

Mobile Phase B

Use Methanol and Buffer solution (80 : 20) % v/v.

Chromatographic conditions

Flow rate : 1.0 ml/min

Column : X-bridge C18 (250 mm x 4.6 mm), 5 µm

Detector wavelength : 254 nm

Injection volume : 20 µl

Column temperature : 25°C

Auto sampler temperature : 25°C

Run time : 120 minutes

Diluent : Water : Methanol (20:80) % v/v

Table 3: Gradient program of Trial – II

Time (minutes)	% Mobile phase A	% Mobile phase B
0	100	0
40	90	10
50	50	50
70	10	90
90	10	90
120	100	0

Trial – II Observation

In this trail the peak of Degradent Impurities i.e Sulfoxide Impurity Isomer 1, Sulfoxide Impurity Isomer required more separation. However Tenofovir (PMPA) impurity is separated. Also Tenofovir peak is eluted at around at 80 minutes.

Trial - III

Mobile phase

Buffer solution

In 1000 mL of purified water dissolve 0.63 g of ammonium formate and mix. Adjust to pH of 3.90 ± 0.05 with diluted formic acid. Filter through 0.45µm membrane filter.

Mobile Phase A

Use buffer solution.

Mobile Phase B

Use Methanol and Buffer solution (80 : 20) % v/v.

Chromatographic conditions

Flow rate : 1.0 ml/min

Column : X-bridge C18 (250 mm x 4.6 mm), 5 µm

Detector wavelength : 254 nm

Injection volume : 20 µl

Column temperature : 30°C

Auto sampler temperature : 25°C

Run time : 100 minutes

Diluent : Water : Methanol (20:80) % v/v

Table 4: Gradient program of Trial – III

Time (minutes)	% Mobile phase A	% Mobile phase B
0	100	0
15	95	5
40	75	25
80	20	80
85	20	80
90	100	0
100	100	0

Trial – III Observation

In this trail the peak of Degradent Impurities i.e Sulfoxide Impurity Isomer 1, Sulfoxide Impurity Isomer 2 is separated. Other degradent impurities are also also well separated. There is no significant interference observed at retention time of analyte as well as at impurities.

FINAL METHODOLOGY

Buffer solution

In 1000 mL of purified water dissolve 0.63 g of ammonium formate and mix. Adjust to pH of 3.90 ± 0.05 with diluted formic acid. Filter through 0.45µm membrane filter.

Mobile Phase A

Use buffer solution.

Mobile Phase B

Prepare a mixture of buffer and methanol (20 : 80) % v/v.

Diluent

Prepare a mixture of methanol and water (30 : 70) % v/v.

Standard preparation

Transfer an accurately weighed quantity about 40 mg of Emtricitabine working standard and 60 mg of Tenofovir Disoproxil Fumarate working standard in to a 200 mL volumetric flask. Add about 150 mL of diluent and sonicate to dissolve. Make up with diluent and mix. Dilute 4.0 mL of this (stock) solution to 100.0 rnL with diluent and mix. (Concentration 8 µg/mL of Emtricitabine and 12 µg/mL of Tenofovir Disoproxil Fumarate)

Placebo/ Sample preparation

Transfer an accurately weighed quantity of placebo powder/sample equivalent to about 200 mg of Emtricitabine or 300 mg of Tenofovir Disoproxil Fumarate into a 100 mL volumetric flask. Add about 60 mL of diluent and sonicate for 10 minutes with vigorous shaking. Keep the flask on bench top to attain room temperature. Make volume up to the mark with diluent and mix. Filter the solution through Millipore PVDF 0.22 µm filter; collect the placebo/sample by discarding first 3 mL volume of the filtrate [5].

Chromatographic conditions

Column : X-bridge C18 (250 mm x 4.6 mm), 5 µm

Detector : 254 nm

Flow rate : 1.0 mL/min

Injection volume : 10 µl

Column temperature : 25°C

Vial thermostat temperature : 25°C

Table 5: Gradient program of final methoddolgy

Time (minutes)	% Mobile phase A	% Mobile phase B
0	100	0
15	95	5
40	75	25
80	20	80
85	20	80
90	100	0
100	100	0

VALIDATION PARAMETERS

System suitability and precision

System suitability and precision were demonstrated by injecting sensitivity solution and six replicate injections of standard solution prepared as per the test method and chromatographed into HPLC system. The signal to noise ratio of Emtricitabine and Tenofovir Disoproxil peak was evaluated from sensitivity solution. The tailing factor and theoretical plates for Emtricitabine and Tenofovir Disoproxil peak were evaluated from standard solution. The precision was evaluated by computing the relative standard deviation for the peak area of these replicate injections [6].

Table 6: System suitability and precision results of % RSD

Injection No.	Peak area	
	Emtricitabine	Tenofovir Disoproxil
1	120.564	143.214
2	122.321	141.234
3	121.456	145.218
4	124.369	142.120
5	118.965	140.365
6	122.354	142.654
Average	120.564	143.214
% RSD	1.5	1.2

- The signal to noise ratio of Emtricitabine peak: 116.7
- The signal to noise ratio of Tenofovir Disoproxil peak: 114.9
- Tailing factor for Emtricitabine peak: 1.1
- Theoretical plates for Emtricitabine peak: 190092
- Tailing factor for Tenofovir Disoproxil peak: 1.0
- Theoretical plates for Tenofovir Disoproxil peak: 782665

Acceptance criteria

1) The signal to noise ratio for both peak is NLT 10.
2) Theoretical plates for both peaks are not less than 10000.
3) Tailing factor for both peaks are not more than 2.0.
4) % RSD of six replicate standard is not more than 5.0.

