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Research Article

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS QUANTITATIVE ESTIMATION OF EFAVIRENZ, LAMIVUDINE AND ZIDOVUDINE IN TABLETS

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ABSTRACT

Objective: To develop a new, simple, accurate, precise, linear and rapid Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method for the simultaneous quantitative estimation of Efavirenz, Lamivudine and Zidovudine in tablets as per ICH guidelines.

Methods: The optimized method uses a reverse phase C18 column, Enable C18G (250 X 4.6 mm;5µ), a mobile phase consisting of acetonitrile:0.02M potassium dihydrogen orthophosphate buffer adjusted to pH 3.2 in the proportion of 30:70v/v, flow rate of 1.0 ml/min and a detection wavelength of 275nm using a UV detector.

Results: The developed method resulted in Efavirenz eluting at 2.01 min, Lamivudine at 2.90 min and Zidovudine at 7.52 min. The linearity of the method was over the range of 75-450µg/ml for Efavirenz, 18.75-112.5µg/ml for Lamivudine and 37.5-225µg/ml for Zidovudine. The method precision was exemplified by relative standard deviations of 0.15 % for Efavirenz, 0.24% for Lamivudine and 0.37% for Zidovudine. Percentage Mean recoveries obtained during accuracy were in the range of 98-102. The limit of detection (LOD) was obtained as 20ng/ml for Efavirenz, 1ng/ml for Lamivudine and 2ng/ml for Zidovudine.. The limit of quantitation (LOQ) was obtained as 50 ng/ml for Efavirenz, 2.5ng/ml for Lamivudine and 5ng/ml for Zidovudine.

Conclusion: A new, simple, accurate, precise, linear and rapid RP-HPLC method was developed and validated for the simultaneous estimation of Efavirenz, Lamivudine and Zidovudine mg in tablets as per ICH guidelines. Hence the method can be used for the routine analysis in various pharmaceutical industries.

Keywords: RP-HPLC, Efavirenz, Lamivudine, Zidovudine, Validation.

INTRODUCTION

Efavirenz (**Figure 1**) (S)-6-chloro(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-1-benzoxazin-2-one) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) and is used as part of highly active antiretroviral therapy (HAART) for the treatment of a human immunodeficiency virus (HIV) type1[1-4]. Lamivudine (**Figure 2**) and Zidovudine (**Figure 3**) are synthetic nucleoside analogues with activity against human immunodeficiency virus (HIV) and form one of the first line regimens in HIV treatment as fixed dose combination. The chemical name of Lamivudine is ((4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one and Zidovudine is 3'-azido-3'deoxythymidine. Lamivudine has very low cellular cytotoxicity and generally less potent than Zidovudine in inhibiting HIV-1 and HIV-2 replication in vitro[5-11].

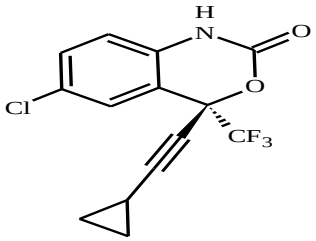

Fig. 1: Structure of Efavirenz

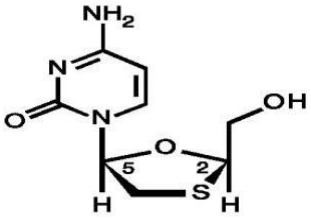

Fig. 2: Structure of Lamivudine.

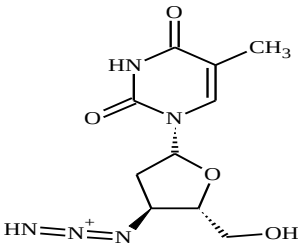

Fig. 3: Structure of Zidovudine

A detailed literature survey reveals that RP-HPLC methods have been reported for the determination of Efavirenz[2], Zidovudine[12], Lamivudine[13]and individually in pharmaceutical dosage forms. Literature is also available concerning RP-HPLC methods in combination of Efavirenz with other substances and similarly Lamivudine and Zidovudine with other drug combinations [1,3-11,14-16]. As per our detailed literature survey as on date, there are few reports [17-19] using RP-HPLC for the simultaneous quantitative estimation of Efavirenz, Lamivudine and Zidovudine in pharmaceutical dosage forms. We here report a totally new, simple, sensitive, precise, accurate, linear and isocratic RP-HPLC method for the simultaneous quantitative estimation of Efavirenz, Lamivudine and Zidovudine in Tablets.

