Optimization and Validation of Rp-Hplc Stability-Indicating Method for Determination of Efavirenz and its Degradation Products

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Abstract: Stability is considered one of the most important criteria in pharmaceutical quality control. With this objective a stability-indicating high performance liquid chromatographic (HPLC) method has been established for analysis of Efavirenz in the presence of the degradation products generated in the stress degradation study. The drug was subjected to stress conditions of hydrolysis, oxidation, photolysis and thermal decomposition. Extensive degradation was found to occur in alkaline medium and under thermal stress. Minimum degradation was observed under acidic medium, in the photolytic conditions and oxidative stress. Successful separation of drug from degradation products formed under stress conditions was achieved on a C-8 column using acetonitrile: potassium dihydrogen phosphate (pH 2.9, 25mM) - (60:40% v/v) as the mobile phase. The flow rate was 1 mL min^{-1} and the detector was set at in a range of wavelength between 220nm to 390nm. The method was validated for linearity, range, precision, accuracy, limit of quantification and limit of detection. Because the method effectively separates the drugs from their degradation products, it can be used as stability-indicating method.

Keywords: Efavirenz; stress degradation; stability indicating method; HPLC

1. Introduction

Efavirenz (Figure1), (4*S*)-6-chloro -4-(cyclopropylethynyl)-1,4-dihydro-4- (trifluoromethyl)- 2*H*-3, 1-benzoxazin-2-one is a non-nucleoside reverse transcriptase (RT) inhibitor of human immunodeficiency virus type 1 (HIV-1) [1-2]. Efavirenz activity is mediated predominantly by non-competitive inhibition of HIV-1 RT. HIV-2 RT and human cellular DNA polymerases alpha, beta, gamma, and delta are not inhibited by Efavirenz [3].
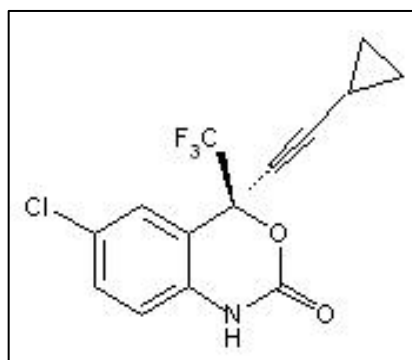

Figure 1. Structure of Efavirenz

Stability is considered as one of the most

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important criteria in pharmaceutical quality control. Only stable preparation would promise precise delivery of the drug to the patients. Expiration dating on any drug product is based upon scientific studies at normal and stressed conditions [4].

Literature survey reveals that there are analytical methods available for determination of Efavirenz from biological matrices [5-10], bulk drug and dosage forms [11-13], and analytical methods for determination of Efavirenz with combination of other antiviral drugs [14-37].

Literature survey further revealed that the drug Efavirenz is still not official in USP or BP and there are no official methods available for studying the impurities or related substances in Efavirenz. Moreover, there is no validated stability indicating analytical method for the determination of degraded substance in Efavirenz bulk drug. Attempts were made to develop a stability indicating HPLC method for the degraded substance determination.

Keeping in view of susceptibility of Efavirenz under variety of conditions, it was felt that a HPLC method of analysis, that separates the drug from the degradation products which are formed under the ICH suggested conditions such as hydrolysis, oxidation, photolysis and thermal stress) would be of remarkable interest. These studies serve to give information on drug's inherent stability and help in the validation of analytical methods to be used in stability studies. Therefore, the objective of the present study was to study degradation of Efavirenz under different ICH recommended stress conditions [38], and to establish an accurate, specific, reproducible and validated stability-indicating HPLC method.

This paper deals with the forced degradation of Efavirenz under stress condition like acid hydrolysis, base hydrolysis, oxidation, heat and UV light. It also deals with the validation of the developed method for the accurate quantification of degradation product.

2. Experimental

2.1. Chemicals and Reagents

The working standard of Efavirenz was procured from Cipla Ltd., India. HPLC grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Deionised and ultra pure water used in all experiments was obtained from Milli-Q System (Millipore). Potassium dihydrogen phosphate of AR (Analytical Reagent) grade was procured from Merck (Darmstadt, Germany). Orthophosphoric acid used for adjusting the pH of buffer solution was of AR grade (S. D. Fine chemicals).

