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DEVELOPMENT AND VALIDATION OF AN RP- HPLC METHOD FOR THE DETERMINATION OF EFAVIRENZ IN HUMAN PLASMA

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ABSTRACT

A rapid, selective and sensitive high performance liquid chromatographic method for the estimation of efavirenz in human plasma has been developed. Chromatography was carried out on a Denali C_{18} column using a mixture of phosphate buffer (pH 3.5 ± 0.05) and acetonitrile in a ratio of 60 : 40 v/v as the mobile phase. The eluates were monitored at 247 nm. Carbamazepine was used as an internal standard. The retention times for efavirenz and carbamazepine were found to be 15.4 and 2.3 min, respectively. The method was found to be linear in the concentration range of 40.0 ng/mL to 4003.9 ng/mL. The method validated as per FDA guidelines and was found to be suitable for bioequivalence and pharmacokinetic studies.

Key words: Efavirenz, Determination, Plasma, HPLC.

INTRODUCTION

Efavirenz, (4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-1H-3, 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) type 1[1,2].

Antiviral activity of efavirenz is dependent on intracellular conversion to the active triphosphorylated form. The rate of efavirenz phosphorylation varies, depending on cell type. It is believed that inhibition of reverse transcriptase interferes with the generation of DNA copies of viral RNA, which in turn, are necessary for synthesis of new virions. It is official in USP and BP. Its empirical formula is $C_{14}H_9ClF_3NO_2$. The structure of efavirenz is shown

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in Fig. 1. A literature survey reveals reports of a few analytical methods for the determination of efavirenz in dosage forms[3] and in biological fluids by HPLC[4-10]. An LC-MS/MS method[11] for the determination of the drug with other anti-retero virals was also reported. The authors now propose a simple, sensitive, accurate and precise RP-HPLC method for the determination of efavirenz, which utilises less plasma sample volume for liquid-liquid extraction of the drug.

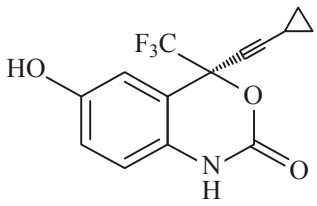

Fig. 1: Structure of efavirenz

EXPERIMENTAL

Chemicals and solvents

HPLC grade acetonitrile and methanol used in the study were obtained from Qualigens, India. HPLC grade water (milli Q) was prepared from Millipore (USA) equipment. Phosphate buffer was prepared by dissolving 1.36 g of potassium dihydrogen phosphate in 1000 mL of milli-Q water. The pH of the solution was adjusted to 3.5 ± 0.05 with ortho-phosphoric acid. The working reference samples of efavirenz and carbamazepine were obtained from Aurobindo Pharma (Hyderabad).

Chromatographic conditions

A Shimadzu HPLC equipment comprising two LC-10AT VP pumps, VP CTO-10AS VP column oven, a Denali C_{18} (4.6 ID X 250 mm, 5µ particle size) column and an SPD-10A variable-wavelength programmable UV-Visible detector was used for chromatographic separation. The detection of the compounds was monitored at 247 nm. A mobile phase consisting of a mixture of phosphate buffer (pH 3.5 ± 0.05) and acetonitrile in a ratio of 60 : 40 v/v was pumped at a flow rate of 1.4 mL/min. The data was acquired and processed with Class VP Software.

The working standard solutions and the calibration curve

The stock solutions of the analyte and the internal standard were prepared in a mixture of acetonitrile and water (50 : 50 v/v) at a free base concentration of 1 mg/mL. The working standard solutions were prepared from the stock solution by using the same diluent.

These diluted working standard solutions were used to prepare the calibration curve standards and quality control samples. The solutions for obtaining the eight point standard calibration curve were prepared by spiking the screened blank plasma with appropriate amounts of efavirenz dilutions. The calibration curve was linear in the range of 40 - 4003.9 ng/mL ($r \geq 0.9900$). The calibration plot was drawn with a weighing factor of $1/X^2$. The quality control samples were prepared at four concentrations of 40.2 ng\mL (LLOQ QC) 120.3 ng\mL (LQC), 2148.1 ng\mL (MQC) and 2902.8 ng\mL (HQC). The results are presented in Tables 1 and 2.

Table 1: Summary of calibration parameters

Calibration curve	Slope	Intercept	Correlation coefficient
1	0.000102	-0.00163	0.9972
2	0.000103	0.00067	0.9999
3	0.000101	0.00132	0.9977
4	0.000104	-0.00968	0.9879
5	0.000097	0.00703	0.9923
6	0.000099	-0.00234	0.9894

Table 2: Precision of plasma calibration curve standards (n = 6)

Nominal concentration (ng\mL)	Mean calculated concentration	Percent coefficient variation	Percent relative error
40.0	39.32	4.3	1.72
80.0	83.75	7.0	-4.67
200.0	202.62	5.3	-1.29
500.1	507.17	4.8	-1.42
1500.0	1563.37	8.9	-4.23
2252.2	2151.85	7.8	4.45
3002.9	2915.37	8.9	2.92
4003.9	4159.43	6.8	-3.89

