ABSTRACT

Background: The crucial aspect to consider during method development and validation, ensuring accurate, precise, and specific estimation of drug substances and drug products, is stability. Various factors, including environmental, instrumental, reagent, and human factors, can pose challenges in achieving suitable method development and validation. Objective: This work aimed to develop and validate a low flow rate, LCMS compatible, simple, and rapid reverse-phase high-performance liquid chromatographic method for estimating efavirenz and its degradation products at different stress conditions. Materials and Methods: The HPLC system employed a Phenomenex Luna 5µ C18 (2) 100A (250 x 4.6 mm) column and a mobile phase of methanol: 20 millimolar ammonium formate solution (90:10) adjusted to pH 4 with formic acid. All analytes were separated within 15 minutes and detected at 247 nm. Method validation was carried out according to ICH guidelines, including linearity, accuracy, precision, ruggedness, robustness, LOD, and LOQ. Results and Discussion: The method was linear in the 10-90 µg/ml range, with a regression coefficient 0.999. Intra- and inter-day precisions, ruggedness, and robustness were within acceptable limits (≤2% RSD) with LOD and LOQ of 0.35 and 1.16 µg/ml, respectively. Degradation study indicates well resolution of the drug and degradants. Conclusion: Purposeful degradation of efavirenz resulted in different degradation products under various stress conditions, and the method demonstrated satisfactory resolution from its degradants.

INTRODUCTION

ICH guideline, ICH Q14, extensively describes the principle of method development of drug substances and products, which can be applied to new and revised analysis procedures. The minimum approaches include the identification of attributes, selecting appropriate technology and instruments, and defining analytical procedures. Advanced and enhanced approaches include evaluation of sample properties, analytical target profile definition, risk assessment evaluation, multivariate analysis, control strategy of procedure, and change of lifecycle management plan. The use of more sophisticated and advanced

Keywords
Efavirenz, NNRTIs, SIAMs, Purposeful Degradation, ICH guidelines

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analytical procedures is to be encouraged [1]. All the analytical procedures need to be validated for standard procedures such as identification tests, impurities quantification, and limit tests for the impurities for their control. The parameters to be validated include accuracy, precision (repeatability, intermediate precision), specificity, detection limit, quantitation limit linearity, and range [2]. Human Immunodeficiency Virus can be effectively controlled by combination therapy. USFDA has approved seven categories for the treatment of AIDS, which include NRTIs, NNRTIs, protease inhibitors, integrase inhibitors, fusion inhibitors, CCR5 antagonists, and post-attachment inhibitors. Efavirenz (DMP-266, sustiva) 6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one, chemically a benzooxazone-2-one derivative (as shown in Figure 1) a new class of NNRTIs on clinical evaluation exhibited HIV-1 reverse transcriptase inhibition [3]. Efavirenz is effectively used to treat retroviral infection. Efavirenz on chronic administration may affect microanatomy of lateral geniculate body of adult Wistar rats. Combination therapy with improved tolerability achieved by the combination of efavirenz [4]. The effect of efavirenz on different cells and organs was evaluated [5]. Pharmacokinetics studies were conducted with combination therapies [6]. Determination of efavirenz plasma concentration using HPLC was described. The reverse phase HPLC method was developed for efavirenz and its related substances in capsule dosage form [7]. Quality by design is utilized to develop stability, indicating the reverse phase chromatographic method [8]. Determination of antiretroviral agents and metabolites using HPLC with UV detection [9,10]. Determination of antiretroviral agents in human serum by capillary electrophoresis was described. Efavirenz intermediate cyclization was achieved catalytically using enzymes lysozyme and novozyme-435 to form benzoxazine derivatives. Plasma efavirenz concentration was determined using high-performance liquid chromatography [11]. Efavirenz estimated simultaneously described in the method development and validation [12]. The bioanalytical method in human plasma was developed and validated using acetonitrile and sodium dihydrogen orthophosphate buffer by HPLC. Simultaneous estimation of some antiretroviral drugs is reported. The reversed-phase high-performance liquid chromatography method was developed based on the design of the experiment strategy to estimate efavirenz and its formulation. A survey of these papers revealed that some methods involved tedious extraction procedures using solid phase cartridges, protein precipitation, and high flow rate. In contrast, others used complex mobile phases and buffers or gradient mobile phases. Some methods include LCMS, Tandem mass spectrometry, MALDI-TOF/TOF technology, liquid chromatography–triple quadrupole mass spectrometry, and gas chromatography. Using phosphate buffers, a common practice in method development, affects column life. The high flow rates in the method may not be suitable for mass detection and consume more mobile phase. Therefore, this study attempted to develop a low flow rate, a simple, rapid, accurate, precise, robust, and rugged high-performance liquid chromatographic method for the routine analysis of efavirenz in the presence of its degradants.

