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Method Development and Validation for Simultaneous Estimation of Dolutegravir, Lamivudine and Tenofovir Disoproxil Fumarate in Bulk and Tablet Dosage Form by UV Spectrophotometry and RP-HPLC

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ABSTRACT
Two methods were described for the simultaneous estimation of Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate in bulk and tablet dosage form. The first method was based on UV spectrophotometric determination of three drugs using simultaneous equation method. It involves absorbance measurement at 257nm (λ_{max} of Dolutegravir), 271nm (λ_{max} of Lamivudine) and 260nm (λ_{max} of Tenofovir disoproxil fumarate) in distilled water (for Lamivudine and Tenofovir disoproxil fumarate) and in methanol followed by further dilutions in distilled water (for Dolutegravir). Linearity was obtained in the range of 1-5 µg/ml for Dolutegravir, 6-30 µg/ml for Lamivudine and Tenofovir disoproxil fumarate with good correlation (> 0.998). The second method was based on RP-HPLC separation of three drugs using Shimadzu C_8 (250mm×4.6mm) 5µm column at 25˚C. The separation was achieved by employing a mobile phase consists of acetonitrile: phosphate buffer (pH 3) in the ratio of 50 : 50 at 260nm with flow rate of 1 ml/min. Retention time was found to be 6.43, 2.56 and 4.79 min for Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate respectively. The method was found to be linear in the range of 3-7µg/ml for Dolutegravir, 18-42µg/ml for Lamivudine and 18-42µg/ml for Tenofovir disoproxil fumarate with correlation > 0.998. Both these methods have been successively applied to pharmaceutical formulation and were validated according to ICH guidelines.

KEY-WORDS: RP-HPLC, UV spectro photometry, Dolutegravir, Lamivudine, Tenofovir disoproxil fumarate.

I. INTRODUCTION

Dolutegravir belongs to the class of HIV-1 antiviral agent. It prevents HIV integrase by binding to the integrase active site. Thus it stops the strand transfer step of DNA synthesis which is specific for HIV replication cycle. Lamivudine belongs to the class of nucleoside synthetic analogue. It is phosphorylated intracellularly into active 5' triphosphate metabolite (Lamivudine triphosphate). This active metabolite involves in prevention of reverse transcriptase via chain termination after infusion of nucleotide analogue. Tenofovir disoproxil fumarate 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. It inhibits HIV-1 reverse transcriptase and Hepatitis B polymerase by competitive binding with natural deoxyribonucleotide substrate (deoxyadenosine 5'-triphosphate) and after integration into DNA causes viral DNA chain termination. A review of literature reveals that no method has been developed using UV spectroscopy though the formulation was available in the market; only a HPLC method using different solvent system have been reported for the simultaneous estimation of Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate in tablet dosage form. So our aim is to develop a simple, rapid, economic, accurate, reproducible, less time consuming and reliable new validated spectrophotometric and RP-HPLC method of analysis for Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate in tablet dosage form.

International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

II. MATERIALS AND METHODS

Apparatus and conditions

Spectroscopic conditions

Shimadzu (UV-1650PC) UV spectrophotometry device equipped with silicon photodiode detector and UV probe software was utilized.

1. Selection of solvent

The initial stock solution of Dolutegravir was made up with methanol and further serial dilutions were done with purified distilled water. The initial stock solutions of Lamivudine and Tenofovir disoproxil fumarate were made up and further dilutions were carried out with purified distilled water.[1-6]

2. Selection of wavelength

In this UV spectroscopic method for the estimation of this combination, methanol and water was selected as a solvent. The UV scanning at 200-400nm for Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate showed that 257, 271 and 260nm is the suitable wavelength for detection as per maximum absorbance.[1-6]

Chromatographic conditions

Shimadzu (CBM-20A) HPLC device with Shimadzu-SPD-M20A diode array detector, Shim pack solar C_8 Column (250mm×4.6mm) 5µm and LC lab solution was utilized.

1. Selection of wavelength

From the UV spectra, the wavelength was selected as 260nm as it shows good absorbance. Hence, from the spectrum it was concluded that 260nm is the detection wavelength for study.

2. Fixed chromatographic conditions

Method development was carried out with mobile phase acetonitrile: phosphate buffer pH 3 (50: 50). The flow rate, column temperature and sample volume were tuned at 1 ml/min, 25˚C and 20µl, respectively at a detection wavelength of 260nm.

Chemicals

Dolutegravir and Lamivudine were obtained from Macleod's Pharmaceuticals Ltd., Himachal Pradesh. Tenofovir disoproxil fumarate was procured from Macleod's Pharmaceuticals Ltd., Baddi. The commercial product of Viropil™ was manufactured by Emcure® pharmaceuticals Ltd., India. Methanol AR grade and Potassium dihydrogen orthophosphate were received from Himedia Laboratory Pvt. Ltd., Mumbai. Acetonitrile HPLC grade was procured from Sigma Aldrich Chemicals Pvt. Ltd, Mumbai. Ortho phosphoric acid was received from Loba Chemie Pvt. Ltd.

UV Spectroscopy

Preparation of stock solution

10 mg of Dolutegravir working standard was taken and transferred into 10ml volumetric flasks and volume was made up to 10 ml with methanol[1]. 10 mg of Lamivudine and 10 mg of Tenofovir disoproxil fumarate working standard was taken and transferred into 10ml volumetric flasks and volume was made up to 10 ml with water[2-6]. Then the mixture was sonicated for 15 min to dissolve it completely. Final concentrations of Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate were 1000 µg/ml.

Construction of Calibration curve

1 ml aliquots of Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate from the stock solutions were transferred into 10ml volumetric flasks and volume was made up to 10 ml with water. From the above solution of Dolutegravir aliquots of 0.1, 0.2, 0.3, 0.4, 0.5 ml were taken and transferred into 10ml volumetric flasks and volume was made up to 10 ml with water to get a final concentration range of 1-5 µg/ml. From the above solutions of Lamivudine and Tenofovir disoproxil fumarate aliquots of 0.6, 1.2, 1.8, 2.4, 3ml were taken and transferred into 10ml volumetric flasks and volumes were made up to 10 ml with water to get a final concentration ranges of 6-30 µg/ml. The standard solutions of Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate were scanned at wavelengths of 257 nm, 271 nm and 260 nm respectively and calibration curves were constructed. The isobestic point was identified as 260nm[1-6].

