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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF INVITRO DISSOLUTION STUDIES OF EFAVIRENZ, LAMIVUDINE AND TENOFOVIR DISOPROXIL FUMARATE IN PHARMACEUTICAL DOSAGE FORM BY RP-HPLC

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ABSTRACT

A simple, specific, precise, accurate and rapid reversed phase HPLC method with PDA detector has been developed and subsequently validation for simultaneous estimation of efavirenz (EFV), lamivudine(LMI) and tenofovir disoproxil fumarate(TDF) in their combined tablet dosage form. The separation was based on the use of Hypersil C_{18} (150x4.6mm, i.d, 5µm). The gradient elution achieved with in 20min,pH 4 phosphate buffer as mobile phase A and degassed mixture of water and acetonitrile (30:70) as mobile phase B. The separation was carried out at 35°C temperature with a flow rate of 1ml/min Quantitation was achieved with PDA detection at 260nm with linear calibration curves at concentration ranges 150 - 900µg/ml of Efavirenz, 75-450µg/ml of Lamivudine and 75-450µg/ml of Tenofovir. The correlation coefficient of Efavirenz, Lamivudine and Tenofovir was found to be 0.997, 0.9997 and 0.993 respectively which indicates a perfect correlation. The recoveries obtained were 98.16-100.93% for Efavirenz, 99.16-100.86% for Lamivudine and 98.2-101.1 for Tenofovir. The good percentage recovery of the sample clearly indicates the reproducibility and accuracy of the developed method. Similarly the %RSD value for precision was also found to be within the acceptable limit. The method was validated according to international conference of harmonization guidelines in terms of accuracy, precision, specificity, robustness, linearity and other aspects of analytical validation.

Keywords: Efavirenz, Lamivudine and Tenofovir disoproxil fumarate, RP-HPLC

INTRODUCTION

Antiretroviral drugs like nucleoside reverse transcriptase inhibitors, non nucleoside reverse transcriptase inhibitors and protease inhibitors are essential in the management of HIV infection. The synthetic non nucleoside reverse transcriptase inhibitor analogues Efavirenz and nucleoside reverse transcriptase inhibitors Lamivudine and Tenofovir Disoproxil Fumarate form one of the fixed dosage combinations used in the effective management of HIV. Efavirenz (EFV), (4S)-6-chloro-4-(cyclopropylethynyl) -4- (trifluromethyl) – 1 – 4 – dihydro - 2H- 3, 1- enzoxazin-2-one, is an antiretroviral drug which is a non-nucleoside reverse transcriptase inhibitor (NNRTI) [1,2Figure] 1. EFV has been determined by UV spectroscopic[3, 24] and RP-HPLC[4] methods in single and in combined dosage form[18,23].

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Tenofovir disoproxil fumarate (TDF), 9-((R)-2- (bis (((isopropoxycarbonyl) oxy) methoxy)phosphinyl) methoxy)propyl) adenine fumarate (1:1), is a nucleotide analogue reverse transcriptase inhibitor (nRTIs)[1,2]figure 2.TDF has been determined in spiked human plasma by HPLC[5,6] and other combination[14-17,20,22,23,] . The estimation of TDF by RP-HPLC has been reported[4,7]. Lamivudine (LMI), (2R,cis)-4-amino- 1-(2-(hydroxylmethyl-1,3-oxathiolan-5-yl)-(1H) pyrimidin-2-one, is nucleoside-reverse transcriptase inhibitor (NRTI)[1,2] figure 3.

It is an analogue of cytidine. The estimation of lamivudine using UV[3,8–10,21] spectroscopy and HPLC has been reported [7,11,25]. Although the combination of EFV, LMI and TDF is not available commercially in the market, it is in phase 3 clinical trial and the safety and efficacy of TDF in combination with LMI and EFV has already been report[12,13]. This study revealed that once daily regimen containing EFV, TDF and LMI is virologically and immunologically effective, well tolerated and safe with benefits in the lipid profile in the majority of patient. Hence, the objective of the work is to develop new spectophotometric methods for estimating EFV, TDF and LMI in pharmaceutical formulation with good accuracy, simplicity, precision and economy.

