VALIDATION AND STABILITY OF RP-HPLC METHOD FOR THE DETERMINATION OF EFAVIRENZ AS BULK DRUG AND IN PHARMACEUTICAL FORMULATIONS

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ABSTRACT

A rapid, precise, accurate, specific and simple RP-HPLC method was developed for the estimation of Efavirenz in its tablet form. A High performance liquid chromatograph 10AT SHIMADZU- SPD10A, using Phenomenex - Luna RP-18(2),250X4.6mm, 5 µm column, with mobile phase composition of Acetonitrile: Phosphate Buffer [58:42 %(v/v)] was used. The flow rate of 1.0 ml min⁻¹ and effluent was detected at 247 nm. The retention time of Efavirenz was 4.611 minutes. Linearity was observed over concentration range of 500-10000ng ml⁻¹. The Limit of detection and limit of quantification was found to be 157.63ng ml⁻¹ and 477.68ng ml⁻¹ respectively. The accuracy of the proposed method was determined by recovery studies and found to be 98.240 to 101.170 %. The proposed method is applicable to stability studies and routine analysis of Efavirenz in bulk and pharmaceutical formulations. The proposed method was validated for various ICH parameters like linearity, limit of detection, limits of quantification, accuracy, precision, range and specificity.
Efavirenz is chemically \((4S)-(6\text{-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-1H-3,1-benzoxazin-2-one})\) (Fig. 1). It is a white powder form and used as antiretroviral agent, for the treatment of HIV infection. It has an empirical formula of \(\text{C}_{14}\text{H}_{9}\text{ClF}_{3}\text{NO}_{2}\) and molecular weight of 315.6750. Efavirenz belongs to a class of antiretroviral drugs known as 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. Literature survey reveals that very few analytical methods has been established for the determination of Efavirenz viz. Development of Rapid UV Spectrophotometric Method for the Estimation of Efavirenz in Formulations, High-performance liquid chromatographic method for the determination of HIV-1 non-nucleoside reverse transcriptase inhibitor efavirenz in plasma of patients during highly active antiretroviral therapy, Development of a competitive immunoassay for efavirenz: Hapten design and validation studies, Simultaneous quantification of a non-nucleoside reverse transcriptase inhibitor efavirenz, a nucleoside reverse transcriptase inhibitor emtricitabine and a nucleotide reverse transcriptase inhibitor tenofovir in plasma by liquid chromatography positive ion electrospray tandem mass spectrometry. Determination of efavirenz, a selective non-nucleoside reverse transcriptase inhibitor, in human plasma using HPLC with post-column photochemical derivatization and fluorescence detection.

The stability of a drug substance or drug product is defined as its capacity to remain within established specifications, i.e. to maintain its identity, strength, quality, and purity until the retest or expiry date. Stability testing of an active substance or finished product provides evidence of how the quality of a drug substance or drug product varies with time under a variety of environmental conditions, for example temperature, humidity, and light. Knowledge from stability studies is used in the development of manufacturing processes, selection of proper
packaging and storage conditions, and determination of product shelf-life\textsuperscript{10}. There was no reported stability-indicating analytical method for analysis of Efavirenz in the presence of its degradation products in bulk and pharmaceutical dosage forms. The objective of this work was to develop a new, simple, economic, rapid, precise, and accurate stability-indicating HPLC method for quantitative analysis of Efavirenz, and to validate the method in accordance with ICH guidelines\textsuperscript{10} with shorter retention time, runtime, and economic mobile phase.

**EXPERIMENTAL**

**MATERIALS AND METHODS**

Pure standard of Efavirenz (Assigned purity 99.98\%) was obtained as a gift sample from Ranbaxy labs Pvt. Ltd, Gurgaon, India. The gift samples were used as standard without further purification. HPLC grade water, Acetonitrile and methanol (Qualigens), Hydrochloric acid, Sodium hydroxide, Hydrogen peroxide, Anhydrous disodium hydrogen phosphate, citric acid monohydrate (S.D. fine chemicals, Mumbai, India), were used throughout the experiment. Commercial pharmaceutical preparation (Sustiva) which was claimed to contain 600mg of Efavirenz was used in analysis. The chemical structure and purity of the sample obtained was confirmed by TLC, IR, Melting point studies.