Figure 1: Chemical structure of efavirenz

MATERIALS AND METHOD
In the present investigation, HPLC-grade methanol, acetonitrile, and water for the study were procured from Merck (India), and Ammonium formate, Ammonium Acetate, Hydrochloric Acid, Sodium Hydroxide, Formic Acid, and Hydrogen Peroxide were procured from Loba Chemie Pvt. LTD., India.

Chromatographic System Selection
The HPLC system consists of an LC-20AT prominence liquid chromatograph pump, an SPD-20A prominence UV-Vis detector, and a rheodyne-type injector fitted with a 20μl capacity loop was used for loading the sample, all from Shimadzu Corporation, Japan. The analytical column was phenomenal luna 5μ C18 (2) 100A (250X 4.6mm); the output signals were monitored and processed using Spinchrom CFR software. Spinchrom CFR software workstation was used for data collection and acquisition. The mobile phase consisted of methanol: 20 mM ammonium formate (90:10) pH adjusted to 4 with formic acid. Before preparation of the mobile phase, the methanol and 20mM were degassed separately using a Millipore vacuum pump. The UV detector was set at 247 nm. The chromatogram was run for 15 minutes at a 0.5 ml/min flow rate at ambient temperature [16]. Unknown concentrations were derived from linear regression analysis of the peak area vs. concentration curve. The linearity was verified using estimates of correlation coefficient ($r$).
Optimization of chromatographic method for analysis
Selection of Mobile Phase: Different efavirenz concentrations were injected into the column with different mobile phases of different ratios with varying rates of flow till sharp peaks without any interference peaks in the chromatogram were obtained. The different mobile phases contained one or the combinations of two or three of the following: methanol, acetonitrile, and water (HPLC grade), 20mM ammonium formate, and 20 mM ammonium acetate solutions [16].

Chromatographic Conditions: A reverse-phase C-18 column was equilibrated with the mobile phase. The mobile phase flow rate was maintained at 0.5 ml/min, and eluents were monitored at 247 nm. The samples were injected using a 20-microliter fixed loop. All determinations were performed at ambient temperature for a run time of 15 min.

Preparation of 20mM Ammonium Formate solutions
Accurately weighed 0.12612g of ammonium formate was transferred into a 100 ml volumetric flask. The volume was made up to the mark with HPLC grade water. Then, the pH was adjusted to 4 with formic acid.

Preparation of Mobile Phase: The mobile phase was prepared by mixing 900 ml of HPLC-grade methanol with 100 ml of 20 mM ammonium formate solution to get a 90:10 v/v proportion. Finally, the pH was adjusted to 4 with formic acid. The mobile phase was sonicated for 10 minutes and filtered through a 0.45µ membrane filter.

Preparation of Standard Drug Stock Solutions: The standard stock solutions of 1000μg/ml of the drug were prepared by dissolving 50mg of pure drug in the mobile phase in a 50ml volumetric flask, and the volume was made up to the mark. The resulting solutions were diluted with mobile phase to obtain a final concentration of 100μg/ml and stored under refrigeration.

Calibration Curve: Aliquots of standard stock solutions of the drug were taken in a 10 ml volumetric flask and diluted up to the mark with mobile phase so that the final concentration of the drug was in the range of 10-90μg/ml. Triplicate injections of 20 microliters were made and chromatographed under the described conditions. Evaluation of the drug was performed, and peak areas were recorded. The linear regression analysis was performed to establish the relationship between concentration and peak area by taking the peak area on the y-axis and the respective concentration of the drug on the x-axis [16].

Estimation of efavirenz in dosage form: Twenty tablets were taken, weighed, and powdered. Powder equivalent to 10 mg of the drug was accurately weighed and transferred to a 100 ml volumetric flask. The drug was extracted four times by adding solvent in portions, 20 ml each, and then the volume was made up to mark with the same mobile phase. The above solutions were filtered using Whatman filter paper No. 41. Appropriate volumes of the aliquots were transferred and diluted with mobile phase to get 30 μg/ml of drug concentration. The solutions were injected at the above chromatographic conditions, and peak areas were measured. The quantification was carried out by keeping these values to the straight-line equation of the calibration curve.