Observation

The results obtained meet the system suitability and precision requirement, which indicates that the system is suitable and precise for analysis.

Figure 1: Typical chromatogram of mobile phase

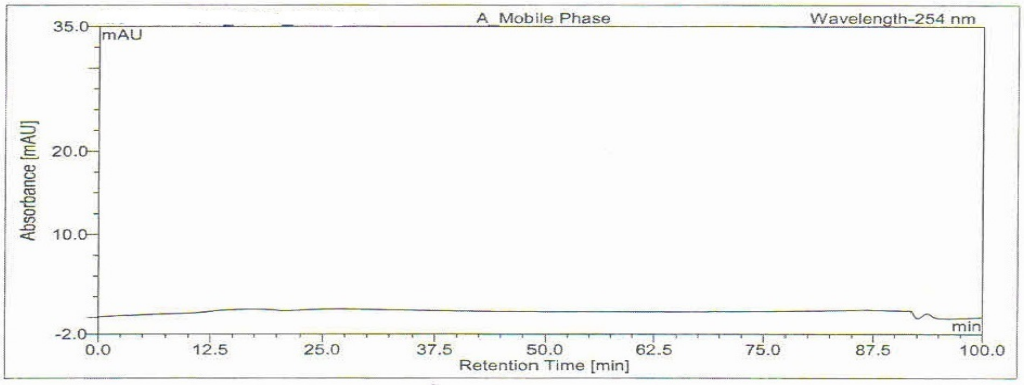
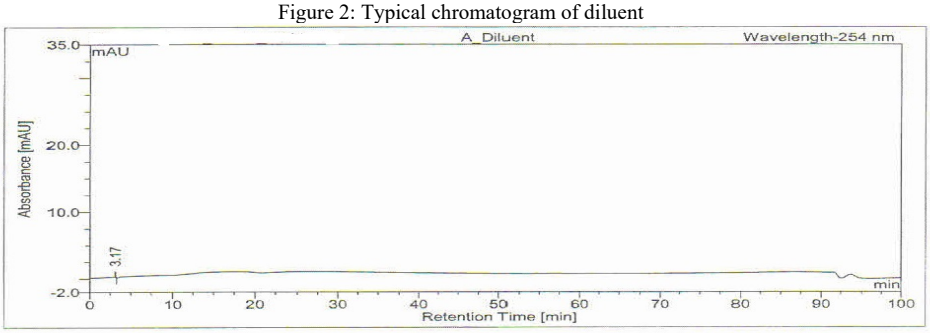


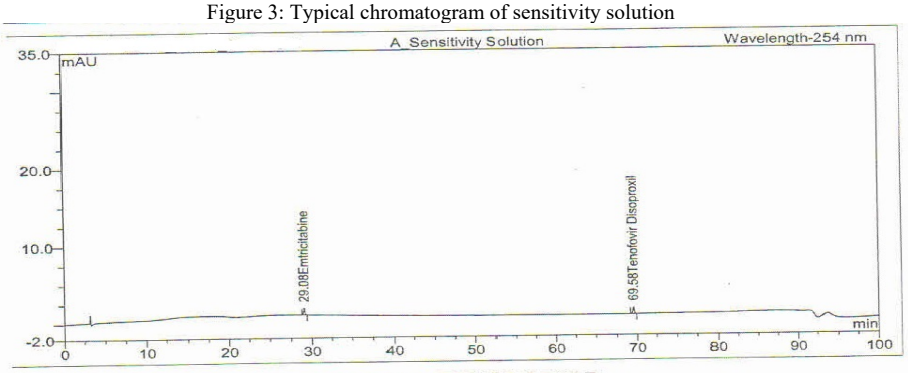
Figure 2: Typical chromatogram of diluent



PEAK RESULT

No.	Ret.Time min	Peak Name	Type	Height mAU	Area mAU*sec	Rel.Area %
1	3.17	n.a.	BMB	0.843	2.956	100.00
Total:					2.956	100.00

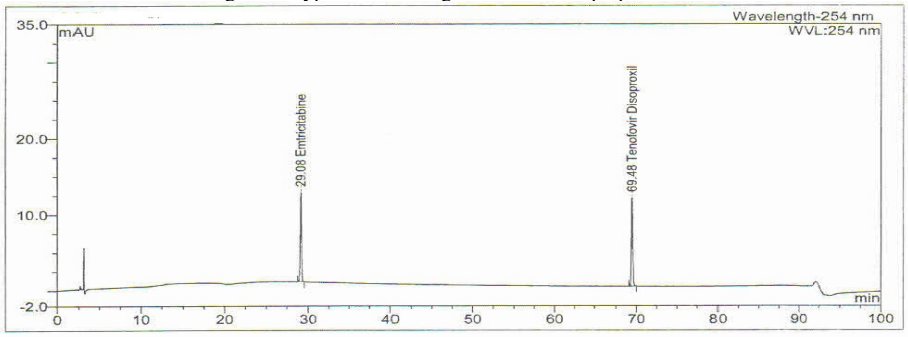
Figure 3: Typical chromatogram of sensitivity solution



SYSTEM SUITABILITY RESULT

No.	Ret.Time min	Peak Name	Type	Height mAU	Noise mAU	S/N Ratio	K'	Area mAU*sec
1	29.08	Emtricitabine	BMB	0.855	0.020	116.7	n.a.	10.175
2	69.58	Tenofovir Disoproxil	BMB	0.841	0.020	114.9	n.a.	11.406

