RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF EFAVIRENZ, LAMIVUDINE AND ZIDOVUDINE IN TABLET DOSAGE FORM

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ABSTRACT

Analytical chemists work to improve the reliability of existing techniques to meet the demands for better chemical measurements, which arise constantly in our society. They adapt proven methodologies to new kinds of materials or to answer new questions about their composition. They carry out research to discover completely new principles of measurement and are at the forefront of the utilization of major discoveries such as lasers and microchip devices for practical purposes.In this paper we are dealing with RP-HPLC procedure was developed and validated as per ICH guidelines for the simultaneous estimation of efavirenz, lamivudine and zidovudine.

No:of Tables : 6	No:of Figures : 11	No:of References:33

INTRODUCTION

NNRTI class of antiretrovirals are both nucleoside and non-nucleoside RTIs inhibit the same target, the reverse transcriptase enzyme, an essential viral enzyme which transcribes viral RNA into DNA. Unlike nucleoside RTIs, which bind at the enzyme's active site, NNRTIs act allosterically by binding to a distinct site away from the active site known as the NNRTI pocket.Efavirenz is not effective against HIV-2, as the pocket of the HIV-2 reverse transcriptase has a different structure, which confers intrinsic resistance to the NNRTI class.As most NNRTIs bind within the same pocket, viral strains which are resistant to efavirenz are usually also resistant to the other NNRTIs, nevirapine and delavirdine. The most common mutation observed after efavirenz treatment is K103N, which is also observed with other NNRTIs.It can be administered orally and highly bound to human plasma protein predominantly albumin. It is principally metabolized by cytochrome P450 system to hydroxylated metabolites with subsequent glucuronidation of these hydroxylated metabolites. CYP3A and CYPB6 are the major isoenzymes responsible for efavirenz metabolism and excretion by urinary excretion.Efavirenz is a non-nucleoside reverse transcriptase inhibitor (NNRTI) of HIV-1. Efavirenz binds directly to reverse transcriptase and blocks the RNA-dependent and DNA-dependent DNA polymerase activities by inducing a conformational change that causes a disruption of the enzyme's catalytic site. The activity of efavirenz does not compete with template or nucleoside triphosphates. HIV-2 reverse transcriptase and eukaryotic DNA polymerases (such as human DNA polymerases α, β, γ, or δ) are not inhibited by efavirenz.Lamivudine is an analogue of cytidine. It can inhibit both types (1 and 2) of HIV reverse transcriptase and also the reverse transcriptase of hepatitis B. It is phosphorylated to active metabolites that compete for incorporation into viral DNA. They inhibit the HIV reverse transcriptase enzyme competitively and act as a chain terminator of DNA synthesis. The lack of a 3'-OH group in the incorporated nucleoside analogue prevents the formation of the 5' to 3' phosphodiester linkage essential for DNA chain elongation, and therefore, the viral DNA growth is terminated.Lamivudine is administered orally, and it is rapidly absorbed with a bio-availability of over 80%. Some research suggests that lamivudine can cross the blood–brain barrier. Lamivudine is often given in combination with zidovudine, with which it is highly synergistic. Lamivudine treatment has been shown to restore zidovudine sensitivity of previously resistant HIV. Lamivudine showed no evidence of carcinogenicity or mutagenicity in *in vivo* studies in mice and rats at doses from 10 to 58 times those used in humans.Lamivudine has a high oral bioavailability and is distributed widely into the body. Lamivudine either enters the cells by passive diffusion or is actively transported by uptake transporters

(SLC22A1, SLC22A2, and SLC22A3). Intracellularly, it is phosphorylated to its active triphosphate from. Phosphorylation of 3TC to 3TC-monophosphate (3TC-MP) is catalyzed by deoxycytidine kinase. Phosphorylation of 3TC-MP to 3TC-DP is catalyzed by cytidine monophosphate/deoxycytidine monophosphate kinase while 3TC-DP is phosphorylated to 3TC-TP by 3'-phosphoglycerate kinase or nucleoside diphosphate kinase. Dephosphorylation of 3TC-MP to 3TC is catalyzed by 5'-nucleosidase or phosphatases. The rate-limiting step is the conversion of the 3TC-DP to 3TC-TP, which is a saturable enzymatic process. 3TC-TP is converted to 3TC-MP by two salvage pathways. 3TC-TP is converted to 3TC-DP-choline by choline cytidyl transferase, which is converted to 3TC-MP by p-choline glyceride transferase. 3TC-TP is converted to 3TC-DP-ethanolamine by p-ethanolamine cytidyl transferase, which is then converted to 3TC-MP. Lamivudine is actively transported out of cell by efflux transporters ABCB1, ABCC1, ABCC2, ABCC3, ABCC4 and ABCG2. However, lamivudine in its monophosphate form is transported out of the cell by ABCC4. Lamivudine is primarily (70 % of the drug) excreted unchanged in urine. However 5-10 % of the drug is excreted as inactive trans-sulfoxide metabolite Lamivudine triphosphate competes with deoxycytidine triphosphate for binding to reverse transcriptase, and the incorporation of this 3TC-triphosphate (3TC-TP) form in viral DNA results in chain termination. Lamivudine when administered orally (150 mg every 12 hours) reaches plasma concentrations which are consistently above its IC50 values.

