# DEVELOPMENT AND METHOD VALIDATION OF NEVIRAPINE BY HPLC

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## ABSTRACT

**Objective:** To create a new reverse phase high-performance liquid chromatography method and validate for selective, sensitive, and precise, utilizing UV detector for the quantification of Nevirapine.

**Method:** The separation and quantification were performed using a Zorbax C18 isocratic column (100 mm × 4.6 mm i.e., 3.5 µm particle size) at ambient temperature. The analysis was conducted with an Agilent 1260 Prominence Liquid Chromatograph, utilizing a mobile phase composed of pH 3.5 Phosphate buffer and Acetonitrile in a ratio of 30:70 (v/v). The flow rate was maintained at 1.5 ml/min, and detection was carried out at a wavelength of 315 nm, Injection volume 5 µL and diluent as Water: Methanol (50%v/v).

**Results and Discussion:** The method underwent validation for linearity, accuracy, and precision. The reported % RSD was less than 2%, demonstrating that the method is both precise and accurate. Mean recovery rates were observed to be between 98% and 105%. The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were calculated to be 0.05 µg/ml and 0.01 µg/ml, respectively, indicating the method's sensitivity. Additionally, no interfering peaks were detected in the chromatogram, confirming that the excipients in the tablet formulations did not affect the drug estimation using the proposed HPLC method.

**Keywords:** Determination, ICH guidelines, Nevirapine, RP-HPLC, Validation.

## INTRODUCTION

Nevirapine (NVP) is chemically identified as 2- cyclopropyl -7-methyl-2,4, 9,15-tetraazatricyclo [9.4.0.0] pentadeca- 1(11),3, 5, 7, 12, 14-hexaen-10-one. It is classified as a Non-Nucleoside Reverse Transcriptase Inhibitor and is effective against HIV-1. NVP works by binding directly to the Reverse Transcriptase enzyme, thereby inhibiting both RNA-dependent and DNA-dependent DNA polymerase activities through disruption of the enzyme's catalytic site. Importantly, Nevirapine does not inhibit HIV-2 Reverse Transcriptase

or human DNA polymerases [1]. The role of reverse transcriptase is to convert single-stranded viral RNA into DNA. NNRTIs, including Nevirapine, prevent HIV replication within cells by attaching to a site close to the active site of reverse transcriptase, thus inhibiting its polymerase function. As an anti-HIV medication, Nevirapine helps lower the viral load in the body, thereby mitigating damage to the immune system and reducing the risk of developing AIDS-related illnesses [2].

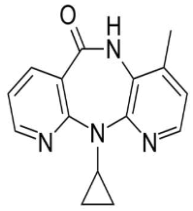



**Figure 1: Structure of Nevirapine**

A review of the literature indicates that there are limited analytical techniques reported for the quantification of NVP in bulk substances, pharmaceutical formulations, and biological fluids using UV spectrophotometry, High Performance Liquid Chromatography (HPLC), and Ion Pair HPLC. However, many of these existing methods present challenges, including extended run times, low sensitivity, high costs, and inadequate symmetry [3-5]. In light of these issues, straightforward, accurate, precise, and dependable HPLC method for measuring NVP in pharmaceutical dosage forms was chosen. The developed method was validated for specificity, linearity, precision, accuracy, robustness, limit of detection (LOD), and limit of quantification (LOQ) in accordance with ICH guidelines established in 1997 [6].

## MATERIALS AND METHODS

### Chemicals and Reagents

An analytically pure sample of NVP Active Pharmaceutical Ingredient (API) was a gift sample from Hetero labs Limited, India. All the chemicals used were of analytical grade. HPLC grade acetonitrile and triethylamine, methanol and water were used which was procure from Merck Private Ltd., Mumbai, India.. A high-performance liquid chromatographic method for estimating nevirapine in plasma and brain samples for pharmacokinetic bio-distribution investigations was developed and validated using HPLC with UV Detector - Agilent (1260). Nevirapine (sigma) served as the internal standard.

### Preparation of standard solution

50 mg of NVP (Nevirapine) and 50 mg of IS (Internal Standard), respectively, were dissolved in 100 ml of diluent (Methanol: Water, 50% v/v) to create stock solutions of NVP (0.5 mg/ml) and IS (0.5mg/ml) [7]. To create 25 ppm NVP solution and 25 ppm IS solution for experimental batches; 5 ml of stock solution was further diluted to 100 ml using diluent. A suitable quantity of the standard solution was spiked in drug-free plasma to create the working standards of NVP in concentrations ranging from 1 to 300 µg/ml [8-11].

**Sample Preparation in plasma**

The 10 ml of whole own blood was withdrawn with the help of experienced medical personnel and was added into EDTA tube. Blood was centrifuged at 2000rpm for 10 minutes and plasma was separated, approximately 4ml plasma was collected for the experimental uses [12-15].

After transferring the 250µl plasma sample to tubes, 50µL of internal standard, 50µL of nevirapine, and 1 ml of ethyl acetate were added. For one minute, the samples were vortexed. After that, the samples were centrifuged at 10,000 RPM (4°C) using a freeze centrifuge (BL-135 R) and the organic phase were removed using nitrogen purging [16]. 100µL of mobile phase was used to reconstitute the solid residue before it was subjected into the HPLC column [17].

**Analytical Method development**

Nevirapine (25 ppm) and internal standard (25 ppm) standard solutions were scanned in the 200–400 nm range to determine the HPLC detection wavelength [18]. To optimize the analytical method to provide good peaks with appropriate retention period, a number of experiments were conducted by adjusting the column, mobile phase, pH of the mobile phase, flow rate, etc. at λmax [19-22].

**Analytical method validation**

According to ICH requirements, the new analytical method was validated for a number of criteria, including linearity, precision, accuracy, specificity, and robustness. The data collected were then statistically analysed [23].

**Linearity and Range**

Nevirapine concentrations ranging from 1 to 300 µg/ml (1, 5, 10, 20, 25, 50, 100, 200, and 300 µg/ml) were shown to be linear. The internal standard concentration used was 25µg/ml. Every experiment was carried out three times. The area ratios (analyte/internal standard) vs. concentration curve were analysed using linear regression. Correlation coefficient estimates were used to confirm the linearity [24-26].

**Precision**

By assaying six samples at 100% test concentration and calculating the standard deviation (SD) and percentage relative standard deviation (RSD), the analytical method's precision (repeatability) was ascertained. By evaluating three samples at three separate times throughout the same day and on three successive days, respectively, and calculating SD & % RSD, the intra-day and inter-day precisions were also ascertained. The data was then statistically analysed using ANOVA [27-30].

**Accuracy**

Three concentration levels were used to evaluate accuracy. Three repetitions each at 50% and 150% concentrations were examined, while six replicates were examined at the 100% concentration level. The accuracy was assessed by calculating the analyte recovery percentage [31].

**Limit of Detection (LOD) and Limit of Quantification (LOQ)**

LOD: Analyte concentration signals for six replicates of 0.01 µg/ml were compared to a blank sample. The ratio of signal to noise was determined and compared to a suitable value of 2:1[32].

LOQ: Measured signals from samples with known low concentrations (six replicates of 0.05µg/ml) of analyte were compared with those of blank samples in order to determine the signal to noise ratio. This comparison was made with the generally accepted signal-to-noise ratio of 10:1 [33].

**Specificity**

The capacity to definitively evaluate the analyte in the presence of potentially predicted components is known as specificity. Analysing the analyte with a placebo present allowed for the assessment of specificity. Three replicate analyses served as the basis for the findings [34].

**Robustness**

The ability of the analytical process to withstand minor but intentional changes in its parameters such as flow rate 1.5ml/min (±0.2), wavelength 315 (±2), and pH of the mobile phase 3.5(±0.2) was used to gauge its resilience and gave a sense of how reliable it was under typical operating conditions. Robustness was assessed in terms of percentage RSD [35].

**Stability of analytical solution**

The analytical solution's stability was evaluated in plasma at 2, 4, 6, 12, 18, 24 hours, and 10 days later. The results were compared to chromatograms of a freshly made sample. Three replications of the experiments were conducted [36].