Figure 4: Typical chromatogram of standard preparation



SYSTEM SUITABILITY RESULT

No.	Ret.Time min	Peak Name	Type	Plates USP	Tailing USP	Resolution USP	K'	Area mAU*sec
1	29.08	Emtricitabine	BMB	190092	1.05	n.a.	40.55	118.381
2	69.48	Tenofovir Disoproxil	BMB	782665	1.04	139.07	98.26	138.250
Total:								256.631

Filter compatibility and saturation study

Table 7: % Impuritie in Filter compatibility and saturation study (A, B, C, D)

A

Volume of sample Discarded (in mL)	% Known impurities					
	5- Fluoro cytosine	Sulfoxide Impurity Isomer 1	Sulfoxide Impurity Isomer 2	5-Fluoro-uracil analogue	Tenofovir (PMPA) impurity	Dimer Impurity
Unfiltered	0.21	0.21	0.30	0.21	0.18	0.23
1	0.21	0.21	0.30	0.21	0.18	0.23
3	0.21	0.21	0.30	0.21	0.18	0.23
5	0.21	0.21	0.30	0.21	0.18	0.23
7	0.21	0.21	0.31	0.21	0.18	0.23
9	0.21	0.21	0.30	0.21	0.18	0.23

B

Volume of sample Discarded (in mL)	% Known impurities					
	5- Fluoro cytosine	Sulfoxide Impurity Isomer 1	Sulfoxide Impurity Isomer 2	5-Fluoro-uracil analogue	Tenofovir (PMPA) impurity	Dimer Impurity
1	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00	0.00
7	0.00	0.00	0.01	0.00	0.00	0.00
9	0.00	0.00	0.00	0.00	0.00	0.00

C

Volume of sample Discarded (in mL)	% Known impurities				% of Maximum individual impurities at RRT 2.64	Total Impurities
	Emtricitabine Acid	Adenine impurity	Monoester impurity	Isopropyl impurity		
Unfiltered	BQL	BQL	0.62	0.13	0.03	2.2
1	BQL	BQL	0.63	0.13	0.03	2.2
3	BQL	BQL	0.65	0.13	0.03	2.2
5	BQL	BQL	0.64	0.13	0.03	2.2
7	BQL	BQL	0.65	0.13	0.03	2.2
9	BQL	BQL	0.65	0.13	0.03	2.2

D

Volume of sample Discarded (in mL)	% Known impurities				% of Maximum individual impurities at RRT 2.64	Total Impurities
	Emtricitabine Acid	Adenine impurity	Monoester impurity	Isopropyl impurity		
1	BQL	BQL	0.01	0.00	0.00	0.0
3	BQL	BQL	0.03	0.00	0.00	0.0
5	BQL	BQL	0.02	0.00	0.00	0.0
7	BQL	BQL	0.03	0.00	0.00	0.0
9	BQL	BQL	0.03	0.00	0.00	0.0

Acceptance criteria

The difference between the unfiltered and filtered samples should not differ by more than 0.05 for each individual impurity and 0.1 for total impurities.

Observation

From the established data the Millipore PVDF 0.45 µm filter proved to be compatible for all discard volume, hence Millipore PVDF 0.45 µm can be used for the analysis. Based on established data it is recommended that first 5 mL of filtrate will be discarded for the analysis.

LOD and LOQ

Limit of detection and quantification were established for known impurities (i.e., 5- Fluoro cytosine, Sulfoxide Impurity Isomer 1, Sulfoxide Impurity Isomer 2, 5-Fluorouracil analogue, Tenofovir (PMPA) Impurity, Monoester Impurity, Dimer Impurity) and unknown impurities (in terms of Emtricitabine and Tenofovir Disoproxil) based on residual standard deviation and slope of the linearity data.

From the linearity data the limit of detection and quantification were calculated using the following formula.

Limit of detection = $3.3\ \sigma / S$
Limit of quantification = $10\ \sigma / S$

Where,
σ = Residual standard deviation of regression line
S = Slope of regression line

Table 8: Estabblished LOD and LOD

Name of Impurity	LOD		LOQ	
	µg/ mL	%	µg/ mL	%
5-Fluorocytosine	0.2042	0.01	0.6126	0.03
Sulfoxide Impurity Isomer 1	0.2214	0.01	0.6642	0.03
Sulfoxide Impurity Isomer 2	0.2065	0.01	0.6195	0.03
5-Fluorouracil analogue	0.2064	0.01	0.6192	0.03
Tenofovir (PMPA) Impurity	0.2096	0.01	0.6288	0.02
Monoester Impurity	0.1512	0.01	0.4536	0.02
Dimer Impurity	0.2994	0.01	0.8982	0.03
Erntricitabine (For unknown impurities)	0.2287	0.01	0.6861	0.03
Tenofovir Disoproxil (For unknown impurities)	0.2901	0.01	0.8703	0.03

Table 9: % RSD and S/N ratio of impurites

Injection Run No.	% Known impurities								
	5-Fluoro cytosine	Sulfoxide Impurity Isomer 1	Sulfoxide Impurity Isomer 2	5-Fluoro uracil analogue	Tenofovir (PMPA) impurity	Dimer Impurity	Monoester Impurity	Emtri-citabine	Tenofovir
1	10.225	8.653	8.632	10.235	42.654	6.231	26.321	11.324	10.235
2	10.695	8.962	8.654	10.654	40.235	6.654	26.954	11.654	10.695
3	9.965	9.214	8.741	11.214	39.654	6.954	25.354	11.21	10.652
4	11.625	9.864	9.012	10.698	42.541	6.214	26.417	11.968	10.365
5	9.564	8.023	8.954	10.654	41.654	6.954	26.954	12.564	11.214
6	10.654	9.621	8.321	10.541	40.654	6.105	25.654	11.222	10.654
Average	10.455	9.056	8.719	10.666	41.232	6.519	26.276	11.657	10.636
% RSD	6.8	7.4	2.9	3.0	3.0	5.9	2.5	4.6	3.2
S/N Ratio	170	202	105	86	425	79	248	102	110

Acceptance criteria

1) The detector response should be positive for LOD solution and the % RSD at LOQ level for impurities shall not be more than 10.0 %.
2) The signal to noise ratio at LOQ concentration shall not be less than 10.

