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Development and Validation of a Method for Simultaneous Determination of Tenofovir Disoproxil Fumarate Emtricitabine and Isoniazid in Bulk and Pharmaceutical Dosage Form Using RP-HPLC with Relevance to Toxicological Aspects

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Abstract

The proposed analytical methods are simple, novel, economical, rapid, sensitive, reproducible and accurate for the simultaneous estimation of Tenofovir Disoproxil Fumarate, Emtricitabine and Isoniazid in bulk and pharmaceutical dosage form by using RP-HPLC. This Method gives reliable assay results with short analysis time using mobile phase of Acetonitrile: 0.02M Potassium Dihydrogen Orthophosphate: water (pH 5.3) in the ratio of 60: 25: 15, respectively. Retention time was found to be 2.3, 3.7 and 4.9 min for Tenofovir Disoproxil Fumarate, Emtricitabine and Isoniazid, respectively. System suitability parameters were in the desired limit. This method has been developed and optimized as per ICH Q2 (R1) guidelines. With Sunfire C18 column, the drugs get eluted with good peak and the pressure was within the limit. Validation parameters, Linearity was found to be 10–50 µg/ml for Emtricitabine and 15–75 µg/ml for both Tenofovir Disoproxil Fumarate and Isoniazid. Correlation coefficient was found to be 0.999, 0.999 and 0.999 for Tenofovir Disoproxil Fumarate, Emtricitabine and Isoniazid, respectively. Accuracy was found to be 100.3%, 100.1% and 100.3% for Tenofovir Disoproxil Fumarate, Emtricitabine and Isoniazid, respectively. Precision was found to be 0.7, 0.6 and 0.7 for Tenofovir Disoproxil Fumarate, Emtricitabine and Isoniazid, respectively. LOD and LOQ were found to be 1.08, 3.2 for Emtricitabine, 1.69, 5.1 for Tenofovir Disoproxil Fumarate and 1.10, 3.3 for Isoniazid. Specificity (Blank interference), no peaks were observed at the retention time of Tenofovir Disoproxil Fumarate, Emtricitabine and Isoniazid in the Chromatogram of blank. System suitability, Tailing factor was 1.38, 1.42 and 1.51, Theoretical plates was 2079, 3388 and 4093 of Tenofovir Disoproxil Fumarate, Emtricitabine and Isoniazid, respectively. Resolution was found to be within 2 min for three drugs. % RSD for all the validation parameters were within the limit (NMT 2.0).

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Keywords: Tenofovir disoproxil fumarate, emtricitabine, isoniazid, pharmaceutical dosage form, RP-HPLC

INTRODUCTION

Emtricitabine is a nucleoside reverse transcriptase inhibitor (NRTI) indicated for the treatment of HIV infection in adults or combined with tenofovir alafenamide for the prevention of HIV-1 infection in high-risk adolescents and adults [1]. Emtricitabine is a cytidine analogue [2]. The drug works by inhibiting HIV reverse transcriptase, preventing transcription of HIV RNA to DNA. IUPAC Name: 4-amino-5-fluoro-1-[(2R,5S)-2-

(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one. Chemical Formula is $C_8H_{10}FN_3O_3S$. Molecular Weight is 247.247 g·mol^{-1}. Emtricitabine is a white to off-white powder with a solubility of approximately 112 mg/mL in water at 25°C. The log P for Emtricitabine is -0.43 and the pKa is 2.65.

Tenofovir disoproxil fumarate (a prodrug of tenofovir), marketed by Gilead Sciences under the trade name *Viread*, belongs to a class of antiretroviral drugs known as nucleotide analogue reverse transcriptase inhibitors (nRTIs) [3]. This drug is prescribed in combination with other drugs for the management of HIV infection as well as for Hepatitis B therapy. Tenofovir belongs to a class of antiretroviral drugs known as nucleotide analog reverse transcriptase inhibitors (ntRTIs), which block reverse transcriptase, an enzyme necessary for viral production in HIV-infected individuals [4]. This enables the management of HIV viral load through decreased viral replication. IUPAC Name (2E)-but-2-enedioic acid; bis({[(propan-2 yloxy) carbonyl]oxy}methyl){[(2R)-1-(6-amino-9H-purin-9-yl)propan-2yl]oxy}methanephosphonate. Chemical Formula is $C_{23}H_{34}N_5O_{14}P$. Molecular Weight is 635.51 g·mol^{-1}.