**Instrumentation and Chromatographic conditions**

- High performance liquid chromatograph, Shimadzu pumpLC-10AT VP equipped with universal injector (Hamilton 25 \(\mu\)L) SPD10A, UV-VIS detector SPD10A-10A VP (Shimadzu) was used. Isocratic elution of mobile phase comprising of Acetonitrile: Phosphate Buffer in the ratio of 58:42 % (v/v) with flow rate of 1.0 ml min\textsuperscript{-1} was performed on C18 column (250x 4.6 mm, 5\(\mu\)m). The effluent was detected at 247 nm. The retention time of Efavirenz was 4.611 minutes. The column temperature was maintained at ambient and the volume of injection was 20 \(\mu\)l. Prior to injection of analyte, the column was equilibrated for 30-40 min with mobile phase.

Different kinds of equipments viz Analytical weighing balance (Shimadzu AX 200), Sonicator (model SONICA 2200MH), Water purification system, Vacuum pump (model XI 5522050 of Millipore), Millipore filtration kit for solvents and sample filtration were used throughout the experiment. The Spinchrom CFR software was used for acquisition, evaluation and storage of chromatographic data.

**Preparation of Mobile Phase**

The HPLC grade solvents of water and Acetonitrile were used for the preparation of mobile phase Mobile Phase: (Acetonitrile 58\% Phosphate Buffer 42\%) I) solvent A, Acetonitrile II) Solvent B, Buffer: (Dissolve 0.900 g of anhydrous disodium hydrogen phosphate in 800 ml water and adjust the pH to 3.6 with 1.298 g of citric acid monohydrate in sufficient water to produce 1000 ml). The contents of the mobile phase were filtered before use through a 0.45\(\mu\)m membrane filter, sonicated and pumped from the solvent reservoir to the column at a flow rate of 1 ml/min.

**Preparation of Standard Solution**

A stock solution of drug was prepared by dissolving 100 mg of Pure Efavirenz in a 100 ml volumetric flasks containing sufficient amount of methanol (HPLC grade) to dissolve the drug, sonicated for about 15 min and then made up to volume with mobile phase. Daily working standard solutions of Efavirenz was prepared by suitable dilution of the stock solution with the mobile phase. Six sets of the drug solution were prepared in the mobile phase containing Efavirenz at a concentration of 6500-9000ng/ml. Each of these drug solutions (20\(\mu\)l) was injected six times into
the column, the peak area and retention times were recorded.

Procedure for sample solution (From formulation)

Twenty tablets were weighed accurately and powdered. An amount of the powder equivalent to 100 mg of Efavirenz was dissolved in 50 ml of mobile phase. The solution was stirred for 10 min using a magnetic stirrer and filtered into a 100 ml volumetric flask through 0.45 µm membrane filter. The residue was washed 3 times with 10 ml of mobile phase, and then the volume was completed to 100 ml with the same solvent. Further add mobile phase to obtain a stock solution of 100µg/ml. An aliquot of this solution (1 ml) was transferred to a 100 ml volumetric flask and made up sufficient volume with the mobile phase to give an expected concentration of 1µg/ml. All determinations were conducted in triplicate.

STABILITY STUDIES

Thermal degradation at different temperature and different time interval:

Expose about 2 to 3 gm of sample at different time intervals viz. 0, 90, and 180 days and at different temperatures viz. -20°C, 25°C, and 40°C. After that sample solution (Efavirenz) in mobile phase was demonstrated by injected the sample solution in HPLC, no degradants were observed in the chromatogram. However, after 180 days and 40°C the chromatographic peak area of Efavirenz decreased insignificantly. Hence, the sample was stable at least for 180 days at 40°C.

Photochemical degradation

The photochemical stability of the Efavirenz was studied by exposing the methanolic stock solution to direct sunlight for 8 h (from 9 AM to 5 PM, at 20°C).

Thermal stress (Test sample exposed to sunlight)

Transfer about 2 to 3 gm of sample into a clean dry watch glass and spread evenly. Expose to sunlight for 10 hours. After the sample got exposed to prescribed time, weigh accurately 100 mg of sample into a clean dry 100 ml volumetric flask, dissolve and dilute to the mark with mobile phase, finally make a concentration of 1000ng/mL with mobile phase and inject 20µl of this sample into HPLC, observe the degradation.