Zidovudine inhibits the activity of reverse transcriptase and blocks the production of DNA and new viruses. Specifically, zidovudine is converted within the body to its active form (zidovudine triphosphate). This active form is similar to the compound thymidine triphosphate, a chemical that is required by the HIV virus to make new DNA. The reverse transcriptase uses zidovudine triphosphate instead of thymidine triphosphate for making DNA, and it is the zidovudine triphosphate that interferes with the reverse transcriptase. Zidovudine does not kill existing HIV virus, and it is not a cure for HIV. Retrovir was approved by the FDA in 1987.Absorbed orally and oral bio availability is 75% due to first pass metabolism and crosses the blood brain barrier. And then elimination is mainly by conjugation in the liver followed by excretion. Metabolism is probably limited by hepatic blood flow. About 14% excreted unchanged following oral administration. In viral infected cells, pharmacologically active form of ZDV, zidovudine triphosphate (ZDV-TP) inhibits the activity of HIV-1 reverse transcriptase by competing with its natural nucleotide counterpart thymidine triphosphate for incorporation into newly synthesized viral DNA. Once incorporated, it leads to DNA chain termination and stops further DNA synthesis. The efficacy of ZDV treatment in HIV infection attributed to its selective affinity for HIV reverse transcriptase as against to human DNA polymerase

however non-specific inhibition of mitochondrial DNA polymerase gamma results in observed mitochondrial toxicity. Specifically interference of mitochondrial DNA replication by ZDV-TP results in reducing mitochondrial DNA subsequently leading to mitochondrial dysfunction with anaerobic respiration, lipoatrophy, myopathy and lactic acidosis with hepatosteatosis. These undesirable side-effects are further potentiated in HIV-patients due to negative impact of HIV infection on mitochondria. Due to these factors the package insert of ZDV includes warnings for risk of hematological toxicity, myopathy and lactic acidosis with hepatosteatosis a rare but life threatening mitochondrial toxicity. Several reverse phase high performance liquid chromatographic (RP-HPLC) methods have been reported for the determination of efavirenz, lamivudine and zidovudine and in combination with other drugs as per literature reports. Yadavalli Rekha et al., (2013).., used a combination of water (pH was adjusted to 2.1 with ortho- phosphoric acid) and methanol in the ratio70:30, v/v as mobile phase for simultaneous estimation of efavirenz, lamivudine and zidovudine in pharmaceutical tablet formulation. T. BHAVYA *et al., (2013)..,* used a combination of acetonitile and 0.02M potassium dihydrogen ortho phosphate buffer adjusted to pH 3.2 in the proportion of 30: 70v/v for the simultaneous estimation of efavirenz, lamivudine and zidovudine in bulk and in tablet dosage form. In these two cases methanol, buffer and acetonitrile were used as mobile phase. Methanol was more economical less expensive and easily available. To suit the analysis of such drug combination it was planned to develop and validate RP-HPLC method for these drugs in combined dosage form as per ICH guidelines. The marketed tablet *DUOVIR-E-kit* contains efavirenz 600mg and lamivudine 150mg and zidovudine 300 mg respectively and the combination of efavirenz, lamivudine and zidovudine is used when the person suffers from viral infections.

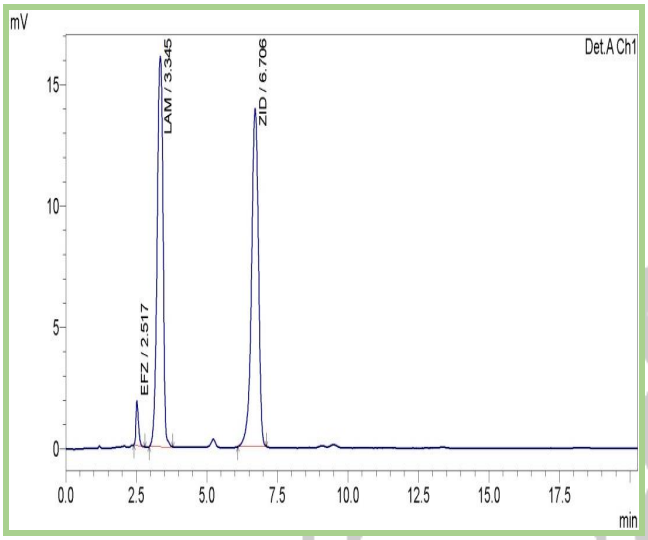
Literature survey reveals that there are no isocratic RP-HPLC methods for the simultaneous estimation of lamivudine and efavirenz in the bulk drugs and in the formulation. Methods have been reported for the estimation of lamivudine, efavirenz & Zidovudine individually combination with other as well as in drugs by HPLC [7-14].

MATERIALS AND METHODS

Selection of chromatographic method in general is done taking into consideration several parameters like the nature of the drugs, molecular weight and solubility. Since both the drugs selected are polar in nature, reversed phase chromatography has been used. C_{18} column was chosen as stationary phase and a methanol organic solvent and water are used to develop a method for the simultaneous estimation of efavirenz, lamivudine and zidovudine.

Optimized chromatographic conditions:

The optimized mobile phase is Methanol: water (35:65 % v/v) at a flow rate of 1 ml/min at 256 nm, under these conditions efavirenz, lamivudine and zidovudine were eluted at 2.517 min, 3.456 min and 6.502 min respectively.



METHOD VALIDATION

Preparation of solutions:

Preparation of mobile phase

Methanol (HPLC grade) of 650 ml was taken and 350 ml water (HPLC) was taken and finally this solution was filtered through 0.45µm membrane filter followed by degassing.

Preparation of standard stock solution:

About 20 mg of efavirenz, lamivudine and zidovudine were accurately weighed into a 20 ml volumetric flask, to this 10 ml of diluent (mobile phase) was added, sonicated and the volume was made up with the diluent and then filtered through 0.45µm membrane filter.

Preparation of sample stock solution:

Twenty tablets (brand name) were taken (each tablet containing 2.5mg of efavirenz and lamivudine, zidovudine) into a mortar and finely powdered with a pestle. An equivalent weight of powder containing 2.5mg of efavirenz and 20 mg of lamivudine, zidovudine were accurately weighed and transferred to 10 ml volumetric flask and diluted with the methanol and shaken mechanically for ten minutes and then centrifuged. The clear supernatant liquid was taken and sonicated in ultrasonic bath for 5 minutes. The solution was filtered through 0.45µm membrane filter and then final volume wad made up with methanol. From this stock solution 1 ml was taken into a 10 ml volumetric flask and made up the volume with diluent.

System suitability:

The system suitability was assessed by six replicate analysis of drugs at concentrations of 20µg/ml for efavirenz, lamivudine and 20µg/ml for zidovudine by taking 2 ml of efavirenz, lamivudine and 2ml of zidovudine from stock solutions in 10mL volumetric flak and made up the volume with mobile phase. The acceptance criteria were ± 2% for percent coefficient variation (CV %) for peak area and retention time for efavirenz, lamivudine and zidovudine.