MATERIALS AND METHODS

Chemicals and Reagents

Analytically pure sample of Efavirenz, Lamivudine and Zidovudine with purities greater than 99% were obtained as gift samples from Chandra Labs, Hyderabad, India and Tablet formulation [CYTOCOM-E was procured from Apollo pharmacy, Hyderabad, India with labelled amount 600mg of Efavirenz,150mg of Lamivudine and 300 mg of Zidovudine. Acetonitrile (HPLC grade) was obtained from Sigma Aldrich (Hyderabad, India), water (HPLC grade), potassium dihydrogen orthophosphate (AR grade), potassium hydroxide (AR Grade) and phosphoric acid (AR Grade) were obtained from SD Fine

chemicals (Hyderabad, India). 0.45µm Nylon membrane filters were obtained from Spincotech Private Limited (Hyderabad, India).

Instrument

HPLC analysis was performed on Waters e2695Alliance HPLC system connected with PDA Detector 2998 and Empower2 Software. The drug analysis data were acquired and processed using Empower2 software running under Windows XP on a Pentium PC. HPLC method development and validation was performed using a reverse phase C18 column, Enable C18G (250X4.6 mm; 5µ). An electronic analytical weighing balance (0.1mg sensitivity, Shimadzu AY 220), digital pH meter (DELUX model 101) and a sonicator (sonica, model 2200 MH) 2.42) were used in this study.

Method

Chromatographic conditions

The separation of the drugs was achieved on a reverse phase C18 column, Enable C18G (250 X4.6mm; 5µ). The mobile phase consists of a mixture of acetonitrile and potassium dihydrogen orthophosphate buffer (20mM, pH adjusted to 3.2 using ortho phosphoric acid) in ratio of 30:70, v/v. The mobile phase was set at a flow rate of 1.0 ml/min and the volume injected was 20 µl for every injection. The detection wavelength was set at 275 nm.

Buffer Preparation

The buffer solution was prepared by weighing 2.736g of potassium dihydrogen orthophosphate (KH_2PO_4) and transferring to 1000 ml of HPLC grade water to get 20 mM buffer strength, which was adjusted to pH 3.2 using 30%v/v ortho phosphoric acid. Later the buffer was filtered through 0.45 µm nylon membrane filter.

Mobile phase Preparation

The mobile phase was prepared by mixing acetonitrile and buffer in the ratio of 30:70, v/v and later sonicated for 10 minutes for the removal of air bubbles.

Preparation of stock and working standard solution

600mg of Efavirenz, 150mg of Lamivudine and 300mg of Zidovudine were taken in 100ml volumetric flask containing 50 ml of diluent then it is sonicated and then it is made up to the mark using the mobile phase. This is considered as standard stock solution. 5ml of the above stock solution was pipetted out and made up to 100 ml to get a concentration, considered as working standard solution, 100% target concentration.

Preparation of Stock and Working Sample Solution

Sample solution was prepared by dissolving tablet powder into diluents (mobile phase). Ten tablets were weighed separately and their average weights were determined, which was weighed and taken in a 100 ml volumetric flask, dissolved in diluents and made up to 100mL using mobile phase and later sonicated for about 10 minutes then filtered through 0.45µ membrane filter to get standard stock sample solution. 5mL of the above stock solution was pipetted out and made up to 100 ml to get a concentration, considered as working sample solution, 100% target concentration.

RESULTS AND DISCUSSION

Method Development

A Reverse phase HPLC method was developed keeping in mind the system suitability parameters i.e. resolution factor (Rf) between peaks, tailing factor (T), number of theoretical plates (N), runtime and the cost effectiveness. The optimized method developed resulted in the elution of Efavirenz at 2.02min, Lamivudine at 2.90min and Zidovudine at 7.52min. **Figures 4 and 5** represent chromatograms of blank solution and mixture of working standard solutions respectively. The total run time is 8 minutes with all system suitability parameters meeting acceptable criteria for the mixture of standard solutions.

