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Research Article

A NEW GRADIENT RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF LAMIVUDINE, TENOFOVIR DISOPROXIL FUMARATE AND EFFAVIRENZ IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT

A new, simple, rapid and accurate gradient RP-HPLC method was developed for the estimation of Lamivudine (LAM), Tenofovir disoproxil fumarate (TDF) and Effavirenz (EFA) in pharmaceutical dosage forms and validated. The HPLC method was developed on SHISEIDO C18 column (250 x 4.6 mm i.d, 5µ) using Acetonitrile: 50mM phosphate buffer (pH 5.0) at 256 nm. The retention times for Lamivudine, Tenofovir disoproxil fumarate and Effavirenz were found to be 2.4, 4.1 and 12.2 min respectively. Linearity was established in the range of 5-25 µg/ml, 5-25 µg/ml and 10-50 µg/ml for LAM, TDF and EFA respectively. The coefficients of regression (R2) values were found to be 0.997, 0.995 and 0.997 for LAM, TDF and EFA respectively. The method was precise with %RSD < 2 for both intraday and interday precision. The accuracy of the method was performed over three levels of concentration and the recovery was in the range of 98-102%. The method was successfully applied for quantifying these drugs in marketed dosage forms.

Keywords: RP-HPLC, Tenofovir disoproxil fumarate, Effavirenz, Phosphate buffer.

INTRODUCTION

Lamivudine (LAM) is a nucleoside reverse transcriptase inhibitor and cytidine analog. It is used to treat human immune deficiency virus type 1 (HIV-1) and Hepatitis B (HBV). Chemically it is 4 - amino-1- [(2*R*, 5*S*) – 2 - (hydroxymethyl) - 1, 3 – oxathiolan – 5 – yl] - 1, 2 – dihydropyrimidin – 2 – one (Fig. 1). Tenofovir disoproxil fumarate (TDF) is a fumaric acid salt of the bis- isopropoxy carbonyl oxymethyl ester derivative of tenofovir. Chemically it is 9-[(R)-2[[bis[[(isopropoxycarbonyl) oxy] methoxy] phosphinyl] methoxy] propyl] adenine fumarate (Fig. 2). Effavirenz (EFA) is a non-nucleotide reverse transcriptase inhibitor (NNRT) and is used as a part of highly active anti-retroviral therapy (HAART) for the treatment of the Human Immuno Deficiency virus (HIV Type-1). Chemically it is (4*S*)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4- dihydro-1*H*-3,1-benzoxazin-2-one[1-3] (Fig. 3).

The present study is to develop a gradient RP-HPLC method for LAM, TDF and EFA. The Literature survey reports different analytical methods for LAM, TDF and EFA based on UV[4-5], HPLC[6-14], stability indicating HPLC[15-19], Fluorimetry[20], Chiral chromatography[21], LC-MS[22,23] were reported. However there were few methods reported for the simultaneous estimation of LAM, TDF and EFA by gradient chromatography, the present aim is to develop a more precise, accurate, simple and gradient RP-HPLC method for the estimation of LAM, TDF and EFA. The molar absorptivity of LAM, TDF and EFA were found maximum at

256nm. The validated method was used for the quantification of marketed formulation containing LAM, TDF and EFA.