RP-HPLC

Preparation of buffer

Phosphate buffer pH 3.0 was prepared by dissolving 6.80 g of potassium dihydrogen orthophosphate in 800 ml of water and the pH was adjusted to 3.0 with ortho phosphoric acid and sufficient water was added to produce 1000 ml.

Preparation of mobile phase

A mixture of acetonitrile and phosphate buffer (pH 3) in the ratio of 50: 50 was prepared and degassed in an ultra sonicator for 15 min.

International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

Preparation of standard stock solution

10 mg of Dolutegravir,10 mg of Lamivudine and 10 mg of Tenofovir disoproxil fumarate working standards were taken and transferred into 10 ml volumetric flasks separately and the volumes were made up to 10 ml with mobile phase. Then the mixture was sonicated for 15 min to dissolve it completely.

From the stock solutions, working standard solutions of drugs were prepared by appropriate dilution with mobile phase. Calibration curves were prepared from 3 to 7 µg/ml for Dolutegravir, 18 to 42 µg/ml for Lamivudine and 18 to 42 µg/ml for Tenofovir disoproxil fumarate[7-10].

III. RESULTS AND DISCUSSION

Method development and optimization of chromatographic conditions

In order to achieve good separation between all the three components different proportions of solvents like acetonitrile and phosphate buffer of different pH conditions were tested. However, in phosphate buffer pH 3 and acetonitrile achieved satisfactory results at a flow rate of 1 ml/ min measured at a detection of 260nm. The chromatogram of optimized standard mixture chromatogram is shown in Fig. The system suitability parameters such as retention time, resolution, tailing factor and theoretical plates for optimized standard mixture chromatogram is tabulated.

Validation

Linearity

Linearity was studied by analyzing five standard solutions in the range of 1 – 5 µg/ml for Dolutegravir, 6 – 30 µg/ml for Lamivudine and 6 – 30 µg/ml for Tenofovir disoproxil fumarate by UV Spectrophotometry and 3 - 7µg/ml for Dolutegravir, 18 - 42µg/ml for Lamivudine and 18 - 42µg/ml for Tenofovir disoproxil fumarate by RP-HPLC. Calibration curves with concentration versus absorbance or peak area was plotted for each method. Linearity was measured by using calibration data regression analysis as well as coefficient of correlation. The regression statistics were displayed in table. The correlation coefficient (r^2) of Dolutegravir was 0.999, 0.998 Lamivudine was 0.998, 0.999 and Tenofovir disoproxil fumarate was 0.998, 0.998 for UV and RP-HPLC methods respectively. This shows better detector response in different concentration for both methods.

Precision

Precision should be measured using a minimum of three determinations per concentration. Precision of the analytical method was demonstrated by, intra day precision, inter day precision and repeatability. % RSD was calculated for both methods and results are represented in table. The coefficient of variation (%RSD) was less than 2% which shows the methods were precise.

Accuracy

Accuracy of the optimized methods were determined by absolute and relative recovery. It was found out by replicate analysis of samples containing known amount of the analyte. Standard addition was used for accuracy determinations at three levels (80%, 100% and 120%). A minimum of three concentrations in the range of expected study sample was recommended. Each concentration range was injected three times. Based on the area/ height of the peak, the % recovery was calculated and results were represented in table. The recovery range for Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate were found to be 102.56%, 100.51%, 101.15% and 100.86%, 99.93%, 100.73% by UV and RP-HPLC methods respectively.

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

The limit of detection and quantitation of the drugs for both methods were calculated with standard deviation and slope. The LOD and LOQ of each method was calculated and represented in table.

System suitability

The determination of the system suitability of RP-HPLC method was accomplished by assaying three samples of the same strength as Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate. The sample concentration of Dolutegravir, Lamivudine and Tenofovir disoproxil fumarate used in this analysis were 5 µg/mL, 30 µg/mL and 30 µg/mL respectively. The retention time, peak area, theoretical plates, and asymmetry factor were evaluated for system suitability. The optimized method is acceptable as system suitability parameters are valid as they passed the criteria of acceptability, as shown in table.

Analysis of formulation

UV Spectroscopy

Ten tablets of formulation (Viropil 650 mg containing 50 mg of Dolutegravir, 300 mg of Lamivudine and 300 mg of Tenofovir disoproxil

International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

fumarate) was accurately weighed to find out average weight and finely powdered. The tablet powder equivalent to 50 mg of Dolutegravir, 300 mg of Lamivudine and 300 mg of Tenofovir disoproxil fumarate was weighed and transferred into 100 ml volumetric flask and the mixture of methanol & water in the ratio of 3.5: 6.5 was added upto the mark. The mixture was sonicated for 15 min and filtered through a Whatman filter paper. From this solution 1ml aliquot was withdrawn and transferred into 10ml volumetric flask and diluted upto the mark with the mixture of methanol & water in the ratio of 3.5: 6.5. Solution contains 50 mg of Dolutegravir, 300 mg of Lamivudine and 300 mg of Tenofovir disoproxil fumarate[1-6].

RP-HPLC

Weigh and powder 10 tablets. Transfer an accurately weighed quantity of finely powdered tablets equivalent to 50 mg of Dolutegravir, 300 mg of Lamivudine and 300 mg of Tenofovir disoproxil fumarate in 100 ml volumetric flask, add about 100 ml of mobile phase and sonicate for 15 min, then filter it with Whatman filter paper (No.41) and dilute to required concentration with mobile phase[7-17].

Abbreviations

DOL- Dolutegravir, LAM- Lamivudine, TDF- Tenofovir disoproxil fumarate, RP-HPLC- Reverse Phase - High Performance Liquid Chromatography, UV- Ultra Violet, ICH- International Council for Harmonization, RSD- Relative Standard Deviation, LOD- Limit of detection, LOQ- Limit of quantitation.

