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Research Article

DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF LAMIVUDINE, TENOFOVIR DISOPROXIL FUMARATE AND EFAVIRENZ IN A COMBINED TABLET DOSAGE FORM

P. VANAJA[1], N. ANUSHA[2]*, V. S. GIRI PRASAD[3]

[1]School of Chemical & Biotechnology, SASTRA University, Thanjavur – 613401, India, [2]School of Chemical & Biotechnology, SASTRA University, Thanjavur – 613401, India, [3]R.G.R. Siddhanthi College of Pharmacy, Secunderabad – 500003. Email:anusha@scbt.sastra.edu

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ABSTRACT

Objective: Development of an accurate, simple, precise and rapid method for estimating Tenofovir disoproxil fumarate, Lamivudine and Efavirenz simultaneously, in a combined tablet form.

Methods: The method uses Reverse Phase High Performance Liquid Chromatography (RP-HPLC). Symmetry C18 (4.6 x 100 mm, 3.5 µm) column operated with a mixture of phosphate buffer of pH 4.0 with ortho phosphoric acid and Acetonitrile (42:58) as mobile phase was found to be suitable for the simultaneous estimation. The flow rate was maintained at 0.5 mL/min. Detection was carried out at 254 nm using a UV detector.

Results: The total run time was less than 11 minutes with the retention times of 2.2 min, 3.3 min and 10.8 min respectively for Lamivudine, Tenofovir disoproxil fumarate and Efavirenz.

Conclusion: Validation of the method was performed for precision, accuracy, linearity, ruggedness, specificity and sensitivity to conform to the ICH guidelines for validation of an analytical method.

Keywords: Lamivudine, Tenofovir disoproxil fumarate, Efavirenz, RPHPLC, Method Development, Validation.

INTRODUCTION

Nucleoside Reverse-Transcriptase Inhibitors (NRTI) are antiretroviral drugs that act by competing with natural deoxynucleotides to be incorporated into the viral DNA chain [1]. There are several such drugs available in the market for treatment of HIV including Tenofovir disoproxil fumarate (TDF) and Lamivudine (LMV). Lamivudine (LMV) has been mentioned in Indian Pharmacopeia, British Pharmacopeia and United States Pharmacopeia [2]. Tenofovir disoproxil fumarate is mentioned in Indian Pharmacopeia [2]. Non-Nucleoside Reverse-Transcriptase Inhibitors (NNRTI) are another class of anti-viral drugs that act by binding to Reverse Transcriptase (RT) causing enzyme conformation changes [3]. Efavirenz (EFV) is an example for NNRTI [4]. A formulation containing TDF, LMV and EFV is considered effective due to the action of both NRTI and NNRTI.

A variety of methods are in vogue for estimation of TDF, LMV and EFV individually as highlighted in the literature [2, 5]. To the best of authors' knowledge only a couple of works including a spectrometric method [6] and a RP-HPLC method [7] have been reported for simultaneous estimation of TDF, LMV and EFV. However, the linearity range (5-30 □ g/mL for TDF & LMV and 1060 □ g/mL for EFV in [6]; 1-6 □ g/mL for TDF & LMV and 212 □ g/mL for EFV in [7]), were lower. Methanol was used as a component of mobile phase in earlier reported RP-HPLC [7], where as acetonitrile has been used in the present study of RP-HPLC based method development for simultaneous estimation of TDF, LMV and EFV in their combined dosage form. Such an initiative is expected to contribute towards the fight of human society in managing HIV treatment. The choice of HPLC for method development is driven by its success for simultaneous estimation of drugs in various dosage forms [8, 9].

MATERIALS AND METHODS

Equipment and Chromatographic Conditions

A Liquid chromatographic system (Shimadzu) equipped with an auto sampler has been used. A mixture of phosphate buffer (42 parts) and acetonitrile (58 parts) was utilized as the mobile phase at 0.5 mL/min. The pH was maintained at 4 with the help of ortho phosphoric acid. All the experiments were performed at 30°C using Symmetry C18 column (4.6 x 100mm, 3.5µm, Make: ACE). A constant injection volume of 20 mL was used, with mobile phase as diluent.