Forced degradation of Efavirenz and tablets of Efavirenz

In order to establish whether the analytical method and the assay were stability indicating, the tablets and pure active pharmaceutical ingredient of Efavirenz were stressed under various conditions to promote degradation. As this drug was freely soluble and stable in methanol and methanol was used as solvent in all forced degradation studies. All solutions that were used in forced degradation studies were prepared by dissolving Efavirenz or drug product in small volume methanol and later diluted with 3% hydrogen peroxide, 0.1N hydrochloric acid and 0.1 N sodium hydroxide to achieve concentration of 100µg/ml. After the degradation, these solutions were diluted with mobile phase to get starting concentration of 10µg/ml with the objective of evaluating stability of Efavirenz. The degradants were observed in the chromatogram and showing good resolution with the Efavirenz.

Hydrolysis (Acid and Alkali)

Initially for hydrolytic degradation the Efavirenz was dissolved in known amount of methanol and diluted with 0.1N HCl or 0.1N NaOH to obtain a concentration of 100µg/ml. After completion of degradation process, both the solutions were neutralized with acid or base, as necessary and diluted with the mobile phase to achieve a concentration of 10µg/ml. The solutions for hydrolysis were prepared in methanol and 0.1 N HCl and 0.1N NaOH (60:40 v/v). The prepared solutions in acid were injected to the chromatographic system at 0 h (immediately after
preparing the solution) and after reflux at 60°C about 2h and the solutions prepared in alkali were injected at 0 h and after reflux at 60°C about 2h. The respective chromatograms were recorded for the study of extent of degradation.

**Peroxide degradation**

The solutions for peroxide degradation were prepared in methanol and 3% hydrogen peroxide (60:40 v/v). The prepared solution was refluxed at 60°C about 2h and injected into chromatographic system after 2 h. The respective chromatogram was recorded for the study of extent of degradation.

**RESULTS AND DISCUSSIONS**

**STABILITY STUDIES**

**Thermal degradation at different temperatures and different time intervals**

Expose about 2 to 3 gm of sample at different time intervals viz. 0, 90, and 180 days and at different temperatures viz. -20°C, 25°C, and 40°C. After that sample solutions (Efavirenz) in mobile phase was demonstrated by injecting the sample solutions in HPLC, no degradants were observed in the chromatogram. However, after 180 days and 40°C the chromatographic peak area of Efavirenz decreased insignificantly. Hence, the sample was stable at least for 180 days at 40°C (Chromatogram No.1-8)

![Stability Chromatogram No.1 (blank)](image1)

![Stability Chromatogram No. 2 (Initial)](image2)
Stability Chromatogram No. 3 (25°C, 90 Days)

Stability Chromatogram No. 4 (25°C, 180 Days)

Stability Chromatogram No. 5 (-20°C, 90 Days)

Stability Chromatogram No. 6 (-20°C, 180 Days)
The result obtained by thermal degradation at different temperature and different time interval viz. Initial, 25°C at 90 days, 25°C at 180 days, -20°C at 90 days, -20°C at 180 days, 40°C at 90 days, 40°C at 180 days was found to be 100.32%, 100.18%, 99.98%, 100.13%, 99.93%, 99.12%, 98.27% respectively. The further study was carried out by employing the following tests: hydrolysis (neutral, acidic and basic), photolysis and thermolysis. No decomposition was observed when the Efavirenz was exposed to sunlight, temperature, UV; whereas significant change i.e., decrease of assay about 20 to 25% observed when sample was treated with 0.1N NaOH and 0.1N HCl. The sample treated with 3% H₂O₂ was almost completely degraded.

VALIDATION OF ANALYTICAL METHOD

Validation of an analytical method is a process to establish by laboratory studies that the performance characteristics of the method meet the requirements for the intended analytical application. Performance characteristics are expressed in terms of analytical parameters.