VALIDATION [17]
Accuracy
The method's accuracy was determined by calculating efavirenz recoveries using standard addition. Known amounts of standard drug corresponding to 80%, 100%, and 120% of the label claim were added to the pre-quantified sample solution, and the amount of efavirenz was estimated by measuring the peak areas and fitting these values to the straight-line equation of the calibration curve.

Precision: The drug's intraday and interday precision studies estimated the corresponding responses on the same day and on three consecutive days, respectively. The results were reported in terms of standard deviation and %RSD.

Specificity: The proposed RP-HPLC method's specificity was determined by the complete separation of two peaks with parameters like retention time (Rt), resolution (Rs), and tailing factor (T).

Robustness: The method's robustness was studied by deliberately varying the analytical parameters, such as flow rate (0.5+0.1 ml/min) and concentration of methanol (90±2%).

Ruggedness: Ruggedness is the degree of reproducibility of the results obtained under various conditions, expressed as %RSD. These conditions include different laboratory conditions and analysts [17].
Detection Limit and Quantification Limit: The linear regression analysis established the relationship between concentration and peak area using efavirenz's expected detection limit range (0.1-5 μg/ml). The standard deviation of the y-intercept of the regression line was determined. Determination of the detection limit and quantification limit were calculated using 3.3σ/s and 10 σ/s, respectively, where σ is the standard deviation of the y-intercept of the regression line, and s is the slope of the calibration curve.

FORCED DEGRADATION STUDIES
The method's specificity can be demonstrated through forced degradation studies conducted on the sample using acid, alkaline, oxidative, thermal, photolytic, and UV degradations. The sample was exposed to these conditions, and the main peak was studied for peak purity, thus indicating that the method effectively separated the degradation products from the pure active ingredient.

Degradation in Neutral Condition
About 10mg of the pure drug was accurately weighed, taken into three sets of different 10-volumetric flasks, and dissolved in a minimum volume of methanol. Then, the volumes were made up to the mark with water and refluxed in round-bottom flasks for 1h, 2h, and 6h. Different solutions were prepared from these samples, and 20 microliters of the sample solutions were injected into the HPLC system [18].

Degradation in Acidic Condition
About 10mg of the pure drug was accurately weighed, taken into three sets of different 10 volumetric flasks, and dissolved in a minimum volume of methanol. Then, the volumes were made up to the mark with 0.1N, 0.5N, and 1N HCl and refluxed for 1h, 2h, and 6h. Different solutions were prepared from these samples, and 20 microliters of the sample solutions were injected into the HPLC system [18].

Degradation in Basic Condition
About 10mg of the pure drug was accurately weighed, taken into three sets of 10 volumetric flasks, and dissolved in a minimum volume of methanol. Then, the volumes were made up to the mark with 0.1N, 0.5N, and 1N NaOH and refluxed for 1h, 2h, and 6h. Different solutions were prepared from these samples, and 20 µl of the sample solutions were injected into the HPLC system [18].

Oxidative Degradation
About 10mg of the pure drug was accurately weighed, taken into three sets of different 10 volumetric flasks, and dissolved in a minimum volume of methanol. Then, the volume was made up to the mark with 1% w/v H₂O₂, 3% w/v H₂O₂, and 6% w/v H₂O₂ and refluxed for 1h, 2h, and 6h. Different solutions were prepared from these samples, and 20 microliters of the sample solutions were injected into the HPLC system [19].

Photolytic Degradation
About 100 mg of pure drug were taken in a clean petri dish and exposed to daylight. Sampling was done at 10h, 1 week, and 2 weeks. Different solutions were prepared from these samples, and 20 microliters of the sample solutions were injected into the HPLC system [19].

UV- Degradation
About 100 mg of the pure drug were taken in a clean petri dish and subjected to UV illumination of 1.2x10⁶ lux hours. Sampling was done at 12h, 24h, and 48h, and different solutions were prepared from these samples. Twenty microliters of the sample solutions were injected into the HPLC system [19].

Thermal Degradation
About 100 mg of pure drug was taken in three clean Petri dishes separately and subjected to dry heat at 70°C. Sampling was done at intervals of 10 days, 20 days, and 30 days. Drug solutions were prepared, and 20 microliters of the sample solutions were injected into the HPLC system [20].