Observation

From the above established data, it can be concluded that the test method shall be capable of detecting and quantifying the impurities, if present in the sample, to the extent that mentioned in table.

Method Precision

Method precision was demonstrated by preparing sample as such and six samples as per the test method, in which the known impurities are spiked at 0.2 % level, representing a single batch. The impurities were quantified for each of these samples. The precision of the method was evaluated by computing the percentage-relative standard deviation for the content of each of known impurities, unknown impurities and total impurities.

Table 10: % content of impurities for as such sample (A, B)

A

Sample Name	% Known impurities (In as such sample)					
	5- Fluoro cytosine	Sulfoxide Impurity Isomer 1	Sulfoxide Impurity Isomer 2	5-Fluoro uracil analogue	Tenofovir (PMPA) impurity	Adenine Impurity
As such	BQL	BQL	0.08	BQL	BQL	0.03

B

Sample Name	% Known impurities (In as such sample)				% Maximum unknown impurity	% Total impurities
	Carbonyl Impurity	Ethyl Impurity-1	Monoester Impurity	Isopropyl Impurity		
As such	BQL	BQL	0.65	0.13	0.02	0.9

Table 11: % content of impurities for spiked sample (A, B)

A

Sample Set No.	% Known impurities							
	5-Fluoro cytosine	Sulfoxide Impurity Isomer 1	Sulfoxide Impurity Isomer 2	5-Fluoro uracil analogue	Tenofovir (PMPA) impurity	Dimer Impurity	Adenine Impurity	Lamivudine
1	0.21	0.22	0.30	0.20	0.20	0.24	0.03	BDL
2	0.21	0.22	0.29	0.20	0.20	0.23	0.03	BDL
3	0.21	0.22	0.29	0.20	0.20	0.24	0.03	BDL
4	0.21	0.22	0.30	0.20	0.20	0.24	0.03	BDL
5	0.20	0.22	0.29	0.20	0.20	0.24	0.03	BDL
6	0.21	0.22	0.30	0.20	0.20	0.24	0.03	BDL
Average	0.21	0.22	0.30	0.20	0.20	0.24	0.03	-
% RSD	1.9	0.0	1.8	0.0	0.0	1.7	0.0	-

B

Sample Set No.	% Known impurities						% of Maximum RRT 2.65	% Total impurities
	Carbonyl Impurity	Ethyl Impurity -1	Ethyl Impurity -2	Monoester impurity	Isopropyl impurity	Emtricitabine Acid		
1	BQL	BQL	BQL	053	0.13	BDL	0.03	2.2
2	BQL	BQL	BQL	0.54	0.13	BDL	0.03	2.2
3	BQL	BQL	BQL	0.54	0.13	BDL	0.03	2.2
4	BQL	BQL	BQL	0.55	0.13	BDL	0.03	2.2
5	BQL	BQL	BQL	0.56	0.13	BDL	0.03	2.1
6	BQL	BQL	BQL	0.57	0.13	BDL	0.03	2.2
Average	-	-	-	0.55	0.13	-	0.03	2.2
% RSD	-	-	-	2.7	0.0	-	0.0	1.9

