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A NOVEL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF LAMIVUDINE, TENOFOVIR DISOPROXIL FUMARATE AND EFAVIRENZ IN BULK AND PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC METHOD

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ABSTRACT

The main objective of the present work was to develop simple, precise, accurate and reproducible method development and validation for simultaneous estimation of Lamivudine, Tenofovir Disoproxil Fumarate and Efavirenzin Bulk and pharmaceutical dosage forms by RP-HPLC method. The separation of these three drugs using RP-HPLC was achieved on a SHISHEDO C18, 250×4.6mm, 5-micron size column with a mobile phase consisting Acetonitrile and Phosphate Buffer PH-5.0 (B:A V/V) and flow rate of 1ml/min and UV detection at 256 nm. The retention times were observed to be 2.4 min, 4.1 min and 12.2 min for Lamivudine, Tenofovir Disoproxil Fumarate and Efavirenz respectively. Linearity was found to be 5-25 µg/ml, 5-25 µg/ml and 10-50 µg/ml for Lamivudine, Tenofovir Disoproxil Fumarate and Efavirenz respectively. The method was statistically validated for Linearity, Recovery, Limit of detection, Limit of quantification, Accuracy, Precision. The developed method was successfully validated for accuracy, precision, linearity, limit of detection, limit of quantification & robustness. Hence, this method can be used for simultaneous estimation of Lamivudine, Tenofovir Disoproxil Fumarate and Efavirenzin bulk and pharmaceutical dosage forms.

Keywords: Lamivudine, Tenofovir Disoproxil Fumarate, Efavirenz and Method Validation.

INTRODUCTION

Lamivudine It is a nucleoside reverse transcriptase inhibitor and cytidine analog. It is used to treat human immune deficiency virus type1(HIV-1) and HepatitisB (HBV). IUPAC Name: 4-amino-1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one. Average weight 229.26 g/mol. Mechanism of Lamivudine is a synthetic nucleoside analogue and is phosphorylated intracellularly to its active 5-tri phosphate metabolite, lamivudine triphosphate (L-TP). This nucleoside analogue is incorporated into viral DNA by HIV reverse transcriptase and HBV polymerase, resulting in chain termination. Tenofovir disoproxil fumarate is a nucleotide reverse transcriptase inhibitor. Tenofovir disoproxil fumarate is converted toTenofovir, anacyclicnucleoside Phosphonate (Nucleotide) analog of adenosine 5-Monophosphate.Mechanism of Tenofovir inhibits the activity of HIV reverse transcriptase by competing with the natural substrate deoxyadenosine 5'-triphosphate

and, after incorporation into DNA, by DNA chain termination. Efavirenz is a non-nucleotide reverse transcriptase inhibitor (NNRT) and is used as apart of highly active antiretroviral therapy(HAART) for the treatment of the Human Immuno Deficiency virus(HIVType-1). Mechanism of Efavirenz inhibits the activity of viral RNA-directed DNA polymerase (i.e., reverse transcriptase). Antiviral activity of efavirenz is dependent on intracellular conversion to the active triphosphorylated form. The rate of efavirenz phosphorylation varies, depending on cell type. A thorough literature survey revealed that there are a few analytical methods developed for the estimation lamivudine, tenofovir disoproxil fumarate and efavirenz by UV and HPLC in pharmaceutical formulations. The aim of the present research work is to develop and validate the assay method for the estimation of lamivudine, tenofovir disoproxil fumarate and efavirenz in pharmaceutical formulations.

MATERIALS AND METHODS

Instruments

UV-3092 LABINDIA Double beam with UV win software UV-Visible spectrophotometer with 1cm matched quartz cells. UFLCSHIMADZU HPLC, Model: LC-20AD, UV-Visible Dual absorbance Detector SPD-20A, with an manual sample injector. The output signal was monitored and integrated using lab solutions software. ASHISEIDO C18(4.6mm i.d x 250mm, 5µm) column was used for separations.