**Results**

**Spectrophotometric Determination of Nevirapine**

Standard solution of Nevirapine was scanned in the UV range (200-400) and from the overlain spectrum; 315nm was selected as λ (lambda) max using UV Spectrophotometer.

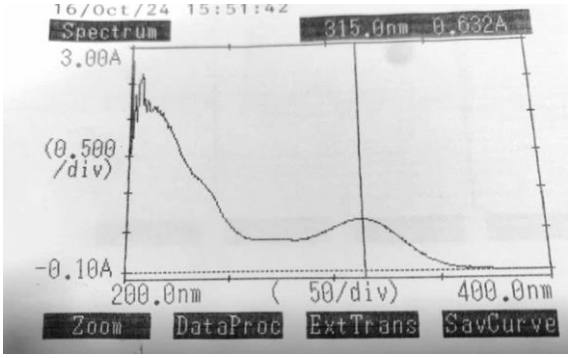



**Figure 2: UV Spectrum of Nevirapine**

## HPLC Method Development &Validation

To choose the chromatographic settings that produced good peak characteristics, a number of experiments were conducted using different columns, mobile phases.

**Table 1: Initial chromatographic conditions for trial batches**

| | |
|---|---|
| **Mobile Phase** | Mobile phase- A:0.1% Orthophosphoric acid in water<br>Mobile phase B: Acetonitrile Ratio (30:70) |
| **Column** | Zorbax ODS,150X4.6mm,5.0µm |
| **Run Time** | 10 minutes |
| **Injection volume** | 5 µl |
| **Sample cooler temperature** | 5 $^{0}$C |
| **Column Temperature** | 30 $^{0}$C |
| **Flow rate** | 1ml /min |
| **Wavelength Detection** | 315 nm |

## Selection and optimization of chromatographic conditions

Several trials were taken for NVP as blank, standard and test to evaluate chromatographic characteristics of NVP along with IS.

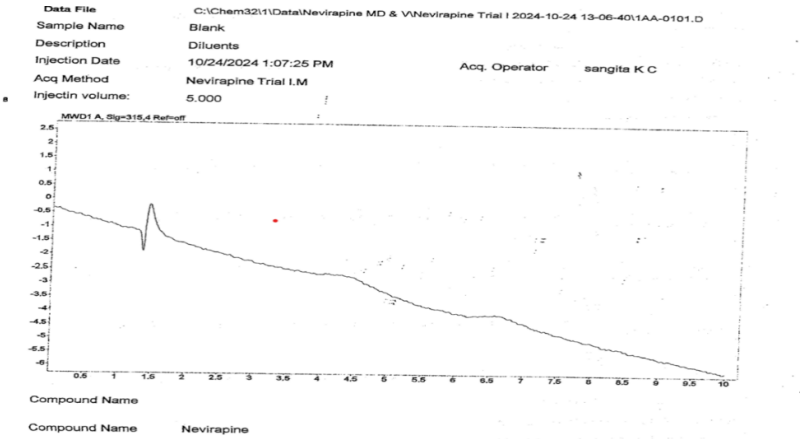
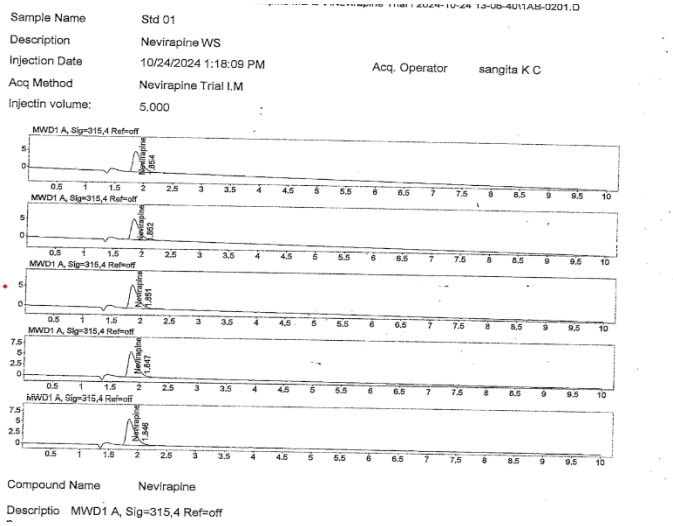



**Figure 3: Chromatogram of NVP blank sample**

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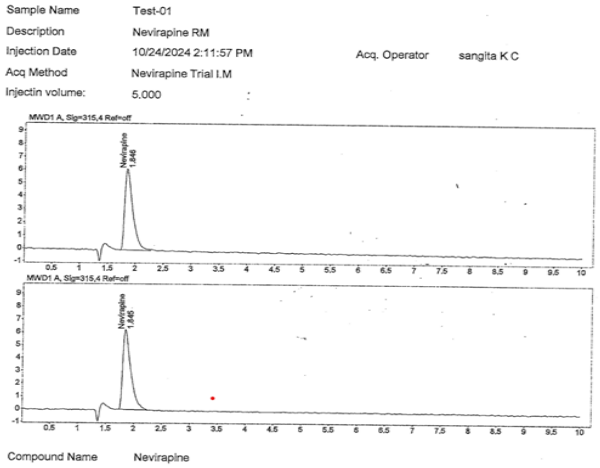
| Sample Name | RT [min] | Area | Peak Area (%) | Peak Height | Theoretical Plates | Tailing Factor | Resolution |
|---|---|---|---|---|---|---|---|
| Std 01 | 1.854 | 61.581 | 100.00 | 6.029 | 846.8134 | 1.86364 | |
| Std 02 | 1.852 | 61.013 | 100.00 | 6.019 | 830.9779 | 1.83929 | |
| Std 03 | 1.851 | 60.987 | 100.00 | 6.043 | 846.0435 | 1.89091 | |
| Std 04 | 1.847 | 61.347 | 100.00 | 5.990 | 794.4443 | 2.06604 | |
| Std 05 | 1.846 | 61.913 | 100.00 | 6.012 | 787.3910 | 2.03704 | |
| Average | 1.850 | 61.368 | | | | | |
| RSD | 0.191 | 0.638 | | | | | |
| Sum | | 306.8416 | | | | | |

**Figure 4: Chromatogram of NVP standard**

Trial 1- Trials were conducted using a 25 ppm solution of NVP. A Zorbax C18 column measuring 150 x 4.6 mm with a particle size of 5.0 µm was employed, along with the chromatographic conditions outlined in Table I. It was noted that the peak base was not satisfactory, indicating the necessity for additional trials to enhance the peak shape.




Compound Name Nevirapine

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| Sample Name | RT [min] | Area | Peak Area (%) | Peak Height | Theoretical Plates | Tailing Factor | Resolution |
|---|---|---|---|---|---|---|---|
| Test-01 | 1.846 | 62.108 | 100.00 | 6.191 | 844.8312 | 1.96296 | |
| Test-02 | 1.845 | 62.316 | 100.00 | 6.237 | 859.0386 | 2.09615 | |
| Average | 1.846 | 62.212 | | | | | |
| RSD | 0.033 | 0.237 | | | | | |
| Sum | | 124.4247 | | | | | |

**Figure 5: Chromatogram of NVP test**

Trial 2- Using the same 25 ppm NVP solution, experiments were conducted with a different column. A Zorbax Symmetry Shield C18 column measuring 150 x 4.6 mm and 5.0 µm was employed, along with the other chromatographic conditions outlined in Table 1. It was noted that the peak base quality was suboptimal, indicating the necessity for further trials to enhance the peak shape.