System-suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (*Rt*), number of theoretical plates (*N*), peak resolution (Rs) and peak Tailing factor (T) were evaluated for six replicate injections of the mixture of standards at working concentration. The results given in **Table 1** were with-in acceptable limits.

Table 1: System suitability studies results.

Drug	*Retention time (min)	*Resolution	*USP plate Count	*USP Tailing
Efavirenz	2.02	-	5317	1.725
Lamivudine	2.90	6.199	6630	1.746
Zidovudine	7.52	14.70	7707	1.520

* Mean of six injections

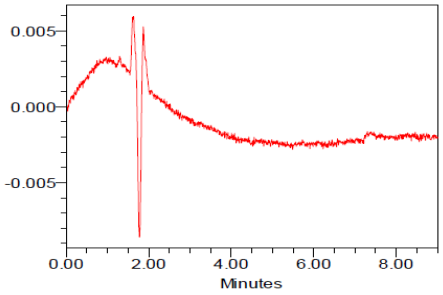

Fig. 4: Typical Chromatogram of Blank solution

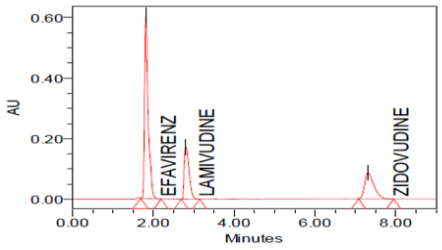

Fig. 5: Typical chromatogram for the mixture of standard solutions

In order to test the applicability of this method developed to a commercial formulation, 'CYTOCOME-E' was chromatographed at a concentration equivalent to working standards concentration and it is shown in **Figure 6.** The sample peaks were identified by comparing the relative retention times with the standard drugs mixture (**Figure 5**). System suitability parameters were within the acceptance limits, ideal for the chromatographed sample. Integration of separated peak area was done and each drug concentration was determined by using the peak area-concentration relationship obtained in the standardization step. The protocol affords reproducible quantification of the two drugs with error less than 10%, which is the standard level in any pharmaceutical quality control.

Method validation

Validation of the analytical method is the process that establishes by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application. RP-HPLC method developed was validated according to International Conference on Harmonization (ICH) guidelines [20] for validation of analytical procedures. The method was validated for the parameters like system suitability, specificity, linearity, accuracy, precision, ruggedness, robustness, limit of detection (LOD) and limit of quantitiation (LOQ).

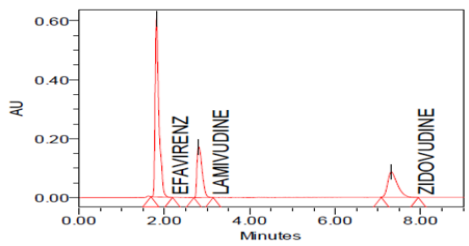

Fig.6: Typical chromatogram for the sample (tablet).

Specificity

Figures 4-6 of blank, mixture of standard drug solution and sample chromatogram reveal that the peaks generated in mixture of

standard solution and sample solution at working concentrations are only because of the drugs as blank has no peaks at the retention times of Efavirenz, Lamivudine and Zidovudine. Hence the method developed is said to be specific.

Precision

System precision

Six replicate injections of the mixture of standard solution at working concentration showed % RSD (% Relative Standard Deviation) less than 2 concerning peak areas for both the drugs, which indicates the acceptable reproducibility and thereby the precision of the system. System precision results are tabulated in **Table 2**.

Method precision

Method precision was determined by performing assay of sample under the tests of (i) repeatability (Intra day precision) and (ii) Intermediate precision (Inter day precision) performed at working concentration by three different analysts on three consecutive days.

Repeatability (Intra day precision)

Six consecutive injections of the sample at working concentration showed % RSD less than 2 concerning % assay for all the drugs which indicate the method developed is precise by the test of repeatability and hence can be understood that the method gives consistently reproducible results (**Table 3**).