Isoniazid is an antibiotic used to treat mycobacterial infections; most commonly used in combination with other antimycobacterial agents for the treatment of active or latent tuberculosis [5]. Isoniazid is a prodrug and must be activated by bacterial catalase. Specifically, activation is associated with reduction of the mycobacterial ferric KatG catalase-peroxidase by hydrazine and reaction with oxygen to form an oxyferrous enzyme complex [6]. Once activated, Isoniazid inhibits the synthesis of mycolic acids, an essential component of the bacterial cell wall [7]. At therapeutic levels Isoniazid is bactericidal against actively growing intracellular and extracellular Mycobacterium tuberculosis organisms. Specifically, Isoniazid inhibits InhA, the enoyl reductase from Mycobacterium tuberculosis, by forming a covalent adduct with the NAD cofactor [8]. It is the INH-NAD adduct that acts as a slow, tight-binding competitive inhibitor of InhA. IUPAC Name pyridine-4-carbohydrazide. Molecular Formula C6H7N3O. Molecular Weight 137.13 as seen in Figures 1–3.

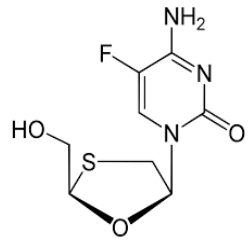

Figure 1. Structure of Emtricitabine.

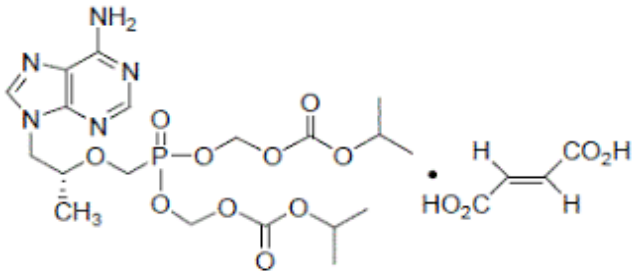

Figure 2. Structure of Tenofovir DF.

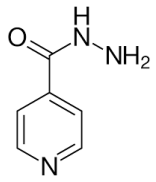

Figure 3. Structure of Isoniazid.

The literature survey revealed that there are really few approaches reported in the literary works for evaluation of Emtricitabine, Tenofovir disoproxil fumarate, Isoniazid alone or in combination with various other drugs in the pure form as well as drugs formulations by RP-HPLC [9–15]. In view of the demand for an appropriate, cost-effective RP-HPLC method for routine analysis of Emtricitabine, Tenofovir disoproxil fumarate, Isoniazid synchronized evaluation of in pharmaceutical dose type. Attempts were made to establish easy, precise, accurate as well as cost-efficient logical method for the

estimate of Emtricitabine, Isoniazid as well as Tenofovir disoproxil fumarate. The recommended approach will be validated according to ICH guidelines. The objective of the recommended work is to establish a brand-new, simple, delicate, exact and economical logical method as well as recognition for the synchronized evaluation of Emtricitabine, Isoniazid and Tenofovir disoproxil fumarate in pharmaceutical dose kind by utilizing RP-HPLC. To verify the established method based on ICH standards for the desired analytical application.

MATERIALS AND METHODS

Chemicals and Reagents

Emtricitabine, Isoniazid and also Tenofovir disoproxil fumarate were purchased from market. NaH_2PO_4 was analytical grade supplied by Finerchem limited, orthophosphoric acid (Merck), and water and methanol for HPLC (Lichrosolv (Merck).

Equipment and Chromatographic Conditions

The chromatography was performed on a Waters 2695 HPLC system, equipped with an auto sampler, UV detector and Empower 2 software. Analysis was carried out at 250 nm with column SunfireTM C18 (250 x 4.6 mm), 5 μm, dimensions at 25ºC temperature. The optimized mobile phase consists of Acetonitrile: 0.02M Potassium Dihydrogen Orthophosphate Buffer: Water. Flow rate was maintained at 0.4 ml/min.

PREPARATION OF SOLUTIONS

Preparation of Mobile Phase

A mixture of Acetonitri: 0.02M Potassium Dihydrogen Orthophosphate buffer: Water (60:25:15) was prepared. The pH of the mobile phase was checked. The resultant mobile phase was degassed in an ultra sonicator for 15 min.

0.02 M Potassium Dihydrogen Orthophosphate buffer was prepared by dissolving 3.12 g of Potassium Dihydrogen Orthophosphate in 1000 ml of water.