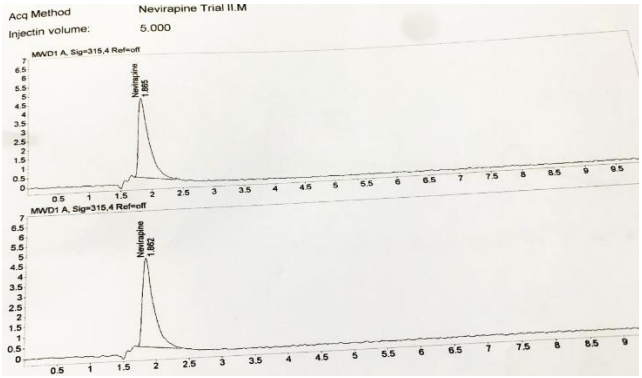


**Figure 6: Trial 2 for HPLC method development**

Trial 3- To enhance the peak shape, the mobile phase was modified from acidic to slightly basic to evaluate any improvements. Consequently, 10 mM ammonium acetate was utilized in place of 0.1% orthophosphoric acid as the mobile phase. The chromatogram produced using a 10 mM ammonium acetate buffer combined with acetonitrile (30:70) on a Zorbax symmetry shield C18 column (150 x 4.6 mm, 5.0 µm) indicated that the peak shape did not show any improvement, necessitating further trials with adjustments to the pH of the mobile phase.

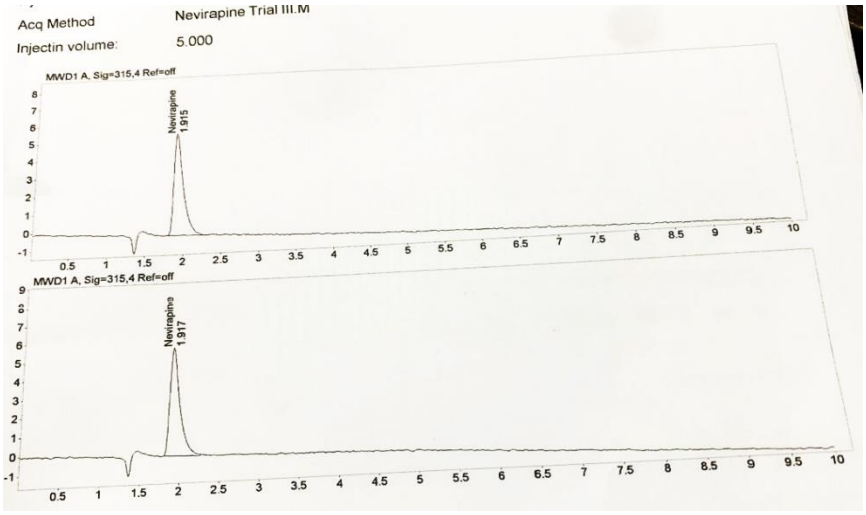


**Figure 7: Trial 3 for HPLC method development**

Trial 4: The experiment involved modifying the pH of the mobile phase to 5.5 while keeping the other chromatographic parameters consistent with those of trial 3. This adjustment resulted in peak splitting when using a mobile phase composed of ammonium acetate buffer at pH 5.5. Therefore, it was determined that additional trials are necessary.

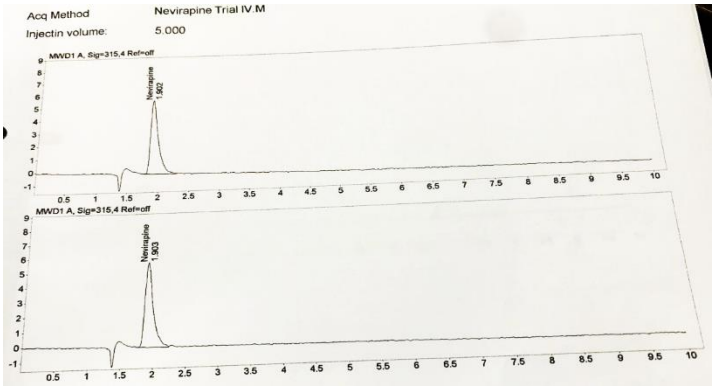


**Figure 8: Trial 4 for HPLC method development**

Trial 5: In this trial, the impact was evaluated by substituting the mobile phase buffer from acetate to a phosphate buffer with an acidic pH. The chromatogram produced using a 10 mM phosphate buffer at pH 2.5 combined with Acetonitrile in a 30:70 ratio revealed, as shown in Figure 8, that the nevirapine peak exhibited a well-defined and symmetrical shape. However, it was noted that the peak eluted close to the void volume. The next trial will involve adjusting the mobile phase pH from highly acidic to a less acidic level.

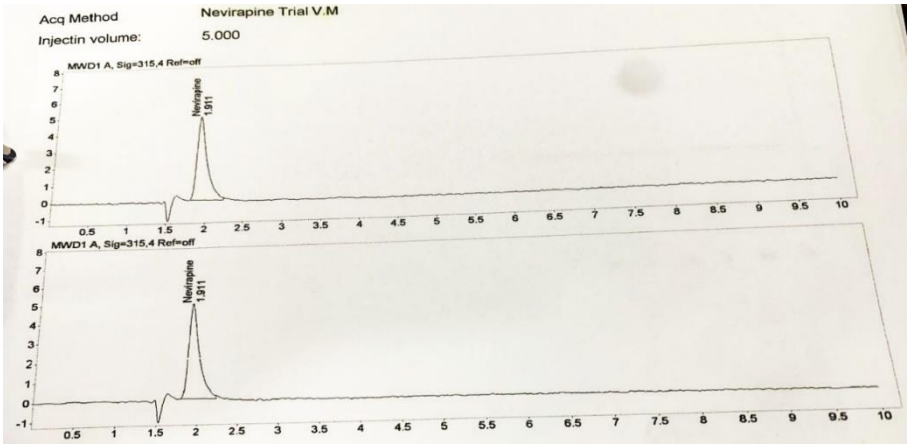


**Figure 9: Trial 5 for HPLC method development**

Trial 6: To enhance retention time, this experiment was conducted using a mobile phase with an elevated pH. Consequently, the mobile phase for this trial consisted of a phosphate buffer at pH 3.5 combined with acetonitrile, while all other parameters remained consistent with those of trial 5. The chromatographic peak for NVP observed in this trial exhibited favorable shape and symmetry, prompting the use of the same chromatographic conditions for subsequent analytical method development studies.

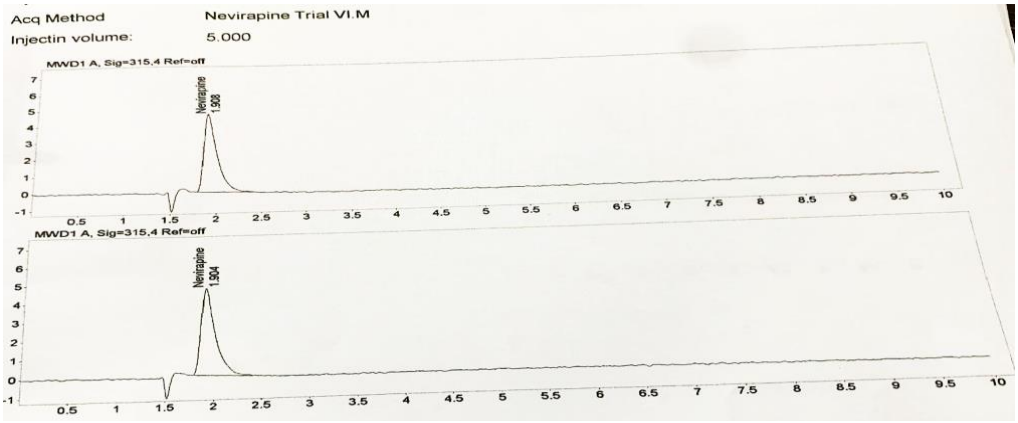


**Figure 10: Trial 6 for HPLC method development**

Trial 7: To assess the peak shape of the internal standard under the designated chromatographic conditions, a 50 ppm solution of the internal standard was created and injected. The chromatogram produced included a sample with 25 ppm of NVP and 25 ppm of the internal standard, formulated using a 50% v/v methanol: water mixture. In the chromatogram from trial 7, both the drug (NVP) and the internal standard displayed acceptable peak shapes; however, the resolution between the two peaks was insufficient. Consequently, further trials are required to enhance the separation of the peaks.