Reagents

All the chemicals and reagents (Potassium di-Hydrogen OrthoPhosphate, Potassium hydroxide pellets, Ortho-phosphoric acid, Methanol, Acetonitrile, Water, Lamivudine, Tenofovir disoproxi fumarate, Efavirenz)are analytical grade and the solvents used in this study are HPLC grade.

Preparation of the solutions

Preparation of standard stock solution

Stock solution of Lamivudine, Tenofovir disoproxil fumarate and Efavirenz was prepared by dissolving each of 100mg of drug in 3 -100 ml volumetric flasks with few ml of water and methanol and sonicate it for about 30 minutes and make upto final volume with methanol and water (50:50v/v) to get 1mg/ml solution. From this, pipette out 10,10,10ml of Lamivudine, Tenofovir disoproxil fumarate and Efavirenz in a 3-100ml volumetric flasks then make up the volume to 100ml with 65:35 ratio of acetonitrile and phosphate buffer to get the concentration 100µg/ml. From this each of 1.5ml,1.5ml, 3ml of LAM, TDF, EFV and make up the volume to 10ml with acetonitrile phosphate buffer 65:35 together required concentration. Inject 20µL of this standard preparation in to HPLC system as per proposed optimized conditions and chromatogram was recorded shown in fig. 1.

Sample Preparation (Assay)

20 Tablets were taken and their average weight was calculated. Tablets were crushed to a fine powder and dose equivalent to 300mg of Lamivudine, Tenofovir disoproxil fumarate,600mg of Efavirenz wastrans-ferredtoa100mlvolumetricflask,dissolved and made upto final volume with methanol and water (50:50) to get 1000µg/ml. From the above solution, 10ml was pipetted out in to 100ml volumetric flask and made upto final volume with mobile phase acetonitrile: phosphate buffer (65:35). From this each of 1.5ml was taken and make up the volume to 10ml with acetonitrile phosphate buffer 65:35 to get the required concentration. Inject 20uL of this sample preparation in to HPLC system as per proposed optimized conditions and chromatogram was recorded shown in fig. 2.

EXPERIMENTAL WORK

Determination of λmax

λmax was determined by preparing 10µg/ml LAM, TDF, EFV solution of water and methanol (50:50).It was scanned in the region of 200-800nmand the spectrum was recorded. From the spectrum wavelength was selected by means of isobestic point shown in fig. 3.

Optimized method chromatographic conditions

Mobile phase Acetonitrile: Phosphate buffer pH 5.0 (B:Av/v), Flow rate: 1ml/min, Column: SHISEIDOC_{18} (250×4.6mm,5µ), Detector wavelength: 256nm, Column temp: Ambient, Injection volume: 20µl, Run time:20 min, observed in table1.

Observation

Good separation and resolution was observed in fig. 4.Tailing was observed 1.1, 1.1 and1.0 within the limits. Theoretical plates were2202, 6681and 29219 and limit is more than 2000. And it was the final optimized trail. Retention time for the drugs was found to be 2.4min, 4.1min, 12.2 min for Lamivudine, Tenofovir disoproxil fumarate and Efavirenz.

Chromatographic conditions

The elution was gradient and the mobile phase consisted of a mixture of acetonitrile and phosphate bufferPH-5(B:Av/v).The buffer was

prepared by dissolving 6.8g of potassium dihydrogen phosphate in 1000 ml water adjusted with 10 M potassium hydroxide pH 5.0 ± 0.1. The buffer was filtered through a 0.5µmembrane filter before prior to use. A SHISEIDO C_{18} (4.6 x 250 mm, 5□m) column was used for determination. The flow rate was 1 ml/ min and the column was operated at ambient temperature. The volume of sample injected was 20 µL. Prior to injection of the solutions, it was filtered through 0.25 u filter. Column was equilibrated for at least 30min with mobile phase flowing through the system. The UV detector was set at wavelength of 256 nm.