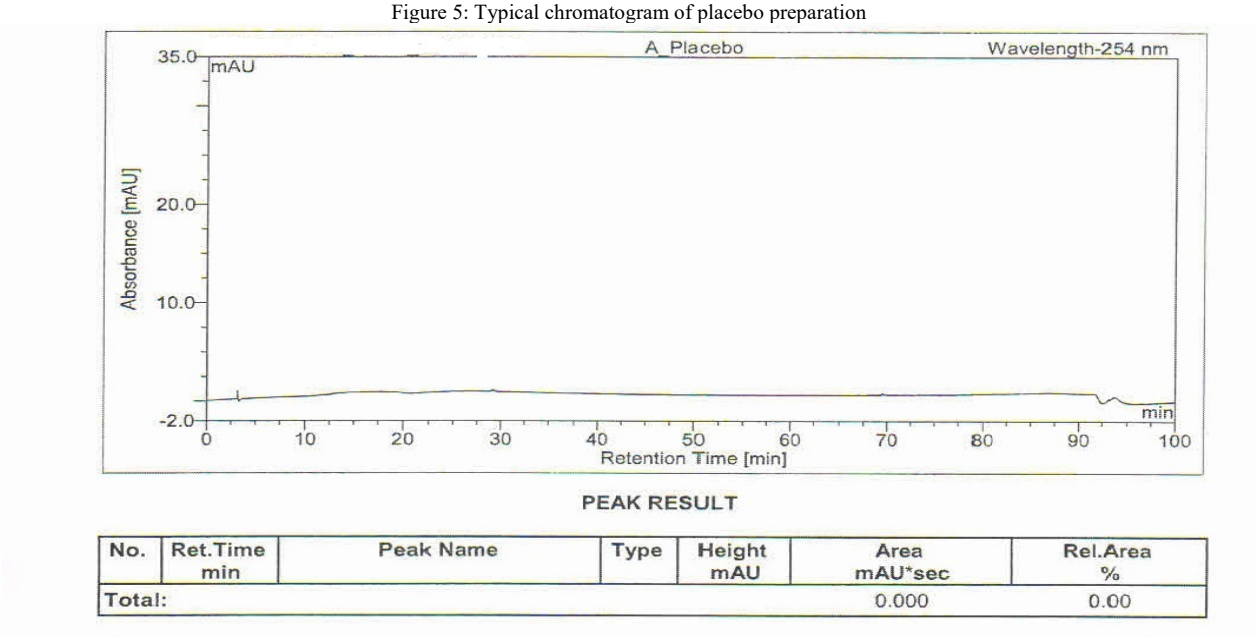
Acceptance criteria

Impurity levels	% RSD
0.05% to 0.10 %	Shall not be more than 25.0 %
0.11% to 0.50 %	Shall not be more than 15.0 %
0.51% to 1.0 %	Shall not be more than 10.0 %
More than 1.0 %	Shall not be more than 5.0 %

Observation

As the precision results obtained for the impurities are found to be within the acceptance criteria, this implies that the method is precise for quantification of impurities in Emtricitabine and Tenofovir Disoproxil Fumarate Tablets.

Figure 5: Typical chromatogram of placebo preparation



PEAK RESULT

No.	Ret.Time min	Peak Name	Type	Height mAU	Area mAU*sec	Rel.Area %
Total:					0.000	0.00

Figure 6: Typical chromatogram of as such sample

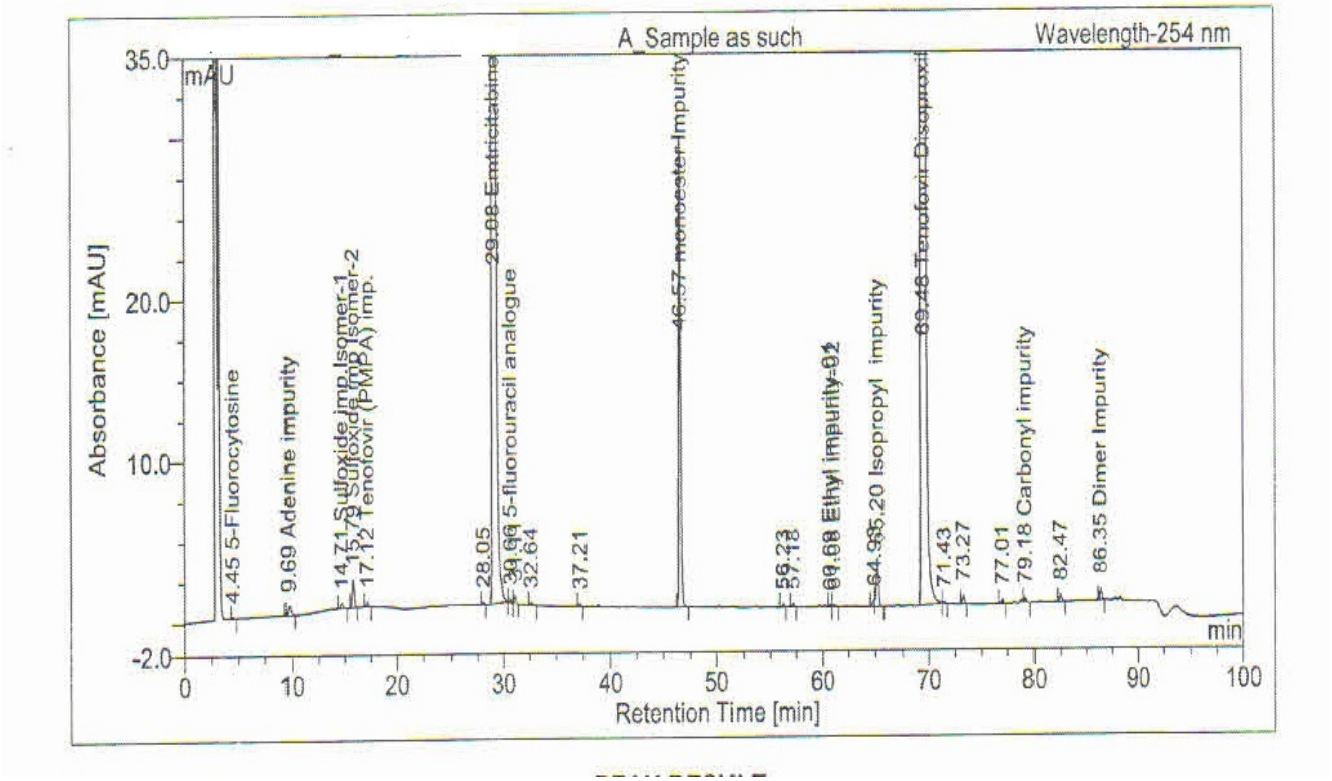
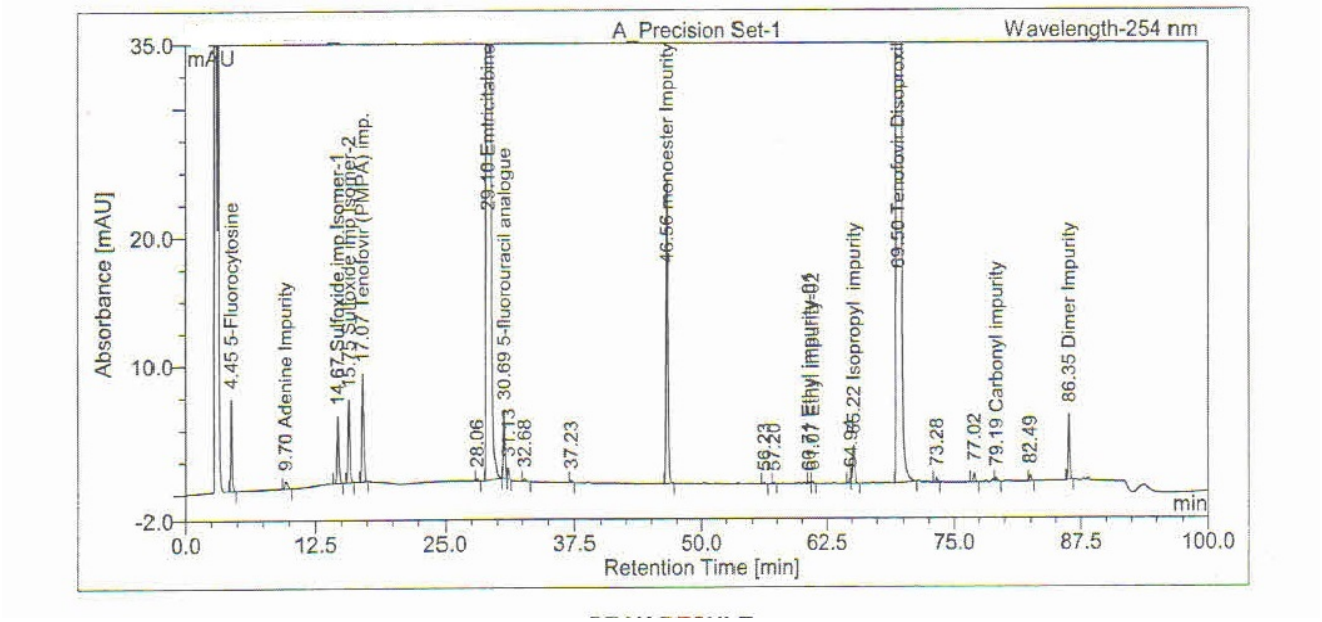