Typical analytical parameters used in validation area:
1. Linearity
2. Accuracy
3. Precision
4. Specificity
5. Limit of detection
6. Limit of quantification
7. Range
8. Ruggedness
9. Robustness
10. System suitability
11. Solution stability

LINEARITY

Acceptance criteria: Coefficient of correlation (r²) should be greater than 0.998

Procedure: A stock solution of drug was prepared by dissolving 100 mg of Pure
Efavirenz in a 100 ml volumetric flasks containing sufficient amount of methanol (HPLC grade) to dissolve the drug, sonicated for about 15 min and then made up to volume with mobile phase. Daily working standard solutions of Efavirenz was prepared by suitable dilution of the stock solution with the mobile phase. Six sets of the drug solution were prepared in the mobile phase containing Efavirenz at a concentration of 6500-9000ng/ml. Each of these drug solutions (20µl) was injected in six concentrations in three replicates times into the column, the peak area and retention times were recorded. (Table No. 1 and chromatogram No. 9)

**Table No. 1**

*For Peak Area of Efavirenz*

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Dilution I</th>
<th>Dilution II</th>
<th>Dilution III</th>
<th>Dilution IV</th>
<th>Dilution V</th>
<th>Dilution VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3733079</td>
<td>4210201</td>
<td>4680931</td>
<td>5049177</td>
<td>5484836</td>
<td>5963214</td>
</tr>
<tr>
<td>2</td>
<td>3742795</td>
<td>4221032</td>
<td>4670452</td>
<td>5031487</td>
<td>5472510</td>
<td>5972532</td>
</tr>
<tr>
<td>3</td>
<td>3752174</td>
<td>4196325</td>
<td>4681234</td>
<td>5151427</td>
<td>5486385</td>
<td>5985248</td>
</tr>
<tr>
<td>Average</td>
<td>3742683</td>
<td>4209186</td>
<td>4677539</td>
<td>5077364</td>
<td>5481244</td>
<td>5973665</td>
</tr>
<tr>
<td>SD</td>
<td>9547.99</td>
<td>12384.73</td>
<td>6139.392</td>
<td>64747.71</td>
<td>7603.128</td>
<td>11060.58</td>
</tr>
<tr>
<td>RSD%</td>
<td>0.26</td>
<td>0.29</td>
<td>0.13</td>
<td>1.28</td>
<td>0.14</td>
<td>0.19</td>
</tr>
</tbody>
</table>

*Average of five readings

**RESULT**

Correlation coefficient ($r^2$) of Efavirenz was found to be 0.999, indicating the linearity and the method is linear between the concentrations of 6500-9000ng mL$^{-1}$.

**ACCURACY**

The accuracy is the closeness of the measured value to the true value for the sample. Accuracy was found out by recovery study from prepared solution (three replicates) with standard solution, of the label claim. Aliquots of 2.5 ml, 3.5 ml and 4.5 ml of sample drug (Efavirenz) solution of 100µg/ml were pipetted into each of three volumetric flasks. To this 4 ml of standard drug (Efavirenz) solution of 100µg/ml was added to each volumetric flask respectively.
The volume was made up to 100 ml with mobile phase. 20 µl of each solution was injected and chromatograms were recorded. The range was found between 98.240 to 101.170 %.

The values of recovery justify the accuracy of the method. The % recovery values were obtained within the standard limit which confirms that the method is accurate and free from any positive or negative interference of the excipients.

The recovery data was generated for Efavirenz are presented in the Table No.2

### RESULT OF RECOVERY STUDIES OF DRUG (TABLE NO. 2)

<table>
<thead>
<tr>
<th>Conc. taken in ng/ml (A)</th>
<th>Std addition in ng/ml (B)</th>
<th>Total drug conc. in ng/ml (A+B)</th>
<th>Peak Area</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>2500</td>
<td>4000</td>
<td>6500</td>
<td>3788493</td>
<td>101.170</td>
</tr>
<tr>
<td>3500</td>
<td>4000</td>
<td>7500</td>
<td>4695260</td>
<td>98.240</td>
</tr>
<tr>
<td>4500</td>
<td>4000</td>
<td>8500</td>
<td>5472491</td>
<td>99.858</td>
</tr>
</tbody>
</table>

*Average of three readings

### RESULT

The percentage recovery by the proposed method was ranging from 98.240 to 101.170 % indicating no interference of the tablet excipients with drug under analysis.

### PRECISION

Precision is measure of repeatability or reproducibility and it was determined by injecting 5 times the expected operating range concentration. The chromatograms were recorded to determine mean standard deviation and relative standard deviation.