MATERIALS AND REAGENTS

Working standards of Lamivudine (99.9%), Tenofovir disoproxil fumarate (99.8%) & Efavirenz (99.6%) were obtained from Aurobindo Pharmaceuticals Limited. Tablets containing 300 mg of LMV, 300mg of TDF and 600 mg of EFV as per label claim in combinations were procured from Aurobindo Pharmaceuticals. HPLC grade methanol, acetonitrile, potassium dihydrogen phosphate and orthophosphoric acid were obtained from Merck, India.

Preparation of Standard Solution

Accurately weighed 10 mg of Lamivudine, 10 mg of Tenofovir disoproxil fumarate and 20 mg of Efavirenz working standards were individually transferred to separate 10 mL clean dry volumetric flasks. To each of the volumetric flasks, 7 mL of diluent was added. They were ultrasonicated and made up using the diluent. These were standard stock solutions-I for each standard drug.

Accurately measured 0.3 mL of LMV, 0.3 mL of TDF & 0.6 mL of EFV of the above stock solutions were diluted to 10 mL in a standard flask. These were standard stock solutions-II that contained a final concentration of 0.03 mg/mL of LMV, 0.03 mg/mL of TDF and 0.06 mg/mL of EFV.

Preparation of Sample Solution

Accurately weighed twenty tablets were ground to obtain fine powders. About 1462 mg of weighed powder equivalent to 300 mg of LMV, 300 mg of TDF & 600 mg of EFV was transferred to a 100 mL volumetric flask to which 70 mLof mobile phase was added and ultrasonicated. Further dilution to 100 mL was performed using the mobile phase. This was sample stock solution-I, from which 0.1 mL was pipetted into a 10 mL volumetric flask and made up using the diluent. This was sample stock solution-II that contained a final concentration of 0.03 mg/mL of LMV, 0.03 mg/mL of TDF and 0.06 mg/mL of EFV

Both standard stock solution-II and sample stock solutions-II were filtered through 0.45 µm syringe filter. Chromatograms were recorded by injection of 20 µL of standard and sample solutions into the column.

Method Validation

Five replicate injections of the standard solutions of LMV, TDF and EFV were performed to estimate number of theoretical plates, %RSD and resolution of peak areas to study the system suitability parameters.

Specificity was evaluated with regard to interference due to presence of any other compounds. The standard solution, sample solution, blank and placebo (tablet excipients without drug added to the mobile phase) were injected separately for analysis. To ascertain the accuracy of the method, known quantities of the pure drug were added to the prepared sample formulation at 50%, 100% and 150% and the chromatograms were recorded.

The linearity of the developed method was studied by applying the method for estimation of Tenofovir disoproxil fumarate, Lamivudine and Efavirenz individually in the concentration ranges of 10-40, 10-50 and 20-100 µg/mL respectively. The ruggedness was tested by inter-analyst, inter-instrument and inter-day estimations.

Test for robustness was performed out by making small changes in the mobile phase ratio of phosphate buffer:Acetonitrile from the original value at the flow rate of 0.5 mL/min. To check the robustness, the flow rate was changed to 0.48mL/min and 0.52mL/min.

The Limit of Quantification (LOQ) was determined as the minimum concentration of the drug for which there was close match between the concentration of drug estimated by the developed method and the actual concentration. For this purpose, a sample of pre-determined concentration was diluted several times and chromatogram recorded each time. For the purpose of determination of Limit of Detection (LOD), the samples prepared for determination of LOQ was diluted further and chromatograms recorded.

RESULTS AND DISCUSSION

After several experiments with different mobile phase composition and flow rates (results not shown to maintain brevity), the optimum mobile phase composition and flow rate were determined as 42:58 (ACN: phosphate buffer with pH 4) and 0.5 mL/min. Under these conditions LMV, TDF & EFV were eluted at 2.217 minutes, 3.275 minutes and 10.801 minutes respectively. Typical chromatograms obtained using the above-mentioned mobile phase at 0.5 mL/min are illustrated in Figures 1 and 2.