Figure 1: Structure of Efavarienz

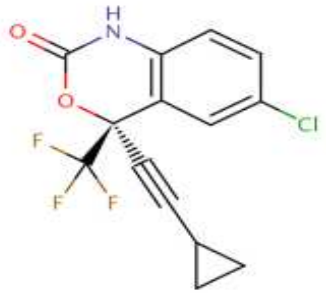


Figure 2: Structure of Tenofovir disoproxil fumarate

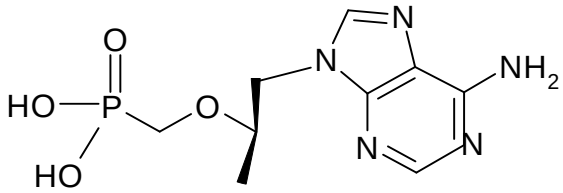
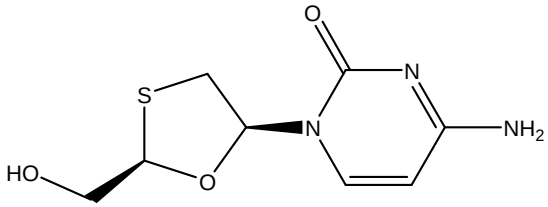


Figure 3: Structure of Lamivudine



MATERIALS AND METHODS

Reagents and Chemicals Used:

All the solvent and reagent used were HPLC and spectroscopic grade. HPLC grade, methanol, acetonitrile and Millipore water obtained from (Milli Q) were used in all experiments, sodium dihydrogen phosphate and sodium lauryl suphate was AR grade are used. Efavirenz, Lamivudine and Tenofovir disoproxil fumarate are used as working of reference standard.

Instrumentation:

The chromatographic separation performed using Waters 2695 HPLC system with PDA detector. Software was used Empower version 2 to monitor and integrate the output single. The dissolution test was carried out using Electro lab dissolution test system. Waters auto injector, thermostatted column compartment and Photo Diode Array detector was used. Waters column (Hypersil BDS C-18 150mm X 4.6 mm X 5µ particle size) was used for the analysis.

Media Preparation:

200g of SLS is accurately weighed & transferred into 10L Demineralized water. Mixed well and sonicated to Dissolve.

Dissolution Parameters:

Medium	: Water+2%SLS
Volume	: 1000ml
Apparatus	: Paddle
Agitation	: 75RPM
Time	: 60min
Temperature	: 37°C
Volume Withdrawn	: 10ml

Preparation of Mobile Phase:

Mobile Phase A:

3.12g of Sodium Di Hydrogen Phosphate is accurately weighed and transferred into 1000ml of MilliQ water, mixed well and filtered with 0.45µm membrane filter and sonicated for degassing (buffer pH 4).

Mobile Phase B:

300ml of MilliQ water is mixed with 700ml of Acetonitrile and sonicated to degas.

Diluent Preparation:

Dissolution media used as diluent.

Preparation of Standard solution:

60.5 mg of Efavarienz, 30.3 mg of Lamivudine and 30.7 mg of Tenofovir were weighed accurately to 100 mL volumetric flask. 70 mL of diluent was added and sonicated for 10 min under cold condition (2º to 8º). Make up to the volume with diluent. Filter through 0.45µ nylon membrane filter.

Sample Preparation:

Each Tablet (1775mg) is transferred into each of six Dissolution Vessels containing 1000ml of dissolution media, which is at 37°C. Run the apparatus as per the dissolution conditions mentioned. Sample is withdrawn at 60min. The sample is filtered.

Optimization of Chromatographic Conditions:

The initial literature search indicated that many HPLC methods are available for individual drugs and their combination with different drugs. Based on literature search, attempts were made to develop a simple method which has less retention time and high selectivity; top priority was given for complete separation of Efavirenz, Lamivudine and Tenofovir disoproxil fumarate. Several mobile phases were tested until good resolution obtained between two drugs.