2.2. Equipment

pH of the mobile phase was checked on a pH/ion analyzer (Lab India PHAN, India). Refluxing of the drug in hydrolysis conditions was carried out in a round bottom flask-condenser assembly. The HPLC system employed in the method development, forced degradation studies and assay method validation was Shimadzu LC-8A pump, Shimadzu SCL-10Avp system controller and Shimadzu SPD-M10 Avp photo diode array detector and Class VP software as data integrator.

2.3. Preparation of Mobile Phase

400 mL of 25 mM potassium dihydrogen phosphate buffer solution was prepared and pH was adjusted to 2.9 with ortho phosphoric acid. The final volume was adjusted by adding this 400 mL of buffer to 600 mL of acetonitrile, which resulted in pH 3.2 for final mobile phase. The mobile phase was sonicated for 15 min.

2.4. Preparation of Standard Solution

A stock solution of Efavirenz (1mg mL^{-1}) was prepared in methanol. Standard solutions

were prepared by dilution of the stock solution with mobile phase to give solution in concentration range of 0.02 to 20.00 μg mL^{-1}. The stock solution used for the degradation studies was 20.00 μg mL^{-1}.

2.5. Optimized Chromatographic Conditions

The chromatographic separation was achieved on RP Spherisorb C-8 (Waters) column (250x4.6 mm, 10 μm), using a mobile phase consisting of mixture of acetonitrile: potassium dihydrogen phosphate (pH 2.9, 25 mM) - (60:40 % v/v). All reagents were filtered through 0.45 μm filter paper and sonicated before use. The injection volume was 100 μL. The UV-Vis detector was set at in a range of wavelength between 220nm to 390nm. The assay was performed at 25^{0}C and the flow was fixed at 1.0 mL min^{-1}.

2.6. Validation of Method

A stock solution of the drug was prepared at strength of 1mg mL^{-1}. It was diluted to prepare solutions containing 0.02 to 20.00 μg mL^{-1} of the drug Efavirenz. The solutions were injected in triplicate into the HPLC column, keeping the injection volume constant (100 μL).

Twelve injections, of three different concentrations [LQC (0.06 μg mL^{-1}), MQC (5.00 μg mL^{-1}) and HQC (18.00 μg mL^{-1})], were given on the same day and the values of relative standard deviation (R.S.D.) were calculated to determine intra-day precision. These studies were also repeated on different days to determine inter-day precision.

Accuracy was evaluated for the known concentration of the drug. The recovery of the added drug was determined. The method is specific as it is well resolved and distinguished from the degradation products.
The LOD and LOQ were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations.

Robustness of method was investigated by varying the chromatographic conditions such as change of flow rate (±10 %), organic content in mobile phase (±2 %), wavelength of detection (±5%) and pH of buffer in mobile phase (±0.2 %). Robustness of the developed method was indicated by the overall % RSD between the data at each variable condition.

The solution stability was carried out by leaving both the test solutions of sample and reference standard in tightly capped volumetric flasks at -20 ^{0}C for 7 days. The sample solution was assayed after 7 days against fresh samples.

2.7. Stress Degradation Studies

Acid induced stress degradation was performed by adding 20 mL of stock solution (1mg mL^{-1}) of Efavirenz to 10 mL each of methanol and 0.1 M HCl and refluxing the mixture at 60°C for approximately 6 h. The solution was then left to reach ambient temperature, neutralized to pH 7 by addition of 0.1 M NaOH, then diluted to 100 mL with mobile phase so as to get concentration 200 μg mL^{-1}. From this solution 10 mL was diluted to 100 mL to get final concentration 20 μg mL^{-1}.

Base induced stress degradation was performed by adding 20 mL of stock solution (1 mg mL^{-1}) of Efavirenz to 10 mL each of methanol and 0.1 M NaOH and refluxing the mixture at 60°C for approximately 6 h. The solution was then left to reach ambient temperature, neutralized to pH 7 by addition of 0.1 M HCl, then diluted to 100 mL with mobile phase so as to get concentration 200 μg mL^{-1}. From this solution 10 mL was diluted to 100 mL to get final concentration 20 μg mL^{-1}.