RESULTS AND DISCUSSION
Optimization of chromatographic method for analysis
The chromatographic method for analysis of Efavirenz was optimized after thorough analytical trials with different mobile phase and column alterations to obtain a peak with good resolution for further quantitation. To optimize the method, the number of theoretical plates, retention or capacity factor, peak shape, asymmetric factor, and retention time were selected.

The method with more theoretical plates is more efficient in separation. Indian Pharmacopoeia suggests that the theoretical plates should be more than 6000 for the pharmacopeial method. A retention or capacity factor of more than 2 and an asymmetric factor of less than 2 were selected for optimizing the method. The optimum separation was achieved with a Phenomenex ODS
C-18 (250x4.6 mm, packed with 5 microns) column and Methanol: Ammonium formate solution (90:10) pH adjusted to 4 with formic acid as mobile phase. The retention and run times were found as desired for further analysis of efavirenz and its degradants. The optimized conditions are disclosed in Table 1.

**Preparation of Calibration curve**

The standard concentration of efavirenz (10μg/ml to 90μg/ml) was injected into the chromatographic system, and the corresponding peak area was obtained. The overlay chromatogram was developed (Figure 2). The linearity data between peak area and efavirenz concentration were recorded (Table 2). The linear regression analysis was performed to establish the relationship between concentration and peak area by taking concentration 10-90 μg/ml on the x-axis and the corresponding peak area on the y-axis. This indicated that efavirenz follows linearity with a regression coefficient of 0.999, as shown in Figure 3.

### Table 1: Optimized Chromatographic Conditions of Efavirenz

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary phase (column)</td>
<td>Phenomenex ODS C-18 (250 x 4.6 mm, packed with 5 micron)</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Methanol: Ammonium formate solution (90:10) pH adjusted to 4 with formic acid.</td>
</tr>
<tr>
<td>Flow rate (ml/min)</td>
<td>0.5</td>
</tr>
<tr>
<td>Run time (minutes)</td>
<td>15</td>
</tr>
<tr>
<td>Column temperature (°C)</td>
<td>Ambient</td>
</tr>
<tr>
<td>Volume of injection loop (µl)</td>
<td>20</td>
</tr>
<tr>
<td>Detection wavelength (nm)</td>
<td>247</td>
</tr>
<tr>
<td>Drug R&lt;sub&gt;t&lt;/sub&gt; (min)</td>
<td>8.087</td>
</tr>
<tr>
<td>Retention or capacity factor (k′)</td>
<td>3.0435</td>
</tr>
<tr>
<td>Asymmetry factor</td>
<td>1.16</td>
</tr>
<tr>
<td>Theoretical plates</td>
<td>8427</td>
</tr>
<tr>
<td>LOD</td>
<td>0.35µg/ml</td>
</tr>
<tr>
<td>LOQ</td>
<td>1.16µg/ml</td>
</tr>
</tbody>
</table>

**Figure 2: Typical HPLC Overlay Chromatogram of Efavirenz**

**Table 2: Calibration Table of Efavirenz for RP-HPLC Method**

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Peak Area</th>
<th>Statistical Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>675.322</td>
<td>Slope=65.88</td>
</tr>
<tr>
<td>20</td>
<td>1352.88</td>
<td>Intercept=56.65</td>
</tr>
<tr>
<td>30</td>
<td>2053.87</td>
<td>SD=1.69</td>
</tr>
<tr>
<td>40</td>
<td>2703.83</td>
<td>%RSD=0.083</td>
</tr>
<tr>
<td>50</td>
<td>3372.24</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>4045.05</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>4735.78</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>5286.93</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>5930.76</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3: Calibration Curve of Efavirenz

Table 3: Validation results of efavirenz

<table>
<thead>
<tr>
<th>SNo.</th>
<th>Parameter</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Accuracy at different levels</td>
<td>Mean % Recovery = 99.7, SD=0.458, %RSD=0.459</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean % Recovery = 99.5, SD=0.8, %RSD=0.804</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean % Recovery = 100.3, SD=0.624, %RSD=0.62</td>
</tr>
<tr>
<td>2.</td>
<td>Precision Data Showing Repeatability</td>
<td>Mean=30.02, SD=0.53, %RSD=1.77</td>
</tr>
<tr>
<td>3.</td>
<td>Intraday Precision</td>
<td>Mean=29.99, SD=0.51, %RSD=1.69</td>
</tr>
<tr>
<td>4.</td>
<td>Inter-day Precision</td>
<td>Mean=30.68, SD=0.24, %RSD=0.79</td>
</tr>
<tr>
<td>5.</td>
<td>Ruggedness</td>
<td>Mean=30.1, SD=0.52, %RSD=1.7</td>
</tr>
<tr>
<td>6.</td>
<td>Robustness at different pH</td>
<td>Mean=30.2, SD=0.41, %RSD=1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean=30.2, SD=0.46, %RSD=1.5</td>
</tr>
<tr>
<td>7.</td>
<td>Robustness at different flow rate</td>
<td>Mean=30.31, SD=0.221, %RSD=0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean=30.04, SD=0.414, %RSD=1.4</td>
</tr>
</tbody>
</table>