Table 2: System precision results

Injection number	EFAVIRENZ		LAMIVUDINE		ZIDOVUDINE	
(N)	RT	Peak Area	RT	Peak area	RT	Peak area
1	2.03	3939072	2.95	1498054	7.53	1497566
2	2.01	3932697	2.94	1498331	7.55	1499369
3	2.06	3937488	2.95	1494546	7.54	1489563
4	2.02	3933983	2.96	1499974	7.51	1498144
5	2.03	3938602	2.94	1491382	7.53	1470728
6	2.01	3948212	2.94	1498393	7.52	1489585
Average		3938342.33		1496780		1490825.33
SD		5470.16		3192.5		10761.30
% RSD		0.14		0.21		0.72

Table3: Method precision results.

N	EFAVIRENZ	LAMIVUDINE	ZIDOVUDINE
	% Assay	% Assay	% Assay
1	99.6	99.59	99.51
2	100	100.2	99.23
3	99.64	99.63	99.36
4	99.73	99.56	98.8
5	99.81	99.86	99.79
6	99.62	99.77	99.79
Average	99.73	99.76	99.41
S.D.	0.152	0.24	0.37
% R.S.D.	0.152	0.24	0.37

Table 4: Inter day precision results.

N	% Assay (Efavirenz)			% Assay (Lamivudine)			% Assay (Zidovudine)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day2	Day3
1	99.36	99.12	99.73	98.37	99.34	99.65	99.45	99.79	98.97
2	98.56	99.56	99.48	99.46	98.65	98.45	98.34	98.49	99.69
3	99.14	99.32	98.70	98.85	98.76	99.32	99.67	99.85	99.93
4	98.48	98.93	99.94	99.54	99.44	99.13	99.38	99.37	99.74
5	99.78	99.23	99.24	99.35	98.93	99.15	98.99	99.78	99.59
6	98.99	98.09	98.84	98.54	98.06	98.24	99.98	98.47	98.59
% RSD	0.49	0.51	0.48	0.50	0.51	0.54	0.57	0.68	0.52

Table 5: Linearity of the chromatography system

Drugs	Linearity range (µg/ml)	R^2	Slope	Intercept
Efavirenz	75-450	0.9999	39304.22	-832.133
Lamivudine	18.75-112.5	0.9999	14857.83	5598.467
Zidovudine	37.5-225	0.9998	14981.92	7868.07

Table 6: Calibration data for Efavirenz

% Level	Actual Concentration (µg/ml)	Peak Area
25	75	983476
50	150	1962758
75	225	2940680
100	300	3936292
125	375	4915675
150	450	5890842

Ruggedness (Intermediate Precision / Inter day precision/): Six consecutive injections of the sample solution at working concentration on three consecutive days by three different analysts, showed % RSD less than 2 for % assay for all the drugs within and between days, which indicate the method developed is inter day precise / rugged (**Table 4)**.

Linearity

Standard solutions of Efavirenz, Lamivudine and Zidovudine at different concentrations level (25%, 50%, 75%, 100%, 125%, 150%) 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 5-8** and **Figures 7-9.** 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 75-450µg/ml for Efavirenz, 18.75-112.5µg/ml for Lamivudine and 37.5-225 µg/ml for Zidovudine.

Table 7: Calibration data for Lamivudine

% Level	Actual Concentration (µg/ml)	Peak Area
25	18.75	374562
50	37.5	749659
75	56.25	1124969
100	75	1487641
125	93.75	1862939
150	112.5	2234179

Table 8: Calibration data for Zidovudine

% Level	Actual Concentration (µg/ml)	Peak Area
25	37.5	359653
50	75	749390
75	112.5	1111284
100	150	1493110
125	187.5	1875266
150	225	2229599

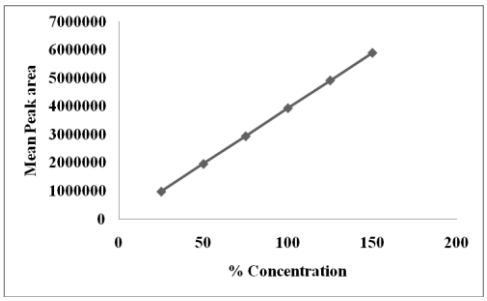

Fig. 7: Calibration curve for Efavirenz.