2.8. Oxidative Degradation

To study the effect of oxidizing conditions, 20 mL of stock solution (1 mg mL^{-1}) of Efavirenz was added to 10 mL 30 % H$_2$O$_2$ solution and the mixture was refluxed at 60°C

for approximately 6 h. The solution was left to reach ambient temperature and then diluted to 100 mL with mobile phase so as to get concentration 200 μg mL^{-1}. From this solution 10 mL was diluted to 100 mL to get final concentration 20 μg mL^{-1}

2.9. Thermal Degradation

To study the effect of temperature, approximately 100 mg Efavirenz was stored at 80 °C for 48 h. Then it was dissolved in 10 mL methanol and volume was adjusted up to 50 mL with mobile phase. Then 1 mL of above solution was further diluted to 100 mL with mobile phase so as to give a solution of final concentration equivalent to 20 μg mL^{-1} of Efavirenz.

2.10. Photolysis

To study the effect of UV light, approximately 100 mg Efavirenz was exposed UV fluorescent lamp having a spectral distribution from 320 nm to 400 nm with a maximum energy emission between 350 nm and 370 nm, for 24 h, then dissolved in 10 mL of methanol and made up volume by mobile phase in 50 mL volumetric flask, and then 1 mL of stock solution was further diluted with mobile phase so as to give a solution of final concentration equivalent to 20 μg mL^{-1} of Efavirenz.100 μL of resulting solution was injected into HPLC and chromatograms were recorded. The stability samples were analyzed using PDA detector to determine the peak purity, as the method was found to be rugged in nature.

3. Results and Discussion

3.1. Degradation behavior

HPLC studies on Efavirenz under different stress conditions suggested the following degradation behavior (Table1).

The percent degradation was calculated by the formula: % degradation = [(actual initial area of untreated stock solution - reduced area of treated stock solution) / actual initial area of untreated stock solution] x 100.

It was observed that on heating at 60 °C in 0.1 M HCl there was no peak of the degradation product eluted in the chromatogram, however reduction in the peak area of the drug was observed in sample (Fig.2C). Only 8.23 % degradation was observed after acid hydrolysis.

The rate of hydrolysis in alkali or thermal was fast and significant reduction in the peak area, with degradation products was observed in sample.

The drug was found to be highly susceptible to alkaline hydrolysis. The reaction in 0.1N NaOH at 60 °C was so rapid that 60.13 % of the drug was degraded in 60 min, forming degradation products at RT 4.79, 6.58, 7.84, 9.02, 14.01 (Figure 2D).

In comparison to alkali and thermal hydrolysis, the drug was reasonably stable to hydrogen peroxide (3%) at 60°C. Only 19.56 % of drug was found to be degraded (Figure 2E).

Efavirenz also proved labile to thermal degradation. After refluxing at 60°C for 60 min, 53.85 % of drug was degraded. Degradation products at RT 7.68, 9.03 (Figure 2F) were formed. 35.41 % degradation product was observed after exposure of drug to UV light for 24hr; with major degradation products at RT 4.56, 7.52, 10.82, 16.88 was formed. (Figure 2G)

Table 1. Percent Degradation of Efavirenz and Retention time of Degradation products

| Sr. No.	Condition	Retention time of drug/ degradation products (Min)	Peak Area (μV. sec)	Mass concentration (μg mL^{-1})	Percent Degradation of drug (n= 5)
1	Untreated Stock Solution (20 μg mL^{-1})	7.70	7438127.5	20	-
2	Acid Hydrolysis	7.70	6825476	18.35	**8.23** %
3	Base Hydrolysis	4.79 6.58 7.84 9.02 14.01	115585 11956 2965345 142324 102563	7.97	**60.13** %
4	Oxidation	6.62 7.73 9.08	3958.5 5982790.5 364472.8	16.08	**19.56** %
5	Thermal degradation	7.68 9.03	3432351.5 230275	9.22	**53.85** %
6	Photolytic degradation	4.56 7.52 10.82 16.88	161837.5 4804187 514495 264234.5	12.91	**35.41** %