RESULTS AND DISCUSSION

System suitability

System suitability and chromatographic parameters were validated such as number of theoretical plates, asymmetry factor and tailing factor were calculated. Optimized conditions and chromatogram was recorded. Atypical chromatogram of Lamivudine, Tenofovir disoproxil fumarate and Efavirenz is observed from the data in table 2. That system suitability parameters, tailing factor is less than 2.0 and theoretical plates are more than 2000. Both parameters are found to be within the acceptance criteria. Hence, it is evident that the preferred system is suitable.

Specificity

Specificity is the ability of a method to discriminate between the analyte(s) of interest and other components that are present in the sample. Studies are designed to evaluate the degree of interference, if any, which can be attributed to the analytes, impurities, degradation products, reagent "blanks" and excipients. This provides the analyst with a degree of certainty that the response observed is due to the single analyte of interest. The degree of specificity testing varies depending on the method type and the stage of validation. Specificity should be evaluated continually through the drug development Process. The chromatogram was shown in fig. 5.

Linearity

Linearity of this method was evaluated by line a regression analysis and calculated by least square method. For this study, standard stock solutions of Lamivudine, Tenofovir disoproxil fumarate and Efavirenz were prepared by dissolving each of 100mg in 100ml volumetric flasks and make upthe volume to 100ml with methanol water (50:50). From this take each of 10mland make up the volume to 100ml with Acetonitrile and Phosphate buffer (65:35) to get 100µg/ml.

Observation

Absorbance of resulting solutions was measured and the calibration curve was plotted between peak area and concentration of the drug. The response was found to be linear in the range 5-25 µg/ml for Lamivudine, 5-25µg/ml for Tenofovir disoproxil fumarate, 10-50 µg/ ml for Efavirenz. The correlation coefficient between the concentration of selected drugs and their chromatographic peak response (area) is highly impressive and found to have R^2 = 0.999. This regression analysis indicates that the method has excellent linearity over the wide concentration range. The linearity plot is given in fig.6, 7, 8.

Precision

Five sample solutions of the same concentration (100%) were prepared and injected into the HPLC system.

Observation

The %RSD for the area of five standard injections results are found to be 0.48, 0.41, 1.4 and they are in limits.

Method precision

Five sample solutions of the same concentration (100%) were prepared and injected into the HPLC system on next day.

Acceptance Criteria

The% RSD for the area of five standard injections results should not be more than 2.

Observation

The %RSD for the area of five standard injections results are found to be 0.5, 0.7, 1.7 and they are in limits.

Accuracy

Accuracy was performed in triplicate for various concentrations of Lamivudine, Tenofovir disoproxil fumarate and Efavirenz equivalent to 50%,100% and150% of the standard amounts were injected into the HPLC system. The average %recovery of LMD, TDF, EFV was calculated observed in table 3.

Preparation sample solutions

For preparation of 50%solution

Lamivudine, Tenofovir disoproxil fumarate and Efavirenz of 0.75ml, 0.75ml and 1.5ml from the 100µg/ml solution and then make up the volume to 10ml with Acetonitrile and Phosphate buffer (65:35).

For preparation of 100% solution
Lamivudine, Tenofovir disoproxil fumarate and Efavirenz of 1.5ml, 1.5ml and 3ml from the 100µg/ml solution and then make up the volume to 10ml with Acetonitrile and Phosphate buffer (65:35).

For preparation of 150% solution
Lamivudine, Tenofovir disoproxil fumarate and Efavirenz of 2.25ml, 2.25ml and 4.5mlfrom the 100µg/ml solution and then make up the volume to 10ml with Acetonitrile and Phosphate buffer (65:35).

Robustness
Robustness was done by small deliberate changes in the chromatographic conditions and retention times of Lamivudine, Tenofovir disoproxil fumarate, Efavirenz were noted. The factors selected were flow rate and variation in the λ max. The results remained unaffected by small variations in these parameters.