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International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

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Tables

Table 1: Regression data

PARAMETERS	UV			RP-HPLC		
	DOL	LAM	TDF	DOL	LAM	TDF
Range	1-5 µg/ml.	6-30 µg/ml	6-30 µg/ml	3-7 µg/ml	18-42 µg/ml	18-42 µg/ml
R^2	0.999	0.998	0.998	0.999	0.999	0.998
Slope	0.070	0.038	0.026	64847	28882	24154
Y-intercept	-0.003	+0.036	-0.015	-31466	+37808	-1177

Table 2: Intra-day precision data

Parameters	UV			RP-HPLC		
	DOL	LAM	TDF	DOL	LAM	TDF
Concentration	3	18	18	5	30	30

International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

(µg/ ml)						
Standard deviation	0.00089	0.00057	0.00186	1075.92	500.39	878.78
%RSD	0.46291	0.08335	0.39989	0.36803	0.04906	0.11686

Table 3: Inter-day precision data

Parameters	**UV**			**RP-HPLC**		
	DOL	**LAM**	**TDF**	**DOL**	**LAM**	**TDF**
Concentration (µg/ ml)	3	18	18	5	30	30
Standard deviation	0.002015	0.001244	0.003359	1598.501	4453.754	4557.076
%RSD	1.12634	0.20017	0.83301	0.55059	0.42776	0.62617

Table 4: Repeatability data

Parameters	**UV**			**RP-HPLC**		
	DOL	**LAM**	**TDF**	**DOL**	**LAM**	**TDF**
Concentration (µg/ ml)	3	18	18	5	30	30
Standard deviation	0.000894	0.000516	0.000753	4088.374	14449.37	9775.65
%RSD	0.43418	0.06984	0.16466	1.40088	1.37693	1.32756

Table 5: Accuracy data

Drug	**Recovery level %**	**% Recovery**	
		UV	**RP-HPLC**
DOL	80 %	102.53	100.56
	100 %	101.60	101.60
	120 %	102.70	100.43
LAM	80 %	99.30	99.59
	100 %	100.43	100.02
	120 %	101.54	100.18
TDF	80 %	100.45	100.74
	100 %	100.79	100.46
	120 %	101.56	100.86

International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

Table 6: LOD and LOQ data

Drugs	UV		RP-HPLC	
	LOD	LOQ	LOD	LOQ
Dolutegravir	5.21	15.81	5.21	15.81
Lamivudine	31.33	94.94	31.32	94.91
Tenofovir disoproxil fumarate	31.32	94.92	31.33	94.95

Table 7: System suitability parameters of RP-HPLC

Parameters	DOL	LAM	TDF
Retention time	6.43	2.56	4.79
Peak area	295078	908739	718120
Theoretical plate	3622	2275	3802
Tailing factor	2.10	1.96	2.11

Table No. 8: Analysis of formulation

Drug		Label claim (mg)	Amount found (mg)	%Label claim
UV	DOL	3	2.99	99.84
	LAM	18	17.99	99.98
	TDF	18	17.99	99.98
RP-HPLC	DOL	5	4.97	99.51
	LAM	30	29.60	98.68
	TDF	30	29.91	99.72

International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

Figures

Fig 1: Overlain spectra of standard solution of DOL

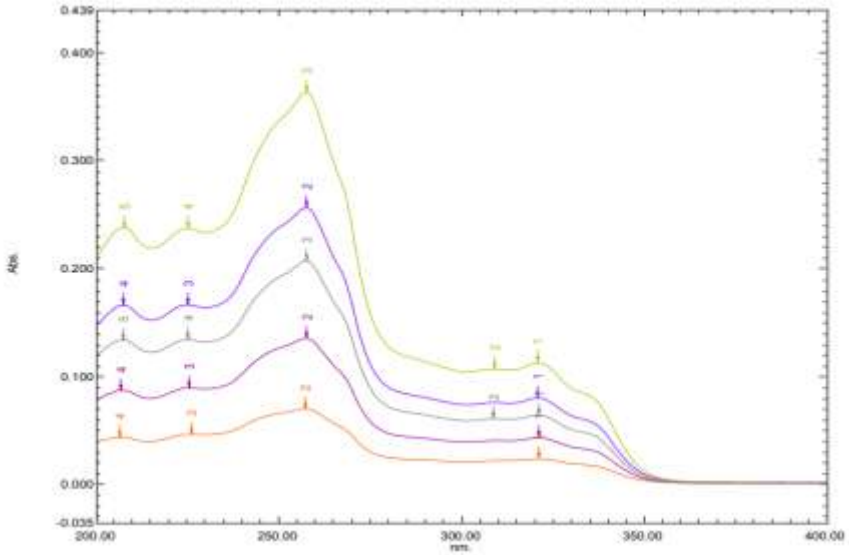
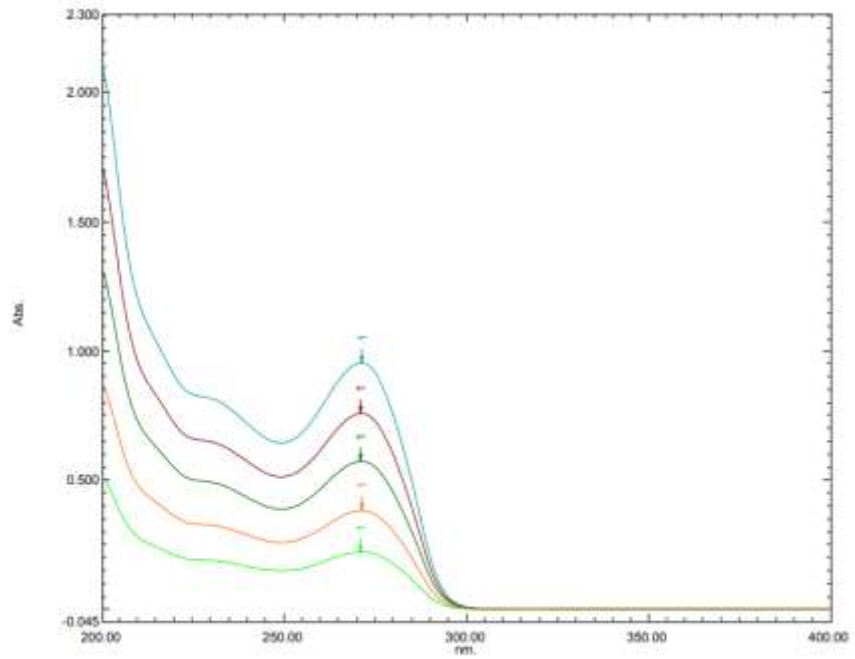