Preparation of Standard Stock Solution

Transfer an accurately weighed quantity of about 10 mg of Tenofovir Disoproxil Fumarate, 10 mg of Emtricitabine and 10 mg of Isoniazid separately in 10 ml Standard flask and added 10 ml with Distilled Water. From the stock solution, working standard solution of drugs was prepared by appropriate dilution with mobile phase. Stock calibration curve was prepared from 15–75 μg/ml for Tenofovir Disoproxil Fumarate, 10–50 μg/ml for Emtricitabine and 15–75 μg/ml for Isoniazid.

Preparation of Sample Solution

Weigh and powder 10 tablets. Transfer an accurately weighed quantity of finely powdered tablets equivalent to 10 mg of Emtricitabine and 15 mg of Tenofovir Disoproxil Fumarate and Isoniazid in 10 ml volumetric flask, add about 10 ml of mobile phase is added and sonicate for 15 min, then filter it with Whatman filter paper (No.41) and dilute to volume with mobile phase and mix.

The Diluents

The mobile phase was used as the diluent.

Procedure

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

METHOD

The developed chromatographic method was validated for system suitability, linearity accuracy, precision, ruggedness and robustness as per ICH guidelines.

System Suitability Parameters

To evaluate system suitability parameters such as retention time, tailing factor and USP theoretical plate count, the mobile phase was allowed to flow through the column at a flow rate of 0.6 ml/min for 12 minutes to equilibrate the column at ambient temperature. Chromatographic separation was achieved by injecting a volume of 20 µL of standard into Sunfire^{TM} C18 (250 x 4.6 mm), 5 µm, the mobile phase of composition Acetonitrile: 0.02M Potassium Dihydrogen Orthophosphate Buffer: Water, Mobile phase ratio: 60: 25: 15 was allowed to flow through the column at a flow rate of 0.6 ml per minute. Retention time, tailing factor and USP theoretical plate count of the developed method are shown in Table 1.

VALIDATION OF ANALYTICAL METHOD

Linearity

A calibration curve is the relationship between the instrument response and known concentration of analyte. It was observed that the optimized methods were linear with in a specific concentration range for individual drugs. The calibration curve was constructed by plotting the Peak area Vs Concentration of calibration standards. The standards were found to be linear in the concentration range of 10–50 µg/ml for Emtricitabine, 15–75 µg/ml for Tenofovir Disoproxil Fumarate (Figure 13) and 15–75 µg/ml for Isoniazid. The results are shown in Tables 2 to 4 and Figures 4 to 6.

Accuracy Studies

Accuracy of the optimized method was determined by absolute and relative recovery. It was found out by replicate analysis of samples containing known amount of the analyte. A minimum of three concentrations in the range of expected study sample was recommended. Based on the calibration curve, accuracy of Emtricitabine, Tenofovir Disoproxil Fumarate and Isoniazid was found by using three concentrations (10, 30, 50 µg/ml in Emtricitabine) and (15, 30, 45 µg/ml in Tenofovir Disoproxil Fumarate and Isoniazid) as LQC (low quality control), MQC (middle quality control), HQC (high quality Control). Each concentration range was injected six times. The results are shown in Table 5.

Precision Studies

Intraday precision was found by carrying out the analysis at three different concentrations in the linearity range (10, 20, 30 µg/ml) for Emtricitabine and (30, 45, 60 µg/ml) for Tenofovir Disoproxil Fumarate and Isoniazid for three times on the same day. The results are shown in Table 6.

LOD and LOQ

The sensitivity of RP-HPLC was determined from LOD and LOQ. Which were calculated from the calibration curve using the following equations as per ICH guidelines. The results are shown in Table 7 and Figures 7–9.

$LOD = 3.3\sigma/S$ and

$LOQ = 10\ \sigma/S$, where

σ= Standard deviation of y intercept of regression line,

S = Slope of the calibration curve

Table 1. System suitability parameters.