Limit of detection and Limit of quantification
The lowest amount of analyte in sample that can be detected, but not necessarily quantified, with acceptable precision and accuracy was determined by comparison of S/N value of standard solution with that of blank. S/N ratio should be 3:1.The lowest amount of analyte in sample that can be quantified, with acceptable precision and accuracy was determined by comparison of S/N value of standard solution with that of blank. S/Nratioshouldbe10:1.LOD, LOQ are shown in the table 4.

SUMMARY
A reverse-phase HPLC column was used to develop a suitable method for the simultaneous estimation of Lamivudine, Tenofovir disoproxil fumarate and Efavirenz. The chromatographic conditions were optimized by changing the mobile phase composition. Different ratios were experimented to optimize the mobile phase. Finally Acetonitrile and Phosphate Buffer (pH=5.0$\pm$ 0.1) combination was used as mobile phase with gradient technique. This mobile phase showed good resolution of Lamivudine, Tenofovir disoproxil fumarate and Efavirenz peaks. The wavelength of detection selected was 256nm, as the drugs showed optimized absorbance at this wavelength and the flow rate was 1ml/min. The retention times of Lamivudine, Tenofovir disoproxil fumarate and Efavirenz were about 2.4, 4.1, 12.2 min respectively and none of the impurities were interfering in its assay. Linearity, precision, accuracy results are within the acceptable range of limits.

The statistical analysis of data and the drug recovery data showed that the method was simple, rapid, economical, sensitive, precise and accurate and can there by easily adopted for routine quality control analysis. The results of this analysis confirmed that the proposed method was suitable for determination of drug in pharmaceutical formulation with virtually no interference of additives. Hence the proposed method can be successfully applied in simultaneous estimation of Lamivudine, Tenofovir disoproxil fumarate and Efavirenz in marketed formulation.

CONCLUSION
A Simple RP-HPLC method for simultaneous analysis of Lamivudine, Tenofovir disoproxil fumarate and Efavirenz was developed and an extensively single laboratory validation for specificity and or selectivity, linearity, accuracy, precision and robustness. Cost implication for the method application is relatively low as it does not involve any costly sample extraction procedure. The good recoveries were obtained in all cases as well as reliable agreement with their respective label claims and non-interference of formulation excipients in the estimation. This method remains largely unaffected by small variations in the flow rate and λmax. The analysis time is also short approximately 15mins.The procedure proved that the applied method is considered accurate, precise, selective with lower limit of detection and quantitation more specific and sensitive according to ICH guidelines. This makes the reported method is suitable for routine analysis in pharmaceutical dosage forms which contains Lamivudine, Tenofovir disoproxil fumarate and Efavirenz.

Table 1: Parameters of optimized method

Parameters	Conditions
Column (Stationary Phase)	SHISEIDOC$_{18}$(4.6x250mm, 5µ,)
Mobile Phase	Acetonitrile(pH4): Phosphatebuffer
Flow rate(ml/min)	1ml/min
Runtime(min)	15
Column temperature(°C)	Ambient
Volume of injection loop(µl)	20µl
Detection wavelength(nm)	256nm

Table 2: System suitability results of LAM, TDF and EFV

INJECTION	LAM	TDF	EFV
Injection1	644232	435876	2049461
Injection2	645986	435804	2048692
Injection3	643612	435356	2046124
Injection4	641869	431956	2015692
Injection5	6399103	431289	1995506
Average	643120.4	434056.2	2031095
Standard Deviation	2324.148	2243	24375.52
%RSD	0.36	0.51	1.2
Theoretical Plates	3929	6345	23876
Tailing factor	1.1	1.1	1.0

Table 3: Accuracy and percentage recovery of each analyte

Accuracy level %	Mean recovery of Lamivudine	Mean recovery of Tenofovir disoproxilfumarate	Mean recovery of Efavirenz
50	99.83	99.8	99.866
100	100.13	100.9	100.5
150	100.93	101.0	101.5