In preliminary experiments all the three Efavirenz, Lamivudine and Tenofovir disoproxil fumarate were subjected to separation by reverse phase HPLC equipped with the Hypersil BDS C-18 (150mm X 4.6 mm X 5µm) column and with flow rate 1mL/min and detection wavelength of 260nm (figure 4). Column temperature was maintained at 35°C. Injection volume is 10µL and runtime is for 20min.

The mobile phase consists of buffer pH 4 and methanol: water (85:15). These drugs were able to be separated on the chromatogram but failed in peak purity. The effect of pH and mobile phase composition was checked. It improved peak purity. Acetonitrile was found to be better than methanol in terms of resolution and peak shape. Finally a method developed with buffer pH 4 and water: acetonitrile (30:70) by gradient program given in the table 1. The chromatogram obtained was better than the previous one in all aspects with good peak shape, tailing factor, resolution and theoretical plate as per USP

requirement. The retention times of Lamivudine, Tenofovir disoproxil fumarate and Efavirenz peaks are about 4.76, 8.00 and 13.78 minutes respectively. The chromatograms were shown in the figure 5 and 6.

Validation:

The method was successfully validated as per ICH guideline kQ2 (R1): validation of analytical procedures: text and methodology, international conference on harmonization, Food and Drug Administration, USA, November 2005. The method was validated and parameters were linearity, range, accuracy, precision, LOQ, LOD.

Specificity:

The method is found specific and there is no blank or placebo interference.

Precision:

To check the system precision (repeatability) for peak response obtained with five replicates of standard at specified concentration. The %RSD found to be within 2.0%. To check repeatability (method precision) of the method six individual sample preparations form same batch were prepared and injected the % RSD with six samples found to be within 5.0%. The results obtained were presented in table 4 and 5.

Accuracy:

The accuracy of an analytical method is established across its range. Accuracy is performed in four different levels for Lamivudine, Tenofovir & Efavirenz. The known quantity of Lamivudine/Tenofovir/Efavirenz is spiked at 25%, 50%, 100% and 125% level into the placebo. The samples is analysed in triplicate for each level. The % recovery values for all the three drugs were found to be in between 98.0% to 102.0% and %RSD values were found to be less than 2.0%.The accuracy results were tabulated in the table No.'s 6 to 8.

Linearity and range:

The Linearity of detector response to different concentration of all the three drugs was studied with a series of working standard solutions prepared by diluting the stock solution with diluents. The Standard plots were constructed between concentrations vs. peak area a linear response of peak area was observed over the concentration range of 150 to 900 µg/mL for EFV, 75 to 450 µg/mL for LMI and 75 to 450µg/mL for TDF. Ten micro-liter of each sample was injected under above chromatographic conditions and peak area was measured. The data of linearity curve was summarized in the tables 2, 3 and figures 7 to 9 and it was found that correlation coefficient (R^2) and regression analysis were within the limits.

Robustness:

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness was done by changing the column temperature (± 5°C), flow rate (± 10%), Changing the wavelength (± 5 nm).

Ruggedness:

This is to prove the lack of influence of operational and environmental variables of the test results by using the method. The average of the six preparations and % RSD for the six observations was calculated and recorded. The method precision was carried out as described above using different analyst, different column and different instrument. The % RSD for the six determinations shall be NMT 5.0.

Solution Stability:

A solution of standard and sample was prepared and stored at room temperature for 36 hrs. The stability of solutions was checked for 36 hours. The different time intervals are 0, 12, 22, 30, 36hr.

Filter Interference:

To establish the suitability of filter and to validate the interference of the filters with the sample or standard, the study was conducted using three different filters namely 0.45 µm PVDF filters, 0.45 µm PTFE and 0.45 nylon filters. The unfiltered standard solutions and the centrifuged sample solutions were compared with the filtered standard and samples. There is no interference of filters with standard and sample solutions as the difference in responses is within the limit. The %RSD was found to be less than 2.0%.