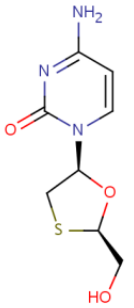

Fig. 1: Chemical structure of LAM

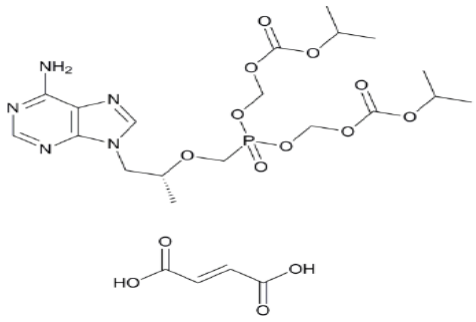

Fig. 2: Chemical structure of TDF

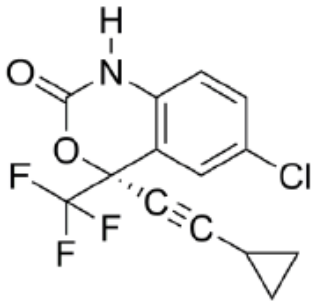

Fig. 3: Chemical structure of EFA

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Lamivudine (LAM), Tenofovir Disoproxil Fumarate (TDF) and Effavirenz (EFA) working standards were procured from Hetero Laboratories Ltd. Commercially available as TELURA (Tenofovir Disoproxil Fumarate /Lamivudine / Efavirenz Tablets IP 300 mg / 300 mg / 600 mg) tablets were purchased from the local Pharmacy of Tadepalligudem. HPLC grade water was purchased from Thermo Fisher Scientifics Ltd., Mumbai. HPLC grade Acetonitrile, Methanol and Orthophosphoric acid were procured from Merck specialties Pvt. Ltd., Mumbai.

Instrumentation and analytical conditions

RP-HPLC method was performed on the HPLC system (Shimadzu) consisting of binary gradient pump with UV detector (LC-20AD). Rheodyne injector with 20 µl fixed loop was used for injecting samples on SHISEIDO C18 column (250 x 4.6 mm i.d, 5µ) in the present study.

Preparation of solutions

Preparation of standard stock solutions

Standard stock solutions of LAM, TDF and EFA were prepared by transferring accurately weighed 100 mg of three drugs in to separate 100 ml volumetric flask. The compounds are then dissolved in few ml of

diluent (Methanol : Water 50:50 v/v) and sonicated for 30 minutes. Then the volume is made up to 100 ml with diluent to obtain a standard solution of LAM (1000 µg/ml), TDF (1000 µg/ml) and EFA (1000 µg/ml).

Preparation of working standard solutions
From the stock solution, 10 ml of three drugs were diluted to 100 ml with diluent in separate volumetric flasks to get the concentration of 100 µg/ml of LAM, 100 µg/ml of TDF and 100 µg/ml of EFA.

Preparation of calibration curve standard solutions
Calibration curve standards were prepared from working standards at concentrations of 5, 10, 15, 20 and 25 µg/ml for LAM, 5, 10, 15, 20 and 25 µg/ml for TDF and 10, 20, 30, 40 and 50 µg/ml for EFA.

Preparation of the sample solution
From the working standard solution pipetted out 1.5, 1.5 and 3.0 ml of LAM, TDF and EFA in to a 10 ml volumetric flask, volume is made up to 10 ml with Acetonitrile : Buffer (65 : 35)

Preparation of the mobile phase
The elution was gradient and the mobile phase consisted of a mixture of acetonitrile and 50 mM phosphate buffer PH-5 (B : A v/v). The buffer was prepared by dissolving 6.8g of potassium dihydrogen orthophosphate in 1000 ml water adjusted the pH with 10 M potassium hydroxide up to 5.0 ± 0.1. The buffer was filtered through a 0.5µ membrane filter before prior to use. Column was equilibrated for at least 30min with mobile phase flowing through the system.

Method validation
The developed method was validated according to International Conference on Harmonization guidelines for validation of analytical procedures. The developed method was validated with respect to parameters such as linearity, LOD and LOQ, precision, accuracy and specificity. Forced degradation studies were done according to ICH Harmonized Tripartite Guideline, Stability Testing of New Drug Substances and Products: Q1A (R2)[22].

System suitability
The system suitability of the HPLC method was determined by making six replicate injections from freshly prepared standard solutions and analyzing each solute for their retention time, theoretical plates number (N) and tailing factor (T).