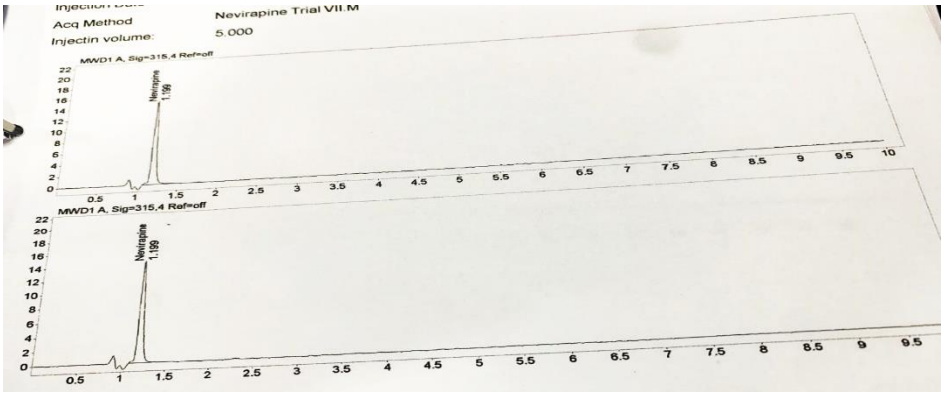


**Figure 11: Trial 7 for HPLC method development**

Trial 8: To enhance the resolution between the peaks of NVP and IS, a new HPLC column featuring a smaller sorbent particle size of 3.5 µm was utilized. The chromatogram produced from the mixed sample of NVP using a 100 x 4.6 mm, 3.5 µm column displayed well-defined peak shapes. However, the IS peak eluted close to the void volume, prompting further experiments with a gradient chromatographic program.

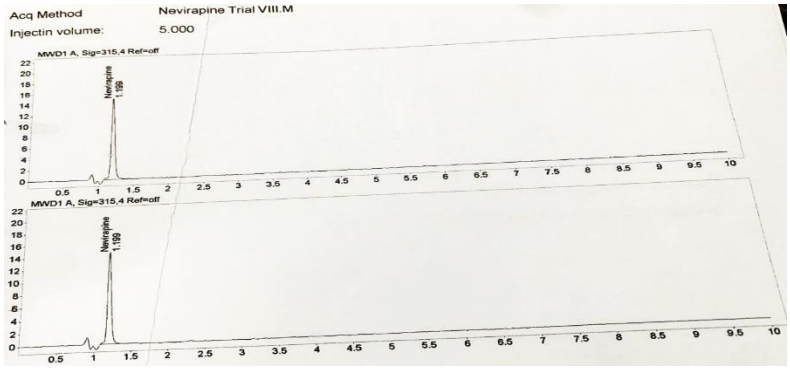


**Figure 12: Trial 8 for HPLC method development**

**Trial 9:** In this experiment, the gradient chromatographic program detailed in Table 2 was employed. The flow rate was adjusted from 1.0 to 1.5 ml/min, and a mixture of the standard NVP and internal standard (IS) was injected. The resulting chromatogram is presented in Figure 13.

**Table 2: Gradient Program used for HPLC method development**

| Time (Minutes) | Mobile phase-A (%v/v) (Phosphate buffer pH 3.5) | Mobile phase-B (%v/v) (Acetonitrile) |
|---|---|---|
| 0 | 95 | 5 |
| 2 | 95 | 5 |
| 10 | 20 | 80 |
| 12 | 20 | 80 |
| 12.2 | 95 | 5 |
| 16 | 95 | 5 |

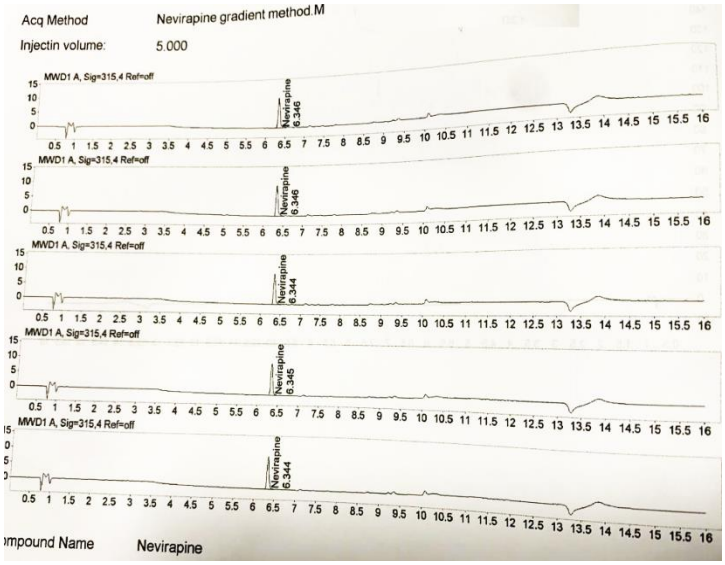


**Figure 13: Trial 9 for HPLC method development Chromatogram of IS**

It can be observed from Figure 13 that peak characteristic including its shape, symmetry, resolution, etc. were found to be good.

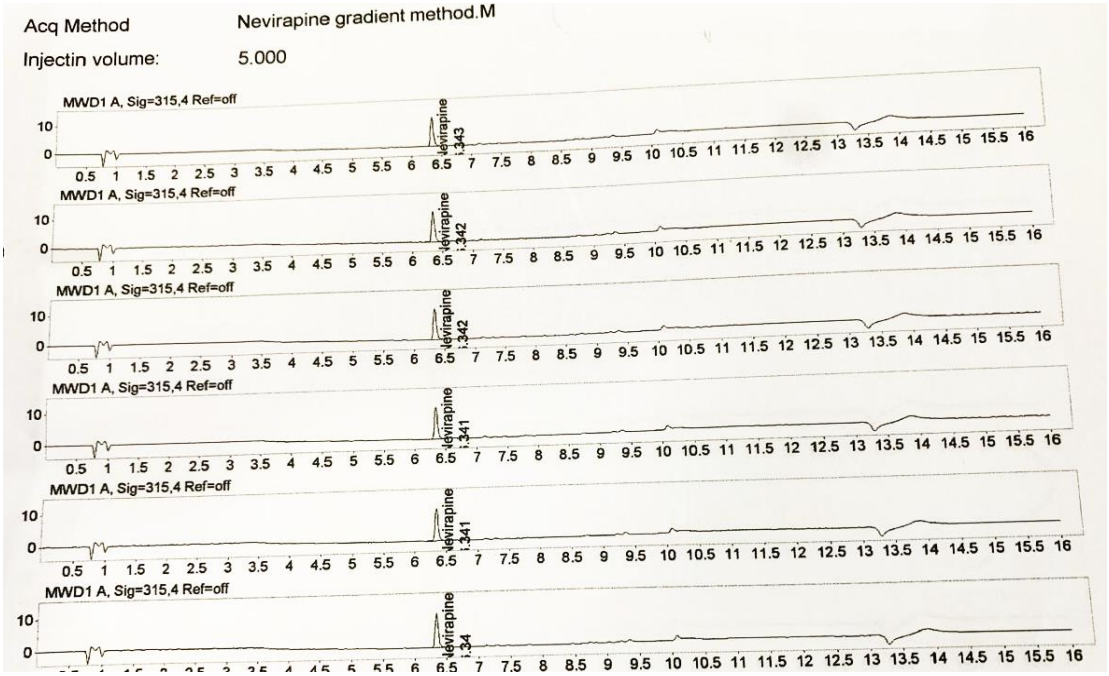


**Figure 14: Chromatogram of NVP in plasma samples**

From the Figure 14, it was concluded that no significant interference was observed with the presence of plasma. The conditions of chromatographic techniques are summarized in the table 2 that is used to estimate NVP in the plasma for development technique validation.
The chromatographic conditions summarized in Table 3 can be used for estimation of NVP from plasma after the validation of the developed method.