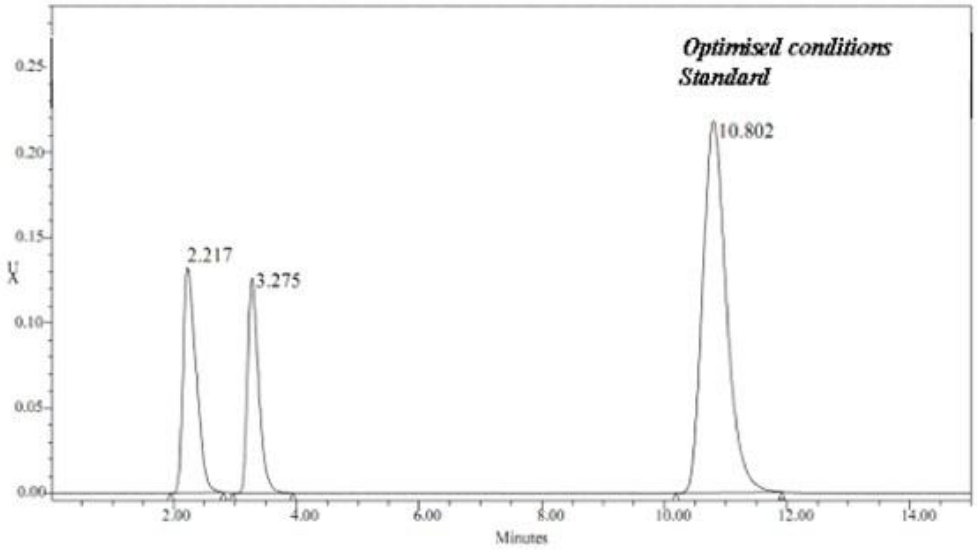

Fig. 1: Chromatogram of the standard solution. The retention times of 2.217 min, 3.275 min and 10.802 min correspond to Lamivudine, Tenofovir disoproxil fumarate & Efavirenz respectively

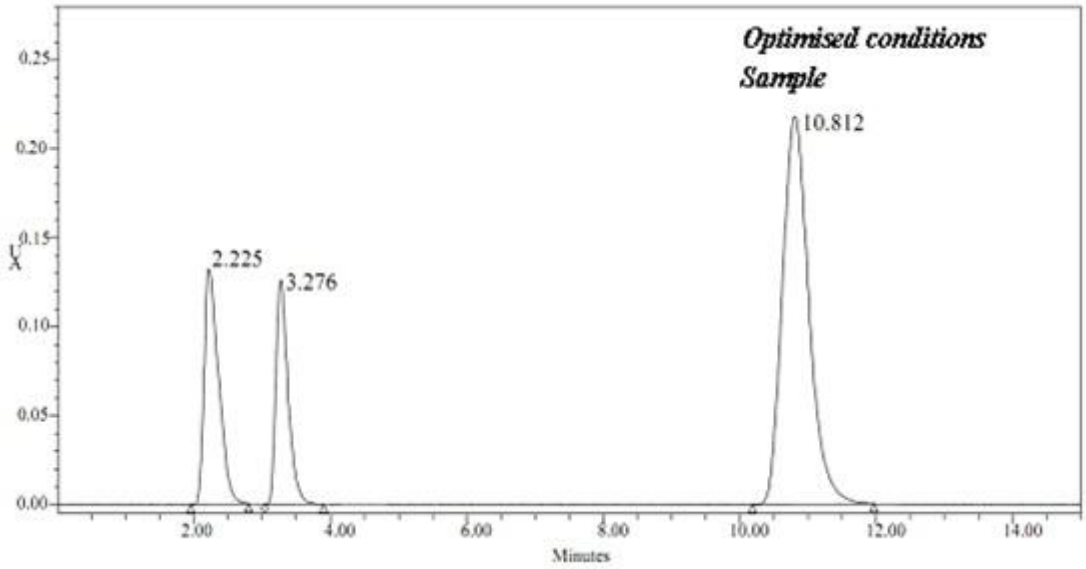

Fig. 2: Chromatogram of the sample solution. The retention times of 2.225 min, 3.276 min and 10.812 min correspond to Lamivudine, Tenofovir disoproxil fumarate & Efavirenz respectively

Tables 1 and 2 show the results of validation, from which it can be concluded that all the system suitability parameters pass the criteria.