Specificity
It is the ability to assess unequivocally the analyte in the presence of impurities, degradants and matrix. To determine this, 20 µl of blank, standard and sample solutions were injected separately in triplicate and respective chromatograms were recorded under the optimized conditions.

Linearity
The calibration curves were obtained with concentrations of the standard solutions of 5-25 µg/ml (50% to 150%), 5-25 µg/ml (50% to 150%) and 10-50 µg/ml (50% to 150%) for LAM, TDF and EFA respectively. Linearity was evaluated by regression analysis, which was calculated by the least square regression method.

Accuracy
To check the degree of accuracy recovery studies were performed in triplicate by the standard addition method at 50%, 100% and 150% levels.

Precision
Precision was checked by analyzing the samples at different time intervals of the same day (intra-day precision) as well as on different days (inter-day precision).

Limit of detection and limit of quantification
Limit of detection (LOD) and limit of quantification (LOQ) were calculated by using the values of Signal to Noise (S/N) ratio for three drugs. For LOD S/N ratio should be 3:1 and for LOQ S/N ratio should be 10:1

Robustness

Robustness was determined by analysis of samples under slight variations in chromatographic conditions. The flow rate of the mobile phase was changed from 0.9 ml/min to 1.1 ml/min. The ratio of the organic phase (Acetonitrile) was changed by +2% and -2%. The effect of retention time and peak parameters were studied.

RESULTS AND DISCUSSION

Method development and optimization

The standard solutions of LAM, TDF and EFA were scanned in UV spectrophotometer in the range of 200-400 nm. The λ_{max} of three drugs were found to be 255.45, 265.14 & 271.50 nm for LAM, TDF and EFA respectively. The iso-absorptive point for three drugs was observed as 256 nm (Fig. 4) which was selected for detection of drugs. Trails were performed using different columns (Hypersil BDS C18, Symmetry C18, Phenomenex C18 and Shiseido C18), buffers (Acetate, Phosphate, Ortho phosphoric acid), pH (3-6), organic phases (Acetonitrile, Methanol). Shiseido C18 column (250mm X 4.6mm I.D. 5 µ) produced good separation with efficient resolution and more theoretical plates. The drugs were eluted at a flow rate of 1.0 ml/min using a mobile phase consisting of Acetonitrile: 50mM phosphate buffer (pH 5.0) in gradient elution. The retention times of the drugs were observed to be 2.4, 4.1 and 12.2 min for LAM, TDF and EFA respectively.

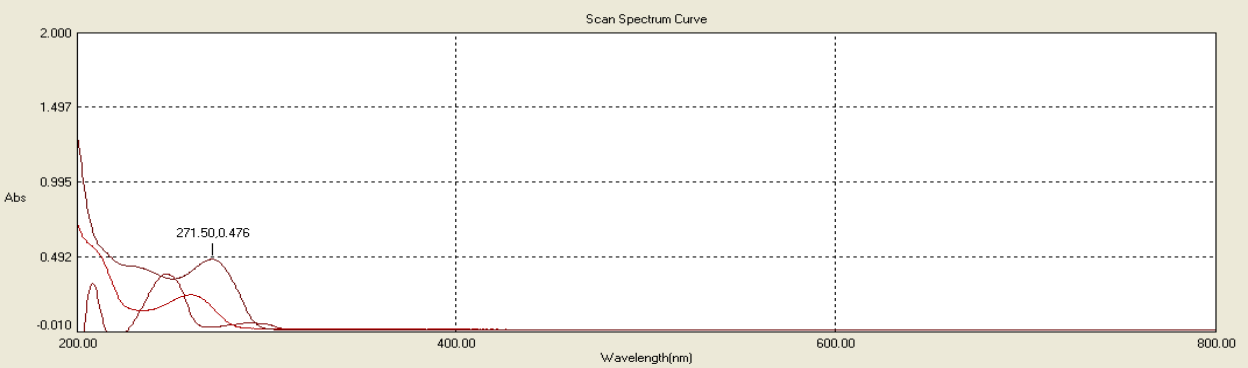

Fig. 4: UV Overlay spectrum of LAM, TDF and EFA

Gradient programming

Time	Mobile phase(B)	Mobile phase(A)
0.01	50	50
2.00	50	50
2.01	60	40
14.00	60	40
14.01	50	50
20	Controller	Stop