**Table 3: Optimized Chromatographic conditions for HPLC method**

| | |
|---|---|
| **Equipment** | **HPLC with UV Detector- (Agilent 1260)** |
| **Column** | Zorbax 100 x 4.6mm, 3.5µm |
| **Mobile phase** | Mobile phase - A: pH 3.5 Phosphate buffer, Mobile phase B: Acetonitrile |
| **Wavelength** | 315 nm |
| **Flow rate** | 1.5 mL/min |
| **Injection volume** | 5 µL |
| **Sample cooler** | 5°C |
| **Column temperature** | Ambient |
| **Diluent** | Water: Methanol (50%v/v) |

In contrast to retention periods stated in the literature, it was found that the time of retention was considerably shortened to 6.34 min using the optimised chromatographic settings displayed in Table 3. This can cut down on sample analysis expenses and run times.

Additionally, all of the reagents utilised are widely accessible. Although the mobile phase's phosphate buffer and acetonitrile composition was comparable to those of several other published techniques, the NVP retention time was found to be significantly less than the reported values (from 13.2 minutes to 6.34 minutes).

This might be because a different dimension of column100 x 4.6 mm, 3.5 µm was used rather than one that was 150 × 4.6 mm, 5 µm.

With a flow rate of 1.5 ml/min, the proper retention time for NVP and IS was noted. A higher flow rate would have also helped to reduce the NVP retention period. The time needed for analysis may be shortened by the shorter retention period. The gradient elution approach was found to boost efficiency, increase detection, improve resolution, and shorten analysis times.

**Analytical Method Validation**

In order to validate the established analytical technique in accordance with ICH requirements, a number of criteria were assessed, including stability, robustness/ruggedness, specificity, (limit of detection, limit of quantification), sensitivity, linearity and range, accuracy, and precision.

**Linearity and Range**

The calibration plot, which includes a math equation and a number that shows how well the points fit the line, is also important. In this study, the method worked well for measuring concentrations between 1 and 300 micrograms per milliliter, with a very high accuracy score of 0.999. The researchers recorded their findings in tables, showing how they prepared the samples and the results they got for different concentrations.

**Table 4: Sample Preparation for linearity Studies**

| Sample | Stock solution | | Dilution | | Conc. (µg/ml) |
|---|---|---|---|---|---|
| | Weight (mg) | Total Volume (ml) | Stock Solution Volume(ml) | Final Volume (ml) | |
| IS | 50 | 100 | 5.0 | 100 | 25 |
| NVP | 50 | 100 | 0.2 | 100 | 1 |
| NVP | 50 | 100 | 1.0 | 100 | 5 |
| NVP | 50 | 100 | 2.0 | 100 | 10 |
| NVP | 50 | 100 | 4.0 | 100 | 20 |
| NVP | 50 | 100 | 5.0 | 100 | 25 |
| NVP | 50 | 100 | 10.0 | 100 | 50 |
| NVP | 50 | 100 | 20.0 | 100 | 100 |
| NVP | 50 | 100 | 40.0 | 100 | 200 |
| NVP | 50 | 100 | 60.0 | 100 | 300 |

**Table 5: Concentration, Area and Area ratio for linearity study**

| Sr. No. | Concentration (µg/ml) | | Area | | Area Ratio (Analyte/IS) |
|---|---|---|---|---|---|
| | Nevirapine (Analyte) | Internal standard (IS) | Nevirapine (Analyte) | Internal standard (IS) | |
| 1 | 1.003 | 25.00 | 2.045 | 36.584 | 0.055899 |
| 2 | 5.017 | 25.00 | 8.597 | 36.584 | 0.234993 |
| 3 | 10.034 | 25.00 | 14.248 | 36.584 | 0.38946 |
| 4 | 20.068 | 25.00 | 28.881 | 36.584 | 0.789443 |
| 5 | 25.085 | 25.00 | 36.300 | 36.584 | 0.992237 |
| 6 | 50.170 | 25.00 | 70.199 | 36.584 | 1.918844 |
| 7 | 100.340 | 25.00 | 136.230 | 36.584 | 3.723759 |
| 8 | 200.680 | 25.00 | 279.868 | 36.584 | 7.650011 |
| 9 | 301.020 | 25.00 | 413.293 | 36.584 | 11.2971 |

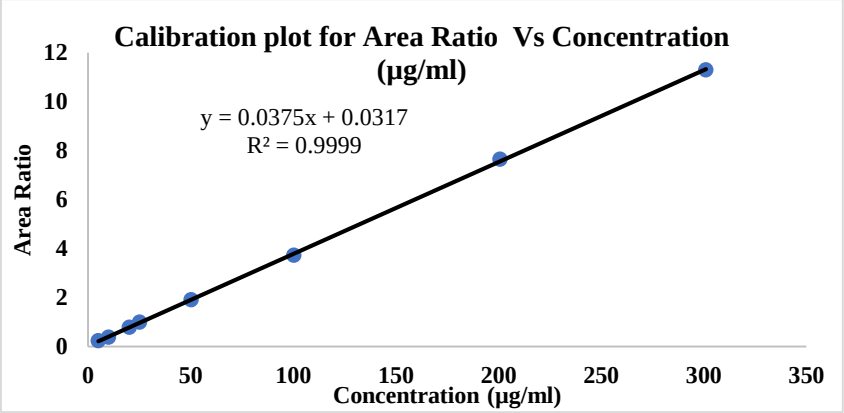


**Figure 14: Calibration plot for linearity**

**Precision**

The precision (repeatability) of the analytical method was evaluated by testing six samples at a concentration of 100%. The chromatograms produced during the precision assessment indicated a % RSD of 1.523, which is below the maximum threshold of 2.0%, as presented in Table 6.

**Table 6: Area ratios and RSD calculation for Precision study (Repeatability):**

| Sample | Stock solution | | Dilution | | Conc. (µg/ml) |
|---|---|---|---|---|---|
| | Wt taken (mg) | Total Volume (ml) | Volume of stock solution taken (ml) | Final Volume (ml) | |
| Nevirapine | 50.08 | 100 | 5.0 | 100 | 25.04 |
| Standard | 50.00 | 100 | 5.0 | 100 | 25 |

| S. No. | Concentration (µg/ml) | Average Area of Triplicate Reading | Area Ratio (Analyte/IS) |
|---|---|---|---|

| | Nevirapine | Internal standard (IS) | Nevirapine | Internal standard (IS) | |
|---|---|---|---|---|---|
| 1 | 25.04 | 25.00 | 35.951 | 36.584 | 0.982697 |
| 2 | 25.04 | 25.00 | 36.821 | 36.584 | 1.006478 |
| 3 | 25.04 | 25.00 | 37.120 | 36.584 | 1.014651 |
| 4 | 25.04 | 25.00 | 36.373 | 36.584 | 0.994232 |
| 5 | 25.04 | 25.00 | 35.762 | 36.584 | 0.977531 |
| 6 | 25.04 | 25.00 | 36.942 | 36.584 | 1.009786 |
| **Mean** | | | 36.49483 | 36.584 | 0.997563 |
| **S.D.** | | | 0.555845 | 0 | 0.015194 |
| **% RSD** | | | 1.523079 | 0 | 1.52309 |

**Table 7: Intraday Precision studies**

| Drug | Conc. (µg/ml) | Average Area of Triplicate Reading | | | Average | SD | %RSD |
|---|---|---|---|---|---|---|---|
| | | at 10 am | at 1 pm | at 4 pm | | | |
| NVP | 25.085 | 35.941 | 36.124 | 36.248 | 36.104 | 0.154 | 0.430 |
| | 25.085 | 36.147 | 36.071 | 36.285 | 36.168 | 0.108 | 0.301 |
| | 25.085 | 36.007 | 35.864 | 36.179 | 36.017 | 0.158 | 0.440 |
| IS | 25 | 36.166 | 36.210 | 37.100 | 36.492 | 0.527 | 1.457 |
| | 25 | 36.044 | 35.831 | 35.866 | 35.914 | 0.114 | 0.319 |
| | 25 | 35.932 | 35.844 | 35.852 | 35.876 | 0.049 | 0.136 |