Figure 7: Typical chromatogram of precision sample
(Impurities spiked at specification level)



Linearity

Table 12: Linearity of impuritiy of all impurities (A, B, C, D, E, F, G)- A

Linearity Level	Concentration of 5-Fluorocytosine in µg/mL	Peak area	RRF	Correlation Coefficient
LOQ	0.6354	12.026	-	0.99998
50 %	2.0037	37.583	0.80	
80 %	3.2059	59.949	0.80	
100 %	4.0073	75.181	0.80	
120 %	4.8088	90.815	0.79	
150 %	6.0110	113.239	0.80	
Average			0.80	

B

Linearity Level	Concentration of Sulfoxide Impurity Isomer 1 in µg/mL	Peak area	RRF	Correlation Coefficient
LOQ	0.6414	10.391	-	0.99997
50 %	1.9972	31.730	0.94	
80 %	3.1955	51.053	0.94	
100 %	3.9943	63.760	0.94	
120 %	4.7932	77.101	0.93	
150 %	5.9915	96.296	0.93	
Average			0.94	

C

Linearity Level	Concentration of 5-Fluorouracil analogue in µg/mL	Peak area	RRF	Correlation Coefficient
LOQ	0.6251	9.525	-	0.99999
50 %	2.0320	30.661	0.99	
80 %	3.2513	49.089	0.99	
100 %	4.0641	61.574	0.99	
120 %	4.8769	73.940	0.99	
150 %	6.0961	92.745	0.99	
Average			0.99	

D

Linearity Level	Concentration of Sulfoxide Impurity Isomer 2 in µg/mL	Peak area	RRF	Correlation Coefficient
LOQ	0.6251	9.393	-	0.99995
50 %	2.0124	29.796	1.01	
80 %	3.2198	47.157	1.02	
100 %	4.0248	59.032	1.02	
120 %	4.8298	71.114	1.02	
150 %	6.0372	88.892	1.02	
Average			1.02	

E

Linearity Level	Concentration Tenofovir (PMPA) Impurity in µg/mL	Peak area	RRF	Correlation Coefficient
LOQ	0.5993	11.358	-	0.99997
50 %	2.9965	55.844	0.62	
80 %	4.7944	88.042	0.63	
100 %	5.9930	111.317	0.62	
120 %	7.1916	133.464	0.62	
150 %	8.9895	166.430	0.62	
Average			0.62	

F

Linearity Level	Concentration of Monoester Impurity in µg/mL	Peak area	RRF	Correlation Coefficient
LOQ	0.4742	8.896	-	0.99996
50 %	45.0657	851.490	0.61	
80 %	72.1052	1341.920	0.62	
100 %	90.1314	1697.998	0.61	
120 %	108.1577	2039.791	0.61	
150 %	135.1972	2560.614	0.61	
Average			0.61	

G

Linearity Level	Concentration of Dimer Impurity in µg/mL	Peak area	RRF	Correlation Coefficient
LOQ	0.8876	7.802	-	1.00000
50 %	3.1153	27.699	1.30	
80 %	4.9844	44.322	1.30	
100 %	6.2305	55.234	1.30	
120 %	7.4766	66.202	1.30	
150 %	9.3458	82.827	1.30	
Average			1.30	

Acceptance criteria

The correlation coefficient is NLT 0.980.

Observation

The study proves that the response for known impurities (i.e., 5-Fluorocytosine, Sulfoxide Impurity Isomer 1, Sulfoxide Impurity Isomer 2, 5-Fluorouracil analogue, Tenofovir (PMPA) Impurity, Monoester Impurity and Dimer Impurity) peak is linear over the range of LOQ to 150 % of shelf life specification limit.