Parameters	Drugs		
	EMT	*TDF*	*INH*
Tailing Factor	1.42	1.38	1.51
Resolution	1.1	1.6	1.3
Theoretical Plate(N)	2079	3388	4093

RESULTS AND DISCUSSION

Tenofovir disoproxil fumarate, sold under the trade name Viread by Gilead Sciences, functions as a prodrug of tenofovir and falls within the category of nucleotide analogue reverse transcriptase inhibitors

(NtRTIs), which play a pivotal role in managing both HIV and hepatitis B infections. By interfering with the activity of reverse transcriptase, a key enzyme essential for viral replication in HIV-infected individuals, tenofovir effectively reduces viral load when combined with other therapeutic agents. Identified by its IUPAC name (2E)-but-2-enedioic acid; bis({[(propan-2-yloxy)carbonyl]oxy}methyl){[(2R)-1-(6-amino-9H-purin-9-yl)propan-2yl]oxy}methanephosphonate, this compound significantly contributes to the management of these viral infections.

In contrast, isoniazid serves as an antibiotic in the treatment of mycobacterial infections, primarily tuberculosis, often administered in conjunction with other antimycobacterial drugs. Operating as a prodrug, it requires activation by bacterial catalase, specifically catalase-peroxidase, facilitated through hydrazine reduction and interaction with oxygen, resulting in the formation of an oxyferrous enzyme complex. Upon activation, isoniazid disrupts the synthesis of mycolic acids, crucial components of the bacterial cell wall. Within therapeutic thresholds, it demonstrates bactericidal effects against both intracellular and extracellular Mycobacterium tuberculosis organisms. Specifically, isoniazid inhibits the activity of InhA, the enoyl reductase from Mycobacterium tuberculosis, through the formation of a covalent adduct with the NAD cofactor, thus acting as a slow, tightly binding competitive inhibitor. This mechanism effectively hampers bacterial growth and proliferation.

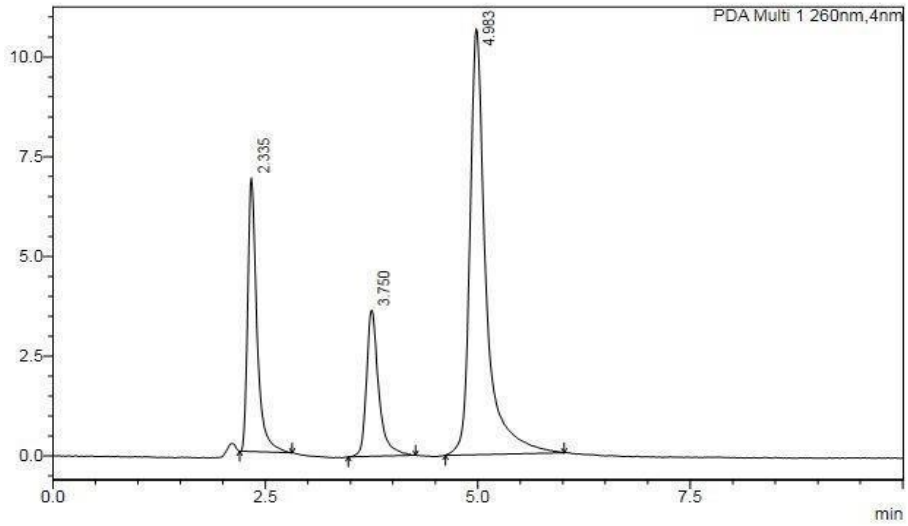

Figure 4. Standard chromatogram.

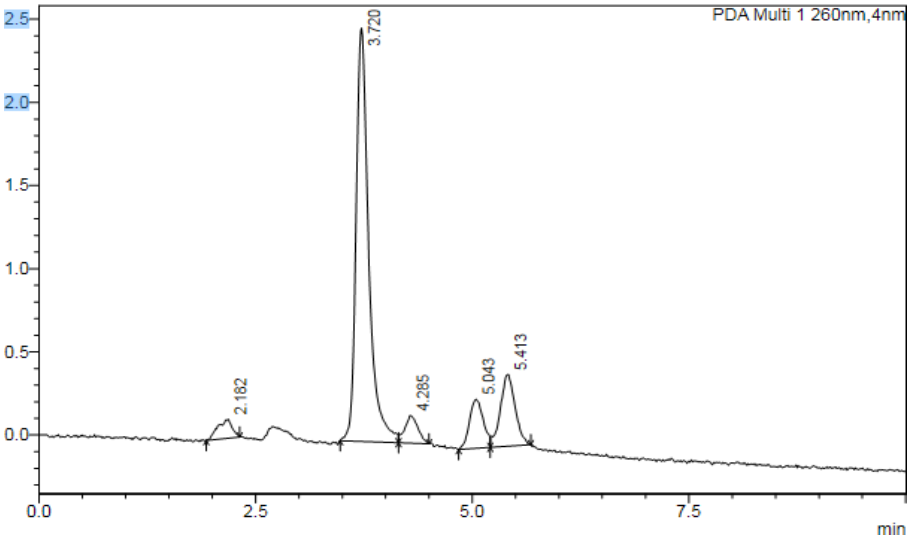

Figure 5. Sample chromatogram.