Fig 2: Overlain spectra of standard solution of LAM



International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

Fig 3: Overlain spectra of standard solution of TDF

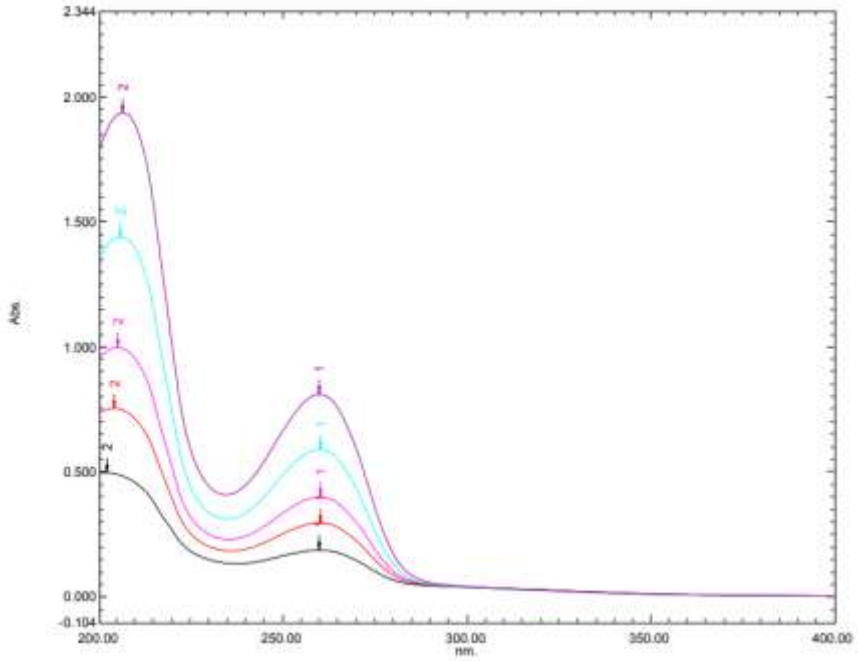
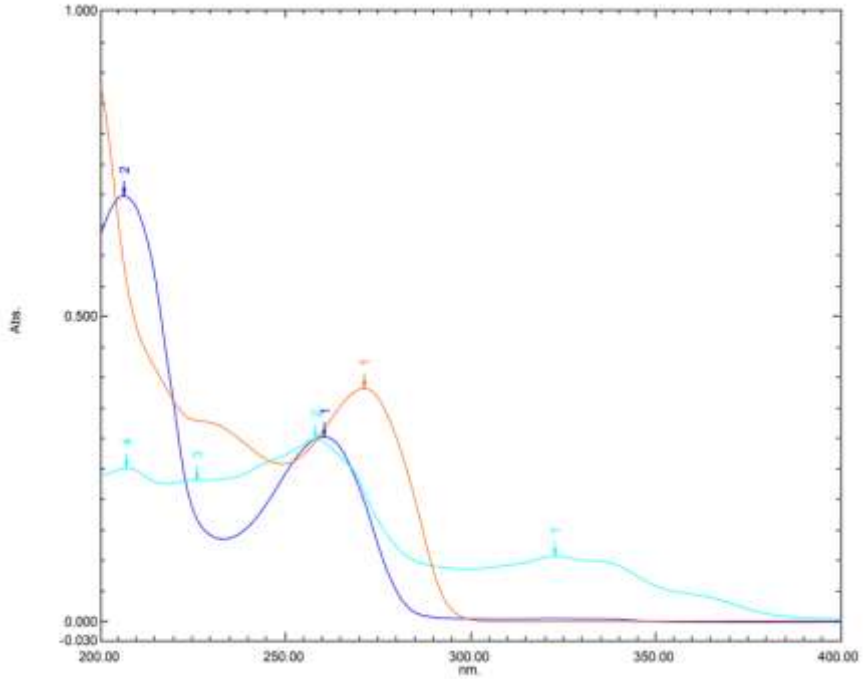