System Suitability:

According to USP system suitability tests are an integral part of chromatographic method validation. The tests were used to verify that the reproducibility of the chromatographic system is adequate for analysis. To ascertain its effectiveness system suitability tests were carried out on freshly prepared standard solution. 10µL of solution was injected into the optimized chromatographic system. For system suitability six replicates of working standard samples were injected and the parameters like retention time (RT), plate number (N), peak area and tailing factors of sample were calculated these results are presented in the table 9.

RESULTS AND DISCUSSION:

To optimize the mobile phase various proportions of buffers with methanol were tested. Mobile phase composition was changed and the method development was started by Hypersil BDS C-18 (150mm X 4.6 mm X 5µm) column and with flow rate 1mL/min and detection wavelength of 260nm. Column temperature was maintained at 35°C. Injection volume is 10µL and runtime is for 20min. The mobile phase consists of buffer pH 4 and acetonitrile: water (70:30) was used by gradient program. The retention times of Lamivudine, Tenofovir disoproxil fumarate and Efavirenz peaks are about 4.76, 8.00 and 13.78 minutes respectively.

Quantitative linearity was observed over the concentration range of 150 to 900 µg/mL for EFV, 75 to 450 µg/mL for LMI and 75 to 450µg/mL for TDF. The regression equations of concentration of Efavirenz, Lamivudine and Tenofovir disoproxil fumarate are found to be y = 7106.x + 172764.22, y = 20483.x -28749.33 and y = 13845 x-431703.75 respectively, where y is the peak area and x is the concentration of drugs (µg/mL).The correlation coefficient of Efavirenz, Lamivudine and Tenofovir disoproxil fumarate was found to be 0.9997, 0.997 and 0.993 respectively.

The numbers of theoretical plates obtained were 30168, 6391 and 26398 for Efavirenz, Lamivudine and Tenofovir disoproxil fumarate respectively which indicates the efficiency of the column. The high percentage recovery indicates that the proposed method is highly accurate. There is no interference of filters with standard and sample solutions as the difference in responses is within the limit. The %RSD was found to be less than 2.0%.

Figure 4: UV Spectrum of Efavirenz, Lamivudine and Tenofovir disoproxil fumarate

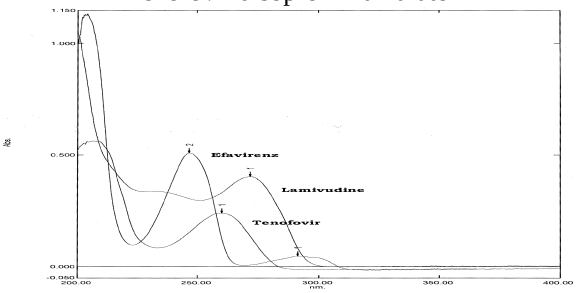


Figure 5: A Blank chromatogram of Efavirenz, Lamivudine and Tenofovir disoproxil fumarate

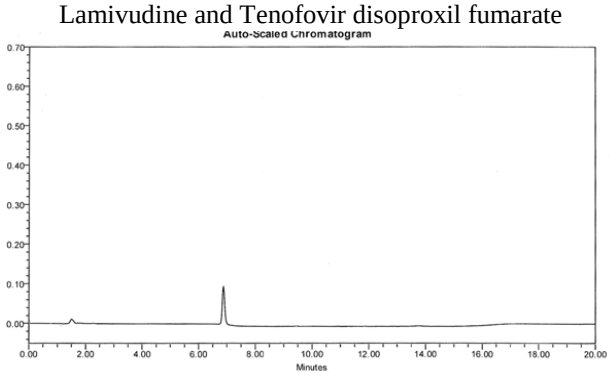


Figure 6: Standard chromatogram of Efavirenz, Lamivudine and Tenofovir disoproxil fumarate