**Table 8: Inter-day Precision studies**

| Drug | Conc. (µg/ml) | Average Area of Triplicate Reading | | | Average | SD | %RSD |
|---|---|---|---|---|---|---|---|
| | | On Day 1 | On Day 2 | On Day 3 | | | |
| NVP | 25 | 35.981 | 36.122 | 36.200 | 36.101 | 0.111 | 0.308 |
| | 25 | 36.127 | 36.146 | 36.101 | 36.125 | 0.022 | 0.062 |
| | 25 | 36.008 | 36.166 | 36.165 | 36.113 | 0.091 | 0.253 |
| IS | 25 | 35.761 | 36.135 | 36.200 | 36.032 | 0.237 | 0.662 |
| | 25 | 36.111 | 36.135 | 36.095 | 36.114 | 0.020 | 0.056 |
| | 25 | 36.005 | 36.146 | 36.155 | 36.102 | 0.084 | 0.234 |

**Table 9: Summary of ANOVA analysis for precision study (intra-day)**

| Groups | Count | Sum (Analyte/IS) | Average | Variance | |
|---|---|---|---|---|---|
| I (at10:00 am) | 3 | 1.385817087 | 0.461939022 | 4.6643 | |
| II (at01:00 pm) | 3 | 1.385046485 | 0.461682254 | 1.75399 | |
| III (at 04:00 pm) | 3 | 1.375885564 | 0.458628453 | 3.54619 | |
| **Variation Source** | **Squares Sum** | **Degree of Freedom** | **Mean of Squares** | **F- value** | **P-value** | **F critical** |

| | | | | | | |
|---|---|---|---|---|---|---|
| Between Groups | 2.0351 | 2 | 1.01755 | 3.06352224 | 0.121112463 | 5.143253 |
| Within Groups | 1.9929 | 6 | 3.3215 | | | |
| Total | 4.028 | 8 | | | | |

**Table 10: Summary of ANOVA analysis for precision study (inter-day)**

| **Groups** | **Count** | **Sum (Analyte/IS)** | **Average** | **Variance** |
|---|---|---|---|---|
| I (Day1) | 3 | 1.381147746 | 0.460382567 | 6.20602 |
| II(Day2) | 3 | 1.384022614 | 0.461340876 | 2.03014 |
| III(Day3) | 3 | 1.386333717 | 0.462111237 | 1.19046 |

| **Variation Source** | **Squares Sum** | **Degree of Freedom** | **Mean of Squares** | **F- value** | **P-value** | **F critical** |
|---|---|---|---|---|---|---|
| Between Groups | 4.5001 | 2 | 2.25006 | 0.81839427 | 0.48497734 | 5.143253 |
| Within Groups | 1.6496 | 6 | 2.74936 | | | |
| Total | 2.0996 | 8 | | | | |

By evaluating three samples at three different times of the same day and on three consecutive days, respectively, the intra-day and inter-day precisions. The RSD was less than 1.5%, as indicated in Tables 7 and 8, respectively, which is within the normal acceptable range (NMT 2.0%). As indicated in Tables 9 and 10, statistical analysis using ANOVA revealed no significant differences between the results obtained for intra-day and inter-day data ($p>0.05$)

**Accuracy**

The observed percent recovery is found to be between 98% and 105%. According to the literature review, the percent recovery obtained through various reported methods ranges from 97% to 105%, with some methods exhibiting even broader ranges.

**Table 11: Data of recovery study for Accuracy Parameter**

| **Sample details** | | **Area of NVP** | **Area of IS** | **Ratio** | **Amount found (µg/ml)** | **Amount added (µg/ml)** | **% Recovery** |
|---|---|---|---|---|---|---|---|
| **Recovery at 50% level** | **Set-1** | 18.405 | 36.239 | 0.508 | 50.78783 | 50.00 | 101.5757 |
| | **Set-2** | 18.983 | 36.239 | 0.524 | 52.38279 | 50.00 | 104.7656 |
| | **Set-3** | 18.361 | 36.239 | 0.507 | 50.66641 | 50.00 | 101.3328 |
| **Recovery at 100% level** | **Set-1** | 35.951 | 36.239 | 0.992 | 99.20528 | 100.00 | 99.20528 |
| | **Set-2** | 36.820 | 36.239 | 1.016 | 101.6032 | 100.00 | 101.6032 |
| | **Set-3** | 37.120 | 36.239 | 1.024 | 102.4311 | 100.00 | 102.4311 |
| | **Set-4** | 36.373 | 36.239 | 1.004 | 100.3698 | 100.00 | 100.3698 |
| | **Set-5** | 35.762 | 36.239 | 0.987 | 98.68374 | 100.00 | 98.68374 |
| | **Set-6** | 36.942 | 36.239 | 1.019 | 101.9399 | 100.00 | 101.9399 |
| **Recovery at 150%** | **Set-1** | 53.908 | 36.239 | 1.488 | 148.7569 | 150.00 | 99.17124 |
| | **Set-2** | 54.300 | 36.239 | 1.498 | 149.8386 | 150.00 | 99.89238 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **level** | **Set-3** | 53.725 | 36.239 | 1.483 | 148.2519 | 150.00 | 98.83459 |

**Limit of Detection (LOD)and Limit of Quantification (LOQ)**

A common signal-to-noise ratio for LOQ is 10:1, and a ratio of 3 to 2:1 is generally regarded as adequate for determining the detection limit at the LOD, the signal at the analyses' known low concentration (six replicates of 0.01 µg/ml each) was compared to the blank sample.

The average signal-to-noise ratio was found to be 4:1, satisfying the acceptance criteria and indicating that detection remained reliable at concentrations as low as 0.01 µg/ml.

With known low analyte concentrations (six replicates of 0.05 µg/ml solution), an average signal-to-noise ratio of greater than 10:1 was obtained, suggesting that even 0.05 µg/ml of nevirapine may be reliably measured.

Nevirapine's therapeutic range has been found to be 1–4 µg/ml. Therefore, even the lowest drug concentration within the effective therapeutic range may be estimated using the presented technique.

Combination of high-performance liquid chromatography with dispersive liquid- liquid extraction yielded findings that were comparable to the stated LOQ and LOD of 0.02 and 0.05 µg/ml, 0.01 and 0.1 µg/ml, respectively.

**Specificity**

Less than 20% of the analyte's peak regions at LOQ should be occupied by molecules that co-elute with one of the analytes. With internal standard are less than 5% should be the peak area for chemicals that co-elute with it. By observing the chromatograph of the placebo and comparing it with the chromatograms of NVP and IS, the specificity of the devised approach was verified.

**Robustness**

The robustness of an analytical procedure demonstrates its ability to survive slight but intentional changes in method parameters, as well as its reliability under usual operating conditions. By varying the mobile phase's pH (±0.2), wavelength (±2 nm), and flow rate (±0.2 ml/min), the method's resilience examined. As indicated in Table 12, the developed method's robustness was assessed in terms of percentage RSD.