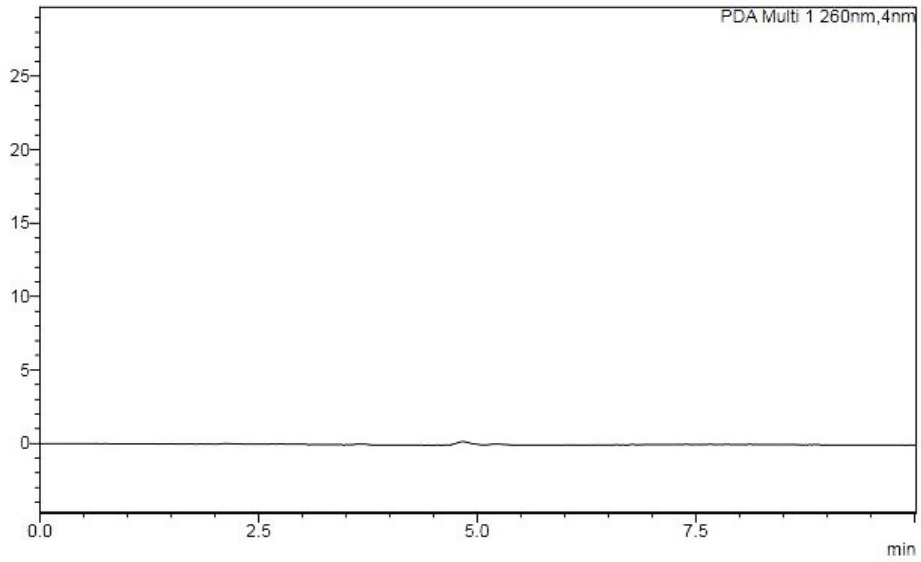

Figure 6. Blank chromatogram.

Table 2. Linearity results of Emtricitabine.

Concentration µg/ml	Peak area
10	61968
20	72678
30	81728
40	91904
50	101024

Table 3. Linearity results of Tenofovir DF.

Concentration µg/ml	Peak area
15	84567
30	94675
45	106789
60	116749
75	127564

Table 4. Linearity results of Isoniazid.

Concentration µg/ml	Peak area
15	94562
30	109562
45	123491
60	136528
75	149562

Table 5. Showing accuracy results for Emtricitabine, Tenofovir DF and Isoniazid.

S.no	Level	% Recovery			% RSD		
		EMT	*TDF*	*INH*	*EMT*	*TDF*	*INH*
1	80%	100.9	100.2	100.4	1.3	0.7	0.8
2	100%	99.8	100.4	100.4			
3	120%	100.3	99.98	100.3			

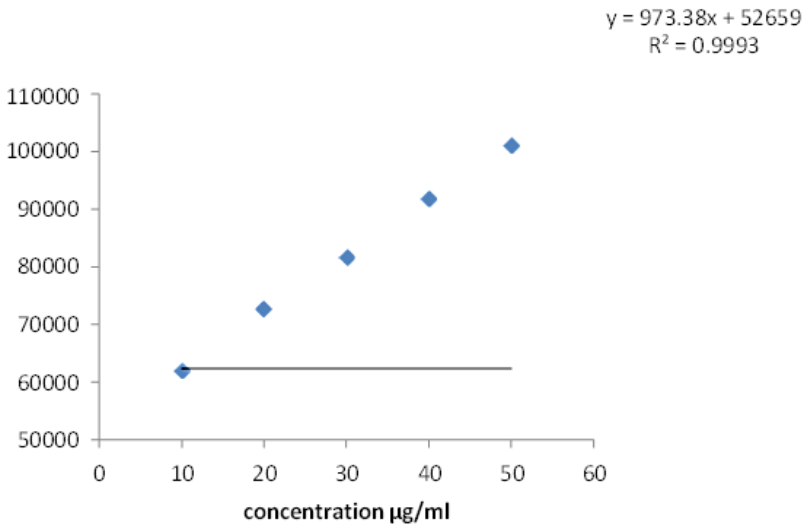

Figure 7. Linearity graph for Emtricitabine.