Table 1: Results of five repetitions of the analysis

Trial No	Lamivudine area in standard	RT of Lamivudine peak (min)	Tenofovir disoproxil fumarate area in standard	RT of Tenofovir disoproxil fumarate peak (min)	Efavirenz area in standard	RT of Efavirenz peak (min)
1	1961718	2.222	1533900	3.273	5909891	10.811
2	1960693	2.223	1532856	3.274	5908621	10.812
3	1961960	2.217	1538982	3.275	5986381	10.802
4	1962851	2.216	1539001	3.274	5897430	10.801
5	1961718	2.222	1533900	3.273	5909891	10.811
Average	1961788	2.22	1535728	3.2738	5904443	10.807
%RSD	0.039	0.145	0.195	0.025	0.117	0.0500

Table 2: Theoretical plates, tailing factor and asymmetry

Serial Number	Parameters	Values obtained for Lamivudine	Values obtained for Tenofovir disoproxil fumarate	Values obtained for Efavirenz
1.	Theoretical plates	2442.7	2712.3	3740.9
2.	Tailing factor	1.0	1.2	1.2
3.	Asymmetry	1.0	1.3	1.4
4.	% RSD of peak retention time	0.145	0.025	0.050

The chromatographs recorded for ascertaining are shown in Figures 3 and 4. It is evident from Figures 3 and 4 that the standard and sample chromatograms are identical. This testifies the specificity of the HPLC method developed in this study for Lamivudine, Tenofovir disoproxil fumarate and Efavirenz only. Also, the comparison of retention time of the analytes (Figures 3 and 4) reveals that there is no specific inference from excipients to the chromatograms of the analytes, again confirming the specificity of method.

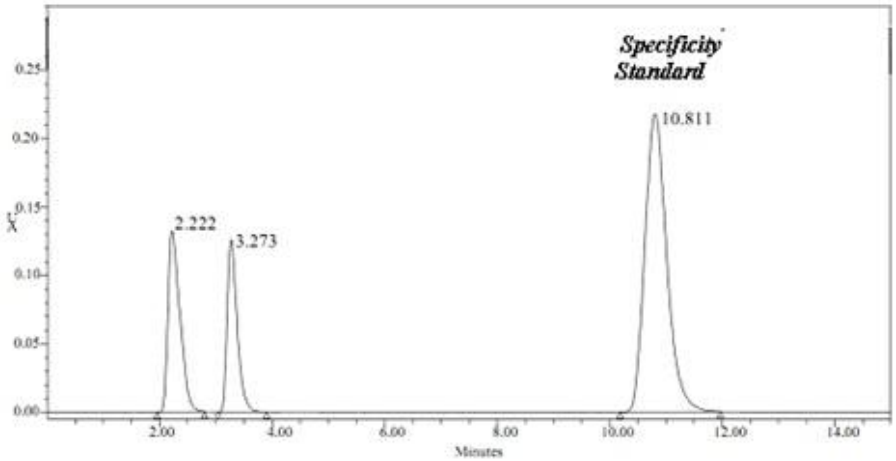

Fig. 3: Chromatogram of the standard solution. The retention times of 2.222 min, 3.273 min and 10.811 min correspond to Lamivudine, Tenofovir disoproxil fumarate & Efavirenz respectively