System suitability

Under optimized chromatographic conditions 20 µl of solution containing 1.5 µg/ml of LAM, 1.5 µg/ml of TDF and 3.0 µg/ml of EFA was injected into the system in six replicates. Chromatograms were recorded and studied for different system suitability parameters like retention time, peak area, number of theoretical plates, tailing factor and resolution. The system suitability parameters were shown in Table 1.

Table 1: System Suitability Parameters of LAM, TDF and EFA

Parameter	LAM	TDF	EFA
Retention Time (minutes)	2.4	4.1	12.2
Peak area	643120.4	434056.2	2031095.5
No. of Theoretical plates	3929	6345	23876
Tailing Factor	1.1	1.1	1.0
Resolution	--	9.89	22.21

Specificity

The HPLC chromatograms were recorded for blank (Fig. 4a) and standard (Fig. 4b) under optimized analytical conditions and compared for additional peaks, however found no additional peaks. The two peaks were completely separated in HPLC chromatogram and the resolution was found to be more than 2.

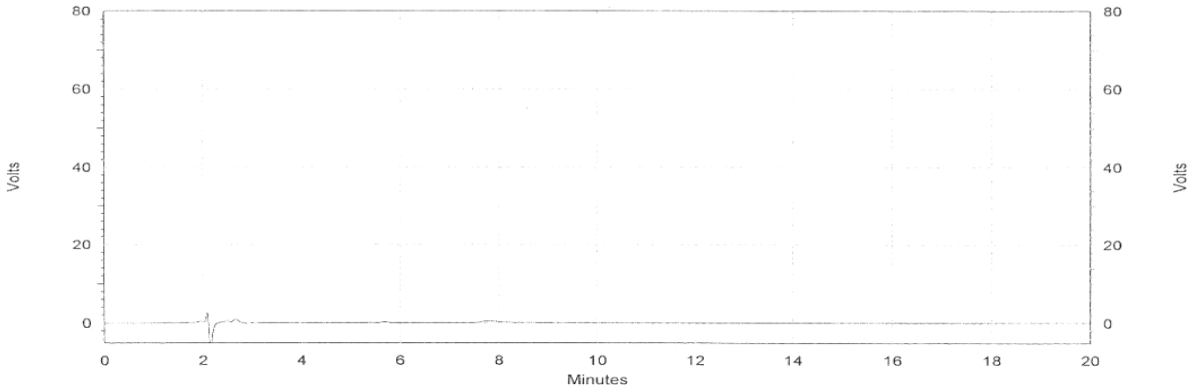

Fig. 4a: Chromatogram of blank

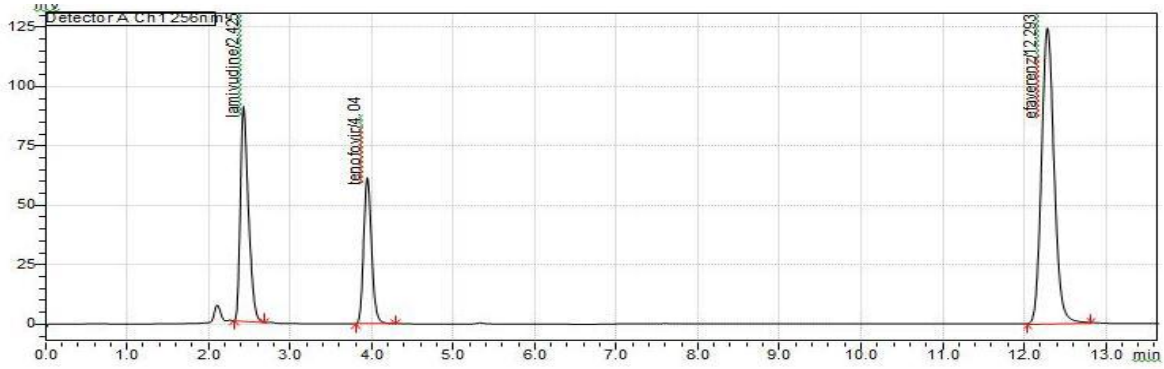

Fig. 4b: Chromatogram of well resolved peaks of LAM, TDF and EFA

Linearity

The calibration curves of LAM, TDF and EFA were constructed in the concentration range of 5-15 µg/ml, 5-15 µg/ml and 10-50 µg/ml for LAM, TDF and EFA respectively. The plots obtained from linear regression were shown in Fig. 5a, 5b and 5c. The result analysis was shown in Table 2.

Table 2: Linearity of LAM, TDF and EFA

S.No	LAM		TDF		EFA	
	Concentration (ug/ml)	Peak area	Concentration (ug/ml)	Peak area	Concentration (ug/ml)	Peak area
blank	0	0	0	0	0	0
Inj 1	5	209829	5	146265	10	679257
Inj 2	10	423969	10	286305	20	1298514
Inj 3	15	642989	15	435785	30	1995471
Inj 4	20	847103	20	587612	40	2627028
Inj 5	25	1050436	25	719527	50	3291528
Regression equation	Y=42189x + 1695		Y=28978x + 356.9		y = 65708x + 1695	
R^2	0.999		0.999		0.999	