Chromatogram for the above discussed system suitability solutions were recorded as shown in the Figure 8.1 and results are listed in the Table 8.1.

Standard solutions of efavirenz, lamivudine and zidovudine at different concentrations level were prepared in triplicate. Calibration curves were constructed by plotting the concentration of drugs versus corresponding mean peak area. The results show that an excellent correlation exists between mean peak area and concentration level for all the drugs and the results are given in tables. The correlation coefficient of efavirenz, lamivudine and zidovudine are 0.9999, 0.9999 & 0.9998 respectively, which meet the method validation acceptance criteria and hence the method is said to be linear in the range of 5-150µg/ml for efavirenz, 1-120µg/ml for lamivudine and 5-150 µg/ml for zidovudine.

Accuracy and precision:

Preparation of LQC sample: From stock solutions 0.5ml of efavirenz, lamivudine and zidovudine were taken into a 10ml volumetric flask and made up the volume with mobile phase to get a concentrations of 12.5 µg/ml of efavirenz, lamivudine and zidovudine.

Preparation of MQC sample: From stock solutions 1ml efavirenz, lamivudine and zidovudine were taken into a 10ml volumetric flask and made up the volume with mobile phase to get a concentration of 25 µg/ml of efavirenz, lamivudine and zidovudine.

Preparation of HQC sample: From stock solutions 1.5ml of efavirenz, lamivudine and zidovudine were taken into a 10ml volumetric flask and made up the volume with mobile phase to get a concentrations of 37.5 µg/ml of efavirenz, lamivudine and zidovudine.

Accuracy of the assay method was determined using the triplicate analysis of QC samples. Precision of the assay was determined by repeatability. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time that was evaluated by assaying the QC samples during same day.

Robustness:

The MQC sample solution prepared was analysed under different chromatographic conditions stated below.

a) Change in flow rate:
   Flow rate – 0.8 ml/min
   Flow rate – 1.2 ml/min
b) Change in mobile phase:
   Methanol: water (30:70 % v/v)
   Methanol: water (40:60% v/v)

RESULTS & DISCUSSION

LIMIT OF DETECTION (LOD):

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Several approaches for determining the detection limit are possible, depending on

whether the procedure is a non-instrumental or instrumental.

Based on Signal-to-Noise - This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio 3:1 is generally considered acceptable for estimating the detection limit.

Based on the Standard Deviation of the Response and the Slope:

The detection limit (DL) may be ex pressed as:

$$DL = 3.3 \sigma / S$$

Where, σ = the standard deviation of y-intercepts of regression lines

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

LIMIT OF QUANTITATION (LOQ):

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified

1. A typical signal-to-noise ratio is 10:1.

2. Based on the Standard Deviation of the Response and the Slope

The quantitation limit (QL) may be expressed as:

$$QL = 10 \sigma / S$$

Where, σ = the standard deviation of the response.

S = the slope of the calibration curve.

Efavirenz is soluble in methanol and partially soluble in water where as lamivudine and zidovudine was found to be soluble in water. Methanol was used as solvent for diluents and determination of absorbance in UV spectrometer. The wavelength selection was done by scanning the drug (pure) in the UV range (200-400) and the maximum absorbance was found to be at 256nm. The drug

solution obeyed Beer's law in the concentration range 10-80µg/ml for efavirenz, zidovudine and 10-60µg/ml for lamivudine.

In chromatographic analysis of substances RP-HPLC is normally advocated because of faster elution of polar compounds than non polar compounds. In the present work the RP-HPLC method was developed and validated which was simple less expensive and more rapid with binary gradient mode.

For the method development six trials were carried out and reported. In first and second trials the clear peaks were observed but time taken for analysis was found to be more than 10 minutes. In third and fourth trials time of analysis was decreased and further trials were conducted to observe system suitability parameters at different proportions of mobile phase. Fourth trial was repeated which lead to the optimized chromatographic conditions for the determinations of efavirenz, lamivudine and zidovudine in combined tablet dosage form. The results were recorded and reported

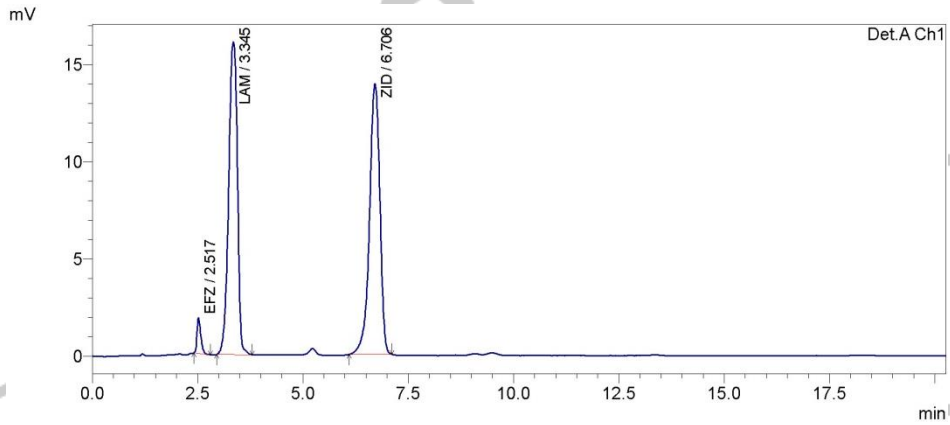


Fig 1: Chromatogram for system suitability

Table 1: system suitability:

System suitability parameters	Efavirenz	Lamivudine	Zidovudine
%RSD for six replicate injections of standard	0.15	0.24	0.37
Tailing factor	1.384	1.024	0.787
Theoretical plates	3719.357	2907.950	6114.518
Resolution	-	4.013	11.304