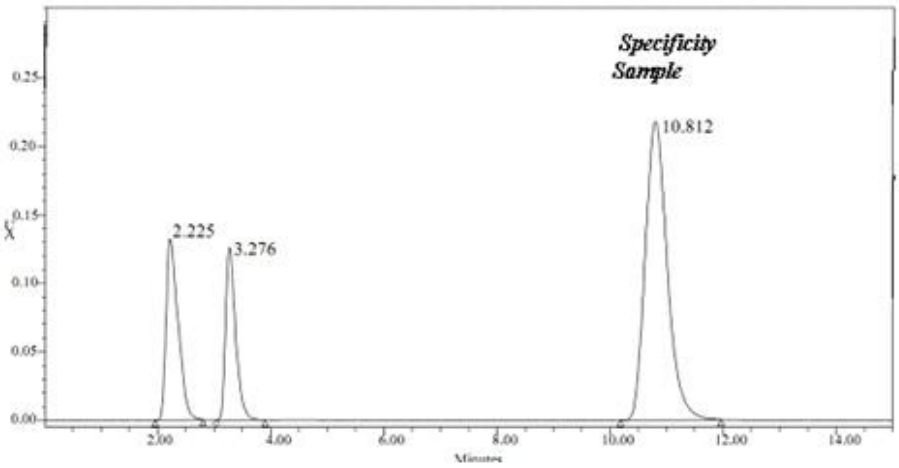

Fig. 4: Chromatogram of sample (pharmaceutical dosage form) solution. The retention times of 2.225 min, 3.276 min and 10.812 min correspond to Lamivudine, Tenofovir disoproxil fumarate & Efavirenz respectively

The lower percentage relative standard deviation of assay for test of accuracy (Table 3) testifies the method's accuracy. From the mean recoveries, which were within the range of 98-102 %, it is clear that the excipients do not interfere with the method. The peak area-concentration data for the three drugs TDF, LMV and EFV are shown in Figs. 5 to 7, from which existence of a linear relationship between peak area and drug concentration is evident. The range of concentrations for which calibration curves were linear for Tenofovir disoproxil fumarate, Lamivudine and Efavirenz are 10-40, 10-50 and 20-100 µg/mL respectively. The correlation coefficient values for all the analytes were found to be greater than 0.98 (Figures 5 to 7) testifying linearity.

Table 3: Recovery studies of LMV, TDF and EFV by standard addition method

Concentration (% at specification Level)	Area for LMV	Recovery For LMV (%)	Area for TDF	Recovery For TDF (%)	Area for EFV	Recovery For EFV (%)
50	1037465	101.6	787847	101.5	3282603	101.5
100	1965894	100.1	1538598	100.1	5909256	99.5
150	2940453	99.8	2307504	100.1	8858473	99.5
% Mean recovery		100.5		100.6		100.2