**Table 12 (a): Robustness data for change in flow rate**

| Drug | Param-eters | Flow rate (ml/min) | Injection 1 | Injection 2 | Injection 3 | Injection 4 | Injection 5 | Injection 6 | Mean | % RSD |
|---|---|---|---|---|---|---|---|---|---|---|
| NVP | RT | 1.3 | 6.660 | 6.661 | 6.660 | 6.661 | 6.660 | 6.660 | 6.660 | 0.01 |
| | | 1.5 | 6.343 | 6.342 | 6.342 | 6.341 | 6.34 | 6.341 | 6.34 | 0.02 |
| | | 1.7 | 6.089 | 6.09 | 6.089 | 6.090 | 6.090 | 6.089 | 6.090 | 0.01 |
| | Area | 1.3 | 42.872 | 42.687 | 42.159 | 41.838 | 42.300 | 41.950 | 41.840 | 0.96 |
| | | 1.5 | 35.951 | 36.820 | 37.120 | 36.373 | 36.410 | 35.762 | 35.760 | 1.40 |
| | | 1.7 | 31.934 | 31.818 | 31.955 | 31.824 | 31.940 | 32.173 | 31.820 | 0.40 |
| | Tailing factor | 1.3 | 1.1333 | 1.12214 | 1.117 | 1.111 | 1.160 | 1.118 | 1.110 | 1.58 |
| | | 1.5 | 1.216 | 1.225 | 1.207 | 1.245 | 1.230 | 1.241 | 1.210 | 1.19 |
| | | 1.7 | 0.841 | 0.860 | 0.846 | 0.861 | 0.845 | 0.853 | 0.840 | 0.99 |
| | Theoretical plates | 1.3 | 69137 | 68497 | 69690 | 69127 | 69254 | 69823 | 68497 | 0.68 |
| | | 1.5 | 79616 | 77800 | 77270 | 77255 | 77856 | 77340 | 77255 | 1.16 |
| | | 1.7 | 69414 | 59016 | 69690 | 69127 | 69414 | 69823 | 59016 | 6.32 |
| IS | RT | 1.3 | 6.662 | 6.661 | 6.664 | 6.662 | 6.660 | 6.661 | 6.66 | 0.02 |
| | | 1.5 | 6.346 | 6.346 | 6.344 | 6.345 | 6.350 | 6.344 | 6.340 | 0.04 |
| | | 1.7 | 6.086 | 6.087 | 6.087 | 6.088 | 6.090 | 6.09 | 6.090 | 0.03 |
| | Area | 1.3 | 43.866 | 44.184 | 43.138 | 44.549 | 43.960 | 44.051 | 43.140 | 1.06 |
| | | 1.5 | 36.239 | 36.867 | 36.865 | 36.523 | 36.620 | 36.584 | 36.240 | 0.64 |
| | | 1.7 | 32.773 | 32.605 | 32.945 | 32.326 | 32.700 | 32.829 | 32.330 | 0.66 |
| | Tailing factor | 1.3 | 1.122 | 1.120 | 1.146 | 1.112 | 1.140 | 1.154 | 1.110 | 1.45 |
| | | 1.5 | 1.217 | 1.257 | 1.27 | 1.23134 | 1.240 | 1.20896 | 1.210 | 1.88 |
| | | 1.7 | 0.858 | 0.847 | 0.87368 | 0.85673 | 0.86 | 0.84212 | 0.840 | 1.28 |
| | Theoretical plates | 1.3 | 69897 | 69827 | 70566 | 69712 | 69895 | 69474 | 69474 | 0.52 |
| | | 1.5 | 82034 | 79797 | 80633 | 81490 | 80925 | 80672 | 79797 | 0.95 |
| | | 1.7 | 31748 | 31466 | 31171 | 32261 | 31458 | 30645 | 30645 | 1.72 |
| Resolution | | 1.3 | 5.6 | 5.5 | 5.6 | 5.4 | 5.5 | 5.7 | 5.40 | 1.89 |
| | | 1.5 | 5.3 | 5.1 | 5.2 | 5.1 | 5.1 | 5.3 | 5.10 | 1.90 |
| | | 1.7 | 5.6 | 5.6 | 5.5 | 5.6 | 5.7 | 5.8 | 5.50 | 1.83 |

**Table 12 (b): Robustness data for change in wavelength**

| Drug | Param-eters | Lambda Max (nm) | Injection 1 | Injection 2 | Injection 3 | Injection 4 | Injection 5 | Injection 6 | Mean | % RSD |
|---|---|---|---|---|---|---|---|---|---|---|
| | RT | 313 | 6.335 | 6.336 | 6.335 | 6.336 | 6.336 | 6.336 | 6.335 | 0.008 |
| | | 315 | 6.335 | 6.336 | 6.335 | 6.336 | 6.336 | 6.336 | 6.335 | 0.008 |
| | | 317 | 6.335 | 6.336 | 6.335 | 6.336 | 6.336 | 6.336 | 6.335 | 0.008 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **NVP** | **Area** | 313 | 40.527 | 40.720 | 40.212 | 40.201 | 39.151 | 40.162 | 39.151 | 1.350 |
| | | 315 | 36.961 | 36.648 | 36.250 | 35.431 | 35.369 | 36.132 | 35.369 | 1.768 |
| | | 317 | 33.438 | 33.973 | 33.309 | 33.431 | 32.429 | 33.316 | 32.429 | 1.499 |
| | **Tailing factor** | 313 | 1.206 | 1.160 | 1.206 | 1.164 | 1.180 | 1.183 | 1.160 | 1.672 |
| | | 315 | 1.209 | 1.173 | 1.200 | 1.164 | 1.159 | 1.181 | 1.159 | 1.686 |
| | | 317 | 1.189 | 1.169 | 1.200 | 1.162 | 1.157 | 1.175 | 1.157 | 1.398 |
| | **Theoretical plates** | 313 | 73855.0 | 74383.000 | 74138.00 | 74896.00 | 75559.00 | 74566.20 | 73855.00 | 0.808 |
| | | 315 | 73744.0 | 74568.000 | 74385.00 | 74636.00 | 75654.00 | 74597.40 | 73744.00 | 0.825 |
| | | 317 | 74131.0 | 74009.000 | 74271.00 | 74636.00 | 75436.00 | 74496.60 | 74009.00 | 0.691 |
| **IS** | **RT** | 313 | 6.337 | 6.336 | 6.336 | 6.336 | 6.337 | 6.336 | 6.336 | 0.008 |
| | | 315 | 6.337 | 6.336 | 6.336 | 6.336 | 6.337 | 6.336 | 6.336 | 0.008 |
| | | 317 | 6.337 | 6.336 | 6.336 | 6.336 | 6.337 | 6.336 | 6.336 | 0.008 |
| | **Area** | 313 | 41.089 | 41.168 | 41.244 | 41.373 | 41.302 | 41.235 | 41.089 | 0.241 |
| | | 315 | 37.149 | 37.328 | 37.181 | 37.123 | 37.249 | 37.206 | 37.123 | 0.199 |
| | | 317 | 34.578 | 34.206 | 33.688 | 34.322 | 34.211 | 34.201 | 33.688 | 0.847 |
| | **Tailing factor** | 313 | 1.183 | 1.145 | 1.147 | 1.129 | 1.139 | 1.149 | 1.129 | 1.594 |
| | | 315 | 1.076 | 1.109 | 1.127 | 1.121 | 1.126 | 1.112 | 1.076 | 1.710 |
| | | 317 | 1.082 | 1.112 | 1.127 | 1.119 | 1.134 | 1.115 | 1.082 | 1.614 |
| | **Theoretical plates** | 313 | 76888.0 | 75686.000 | 75608.00 | 74517.00 | 74340.00 | 75407.80 | 74340.00 | 1.222 |
| | | 315 | 76843.0 | 75196.000 | 75614.00 | 74561.00 | 74474.00 | 75337.60 | 74474.00 | 1.144 |
| | | 317 | 76245.0 | 75274.000 | 75942.00 | 74548.00 | 74249.00 | 75251.60 | 74249.00 | 1.023 |
| **Resolution** | | 313 | 5.500 | 5.500 | 5.600 | 5.400 | 5.500 | 5.500 | 5.400 | 1.150 |
| | | 315 | 5.300 | 5.100 | 5.100 | 5.100 | 5.100 | 5.140 | 5.100 | 1.556 |
| | | 317 | 5.600 | 5.700 | 5.600 | 5.700 | 5.800 | 5.680 | 5.600 | 1.317 |