3.2. Establishment of a stability-indicating method

Efavirenz is weakly acidic in nature (ionizes in basic medium) so reverse phase chromatography was considered as the best choice. Separation of Efavirenz from its degradation products has been performed on RP C8 column. The mobile phase was optimized with different ratios of potassium dihydrogen phosphate buffer and acetonitrile solution. Proportion of acetonitrile in mobile phase was altered to get good resolution and desired retention time. Increasing the acetonitrile ratio was accompanied by decrease in retention time of different components; however the separation was still achieved. Since pKa of Efavirenz is 10.2 so in the acidic pH probability of drug remaining in unionized form is more, which in turn has an effect on peak shape and retention time. This statement was supported when improved peak shape, tremendous decrease in tailing and reproducible response was observed between the pH ranges of 2.7 to 3.5. In order to ensure complete separation and high resolution, the chosen ratio was acetonitrile: potassium dihydrogen phosphate (pH 2.9, 25mM) - (60:40% v/v) as the mobile phase. Final mobile phase pH was 3.2.
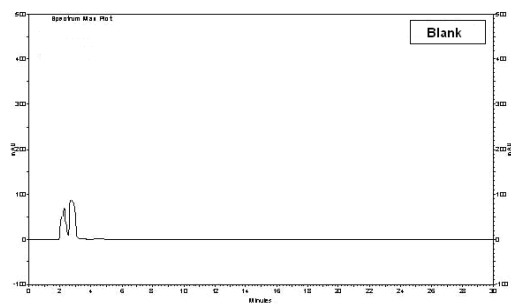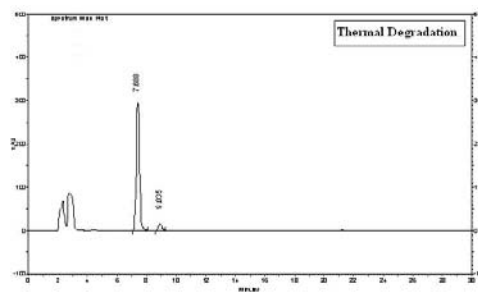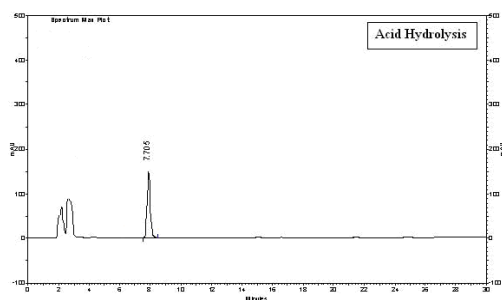
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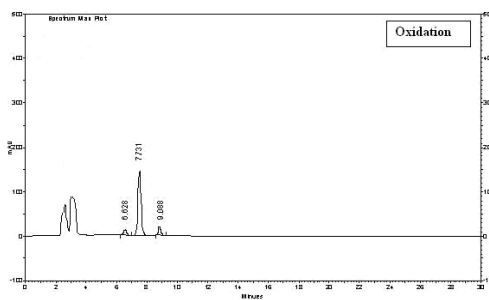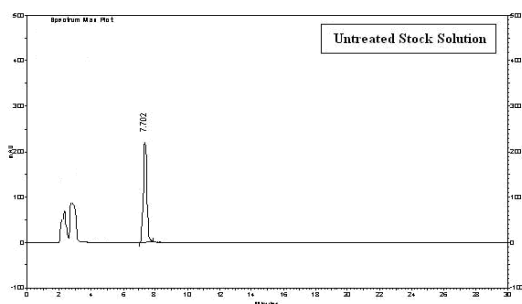
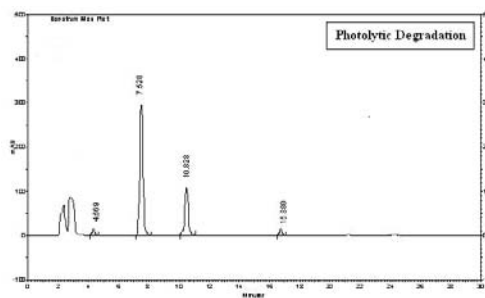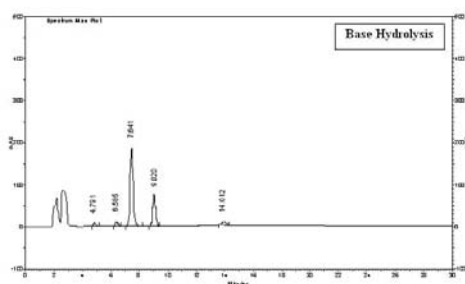(B) Untreated Stock Solution