Fig 4: Overlain spectra of standard solution of DOL, LAM and TDF



International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

Fig 5: Spectra of API mixture

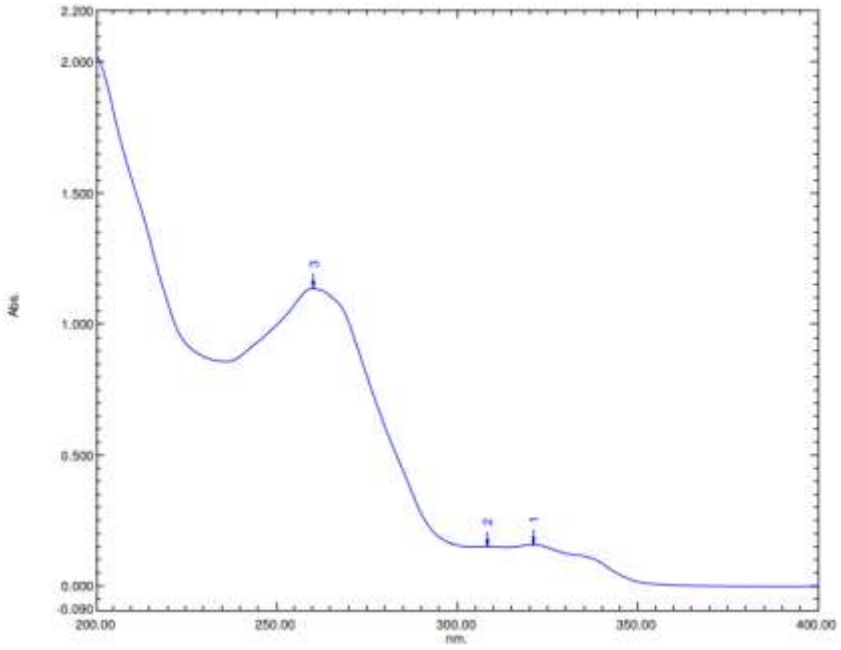
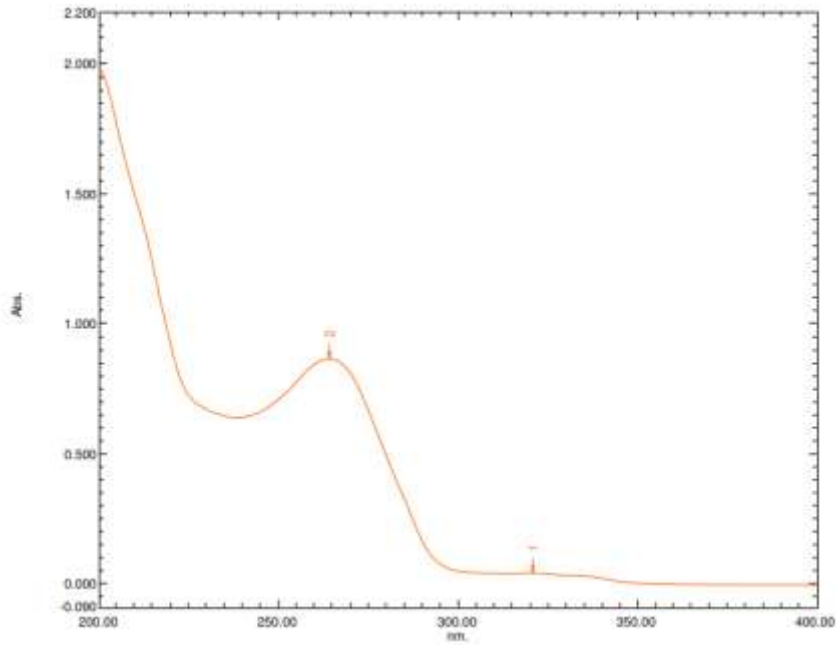


Fig 6: Spectra of formulation



International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

Fig 7: Linearity graph of DOL at 257nm

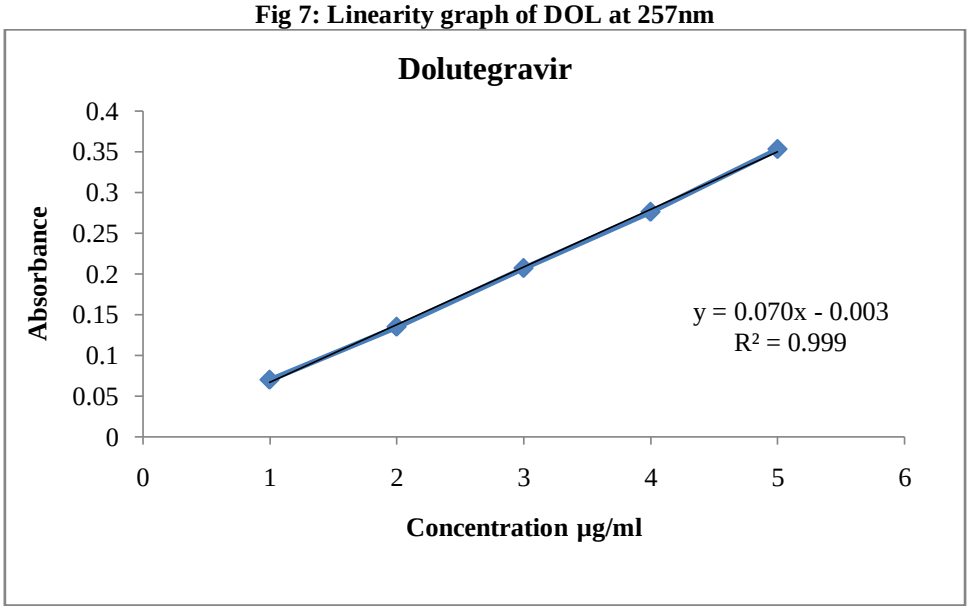
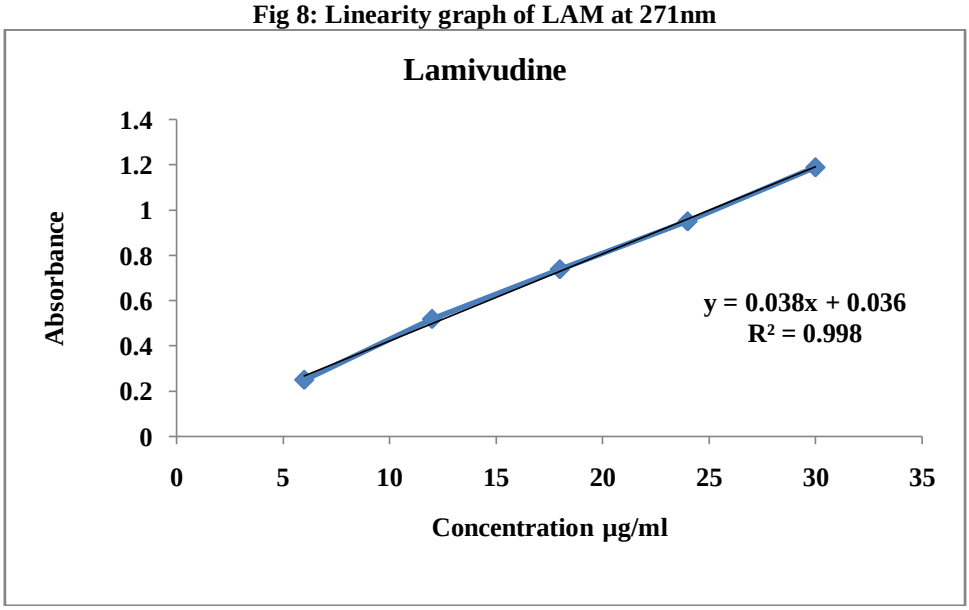