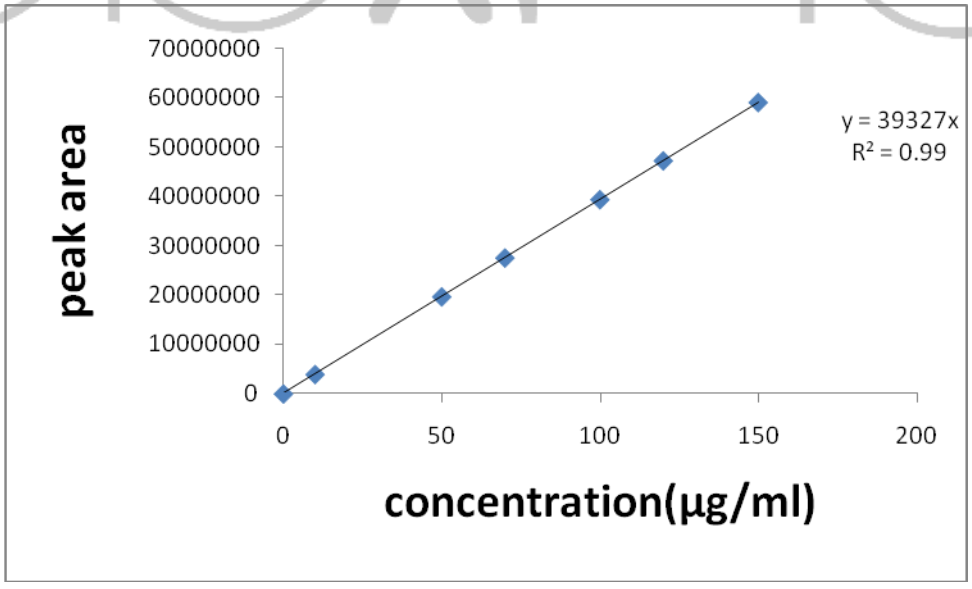


Fig 2: Linearity graph of efavirenz

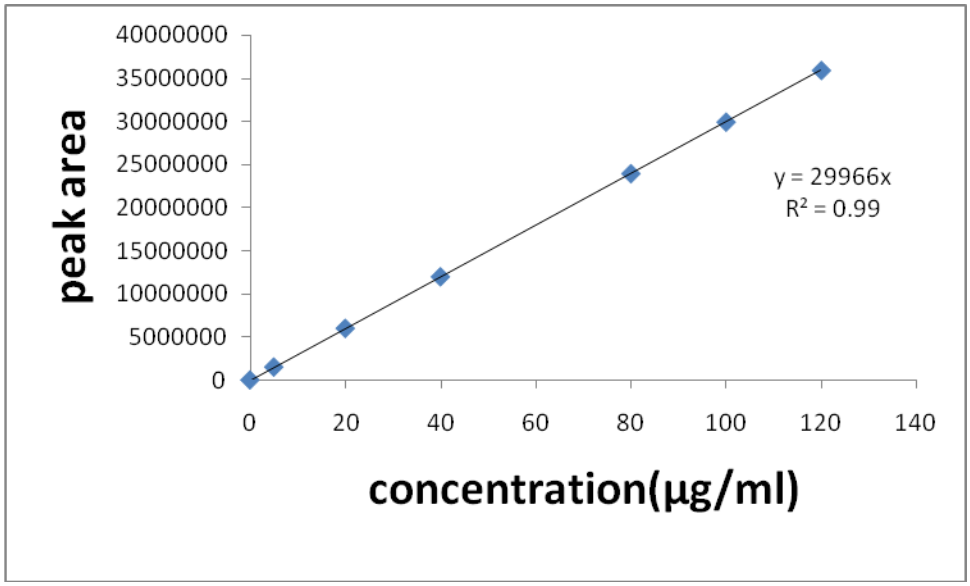


Fig 3: Linearity graph of lamivudine

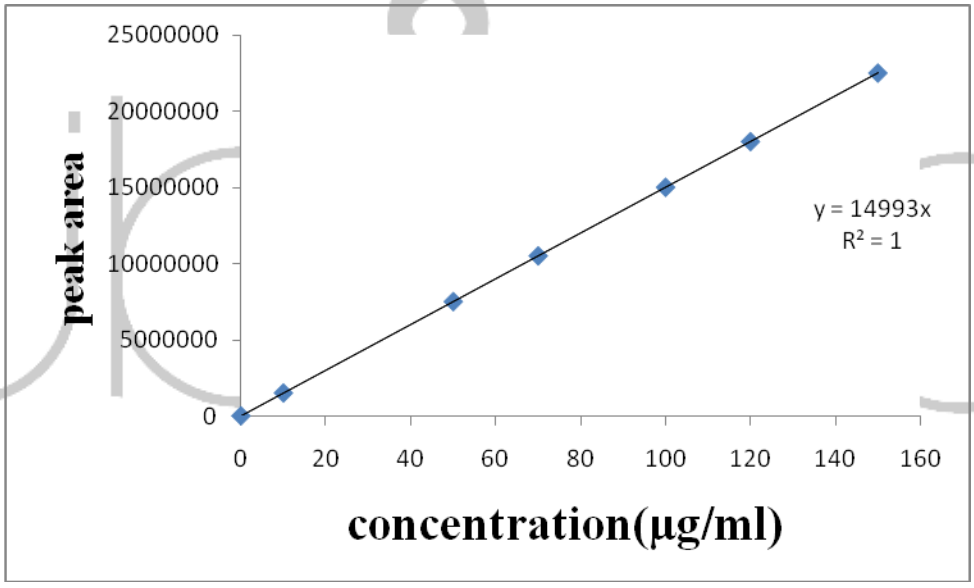


Fig 4: Linearity graph of zidovudine

QC Sample (n = 6)	Nominal Concentration (µg/mL)	Practical Concentration (µg/mL) Mean ± S.D	Accuracy (%)	% C.V