(C) Acid Hydrolysis

(D) Base Hydrolysis

(E) Oxidation

(F) Thermal Degradation

(G) Photolytic Degradation

Figure 2. Representative Chromatograms of Efavirenz for Stability Method

Efavirenz showed maximum wavelength at 247 nm. But the UV-Vis photo diode array detector was set at range of wavelength between 220-390 nm to scan at multiple wavelengths and to check for presence of any degradation products detectable within this range. The specificity of the method is illustrated in Fig.2 and the average retention time of Efavirenz for 10 replicates was 7.70 ±0.10 min. Construction of calibration curve was performed by transferring aliquots of Efavirenz stock and working standard solutions into a series of 10mL volumetric flasks and diluting to volume with the mobile phase to obtain solutions in the concentration range of 0.02 to 20.00 μg mL^{-1}. A 100 μL volume from each solution was injected in triplicate. Chromatographic separation was run under the previously mentioned conditions.

All determinations were performed at ambient temperature. The average peak area obtained for each concentration was plotted versus concentration.

3.3. Validation of the method

The method was validated with respect to linearity, precision, accuracy, specificity and robustness. The response for the drug was linear in the studied concentration range ($r^2 = 0.999$). The mean (±R.S.D.) values of slope and correlation coefficient were 776019.62 (±4124.04) and 0.999 (±0.001), respectively. (Table 2).

Table 2. Linearity and range

Linearity and range	Efavirenz
Range (μg mL^{-1})	0.02 - 20.00
r^2	0.999
Slope	776019.62
Intercept	13.08

Table 3 provides data obtained from the precision experiments. The R.S.D. values for intra- and inter-day precision were < 3 %, thereby indicating that the method was sufficiently precise. The method was found to be specific to the drug. The drug peak was free from any coeluting peak. The result indicated that the method was highly precise. Good separations were always achieved which suggested that the method was selective for all components under the test. The LOD and LOQ concentrations were found to be 0.006 μg mL^{-1} and 0.020 μg mL^{-1}.

Influence of small changes in chromatographic conditions such as change in flow rate (±10 %), organic content in mobile phase (±2 %), wavelength of detection (±5 %) and pH of buffer in mobile phase (±0.2 %) studied to determine the robustness of the method are also in favor (% R.S.D. <2 %) of the developed HPLC method for the analysis of Efavirenz.

The % R.S.D. of the assay of Efavirenz during solution stability experiments were within 2%. No significant changes were observed during solution stability. The solution stability data confirms that the sample solutions were stable at least for 7 days.

4. Conclusion

The study shows that the developed HPLC Method is fast, precise, specific, accurate and stability indicating. The stability-indicating method resolved the drug peak and also the peaks of degradation products formed under variety of conditions. After exposure of Efavirenz to stress conditions, it was concluded that the drug is susceptible to acid, base hydrolysis; oxidation, thermal degradation and photolysis with maximum degradation observed in base hydrolysis followed by thermal degradation. Therefore this method can be employed for monitoring the stability of Efavirenz drug substance commercially.

Table 3. Precision and Recovery data

Precision			% Recovery	
Actual Concentration (μg/mL)	Measured Concentration (μg/mL) ± S.D.; % R.S.D.			
	Intra-day	Inter-day	Intra-day	Inter-day
0.060	0.060 ± 0.002; 2.37	0.060 ± 0.002; 2.25	100.52	100.60
5.000	4.931 ±0.098; 1.99	4.931 ±0.130; 2.63	98.62	98.62
18.000	17.717 ±0.252; 1.42	17.733 ±0.342; 1.93	98.43	98.52

5. Acknowledgement

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