Fig 8: Linearity graph of LAM at 271nm



International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

Fig 9: Linearity graph of TDF at 260nm

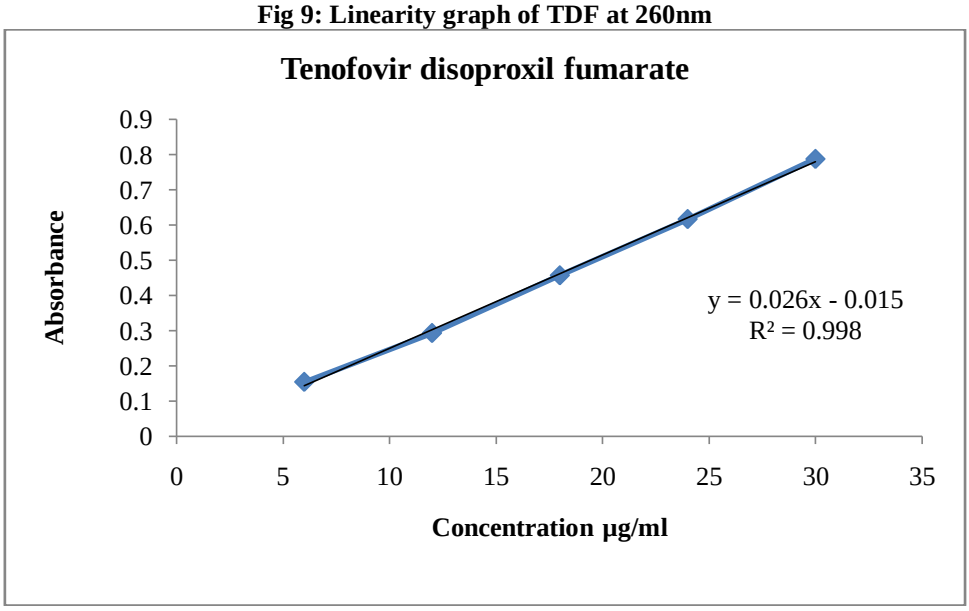
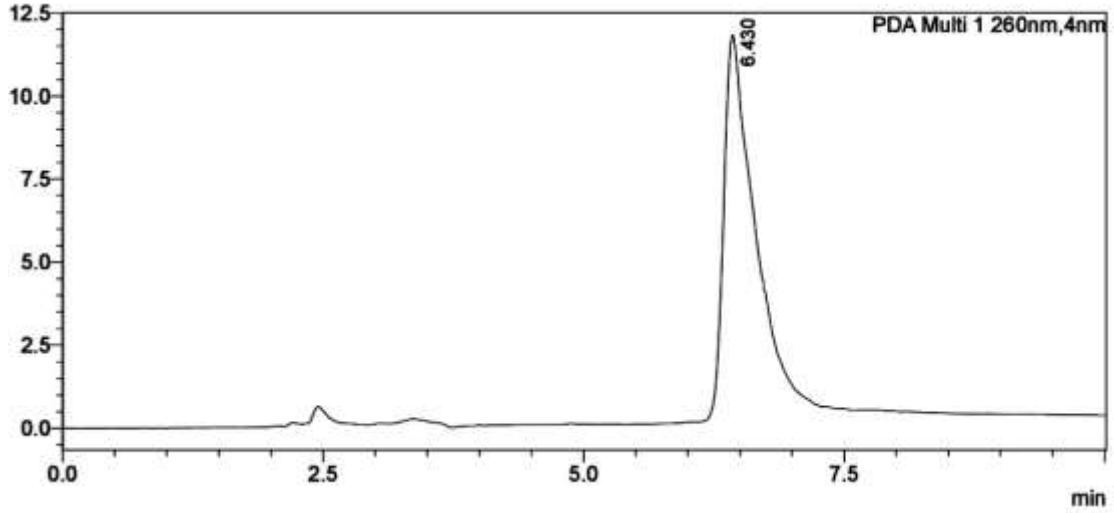