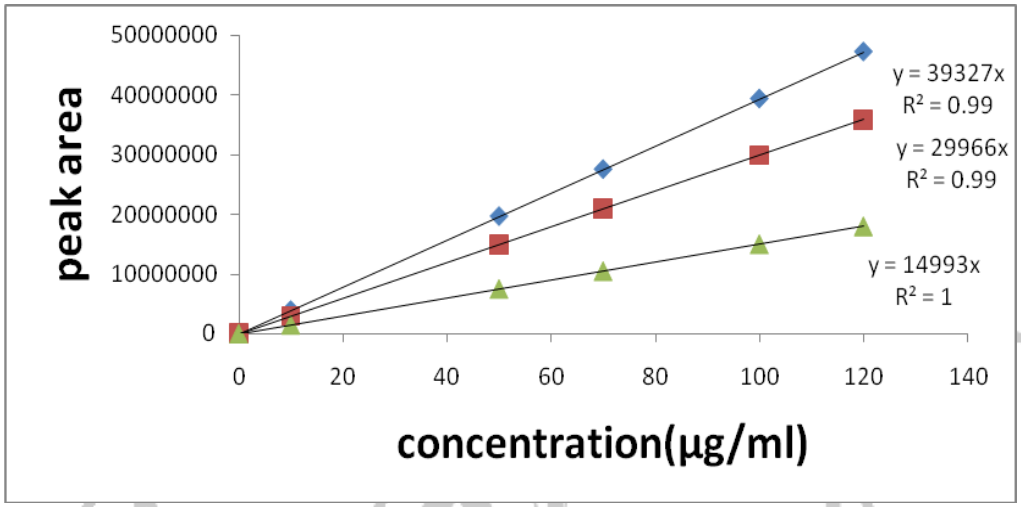


Table 2: linearity:

Parameters	Efavirenz	Lamivudine	Zidovudine
Slope	39327.22	29966.83	14993.92
Intercept	8321.33	5598.467	7868.07
Correlation coefficient	0.9929	0.9963	1

	EFV, LAM & ZID	EFV	LAM	ZID	EFV	LAM	ZID	EFV	LAM	ZID
QC 1	20	21.52±0.152	20.32±0.61	21.54±0.007	99.6	99.59	99.51	1.975	1.995	0.489
QC 2	80	80.53±0.81	80.65±0.51	79.7±0.074	100.27	100.81	99.56	0.182	0.765	0.935
QC 3	180	179.87±0.153	181.18±0.42	24.92±0.118	99.11	100.65	99.48	1.031	1.650	0.794