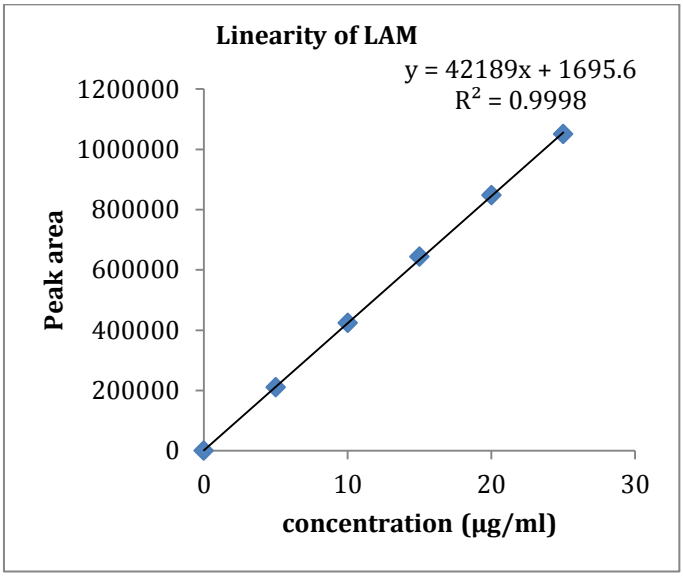

Fig. 5a: Linearity graph of LAM

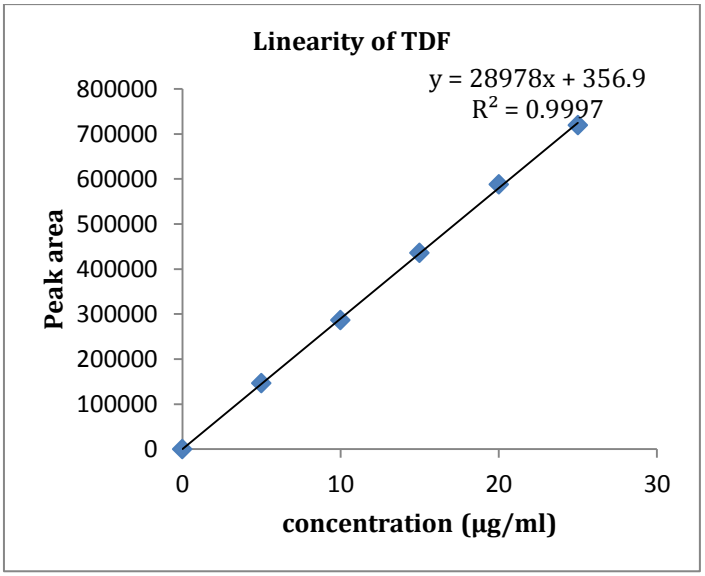

Fig. 5b: Linearity graph TDF

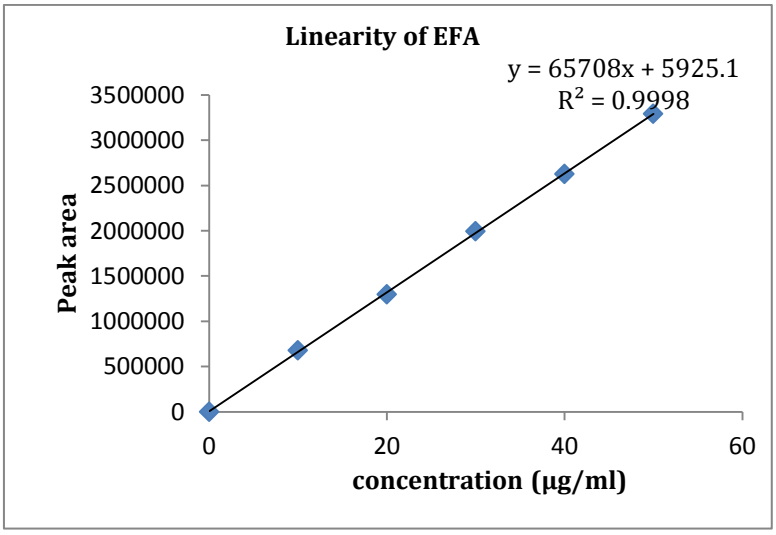

Fig. 5c: Linearity graph of EFA

Accuracy

The accuracy for proposed method was determined, recovery studies were performed in mentioned levels and recorded (Table 3), Obtained results were found to be within the limits of 98-102%, indicating an agreement between the true value and found value.

Table 3: Accuracy of LAM, TDF and EFA

Drug	Concentration level (%)	Concentration of sample (µg/ml)	Concentration of standard added (µg/ml)	Amount drug recovered (µg/ml)	Percentage recovery
LAM	50	5	5	9.8	98.0
	100	15	5	19.7	98.5
	150	25	5	30.2	100.6
TDF	50	5	5	9.9	99.0
	100	15	5	19.8	99.0
	150	25	5	30.1	100.3
EFA	50	10	10	19.9	99.5
	100	30	10	39.5	98.75
	150	50	10	60.8	101.3

Precision

Precision was calculated as intra-day and inter-day variations for the drugs. Percent relative standard deviations for estimation of LAM, TDF and EFA under intra-day and inter-day variations were found to be less than 2. Results were showed in Table 4.

Table 4: Intraday Precision of LAM, TDF and EFA

level	SYSTEM PRECISION (peak areas)			METHOD PRECISION (peak areas)		
	LAM	TDF	EFA	LAM	TDF	EFA
Injection1	645989	435885	2049471	646589	436589	2049471
Injection2	644325	433804	2049119	644135	433154	2051634
Injection3	639011	435735	1998912	649011	435785	2091478
Injection4	641291	431962	2026216	641291	431962	2015645
Injection5	639142	432501	1985906	641957	439841	1998763
Average	641933.6	433997.4	2021925	644596.6	435466.2	2041398.2
S.D	3102.026	1782.72	28912.6	3223.512	3087.34816	35912.7959
% RSD	0.48	0.41	1.4	0.5	0.7	1.7

*Average of 5 determinations

Limit of detection (LOD) and Limit of quantitation (LOQ)

The LOD and LOQ were calculated according to the S/N ratio of the respective drugs. The concentration of the drugs were reduced gradually with regular intervals from 10 µg/ml and injected in to HPLC. The concentration with S/N 3:1 is taken as LOD and concentration with S/N 10:1 is taken as LOQ.