Fig 10: Representative chromatogram of DOL



International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

Fig 11: Representative chromatogram of LAM

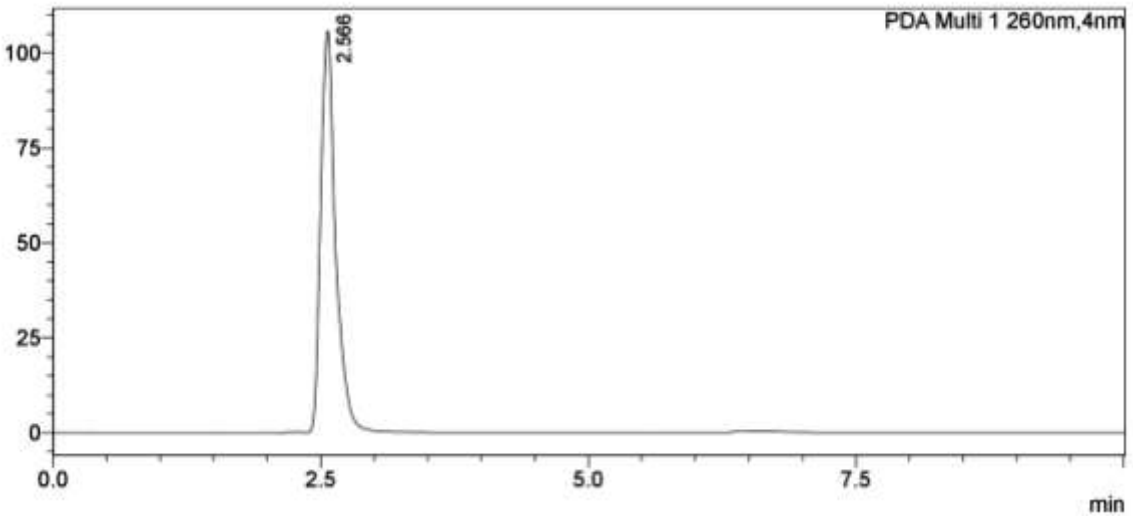
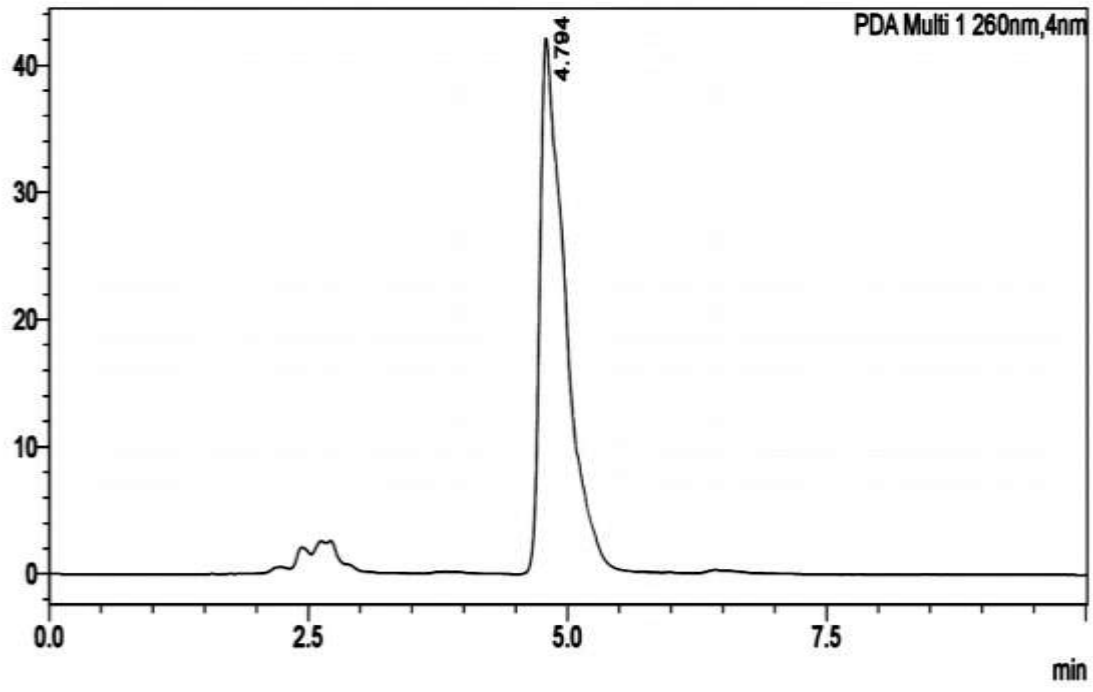


Fig 12: Representative chromatogram of TDF



International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

Fig 13: Representative chromatogram of bulk (DOL, LAM and TDF)

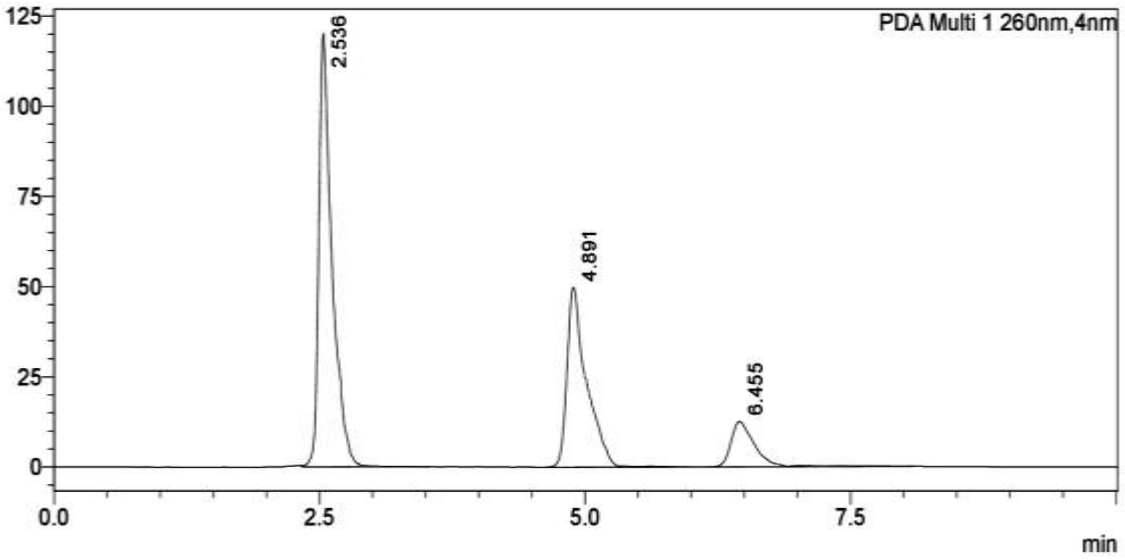
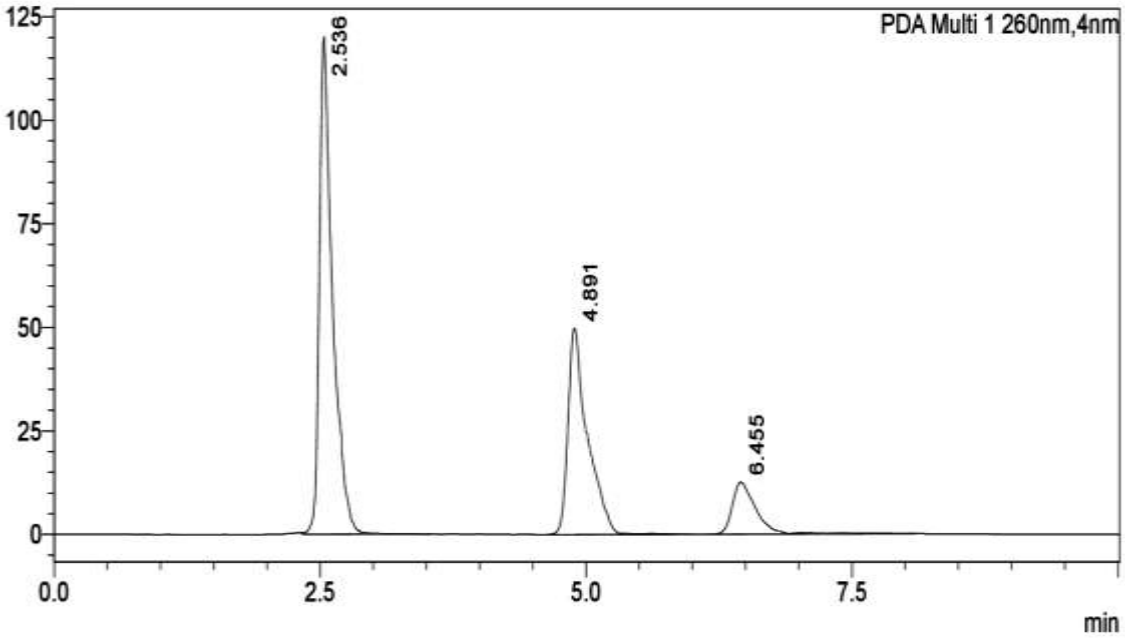