Table 3: Accuracy and precision of Efavirenz, Lamivudine, Zidovudvudine

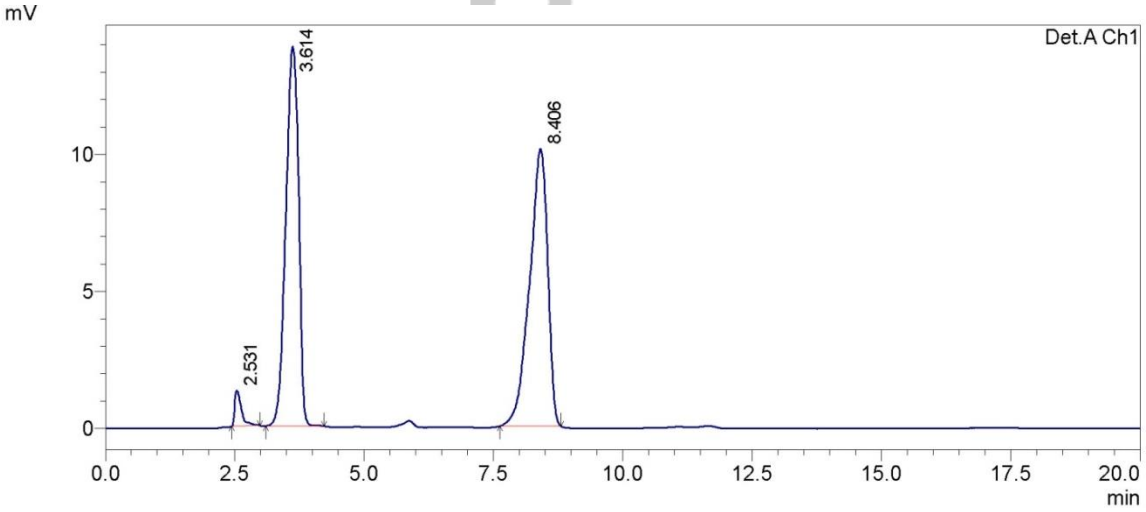


Fig 6: Chromatogram for 0.8 ml/min flow rate

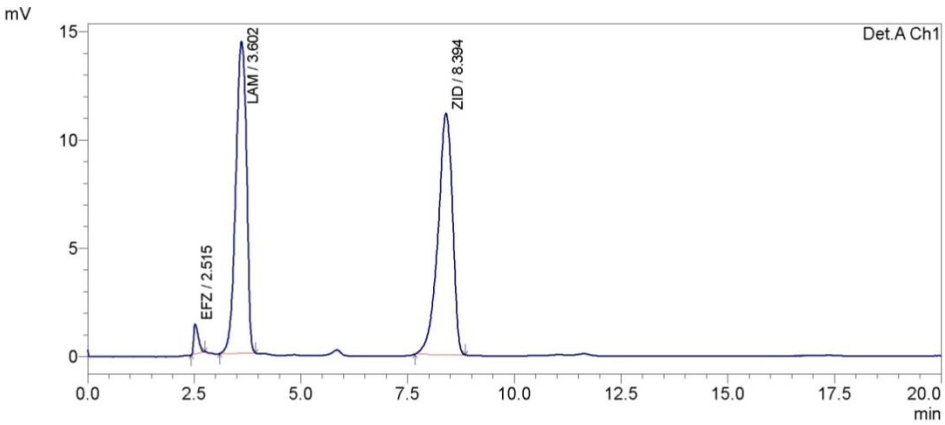


Fig.7: Chromatogram for 1.2 ml/min flow rate

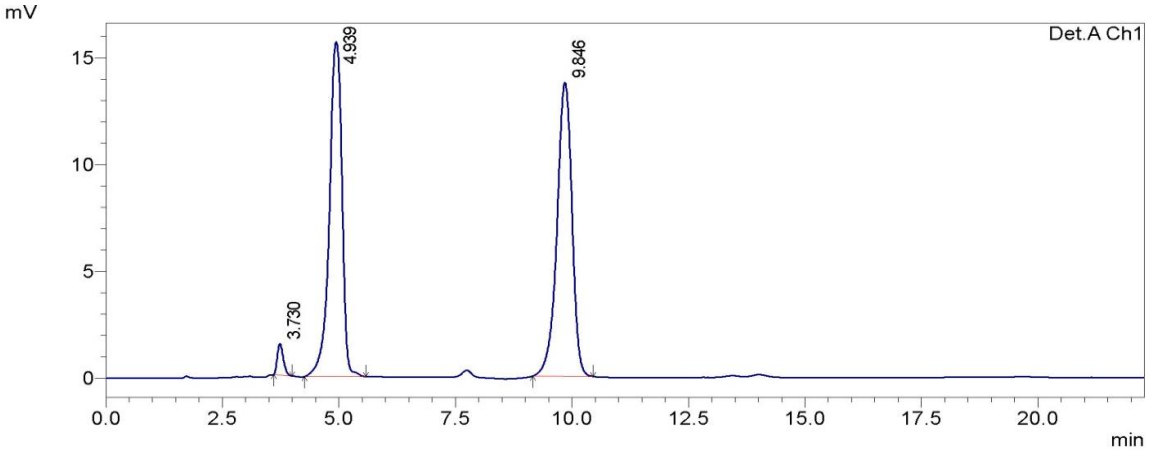


Fig. 8: Chromatogram for 0.8 ml/min flow rate

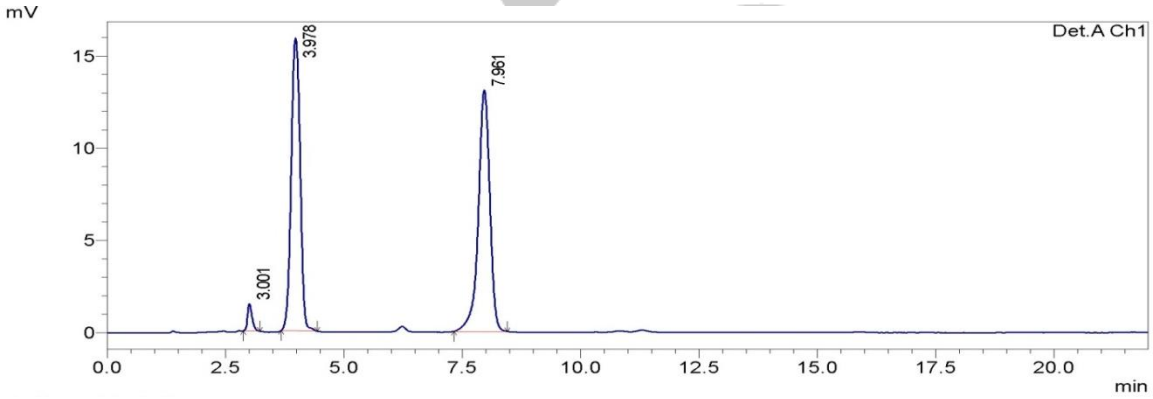


Fig 9: Chromatogram for 1.2 ml/min flow rate

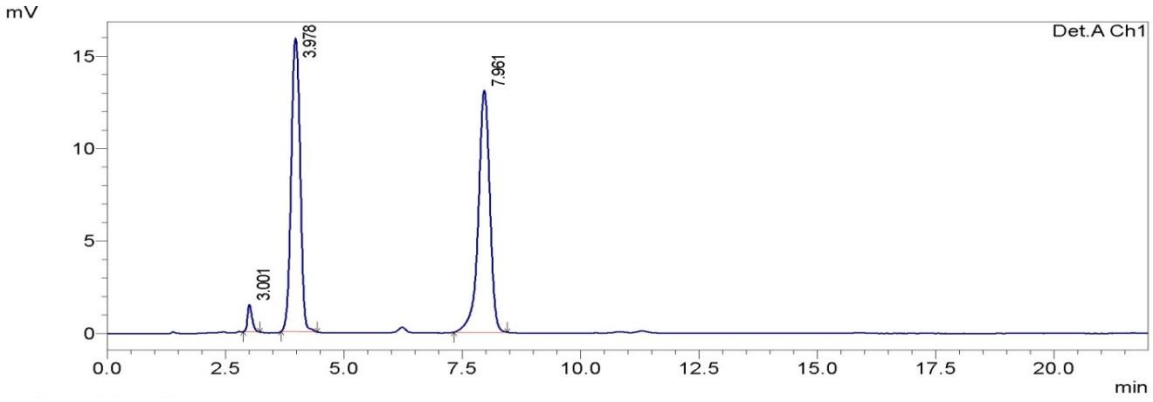


Fig.10: Chromatogram for 40% organic phase

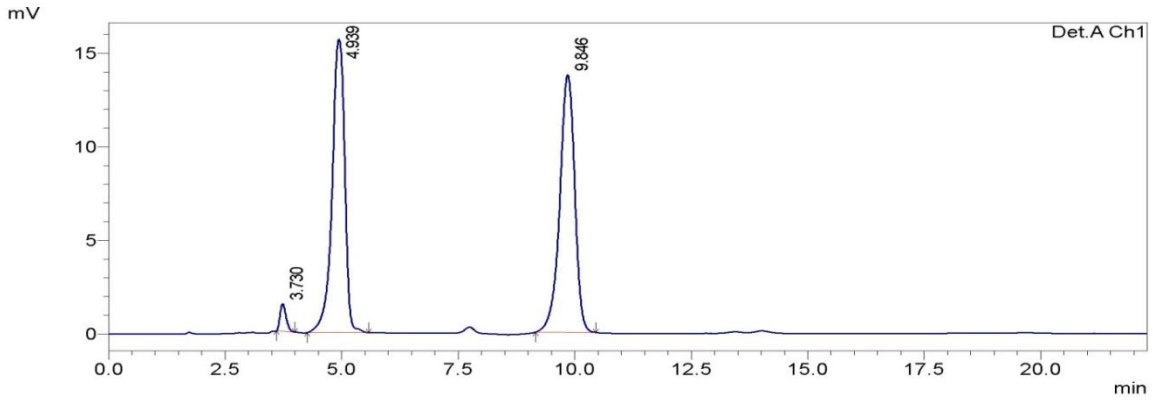


Fig.11: Chromatogram for 30% organic phase

Table 4: Robustness:

	Parameters	Variation	Rt	Tailing factor	Plate count
Efavirenz	**Flow rate**	0.8 mL/min	2.53	2.71	1909.4
		1.2mL/min	2.51	1.93	2168.2
	Mobile phase	40% organic phase	3.0	1.38	3664.8
		30% organic phase	3.7	1.20	3329.58
Lamivudine	**Flow rate**	0.8 mL/min	3.61	0.89	971.276
		1.2mL/min	3.60	0.88	1005.8

	Mobile phase	40% organic phase	3.97	0.92	1970.40
		30% organic phase	4.9	0.78	1700.61
Zidovudine	**Flow Rate**	0.8ml/min	8.4	0.78	2829.44
		1.2ml/min	8.3	0.83	3085.68
	Mobile Phase	40% organic phase	7.9	0.92	4982.29
		30%organic phase	9.8	0.88	4236.07

Table 5: LOD & LOQ:

Drug name	Parameter	Peak area	Theoretical plates	Tailing factor
Efavirenz	LOD	3938342.33	3725.36	1.28
	LOQ	3938342.33	3736.24	1.21
Lamivudine	LOD	1496780	2915.95	1.09
	LOQ	1496765	2956.14	0.96
Zidovudine	LOD	1490825.35	6114.193	0.72
	LOQ	1491562.31	6123.87	0.68

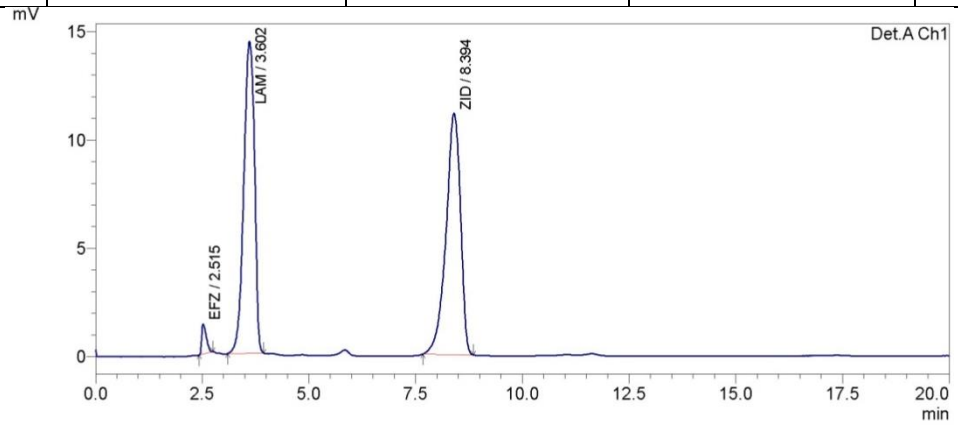


Fig. 11: Chromatogram for formulation

Table 6: Assay of formulation

Formulation tablets	Drug and label claim in mg	Amount of drug taken for assay in mg.	Amount recovered in mg Mean ± S.D (n=6)	Peak areas	%CV	% Assay
DUOVIR-E KIT (Cipla)	EFV(600mg)	50	599.496±2.424	3938342	0.13	99.6
	LAM(150mg)	25	301.496±0.723	1496780	0.21	99.59
	ZID(300mg)	25	300.162±0.784	1490825	0.721	99.51

The robustness of the method was checked by changing flow rate and temperature, and standard was able to give system suitability parameters within limit, which indicates that the method is robust Limit of detection (LOD) and quantification (LOQ) were estimated from signal to noise ratio. The limit of detection (LOD) value for efavirenz, lamivudine and were 0.45 µg/ml,0.70 and, for zidovudine are 2.37 µg/ml and limit of quantification (LOQ) value for efavirenz was 1.39 µg/ml, for lamivudine 2.14 µg/ml and for zidovudine 7.18 µg/ml respectively.

The developed method was applied for the estimation of efavirenz, lamivudine and zidovidine in the marketed formulations and was found to be suitable. A simple and reproducible RP-HPLC procedure was developed and validated as per ICH guidelines for the simultaneous estimation of efavirenz, lamivudine and zidovudine.Quantitative estimation of efavirenz, lamivudine and zidovudine was estimated by RP-HPLC using methanol and water (35:65v/v) as a mobile phase and column ODS C18 , (4.6mm, 250mm, 3µ) as a stationary phase and the peaks were observed at 256nm which was selected as a wavelength for quantitative estimation.

After development of the method it was validated for system suitability, accuracy, linearity, precision, and robustness. The value of theoretical plates (>2500), tailing factor (<2), retention time (minimum) and resollution (<1) was found to be within limits, hence it is concluded that the system is suitable to perform assay.The linearity studies were performed for the formulation. It was found to be linear. In the range of 5-150 µg/ml for efavirenz and 1-120µg/ml for lamivudine and 5-150µg/ml for zidovudine

respectively. The precision was checked and found to be within limits (0.162-1.975), hence the method is precise. The accuracy has been determined from lower limit of quality control sample to high quality control sample and the prescribed limits for recovery are 98%-102%. From accuracy studies, % recovery was calculated and found to be within limits (2%). The robustness of the method was checked by changing flow rate and temperature, and organic phase and it was able to give system suitability parameters within limit, which indicates that the method is robust.Therefore it was concluded that the proposed method can be used for routine analysis of efavirenz, lamivudine and zidovudine in tablet dosage forms.

REFERENCES:

Deepali G., Elvis, M. UV spectro photometric method for assay of the anti-retroviral agent lamivudine in active pharmaceutical ingredient and in its tablet formulation. Pharm analysis. 2010; 2 (4):417-419.

Anand Kumar Y., Rama Rao N. Development of rapid UV Spectrophotometric method for the estimation of efavirenz in formulations. E-Journalof Chemistry. 2010; 7(3):856-860.

A.G. Gilman, Antiretroviral agents The Pharmacological Basis of Therapeutics, McGraw Medica. Hardman J and Limbird LE, New York, 1349 (2001).

Anonymous, Indian Pharmacopoeia. Addendum, Government of India, New Delhi, 920 (2007).