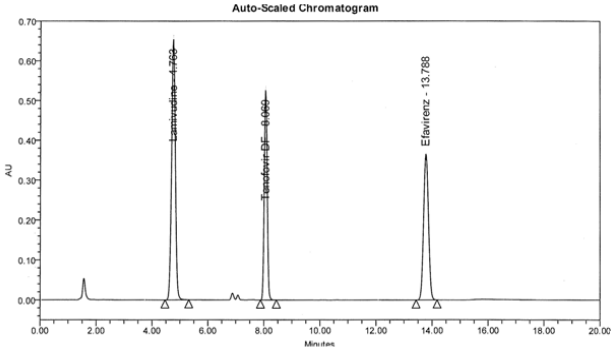


Figure 7: Linearity graph of Efavirenz

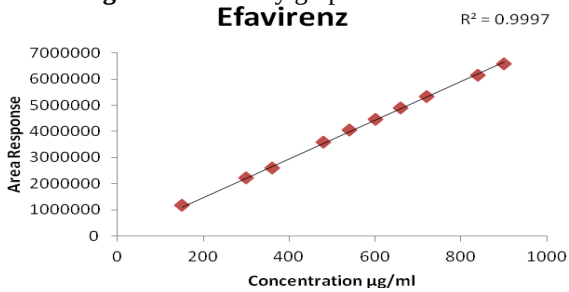


Figure 8: Linearity graph of Lamivudine

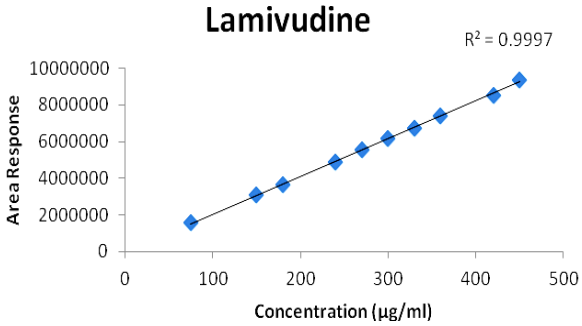


Figure 9: Linearity graph of Tenofovir DF

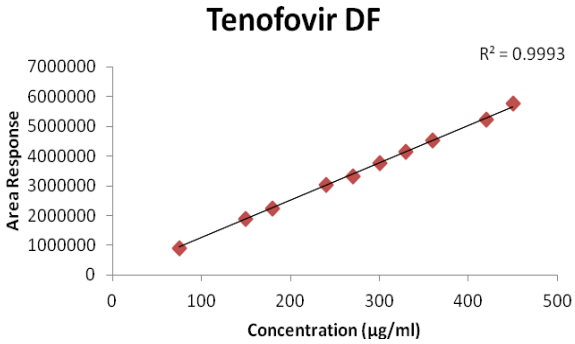


Table 1: Gradient program

Time (minutes)	Mobile Phase-A (%v/v)	Mobile Phase-B (%v/v)
0.0	93.0	7
4.0	93.0	7
5.0	20.0	80
12.0	20.0	80
13.0	93.0	7
20.0	93.0	7

Table 2: Linearity data showing equation of regression line and coefficient of determination

Drug	Conc. Range (µg/mL)	Equation	R^2
Efavirenz	150 - 900	y = 7106.x + 172764.22	0.9997
Lamivudine	75 – 450	y = 20483.x -28749.33	0.9997
Tenofovir DF	75- 450	y = 13845. x-431703.75	0.9993

Table 3: Linearity data of Efavirenz, Lamivudine and Tenofovir DF

S No	Efavirenz		Lamivudine		Tenofovir	
	Concentration (µg/ml)	Average area Response	Concentration (µg/ml)	Average area Response	Concentration (µg/ml)	Average area Response
1	150	1192027	75	1551348	75	655870
2	300	2336345	150	3103275	150	1584462
3	360	2725449	180	3652098	180	1934060
4	480	3589580	240	4882408	240	2832967
5	540	4048512	270	5539840	270	3334821
6	600	4453528	300	6153149	300	3749033
7	660	4887987	330	6754174	330	4138809
8	720	5326678	360	7419842	360	4528778
9	840	6051923	420	8543907	420	5242104
10	900	6593820	450	9370579	450	5761175