Table 5: LOD and LOQ of LAM, TDF and EFA

Drug	LOD (µg/ml)	S/N	LOQ (µg/ml)	S/N
LAM	0.05	2.89	0.2	8.95
TDF	0.1	2.56	0.5	9.05
EFA	0.1	3.01	0.3	8.79

Robustness

For robustness studies, conditions like flow rate and concentration of organic phase were changed and method was performed. In all deliberately varied conditions, percent relative standard deviations for peak areas, retention times, theoretical plates and tailing factor were found to be less than 2% (Table 6).

Table 6: Robustness Parameters of LAM, TDF and EFA

S.No.	Parameter	Retention time* (min)			No of theoretical plates*			Tailing factor*		
		LAM	TDF	EFA	LAM	TDF	EFA	LAM	TDF	EFA
1	Initial conditions	2.4	4.1	12.2	3929	6345	23876	1.0	1.6	1.0
2	Flow 0.9 ml/min	2.5	4.3	12.5	3825	6455	24876	1.1	1.5	1.1
3	Flow 1.1 ml/min	2.2	3.9	11.8	3954	6546	23843	1.0	1.5	1.0
5	Organic phase, 2% less (33%)	2.5	4.3	11.5	3876	6464	23656	1.1	1.5	1.2
6	Organic phase, 2% more (37%)	2.1	3.8	12.6	3944	6375	25646	1.0	1.5	1.0
7	Less pH (4.8)	2.3	4.5	11.3	3899	6322	25736	1.2	1.5	1.1
9	More pH (5.2)	2.0	4.0	12.8	3932	6445	24364	1.2	1.5	1.0

*Average of 3 determinations

Assay

20 tablets were taken and their average weight was calculated, tablets were crushed to fine powder. Quantity of powder equivalent to 10 mg of LAM, 10 mg TDF and 20 mg EFA were taken and dissolved using diluent in a 10 ml volumetric flask to obtain a concentration of LAM (1000 µg/ml), TDF (1000 µg/ml) and EFA (2000 µg/ml). 1 ml of the above solution was diluted to 10ml with diluent to obtain a concentration of 100 µg/ml of LAM, TDF and 200 µg/ml EFA. 1.5 ml of the above solution was diluted to 10ml with mobile phase to obtain a concentration of 15 µg/ml of LAM, TDF and 30 µg/ml of EFA.

20 µl of the above solution was injected in to HPLC and the percent of assay was calculated using peak areas of standard and sample. The experimental values obtained for the determination of LAM, TDF and EFA in pharmaceutical formulation was within the claimed limits (Table 7).

Table 7: Assay data of marketed formulation

Drug	Amount labeled (mg)	Amount found*	% Assay
LAM	300	298.72±0.51 mg	99.57%
TDF	300	299.15±0.69 mg	99.71%
EFA	600	601.45±0.35 mg	100.24%

*values are expressed as mean ±SD (n= 3)

CONCLUSION

In the proposed HPLC method, different proportions of Acetonitrile and phosphate buffer were tried for selection of the mobile phase. Ultimately, 50 mM phosphate buffer (pH 5) in water and Acetonitrile in a gradient run. The elution order was LAM (R_t=2.4 min), TDF (R_t=4.1 min) and EFA (Rt=12.2 min), at a flow rate of 1.0 ml/min. The chromatogram was recorded at 256 nm. The developed method was validated as per ICH guidelines. Parameters like precision, accuracy, specificity, ruggedness, robustness were done and found to be within the acceptance criteria. Hence the RP-HPLC method could selectively quantify LAM, TDF and EFA in pharmaceutical formulations.

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