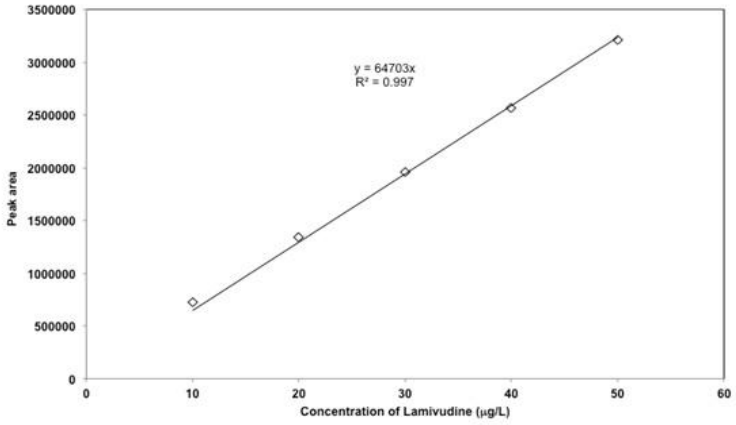

Fig. 5: Linearity graph for Lamivudine

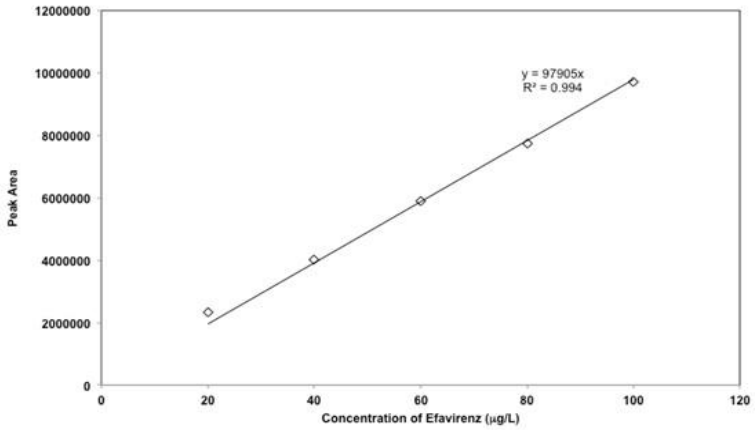

Fig. 6: Linearity graph for Efavirenz

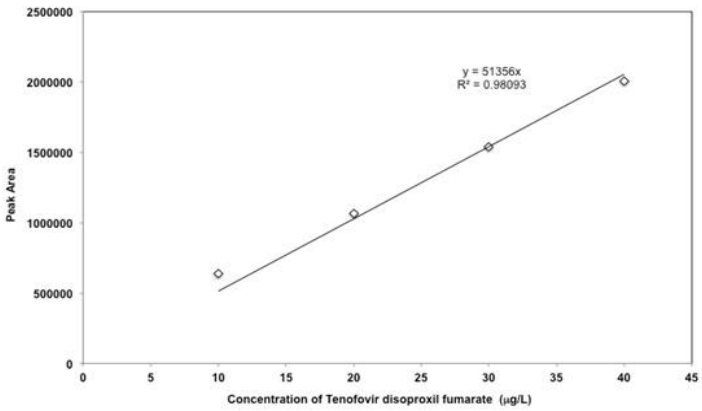

Fig. 7: Linearity graph for Tenofovir disoproxil fumarate

The assay values obtained for the 'Precision' are shown in Table 4. The relative standard deviations obtained for Lamivudine, Tenofovir disoproxil fumarate and Efavirenz were 0.17, 0.16 and 0.05 respectively, confirming the method's precision. The sample chromatograms recorded by two different analysts for ascertaining ruggedness of the method are shown in Figures 8 and 9. Table 5 shows % RSD values for the estimation of these three drugs on different days by different analyst. The lower values of % RSD (< 1 %) for retention times, sample areas, standard areas and assay values and high reproducibility of chromatograms (Figures 8 and 9) indicate the ruggedness of the method (as per ICH guidelines) developed in the present work.

Table 4: Results of Precision for LMV, TDF & EFV

Drug assayed	Assay 1 (%)	Assay 2 (%)	Assay 3 (%)	Assay 4 (%)	Assay 5 (%)	Assay 6 (%)	Average (%)	RSD (%)
LMV	99.9	99.9	100.0	99.9	99.8	100.3	99.9	0.17
TDF	99.4	99.7	99.4	99.2	99.4	99.5	99.4	0.16
EFV	99.5	99.4	99.4	99.4	99.5	99.5	99.5	0.05

Table 5: Comparison of % RSD values for LMV, TDF and EFV on different days by different analysts

Day 1 Analyst 1	% RSD of retention time	% RSD of standard area	% RSD of sample area	% RSD of assay values
LMV	0.071	0.03	0.210	0.283
TDF	0.048	0.276	0.017	0.071
EFV	0.015	0.075	0.017	0.002
Day II Analyst II				
LMV	0.103	0.190	0.230	0.188
TDF	0.546	0.276	0.018	0.152
EFV	0.015	0.078	0.075	0.048

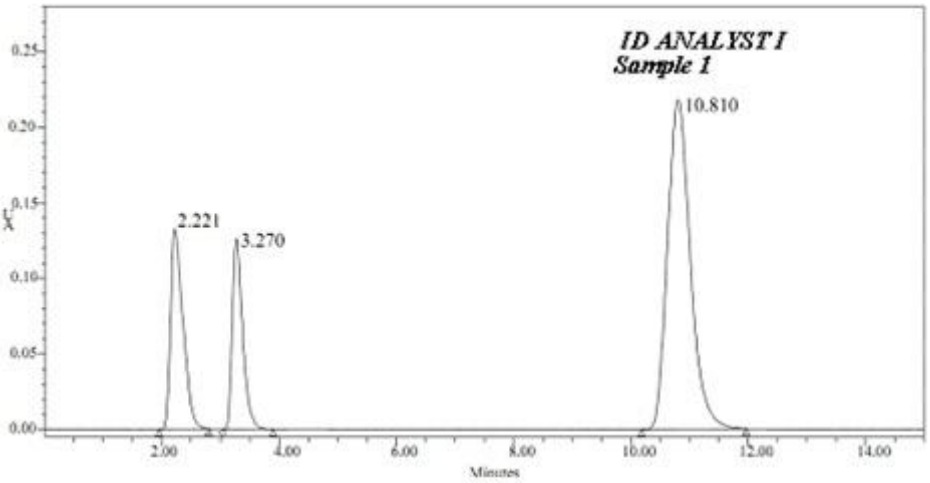

Fig. 8: A representative chromatogram of the analysis performed by analyst I to check ruggedness of the method