**Validation**

The validation protocol was followed according to the ICH guidelines for accuracy, precision, ruggedness, and robustness. The accuracy was studied at three levels (80%, 100%, 120%) in triplicate by standard addition procedure. A statistical analysis was carried out, and the result is presented in Table 3. Precision data showing repeatability, Intraday Precision, Inter-day Precision, Ruggedness, Robustness at different pH, and Robustness at different flow rates were studied by injecting six standard concentrations of 30 μg/ml for the respective parameters. The concentration was calculated from the peak area and subjected to statistical analysis. The results are discussed in Table 3. The study protocol was followed according to ICH guidelines. Ruggedness was determined by changing the analyst, and robustness study results were obtained by changing the mobile phase's pH and flow rate. The data were collected and statistically analyzed. The statistical analysis was performed using Microsoft Excel, and the results indicated that the standard deviation and percentage relative standard deviation were less than 2%, as per the regulatory guidelines.

**Detection Limit and Quantification Limit:**

Determination of the detection limit and quantification limit was carried out by the calibration plot in the range of 0.1-5μg/ml for efavirenz three times. The standard deviation of the y-intercept of the regression line and the slope's mean were calculated. Determination of the detection limit and quantification limit were calculated using 3.3σ/s and 10σ/s, respectively, where σ is the standard deviation of the y-intercept of the regression line, and s is the slope of the calibration curve. The detection and quantification limits were 0.35μg/ml and 1.16μg/ml, respectively.
FORCED DEGRADATION STUDIES

Hydrolytic Degradation in Neutral Condition

Samples were withdrawn according to the protocol. From the drawn samples, 20μg/ml solutions were prepared and subjected to analysis. The representative chromatogram, shown in Figure 4a, indicated 31.79% degradation after 6h. The chromatogram also revealed that the drug was analyzed in the presence of degradants with the required resolution. The retention time of the drug, degradants, and %degradation were tabulated below in Table 4.

![chromatogram](a)

Hydrolytic degradation of efavirenz in acidic condition

Samples were withdrawn according to the experimental protocol. From the drawn samples, 10μg/ml solutions were prepared and subjected to analysis. The representative chromatogram, shown in Figure 4b, indicated 40.43% degradation after 2h. The chromatogram was analyzed. The drug's retention time, degradants, and %degradation are shown in Table 4. The chromatogram indicated the efavirenz resolution from its degradants.

![chromatogram](b)

Hydrolytic degradation of efavirenz in basic condition

The chromatogram obtained from the hydrolytic degradation of efavirenz in basic stress conditions is shown in Figure 4c. ICH protocol was followed for the sample preparation. Samples were withdrawn according to the protocol. From the drawn samples, 20μg/ml solutions were prepared and subjected for analysis. The representative chromatogram indicated 47.37% degradation after 2h. The chromatogram also revealed that the drug can be

![chromatogram](c)

Table 4 Results of forced degradation studies of efavirenz

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Stress Condition</th>
<th>Rt of Efavirenz and Degradants</th>
<th>% degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hydrolytic Degradation in Neutral Condition</td>
<td>8.603 and 5.423, 6.697, 7.713</td>
<td>31.79</td>
</tr>
<tr>
<td>2</td>
<td>Hydrolytic Degradation in Acidic Condition</td>
<td>8.633 and 5.43, 7.733</td>
<td>40.43</td>
</tr>
<tr>
<td>3</td>
<td>Hydrolytic Degradation in Basic Condition</td>
<td>8.603 and 5.33, 6.463, 7.023</td>
<td>47.37</td>
</tr>
<tr>
<td>4</td>
<td>Oxidative Degradation</td>
<td>8.023 and 5.41, 6.58, 7.067</td>
<td>21.6</td>
</tr>
<tr>
<td>5</td>
<td>Photolytic Degradation</td>
<td>7.767 and 6.123, 6.973, 11.52</td>
<td>12.6</td>
</tr>
<tr>
<td>6</td>
<td>UV degradation</td>
<td>7.983 and 6.18, 6.56, 7.043</td>
<td>8.64</td>
</tr>
<tr>
<td>7</td>
<td>Thermal degradation</td>
<td>8.03 and 5.37, 6.173, 7.023, 12.04</td>
<td>7.33</td>
</tr>
</tbody>
</table>