**Acceptance criteria:** RSD<2.0% for peak area and retention time

#### Table No.3

(Precision of Efavirenz)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Area Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4568142</td>
</tr>
<tr>
<td>2</td>
<td>4583698</td>
</tr>
<tr>
<td>3</td>
<td>4582587</td>
</tr>
<tr>
<td>4</td>
<td>4572365</td>
</tr>
<tr>
<td>5</td>
<td>4572365</td>
</tr>
<tr>
<td>Average</td>
<td>4563831.4</td>
</tr>
<tr>
<td>S.D</td>
<td>29523.924</td>
</tr>
<tr>
<td>R.S.D</td>
<td>0.65</td>
</tr>
</tbody>
</table>

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RESULT
From the above analytical data it is observed that RSD for the assay is 0.65 which indicates that the method is precise and reproducible.

SPECIFICITY

Specificity is the ability to assess the analyte in the presence of components that may be expected to be present in the sample matrix (USP 2004). For demonstrating the specificity of the method for drug formulation the drug was spiked and the representative chromatogram (chromatogram No. 10).

RESULT
The excipients used in different formulation products did not interfere with the drug peak and thus, the method is specific for Efavirenz.

SOLUTION STABILITY
The solution stability of the standard sample and the sample prepared in mobile phase were studied for 5 days. The solutions under study were compared with freshly prepared standard solution, the samples were found to be stable for period of more than 72 hours.

RANGE
The specific range derived from the linearity studies. The range was calculated from the linearity graph. From the lower to higher concentration between which the response is linear, accurate and precise.

Acceptance criteria: RSD < 2.0

The range for Efavirenz was found to be 500-10000ng/mL.

LIMITS OF DETECTION AND QUANTIFICATION
The detection limit (LOD) is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. It may be expressed as a concentration that gives a signal-to-noise ratio of 2:1 or 3:1. The lower limit of detection for Efavirenz is 157.63ng/ml in reference material and formulation. Limit of Quantification (LOQ) is the lowest amount analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. A signal-to-noise ratio of 10:1 can be taken as LOQ of the method. The LOQ values were found to be 477.68ng/ml for raw material and formulations.
SYSTEM SUITABILITY

To know reproducibility of the method system suitability test was employed to establish the parameters such as tailing factor, theoretical plates, limit of detection and limit of quantification. The values were shown in table 4. A solution of 7500ng mL\(^{-1}\) (Approx.) of Efavirenz (in five replicates) was prepared and same was injected, then the system suitability parameters were calculated from the following chromatogram. (Chromatogram No. 11)

**Theoretical plates per column**

Theoretical plates column were calculated from the data obtained from the peak.

\[ n = \frac{(5.54V_r^2)}{W_{h2}} \]

Where, ‘n’ is number of theoretical plates per meter, ‘V\(_r\)’ is the distance along the base line between the point of injection and a perpendicular dropped from the maximum of the peak of interest and ‘W\(_{h2}\)’ is the width of the peak of interest at half peak height.

**Tailing Factor (USP Method)**

A measure of the symmetry of a peak, given by the following equation where \(W_{0.05}\) is the peak width at 5% height and \(f\) is the distance from peak front to apex point at 5% height. Ideally, peaks should be Gaussian in shape or totally symmetrical.

\[ T = \frac{W_{0.05}}{2f} \]

The accuracy of quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where/when the peak ends and hence the calculation of the area under the peak. Integrator variables are preset by the analyst for optimum calculation of the area for the peak of interest.

Recommendations:

\[ T \leq 2 \]

\[ T = \frac{(a+b)}{2a} \]

Where:

- \(T = \) tailing factor (measured at 5% of peak height)
- \(b = \) distance from the point at peak midpoint to the trailing edge
- \(a = \) distance from the leading edge of the peak to the midpoint

**Table 4.**

**Results of system suitability parameters**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Data obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical plates per column</td>
<td>6025</td>
</tr>
<tr>
<td>Symmetry factor/Tailing factor</td>
<td>1.17</td>
</tr>
</tbody>
</table>

**Chromatogram No. 11 (showing system suitability)**

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CONCLUSION

The proposed RP-HPLC method is found to be accurate, precise, linear, stable, specific, and simple, for quantitative estimation of Efavirenz in raw material and pharmaceutical formulations. Hence the present RP-HPLC method is suitable for routine assay of Efavirenz in raw materials and in pharmaceutical formulations in the quality control laboratories.

ACKNOWLEDGEMENT

The authors thank Ranbaxy labs Pvt. Ltd, Gurgaon, India, for providing a sample of Efavirenz as a gift.

REFERENCES