Accuracy

Table 13: %Recovery of 5-Fluorocytosin (A, B, C, D)- A

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
LOQ Level	1	0.0159	0.0152	97.4
	2		0.0149	95.5
	3		0.0156	100.0
Average				97.6

B

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
50% Level	1	0.0502	0.0501	99.8
	2		0.0511	101.8
	3		0.0498	99.2
Average				100.3

C

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
100% Level	1	0.1008	0.09965	98.9
	2		0.09546	94.7
	3		0.09852	97.7
Average				97.1

D

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
150% Level	1	0.1512	0.1527	101.0
	2		0.152	100.5
	3		0.1498	99.1
	Average			100.2

Table 14: %Recovery of Sulfoxide Impurity Isomer 1 (A, B, C, D)

A

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
LOQ Level	1	0.0149	0.0149	100.0
	2		0.0151	101.3
	3		0.0156	104.7
	Average			102.0

B

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
50% Level	1	0.0503	0.0509	98.2
	2		0.0501	97.6
	3		0.0498	98.2
	Average			99.9

C

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
100% Level	1	0.1006	0.1001	99.5
	2		0.1009	100.3
	3		0.1024	101.8
	Average			100.5

D

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
150% Level	1	0.1509	0.1527	101.2
	2		0.1569	104.0
	3		0.1498	99.3
	Average			101.5

Table 15: %Recovery of Sulfoxide Impurity Isomer 2 (A, B, C, D)

A

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
LOQ Level	1	0.0149	0.0156	100.0
	2		0.0149	95.5
	3		0.0152	97.4
	Average			97.6

B

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
50% Level	1	0.0502	0.0502	100.0
	2		0.0506	100.8
	3		0.0521	103.8
	Average			101.5

C

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
100% Level	1	0.1004	0.1011	100.7
	2		0.1021	101.7
	3		0.1017	101.3
	Average			101.2

D

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
150% Level	1	0.1506	0.1521	101.0
	2		0.1535	101.9
	3		0.1534	101.9
	Average			101.6

Table 16: %Recovery of Tenofovir (PMPA) Impurity (A, B, C, D)-A

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
LOQ Level	1	0.0150	0.0154	102.7
	2		0.0151	100.7
	3		0.0146	97.3
	Average			100.2

B

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
50% Level	1	0.0749	0.0765	102.1
	2		0.0777	103.7
	3		0.0736	98.3
	Average			101.4

C

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
100% Level	1	0.1498	0.1521	101.5
	2		0.1492	99.6
	3		0.1534	102.4
	Average			101.2

D

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
150% Level	1	0.2247	0.2256	100.4
	2		0.2269	101.0
	3		0.2284	101.6
	Average			101.0

Table 17: %Recovery of Monoester Impurity (A, B, C, D)

A

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
LOQ Level	1	0.0135	0.0141	104.4
	2		0.0138	102.2
	3		0.0132	97.8
	Average			101.5

B

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
50% Level	1	1.1280	1.1295	100.1
	2		1.2564	111.4
	3		1.2065	107.0
	Average			106.2

C

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
100% Level	1	2.2560	2.2854	101.3
	2		2.2654	100.4
	3		2.2965	101.8
	Average			101.2

D

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
150% Level	1	3.3840	3.3965	100.4
	2		3.2541	96.2
	3		3.6521	107.9
	Average			101.5

Table 18: %Recovery of Dimer Impurity (A, B, C, D)

A

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
LOQ Level	1	0.0239	0.0232	97.1
	2		0.0245	102.5
	3		0.0262	109.6
	Average			106.1

B

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
50% Level	1	0.0768	0.0785	102.2
	2		0.0796	103.6
	3		0.0724	94.3
	Average			100.0

C

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
100% Level	1	0.1536	0.1596	103.9
	2		0.1621	105.5
	3		0.1601	104.2
	Average			104.6

D

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
150% Level	1	0.2304	0.2365	102.6
	2		0.2398	104.1
	3		0.2347	101.9
	Average			102.9

Table 19: %Recovery of 5- Fluorouracil analogue Impurity (A, B, C, D)

A

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
LOQ Level	1	0.0159	0.0152	95.6
	2		0.0171	107.5
	3		0.0165	103.8
	Average			102.3

B

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
50% Level	1	0.0490	0.0496	101.2
	2		0.0478	97.6
	3		0.0512	104.5
	Average			101.1

C

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
100% Level	1	0.0980	0.0982	100.2
	2		0.0962	98.2
	3		0.0987	100.7
	Average			99.7

D

Recovery level	Sample No.	Amount Spiked (mg)	Amount Recovered (mg)	% Recovery
150% Level	1	0.1470	0.1496	101.8
	2		0.1462	99.5
	3		0.1421	96.7
	Average			99.3

Acceptance criteria

Impurity levels	% Recovery
Up to 0.10 %	Between 50.0% to 150.0%
0.11 % to 0.50 %	Between 70.0% to 130.0%
0.51 % to 1.0 %	Between 80.0% to 120.0%
More than 1.0 %	Between 90.0% to 110.0%

Observation

As the recovery results obtained for known impurities [i.e., 5-Fluorocytosine, Sulfoxide Impurity Isomer 1, Sulfoxide Impurity Isomer 2, 5-Fluorouracil analogue, Tenofovir (PMPA) Impurity, Monoester Impurity and Dimer Impurity] were within the acceptable limits of recovery, the study proves that the method is accurate for quantification of impurities in the range of LOQ to 150 % of shelf-life specification level.