Table 4: LOD, LOQ data of LAM,TDF, EFV

	LOD	S/N	LOQ	S/N
Lamivudine	0.05µg/ml	2.89	0.2µg/ml	8.95
Tenofovir disoproxil fumarate	0.1µg/ml	2.56	0.5µg/ml	9.05
Efavirenz	0.1µg/ml	3.01	0.3µg/ml	8.79

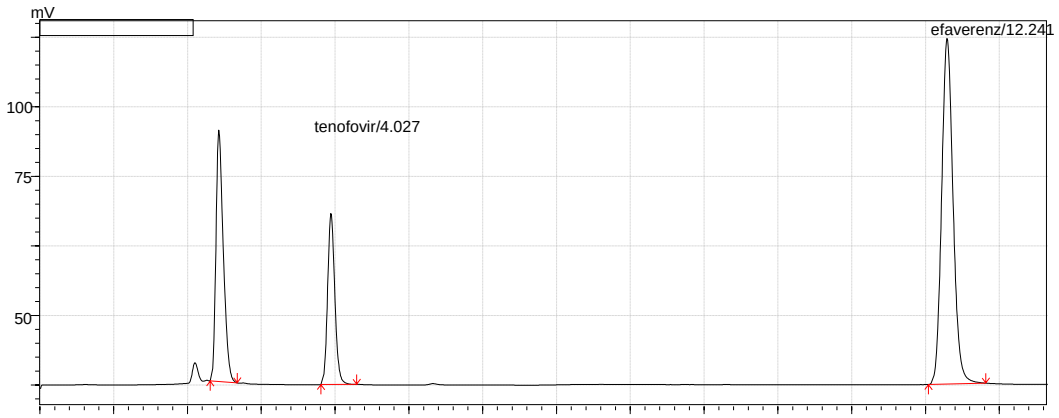

Fig.1: Chromatogram of standard LAM, TDF, EFV

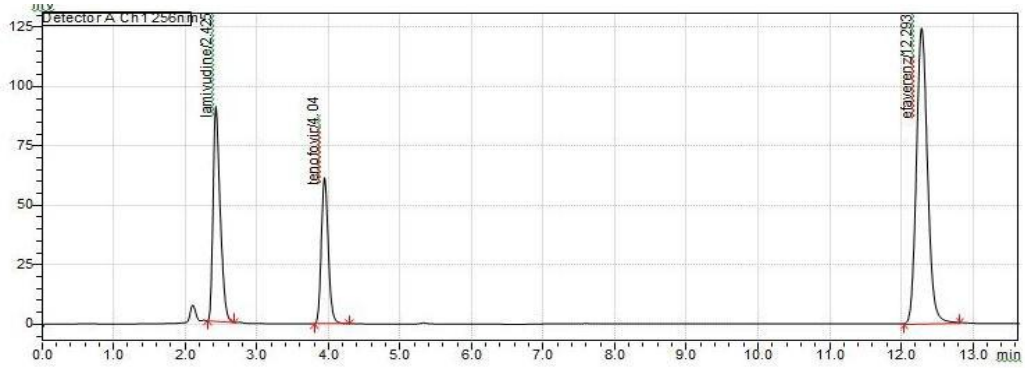