Sample extraction procedure

Two hundred micro liters, each of spiked plasma calibration curve standards and quality control samples, were transferred to pre-labeled polypropylene tubes containing 50 µL of the internal standard, carbamazepine (50 μgmL^{-1} dilution). The tubes were vortexed for ten seconds. Each of the tubes was added 2.5 mL of extraction solvent (methyl tertiary butyl ether). The tubes were vortexed for 10 minutes at 2000 rpm on a vibramax unit and were then centrifuged at 4000 rpm for 5 minutes in a refrigerated centrifuge at 10°C temperature. From the centrifuged polypropylene tubes, approximately 2.0 mL of supernatant extracted solvent was transferred to a new set of pre-labeled polypropylene tubes. The contents of the tubes were evaporated in a stream of nitrogen at 40°C for 10 minutes and the residues of the dried tubes were reconstituted with 0.15 mL mobile phase. The contents of the tubes were vortexed and transferred into auto-sampler vials and then analyzed with HPLC unit by injecting 50 µL of sample volume.

Validation

The proposed method has been validated[12] for selectivity, sensitivity, linearity, precision, accuracy, recovery, stability and dilution integrity. Selectivity was determined by testing different blank plasma samples (from different donors) for interference at the retention times of the analyte and the internal standard. Sensitivity was determined by analyzing six replicates of blank human plasma and the plasma spiked with the analyte at the lowest value in the calibration curve. The intra run and inter run accuracy was determined by replicate analysis (n = 6) of the quality control samples at each level and at the limit of quantification value (LOQ) that was derived from the sample batch. Inter run precision and accuracy of the calibration standards were computed from the six calibration curves used for assay validation. The results were presented in Tables 3, 4 and 5.

Table 3: Stability data

Details of stability experiment	n	Spiked concentration (ng\mL)	Mean calculated comparison sample concentration	Mean calculated stability sample concentration	Mean percent change
Bench top stability HQC	6	2902.8	3087.57	2966.52	4.08
Bench top stability LQC	6	120.3	128.82	123.60	4.22

Cont…

Details of stability experiment	n	Spiked concentration (ng\mL)	Mean calculated comparison sample concentration	Mean calculated stability sample concentration	Mean percent change
Auto-sampler stability HQC	6	2902.8	2896.77	3106.22	-6.74
Auto-sampler stability LQC	6	120.3	121.37	117.82	3.01
Freeze-thaw stability HQC	6	2902.8	3087.57	2939.13	5.05
Freeze-thaw stability LQC	6	120.3	128.82	124.65	3.34
Dry extract stability HQC	6	2902.8	2896.77	2754.38	5.17
Dry extract stability LQC	6	120.3	121.37	118.43	2.48
Long term stability HQC	6	2902.8	2859.28	2848.03	0.40
Long term stability LQC	6	120.3	117.87	124.37	-5.23

Table 4: Precision and accuracy of intra batch

QC ID	Nominal concentration (µg/mL)	Intra batch			
		n	Mean concentration observed (µg/mL)	% CV	% RE
LLOQ QC	40.2	6	40.85	5.7	-1.67
LQC	120.3	6	122.98	5.2	-2.24
MQC	2148.1	6	2078.60	5.8	3.23
HQC	2902.8	6	2848.42	3.5	1.87

Table 4: Precision and accuracy of intra batch

QC ID	Nominal concentration (ng/mL)	Inter batch			
		n	Mean concentration observed (µg/mL)	% CV	% RE
HQC	40.2	36	41.04	6.2	-2.1
MQC	120.3	36	125.36	4.7	-4.2
LQC	2148.1	36	2121.89	5.5	1.2
LLOQ QC	2902.8	36	2888.46	6.0	0.5

Accuracy is defined as the percent relative error (% RE) and is calculated using the formula % RE = (E - T) x (100/T) were E is the experimentally determined concentration and T is the theoretical concentration of the drug. Assay precision was calculated using the formula % RSD = (SD/M) x (100), where M is the mean of the experimentally determined concentration and SD is standard deviation of M. Dilution integrity was performed with samples falling above the upper concentration limits of the calibration curve. For this, a concentration of double the uppermost calibration standard was diluted two fold and four fold with blank plasma. Six replicates each of the diluted samples were processed and analyzed for accuracy and precision.

The auto-sampler or wet extracted stability of the processed sample was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were injected after keeping in auto-sampler at 10^{o}C for 20.0 hrs. The stability of spiked human plasma samples stored at room temperature (Bench top stability) was evaluated for a period of nine hours and compared with that of the freshly prepared samples. The freeze-thaw stability was assessed by comparing the stability of the samples that had been frozen and thawed three times, with that of the freshly spiked quality control samples. The stability of spiked human plasma stored at -70^{o}C (long-term stability) was evaluated by analyzing the quality control samples that were stored at -70^{o}C for 9 days together with the freshly spiked calibration standards and the quality control samples. All stability evaluations were based on back-calculated concentrations. Analytes were considered stable if the deviations of the mean test responses were within 15% of the freshly prepared or comparison samples.