Anonymous, Indian Pharmacopoeia Addendum, Government of India, New Delhi, 913 (2007).

Appala Raju N, Begum S. Simultaneous RP-HPLC Method for the Estimation of the Emtricitabine, Tenofovir Disoproxil Fumerate and Efavirenz in Tablet Dosage Forms. Research journal of pharmacy and technology 2008: 1(4):522-525.

Balint, G.A., Antiretroviral therapeutics possibilities for human immunodeficiency virus/acquired immunodeficiency syndrome. Pharmacol. Ther. 89, 17–27.,2001.

Beckett AH, Stenlake JB. Practical Pharmaceutical chemistry, 4th edn., part II, S.K. Jain for CBS publisher and Distributors. New Delhi, 1998, 275-300.

Bhavsar DS, Patel BN, Patel CN. RP-HPLC method for simultaneous estimation of Tenofovir Disoproxil Fumarate, Lamivudine, and Efavirenz in combined tablet dosage form. Pharmaceutical methods 2012; 3(2):73-78.

British Pharmacopoeia, H. M. Stationary Press, London, **2002**, 1814–1815.

Camille Roucairol, Stephane Azoulay, Marie-Claire Nevers, Christophe Creminon, Jacques Grassi, Alain Burger, Daniele

Duval. Analytica Chimica Acta, 589, (1):142-149, (2007).

Dos Santos JV, de Carvalho LA, Pina ME. Development and validation of a RP-HPLC method for the determination of Zidovudine and its related substances in sustained release tablets. Anal Sci. 2011; 27(3):283-289

Estrela, R.C., Salvadori, M.C., Suarez-Kurtz, G., 2004. A rapid and sensitive method forsimultaneous determination of lamivudine and zidovudine in human serum by on-line-phase extraction coupled to liquid chromatography /tandem mass spectrometry detection.Rapid Commun. Mass Spectrom. 18, 1147–1155.

FDA, Guidance for Industry: Impurities in Drug Product, Draft Guidance, Center for Drug Evaluation and Research (CDER), (1998).

Goodman & Gilman's, The Pharmacological Basis of Therapeutics, (CDROM) 9th Ed.; Hardman, J.G., Limbird, L.E., Eds.; McGraw- Hill Companies: Hightstown, NJ, 1996.

International Conference on Harmonization (ICH), Guidance for Industry, Q1A (R2): Stability Testing of New Drug Substances and Products, IFPMA, Geneva, (2003).

International Conference on Harmonization (ICH), Validation of Analytical Procedures: Text and 1.Methodology Q2 (R1), November (2005).

International Conference on Harmonization (ICH), Guidance for Industry, Q1A (R2): Stability Testing of New Drug Substances and Products, IFPMA, Geneva, (2003).

Jadhav SD, Bhatia MS, Thamake SL, Pishawikar SA. Development and validation of method for simultaneous estimation of Lamivudine, Zidovudine and Nevirapine. Asian journal of research in chemistry 2010; 3(4):1-3.

Krishna reddy NV, Phani RSCh, Ramesh raju R. New RP-HPLC method development for analysis and assay of Lamivudine in formulation. International journal of research in pharmaceutical and biomedical sciences 2011; 2(1):220-223.

K D Tripathi, Essentials of Medical Pharmacology, Jaypee Brothers Medical Publishers Ltd, New Delhi, **2003**, 5, 728-329.

Lakshmi Kusuma S, Yadav KKS, Visala G, Venkatesh BK. Method development and validation of RP-HPLC for determination of Zidovudine. International journal of research in pharmacy and chemistry 2011; 1(3):677-680.

Mallepelli S, Narasimharao R, Swapna J. Analytical method development and method validation for the simultaneous estimation of Lamivudine and Stavudine in tablet dosage form by RP-HPLC. International journal of pharma and biosciences 2011; 1(4):551-559

Mahor RP, Parcha V, Singh Y, Sharma R, Bhandari A. Development and validation of a HPLC Method for simultaneous estimation of Lamivudine and Zidovudine in tablet dosage forms. Der Chemica Sinica 2011; 2(6):12-19.

Manikanta Kumar A, Naga Sandhya B, Mahesh N, Prasad VLNN, Diwan PV. Development and validation of RP-HPLC method for simultaneous estimation of Lamivudine and Efavirenz in the pharmaceutical dosage form. Journal of advanced pharmaceutical technology and research 2012; 2(4):232-238.

Namita Kapoor, Sateesh Khandavilli and Ramesh Panchagnula, Simultaneous determination of lamivudine and stavudine in antiretroviral fixed dose combinations by first derivative spectrophotometry and high performance liquid chromatography. Journal of Pharmaceutical and Biomedical Analysis 41, 761– 7659 (2006).

Nandi U, Das A, Roy B, Choudhury H, Gorain B, Pal TK. Development and validation of an HPLC-UV method for simultaneous determination of Zidovudine, Lamivudine and Nevirapine in human plasma and its application to pharmacokinetic study in human volunteers. Drug testing and analysis 2013; 5(6):485–491.

Santosh Kumar M, Venkateswara Rao J, Rajesh P. Development and validation of analytical technique for antiretrovirals by High Performance Liquid Chromatography (HPLC). International journal of pharma and biosciences 2011;1(4):601-605.s

Sunitha T, Sindhuri P, Palavan C. A New RP-HPLC Method for the simultaneous estimation of Lamivudine, Zidovudine and Nevirapine in tablet dosage forms. Journal of pharma and bioanalytical science 2013; 2(1):13-18.

Tarinas A, Tápanes RD, Ferrer G, Pérez J. Validation of high-performance liquid chromatography methods for determination of Zidovudine, Stavudine, Lamivudine and Indinavir in human plasma. *Farmacia Hospitalaria,* 31(4), 2007, 243-247.

Verweij VW, Aarnoutse RE, Burger DM. Simultaneous determination of HIV nucleoside analogues of reverse transcriptase inhibitors Lamivudine, Didanosine, Stavudine, Zidovudine and Abacavir in human plasma using reverse phase high performance liquid chromatography. *Journal of Chromatography B,* 816, 2005, 121