Robustness

Conditions

1) Column Temperature was changed by ± 5°C (i.e., 20°C and 30°C).
2) Organic phase ratio of mobile phase was changed by ± 2 % absolute. [i.e., Mobile phase A as such and Buffer solution: Methanol (22:78) % v/v for - 2 % and [i.e., Mobile phase A as such and Buffer solution: Methanol (18:82) % v/v for + 2 %]
3) Flow rate was changed by ± 10 % (i.e., 0.9 mL / min and 1.1 mL/min).
4) Mobile phase buffer pH was changed by ±0.1 units (i.e., 3.80 and 4.00).

Acceptance criteria

System suitability Criteria are within the limit for each altered condition.

Assessment of Robustness study

Since the system suitability requirement i.e., the signal to noise ratio for both peak is NLT 10, theoretical plates for both peak are NLT 10000, tailing factor for both peak are NMT 2.0 and % RSD of six replicate standard injections for both peak is NMT 5.0% is met for all the above mentioned changed conditions except pH, method is sensitive for change in pH of buffer solution, hence care should be taken while pH measurement it proves that the method is robust.

Table 20: Robustness data of Emtricitabine

Conditions	% RSD	Tailing factor	Theoretical Plates	RT (minutes)
Normal condition	1.4	1.0	145467	28.94
Column oven temperature was changed by -5°C (i.e.20°C)	1.1	1.0	158408	30.08
Column oven temperature was changed by + 5°C (i.e.30°C)	0.5	1.0	132938	27.82
Organic phase ratio of mobile phase was changed by - 2 % [i.e., Mobile phase A as such and Buffer solution: Methanol (22:78) % v/v]	2.0	1.0	142494	28.96
Organic phase ratio of mobile phase was changed by + 2 % [i.e., Mobile phase A as such and Buffer solution: Methanol (18:82) % v/v]	0.9	1.0	149606	28.75
Flow rate was changed by - 10 % (i.e., 0.9 mL/min)	0.7	1.1	154174	30.02
Flow rate was changed by + 10 % (i.e., 1.1 mL/min)	1.6	1.1	138112	27.93
Mobile phase buffer pH was changed by - 0.1(i.e., pH 3.80)	6.4	1.1	133125	28.31
Mobile phase buffer pH was changed by + 0.1(i.e., pH 4.00)	0.6	1.0	136879	28.55

Table 21: Robustness data of Tenofovir Disoproxil fumarate

Conditions	% RSD	Tailing factor	Theoretical Plates	RT (minutes)
Normal condition	0.6	1.1	659947	69.63
Column oven temperature was changed by -5°C (i.e.20°C)	0.7	1.1	645807	70.38
Column oven temperature was changed by + 5°C (i.e.30°C)	0.3	1.1	667762	68.85
Organic phase ratio of mobile phase was changed by - 2 % [i.e., Mobile phase A as such and Buffer solution: Methanol (22:78) % v/v]	0.9	1.1	647166	70.49
Organic phase ratio of mobile phase was changed by + 2 % [i.e., Mobile phase A as such and Buffer solution: Methanol (18:82) % v/v]	0.5	1.1	651285	68.53
Flow rate was changed by - 10 % (i.e., 0.9 mL/min)	0.2	1.1	664274	70.60
Flow rate was changed by + 10 % (i.e., 1.1 mL/min)	0.7	1.1	642257	68.62
Mobile phase buffer pH was changed by - 0.1 (i.e., pH 3.80)	2.7	1.1	646787	69.13
Mobile phase buffer pH was changed by + 0.1(i.e., pH 4.00)	0.3	1.1	672029	69.41

REFERENCES

1. Tripathi K D, 2001. Essential of Medical Pharmacology, Jaypee brother, New Delhi, 4th edition.

2. Satoskar RS, Bhandarkar SD, Ainapure SS, 2003. Pharmacology and Pharmacotherapeutics, Mumbai: Popular prakashan, 16th edition, 861.
3. Torchilin VP, 2005. Recent advances in HPLC, International Journal of Pharmaceutics, 4th edition, pp.145-160.
4. Y Haribabu, K Nihila, VK Sheeja, MB Akhil 2021. Method development and validation for simultaneous estimation of lamivudine, dolutegravir and tenofovir disoproxil fumarate in bulk and pharmaceutical dosage form using RP-HPLC and its application to in-vitro dissolution study. Jour. of Med. P'ceutical & Allied. Sci. V 10 - I 4, 1165 P- 3202-3207. doi: 10.22270/jmpas.V10I4.1165.
5. Sunita Shailajan, Sasikumar Menon, Ashish Singh, Mandar Mhatre, Neelam Sayed, Harshvardhan Joshi, Bhavesh Tiwari, 2019. Estimation of psoralen from herbal formulations containing *Psoralea corylifolia* using the RP‑HPLC‑DAD method and its application to a pharmacokinetic study, Volume 6 Issue 3, page- 217-223, DOI: 10.4103/0973-8258.104935.
6. Saravanan V, Revathi R, Meera N. 2016. Method development and validation for the simultaneous estimation of lycopene and ubidecarenone by rp-hplc in combined pharmaceutical dosage form. Jddt, 6(5):46-1. DOI: 10.22270/jddt.v6i5.1295.

How to cite this article

Jay Bhatt*, Ujashkumar A Shah, Hirak kumar V Joshi, J K Patel, 2021. Analytical method development and validation of related substances by rphplc of emtricitabine and tenofovir disoproxil fumarate tablets. Jour. of Med. P'ceutical & Allied. Sci. V - 10 - I 5, 1184, P-3656-3665. doi: 10.22270/ jmpas.V10I5.1184.