The representative chromatogram obtained for extracted blank plasma sample (Fig. 3) shows that the assay procedure is specific as there were no interfering peaks observed at the

retention times corresponding to the drug and the internal standard.

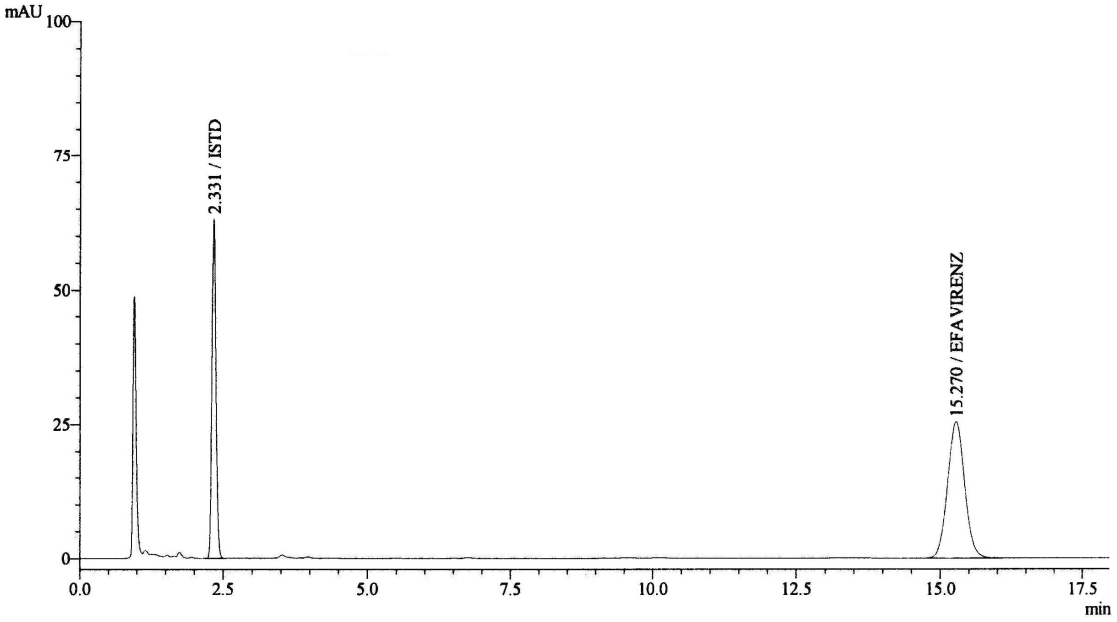

Fig. 2: Representative chromatogram of efavirenz extracted standard

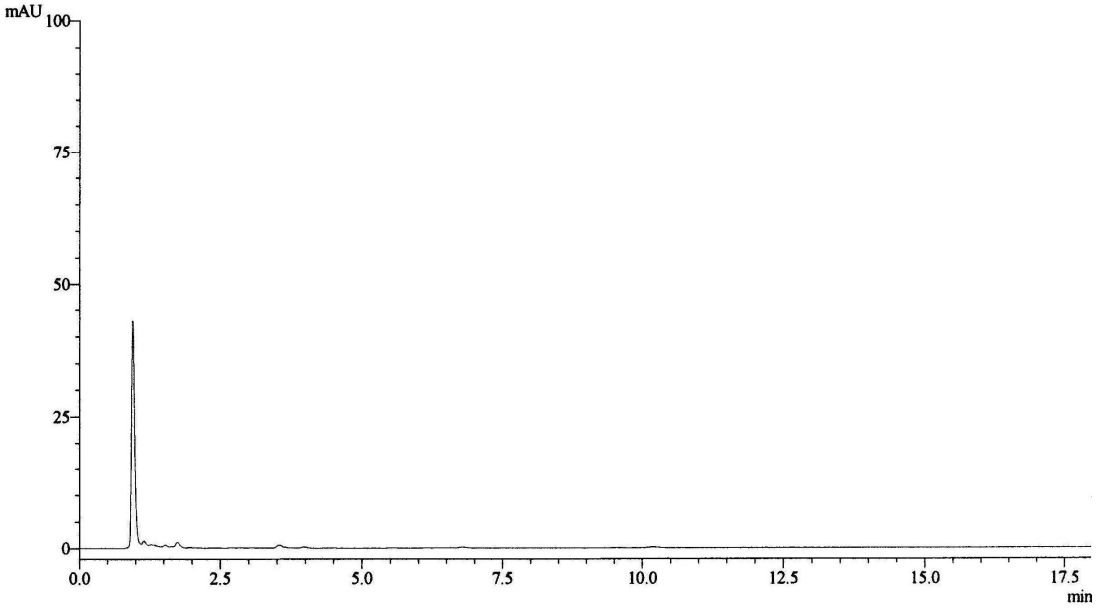

Fig. 3: Representative chromatogram of extracted blank sample

CONCLUSION

The validated HPLC method presented here is simple, selective, precise and accurate for quantification of efavirenz in human plasma. The method can also be applicable for bio-availability and bio-equivalence studies.

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