Fig. 2: Chromatogram of formulation of LAM, TDF, EFV

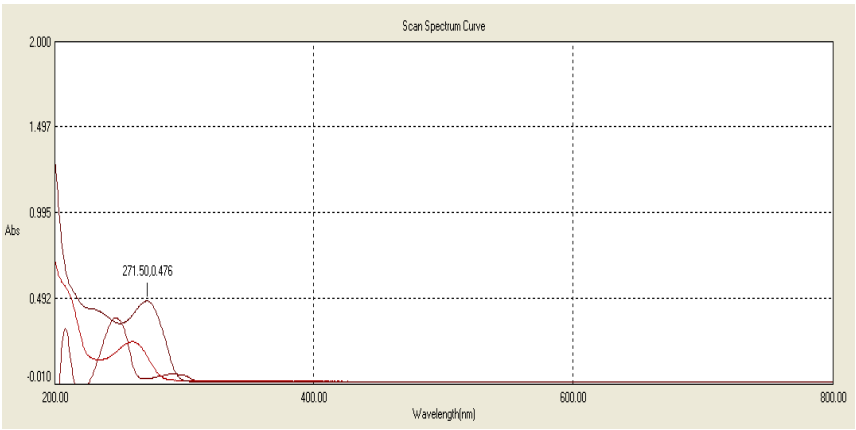

Fig. 3: Isobestic point of LAM, TDF, EFV

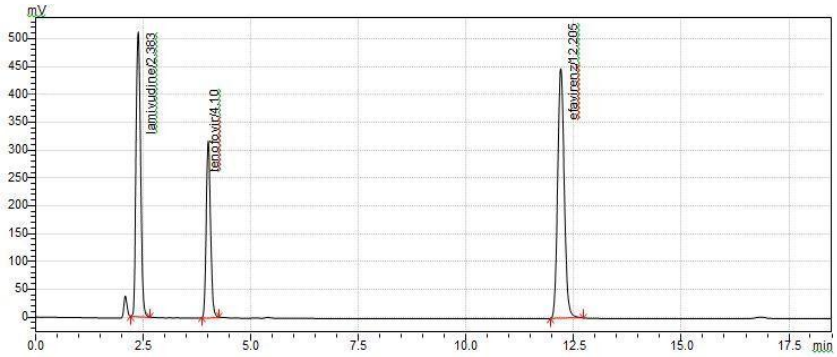

Fig. 4: Optimized chromatogram of LAM, TDF, EFV

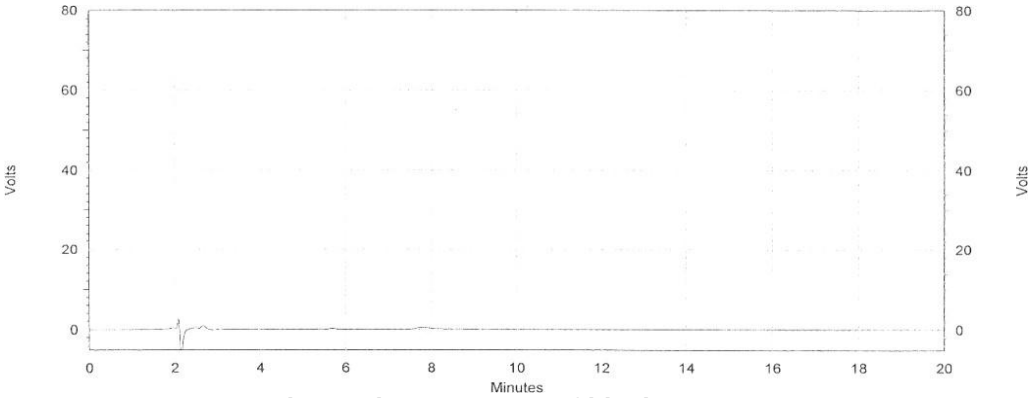

Fig. 5: Chromatogram of blank