![chromatogram](g)
analyzed with its degradants. It was observed that the degradant at retention time 7.023 min was the major degradant. The retention time of the drug, degradants, and % degradation were presented in Table 4.

Oxidative Degradation of Efavirenz
Oxidative degradation was studied using hydrogen peroxide as stress. Protocol in the experimental part followed ICH guidelines, and samples were withdrawn. From the drawn samples, 30 μg/ml solutions were prepared and subjected for analysis. The representative chromatogram indicates 21.6% degradation after 6h. A blank was carried out simultaneously to determine the influence of the reagent (H2O2) on drug retention time and peak area, but it was found that the reagent (H2O2) had a retention time of 5.410 min. Since the chromatogram showed proper resolution between the drug, degradants, and the reagent (H2O2), the proposed method can be utilized to analyze the drugs in the presence of their degradants. The retention time of the drug, degradants, and % degradation were tabulated in Table 4, and the chromatogram is shown in Figure 4d.

Photolytic Degradation of Efavirenz
Samples were withdrawn according to the protocol. From the drawn samples, 20 μg/ml solutions were prepared and subjected to analysis. The representative chromatogram in Figure 4e indicates 12.6% degradation after 2 weeks of exposure. The chromatogram also revealed that the drug can be analyzed with its degradants. The retention time of the drug, degradants, and % degradation were tabulated below in Table 4.

UV- Degradation of Efavirenz
UV-degradation was carried out to study the effect of ultraviolet light on intrinsic stability. Samples were withdrawn, and from the drawn samples, 50 μg/ml solutions were prepared and subjected to analysis. The representative chromatogram indicated 8.64% degradation after 48h. The chromatogram also revealed that the drug was analyzed in the presence of their degradants, as shown in Figure 4f. The retention time of the drug, degradants, and % degradation are shown in Table 4.

Thermal degradation of efavirenz
Samples were withdrawn according to the protocol. From the drawn samples, 70 μg/ml solutions were prepared and subjected for analysis. The representative chromatogram indicates 7.33% degradation after 1 month at 70°C. The chromatogram also reveals that the drug was analyzed with their degradants, presented in Figure 4g. The retention time of the drug, degradants, and % degradation were tabulated in Table 4.

These stability studies of efavirenz suggest that the drug is more prone to hydrolytic degradation. This may be due to a carbonyl group in the benzoxazine-2-one nucleus.

CONCLUSIONS
In the present research endeavor, an RP-HPLC method was successfully developed to estimate efavirenz in the presence of its degradants. The interference in the analysis of efavirenz was resolved using a suitable mobile phase in combination with volatile buffers with reduced flow rate and suitable columns. The developed method can also be applied to analyze efavirenz in its pure form and dosage forms at different stress conditions. The method was fast, simple, reliable, sensitive, specific, economical, accurate, and precise.

This method has the scope to be used in routine analysis. So, measures should focus on reducing efavirenz's exposure to moisture during the study. Stability in the aqueous mobile phase must be an important attribute to be considered during the method development. Furthermore, the research can be extended to analyze efavirenz in biological samples by developing suitable extraction procedures for pharmacokinetic studies.

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FINANCIAL ASSISTANCE
Nil

CONFLICT OF INTEREST
The authors declare no conflict of interest

AUTHOR CONTRIBUTION
The present research investigation was carried out under the supervision of P. N. Murthy, who also provided the requisite facilities. Sudhir Kumar Sahoo designed and developed the method for estimating Efavirenz and skillfully drafted the
article. Prasanta Kumar Choudhury and Uma Shankar Mishra performed the stability studies and analyzed the data obtained. Saroj Kanta Bisoyi and Lokesh Kumar made valuable contributions by meticulously analyzing all the experimental results and preparing the draft for the manuscript. All authors reviewed the final manuscript and provided necessary corrections.

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