Table 4: System precision of Efavirenz, Lamivudine and Tenofovir DF

Injections	Efavirenz (Area)	Lamivudine (Area)	Tenofovir (Area)
1	4416022	6105371	3810374
2	4415373	6089848	3803005
3	4417435	6103935	3802905
4	4411915	6090946	3790045
5	4411915	6088055	3794309
Mean	4414450	6095632	3800127
SD	2614	8315	8007.8
% RSD	0.1	0.1	0.2

Table 5: Method precision of Efavirenz, Lamivudine and Tenofovir DF

Injection	Efavirenz		Lamivudine		Tenofovir	
	Area	% Drug Release	Area	% Drug Release	Area	% Drug Release
1	4279717	97.4	6018478	99.6	4030670	106.7
2	4491013	102.2	6230896	103.1	4029635	106.7
3	4227749	96.2	5953108	98.5	4050181	107.2
4	4431115	100.8	6157577	101.9	3920251	103.8
5	4208517	95.7	5946405	98.4	4018080	106.4
6	4161298	94.7	5912285	97.9	4033598	106.8
Average	--	97.8	--	99.9	--	106.27
SD	--	3.0031	--	2.1232	--	1.2356
% RSD	--	3.1	--	2.1	--	1.2

Table 6: Recovery studies of Efavirenz

S. No	Amount added (mg)	Amount Recovered (mg)	% Recovery	% RSD
1	150.1	149.0	99.3	0.8
2	150.2	149.1	99.2	
3	149.6	150.2	100.4	
4	299.0	302.9	101.3	0.2
5	299.1	303.0	100.9	
6	299.6	304.3	100.6	
7	598.4	594.4	99.3	0.2
8	598.6	593.8	99.1	
9	598.2	595.9	99.6	
10	747.7	734.4	98.2	0.1
11	747.8	734.5	98.1	
12	747.9	734.4	98.2	

Table 7: Recovery studies of Lamivudine

S. No	Amount added (mg)	Amount Recovered (mg)	% Recovery	% RSD
1	75.2	75.8	100.8	0.1
2	75.3	75.8	100.9	
3	75.1	75.8	100.9	
4	150.2	150.1	99.9	0.1
5	150.3	150.1	99.8	
6	150.3	150.1	99.9	
7	300.1	298.4	99.4	0.3
8	300.2	299.7	99.8	
9	300.2	299.7	99.8	
10	374.7	371.8	99.2	0.1
11	374.7	371.8	99.2	
12	375.3	371.8	99.1	

Table 8: Recovery studies of Tenofovir

S. No	Amount added (mg)	Amount Recovered (mg)	% Recovery	% RSD
1	74.2	72.9	98.2	0.1
2	74.1	72.7	98.4	
3	74.5	73.0	98.0	
4	148.2	146.8	99.1	0.6
5	148.2	145.5	98.2	
6	148.2	145.5	98.2	
7	295.6	298.6	101.0	0.3
8	295.6	298.6	101.0	
9	295.1	299.3	101.4	
10	369.3	369.4	100.0	0.4
11	369.5	370.2	100.3	
12	369.3	371.5	100.6	

Table 9: System suitability of Efavirenz, Lamivudine and Tenofovir DF

S. No	System Suitability Parameter	Observations		
		Efavirenz	Lamivudine	Tenofovir
1	RT (retention time)	13.76	8.0	4.78
2	% RSD (Relative standard deviation)	0.1	0.1	0.2
3	Tailing factor	1.0	1.0	1.0
4	USP plate count	30168	6391	26398
5	Resolution	22.0	--	15.0

CONCLUSION:

A simple, specific, accurate, precise, stability indicating reverse phase high performance liquid chromatography method has been developed which can be used accurately for quantitative estimation of Efavirenz, Lamivudine and Tenofovir disoproxil fumarate for routine analysis of individual and combination of drugs. Method was validated as per ICH Q2 (R2) so it can be used by analytical department.

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