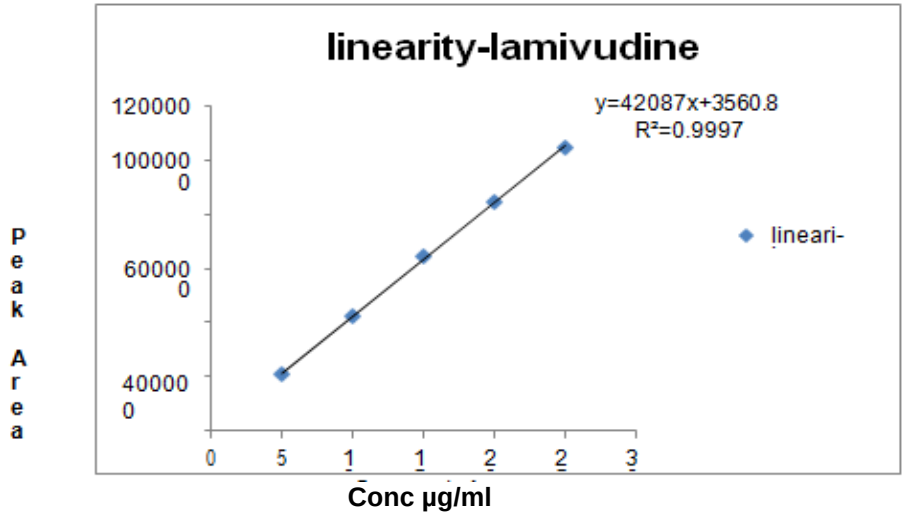

Fig. 6: Linearity plot of lamivudine

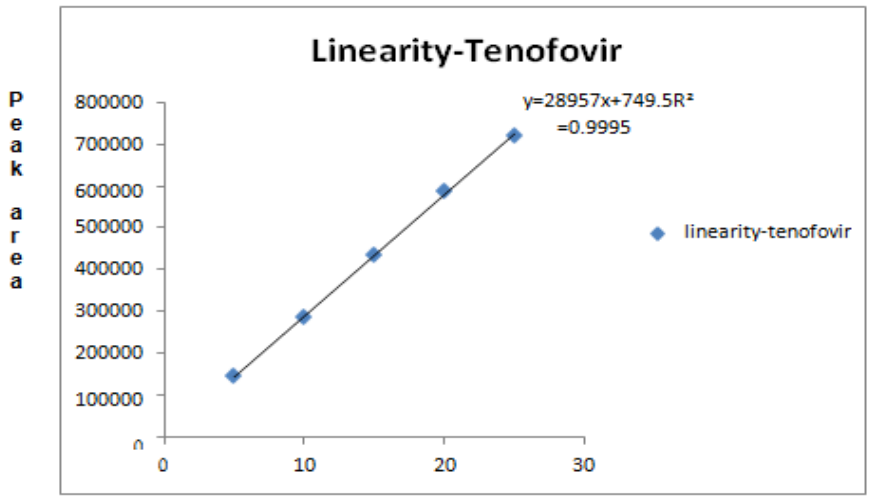

Fig. 7: Linearity plot of Tenofovir disoproxil fumarate

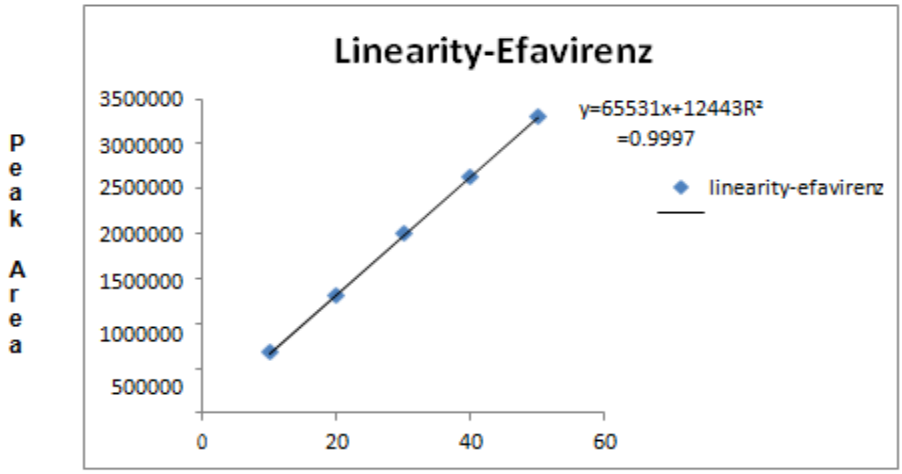

Fig. 8: Linearity plot of Efavirenz

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