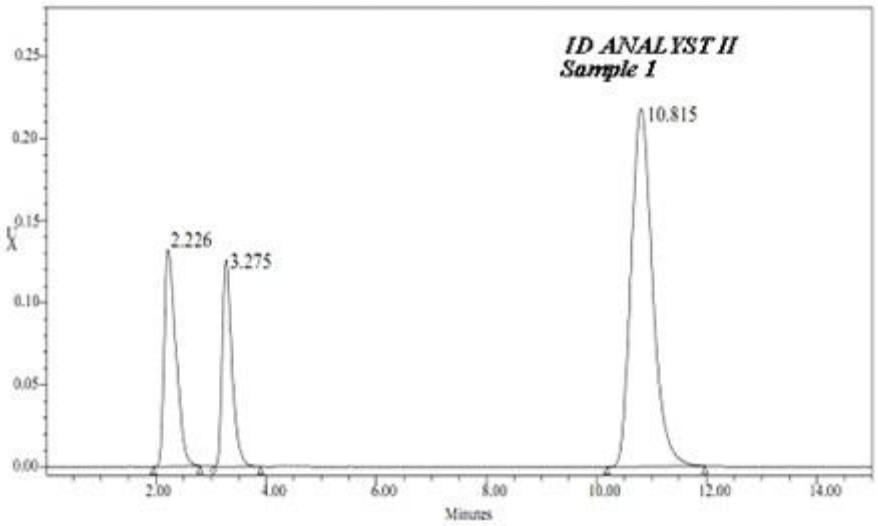

Fig. 9: A representative chromatogram of the analysis performed by analyst II to check ruggedness of the method

Small changes were made in the mobile phase ratio to (40:60) of phosphate buffer:Acetonitrile from the original (42:58) at 0.5 mL/min for the purpose of ascertaining robustness. Also, the flow rate was changed to 0.48 mL/min and 0.52 mL/min from the actual 0.5 mL/min. The % RSD of retention time, area and assay values for LMV, TDF and EFV were within ±2%. The Limit of Detection **(**LOD) of LMV, TDF & EFV were found to be 0.02 µg /mL, 0.03 µg /mL and 0.03 µg / mL. The Limit of Quantification (LOQ) of LMV, TDF & EFV were found to be 0.09, 0.102 and 0.111 µg /mL respectively. This testifies the sensitivity of method.The method developed here was utilized for simultaneous estimation of Lamivudine, Tenofovir disoproxil fumarate and Efavirenz in three tablet dosage forms. The mean assay results are tabulated in Table 6. Close match between label claim and the estimated value and assay percentages close to 100 demonstrate the suitability of the developed method for simultaneous estimation of these drugs in tablet dosage forms.

Table 6: Mean results of assay for determination of LMV, TDF and EFV in three-tablet formulations

Drug name	Label claim per tablet dosage form (mg)	Content of analyte found per vial ± S.D (mg)	Assay in %
Lamivudine	300	299.7 ± 0.05	99.9
Tenofovir disoproxil fumarate	300	298.2 ± 0.07	99.4
Efavirenz	600	598.8 ± 0.15	99.8

CONCLUSIONS

Hence, it may be concluded that a new RP-HPLC for simultaneous determination of Lamivudine, Tenofovir disoproxil fumarate & Efavirenz in combined pharmaceutical dosage form has been developed. The accuracy, precision, sensitivity and ruggedness of the method were confirmed. This method, which can be run in 11 minutes, may be suitable for analysis in Quality control units of Pharmaceutical industries.

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