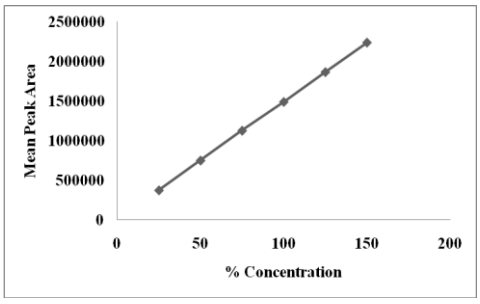

Fig. 8: Calibration curve for Lamivudine.

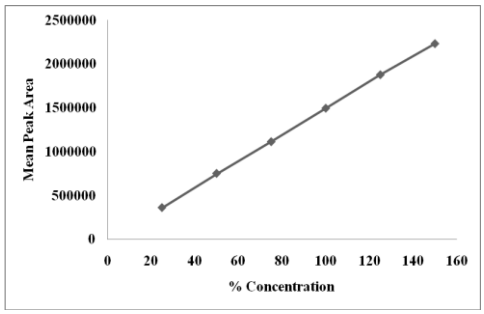

Fig.10: Calibration curve for Zidovudine

Accuracy

Accuracy was determined by means of recovery experiments, by addition of active drug to preanalyzed sample at different spiked levels (50-150%). At each level, three determinations were performed and results obtained. The accuracy was calculated from the test results as the percentage of the analyte recovered by the assay. The amounts recovered, values of percent mean recovery were calculated as shown in **Tables 9-11.** The accepted limits of recovery are 98%-102% and all observed data are within the required range that indicates good recovery values and hence the accuracy of the method developed.

Table 9: Results of Accuracy studies for Efavirenz

	AMOUNT ADDED (µg/ml)	*AMOUNT RECOVERED (µg/ml)	*% RECOVERY
50	150	152.5	101.6
100	300	298.3	99.4
150	450	451.5	100.33

*Mean of three replicates

Table 10: Results of Accuracy studies for Lamivudine

Concentration level (%)	Amount added (µg/ml)	*Amount recovered (µg/ml)	*% Recovery
50	37.5	38.1	101.6
100	75	74.4	99.2
150	112.5	111.1	98.75

*Mean of three replicates

Table 11: Results of Accuracy studies for Zidovudine

Concentration level (%)	Amount added (µg/ml)	*Amount recovered (µg/ml)	*% Recovery
50	75	75.4	100.5
100	150	151.4	100.93
150	225	226.5	100.66

*Mean of three replicates

Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. It is concluded that the method is robust as it is found

that the % RSD is less than 1 concerning % assay despite deliberate variations done concerning flow rate (± 0.2), pH (± 0.2) and % organic phase (± 5%).

Sensitivity

The sensitivity of measurement of Efavirenz,Lamivudine and Zidovudine by use of the proposed method was estimated in terms of the limit of quantitation (LOQ) and the limit of detection (LOD).The limit of detection (LOD) was obtained as 20ng/ml for Efavirenz, 1ng/ml for Lamivudine and 2ng/ml for Zidovudine.. The limit of quantitation (LOQ) was obtained as 50 ng/ml for Efavirenz, 2.5ng/ml for Lamivudine and 5ng/ml for Zidovudine.

CONCLUSION

A reverse phase HPLC isocratic method developed has been validated as per ICH guidelines in terms of specificity, accuracy, precision, linearity, robustness, limit of detection and limit of quantitiation, for the simultaneous quantitative estimation of Efavirenz,Lamivudine Zidovudine. A good linear relationship was observed for both the drugs between concentration ranges of 50 and 150 µg/ml. The correlation coefficients were greater than 0.999 for both the drugs. The inter day and intraday precision results were good enough to say that the method developed is precise and reproducible. Accuracy studies revealed that mean recoveries after spiking experiments were between 98 and 102%, an indicative of accurate method. Accordingly it can be concluded that the developed reverse phase HPLC method is accurate, precise, linear and robust and therefore the method can be used for the routine analysis of Efavirenz, Lamivudine and Zidovudine in tablets.

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