Fig 14:Representative chromatogram of Pharmaceutical dosage form (DOL, LAM & TDF)



International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

Fig 15: Linearity graph of DOL

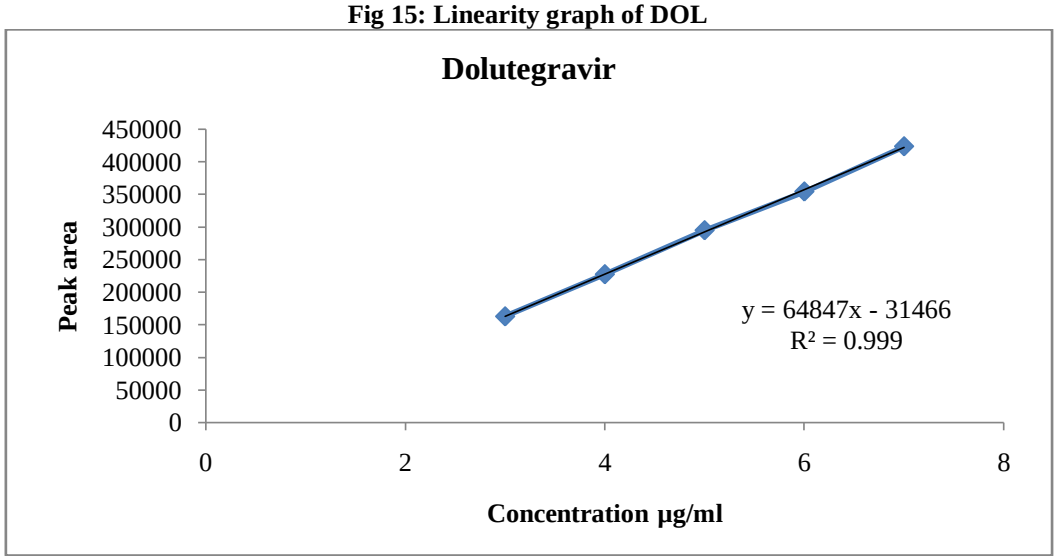
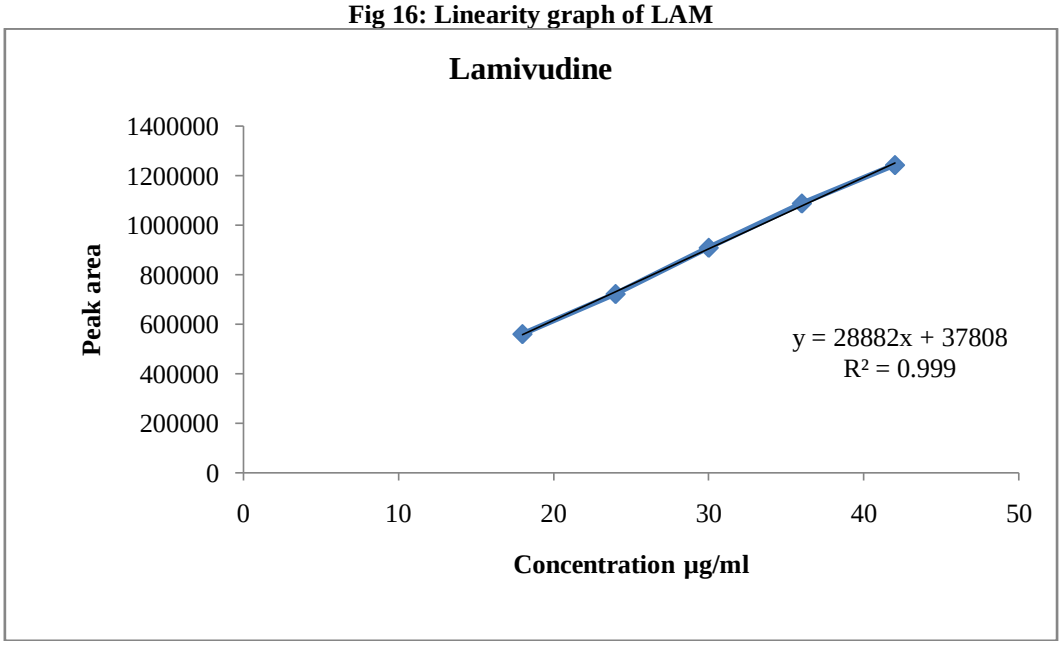


Fig 16: Linearity graph of LAM



International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 3 May-June 2022, pp: 174-189 www.ijprajournal.com ISSN: 2456-4494

Fig 17: Linearity graph of TDF