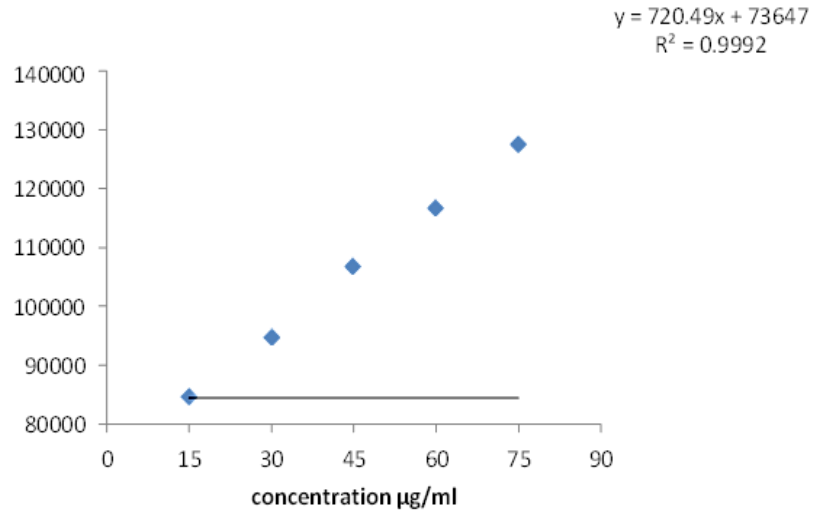

Figure 8. Linearity graph for Tenofovir DF.

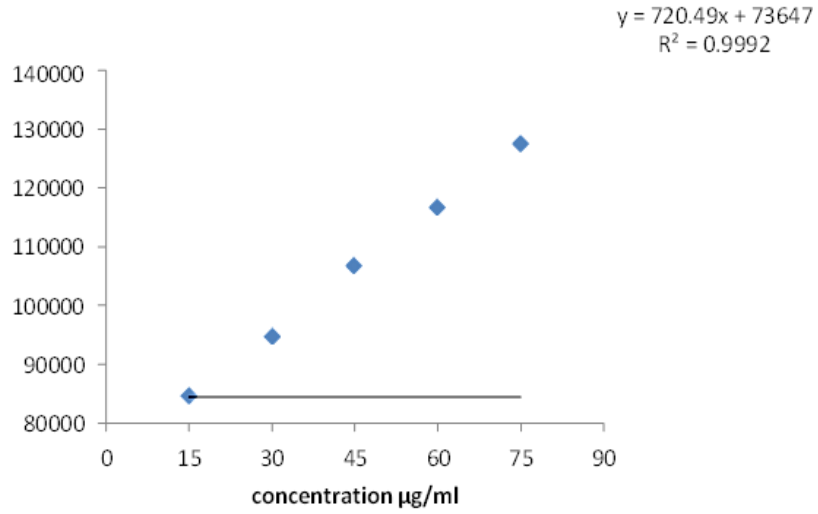

Figure 9. Linearity graph of Isoniazid.

Table 6. Precision results for Emtricitabine, Tenofovir DF and Isoniazid.

Level	Concentration (µg/ml)			Peak area			%RSD		
	EMT	*TDF*	*INH*	*EMT*	*TDF*	*INH*	*EMT*	*TDF*	*INH*
1	20	30	30	72678	94675	109562	0.2	0.3	0.2
				72492	94219	109259			
				72798	94751	109757			
2	30	45	45	81728	106789	123491	0.2	0.2	0.1
				81509	106243	123270			
				81376	106575	123511			
3	40	60	60	91904	116749	136528	0.2	0.2	0.1
				91722	116453	136279			
				92094	116997	136798			

Table 7. LOD, LOQ of Emtricitabine, Tenofovir DF and Isoniazid.

Drugs	Parameters	
	LOD	*LOQ*
EMT	1.08	3.2
TDF	1.69	5.1
INH	1.10	3.3

CONCLUSION

The developed HPLC method was validated and it was found to be simple, precise, accurate and sensitive for the simultaneous estimation of Emtricitabine, Isoniazid and Tenofovir DF in its pure form and in its pharmaceutical dosage forms. Hence, this method can easily and conveniently adopt for routine quality control analysis of Emtricitabine, Isoniazid and Tenofovir DF in pure and its pharmaceutical dosage forms.

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