**Table 12 (c): Robustness data for change in pH of mobile phase**

| **Drug** | **Param-eters** | **pH** | **Injection 1** | **Injection 2** | **Injection 3** | **Injection 4** | **Injection 5** | **Injection 6** | **Mean** | **% RSD** |
|---|---|---|---|---|---|---|---|---|---|---|
| **NVP** | **RT** | 3.3 | 6.335 | 6.335 | 6.335 | 6.335 | 6.335 | 6.335 | 6.335 | 0.000 |
| | | 3.5 | 6.343 | 6.342 | 6.342 | 6.341 | 6.341 | 6.342 | 6.342 | 0.013 |
| | | 3.7 | 6.334 | 6.334 | 6.334 | 6.334 | 6.334 | 6.334 | 6.334 | 0.000 |
| | **Area** | 3.3 | 36.769 | 36.999 | 36.256 | 36.347 | 36.717 | 36.618 | 36.618 | 0.844 |
| | | 3.5 | 35.951 | 36.820 | 37.120 | 36.373 | 35.762 | 36.405 | 36.405 | 1.568 |
| | | 3.7 | 37.336 | 37.511 | 36.552 | 36.394 | 35.780 | 36.715 | 36.715 | 1.937 |
| | **Tailing factor** | 3.3 | 1.111 | 1.102 | 1.114 | 1.106 | 1.109 | 1.108 | 1.108 | 0.432 |
| | | 3.5 | 1.216 | 1.225 | 1.207 | 1.245 | 1.241 | 1.227 | 1.227 | 1.317 |
| | | 3.7 | 1.084 | 1.125 | 1.080 | 1.115 | 1.116 | 1.104 | 1.104 | 1.857 |
| | **Theoretical plates** | 3.3 | 72921.0 | 72066.000 | 73333.00 | 72506.00 | 72350.00 | 72635.20 | 72635.20 | 0.685 |
| | | 3.5 | 79616.0 | 77800.000 | 77270.00 | 77255.00 | 77340.00 | 77856.20 | 77856.20 | 1.296 |
| | | 3.7 | 66612.0 | 66908.000 | 67104.00 | 67094.00 | 67337.00 | 67011.00 | 67011.00 | 0.403 |
| **IS** | | 3.3 | 6.335 | 6.335 | 6.334 | 6.336 | 6.335 | 6.335 | 6.335 | 0.011 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **RT** | 3.5 | 6.346 | 6.346 | 6.344 | 6.345 | 6.344 | 6.345 | 6.345 | 0.016 |
| | | 3.7 | 6.334 | 6.335 | 6.334 | 6.333 | 6.333 | 6.334 | 6.334 | 0.013 |
| | **Area** | 3.3 | 37.608 | 37.743 | 37.719 | 37.598 | 37.173 | 37.568 | 37.568 | 0.613 |
| | | 3.5 | 36.239 | 36.867 | 36.865 | 36.523 | 36.424 | 36.584 | 36.584 | 0.758 |
| | | 3.7 | 37.341 | 37.325 | 37.586 | 37.417 | 37.474 | 37.429 | 37.429 | 0.285 |
| | **Tailing factor** | 3.3 | 1.098 | 1.091 | 1.110 | 1.102 | 1.137 | 1.108 | 1.108 | 1.607 |
| | | 3.5 | 1.217 | 1.257 | 1.240 | 1.231 | 1.208 | 1.231 | 1.231 | 1.564 |
| | | 3.7 | 1.140 | 1.142 | 1.096 | 1.142 | 1.117 | 1.127 | 1.127 | 1.817 |
| | **Theoretical plates** | 3.3 | 73837.0 | 73041.000 | 73394.00 | 73013.00 | 73054.00 | 73267.80 | 0.484 | 0.484 |
| | | 3.5 | 82034.0 | 79797.000 | 80633.00 | 80490.00 | 80672.00 | 80725.20 | 1.007 | 1.007 |
| | | 3.7 | 71112.0 | 70391.000 | 69468.00 | 68195.00 | 68064.00 | 69446.00 | 1.924 | 1.924 |
| **Resolution** | | 3.3 | 5.300 | 5.100 | 5.200 | 5.100 | 5.100 | 5.160 | 5.160 | 1.733 |
| | | 3.5 | 5.300 | 5.100 | 5.200 | 5.200 | 5.100 | 5.180 | 5.180 | 1.615 |
| | | 3.7 | 5.300 | 5.100 | 5.200 | 5.200 | 5.100 | 5.180 | 5.180 | 1.615 |

**Table 12 (d): Summary of robustness data for change in different parameters**

| **Parameters** | | **% RSD** | |
|---|---|---|---|
| | | **NVP** | **IS** |
| Change in flow rate (±0.2 ml/min) | Retention time | 0.013 | 0.03 |
| | Area | 0.900 | 0.786 |
| | Tailing factor | 1.25 | 1.53 |
| | Resolution | 1.87 | 1.87 |
| Change in wavelength (±2 nm) | Retention time | 0.008 | 0.008 |
| | Area | 1.539 | 0.429 |
| | Tailing factor | 1.585 | 1.639 |
| | Resolution | 1.341 | 1.340 |
| Change in pH of mobile phase (±0.2) | Retention time | 0.013 | 0.013 |
| | Area | 1.449 | 0.552 |
| | Tailing factor | 1.202 | 1.662 |
| | Resolution | 1.654 | 1.650 |

Changing the optimal experimental parameters (flow rate, wavelength, and mobile phase pH) did not result in any appreciable changes to the chromatographic parameters. The created method's robustness was assessed using the percentage RSD, as indicated in Table 12(a), 12(b), and 12(c), which are compiled in Table 12(d). In every investigation, the RSD was less than two. Several published studies have not examined robustness with regard to changes in pH, wavelength, or flow rate. RSD values as high as 7.5 have been documented.

**Stability of analytical solution**

The analytical solution's stability was evaluated at 2, 4, 6, 12, 18, 24 hours, and 10 days, and the results were compared to the chromatograms of a newly made sample. Based on the

anticipated length of analysis, no significant change ($p > 0.05$) was seen, suggesting that the analytical solution was stable for ten days. Similar studies have indicated that the samples remain stable for up to 6 hours at room temperature. Nevirapine has been shown to remain stable in human plasma for up to 30 days when kept at -20°C.

**Table 13: Summary of validation Parameters**

| S. No. | Parameters | Results |
|---|---|---|
| 1. | Linearity range | 1-300 µg/ml |
| 2. | Retention time | 6.45 + 0.21 min |
| 3. | LOQ | 0.05 µg/ml |
| 4. | LOD | 0.01 µg/ml |
| 5. | Correlation coefficient | 0.999 |

Therefore, it can be said that a straightforward and sensitive reversed-phase HPLC gradient method has been created and verified for the UV detector-based quantification of NVP in plasma. Table 13 provides an overview of the validation parameters. With retention times of 6.60 minutes and 6.343 minutes, respectively, a good resolution between NVP and IS as internal standards was achieved. Around the NVP and IS retention times, no interference peaks were seen. $R^2 = 0.999$ indicated that the technique was linear in the analytical range of 1–300 µg/ml. The outcomes demonstrated that the medication was stable in plasma and that the procedure was precise and repeatable.

In order to assess the pharmacokinetic properties of NVP, the proposed chromatographic method can be utilised to estimate NVP in plasma with good resolution.

## RESULTS AND DISCUSSION

In this research, we refined the necessary conditions for the development and validation of a highly precise, sensitive, rapid, and accurate HPLC method for the quantification of NVP. To optimize retention time and peak asymmetry, we employed a C18 stationary phase column (100mm x 4.6mm, 3.5 µm particle size) along with a mobile phase composed of pH 3.5 Phosphate buffer and Acetonitrile in a ratio of 30:70 (v/v) and the flow rate was maintained at 1.5 mL/min. UV spectral analysis revealed that NVP has a maximum absorption at 315 nm. Minor modifications in the mobile phase ratio of up to ±5% led to variations in peak asymmetry, plate count, and retention time, all of which remained within acceptable limits, thereby confirming the robustness of the method. All system suitability parameters were found to comply with standard criteria. Chromatographic comparisons between the standard and sample showed no interference, demonstrating the method's specificity. The precision and accuracy of the method were assessed through % RSD and % recovery of the active pharmaceutical ingredient (API). The low % RSD and high % recovery values indicate the method's exceptional precision and accuracy. Precision studies yielded % RSD values of 0.430, 0.301, and 0.440 for intra-day precision, and 0.308, 0.062, and 0.253 for inter-day precision, all of which are within acceptable limits. The method's accuracy was validated,

with overall % RSD for recovery at 50%, 100%, and 150% levels remaining within acceptable thresholds. Validation in accordance with ICH guidelines confirmed that the developed method exhibits high sensitivity.

## CONCLUSION

A High-Performance Liquid Chromatography (HPLC) method has been established for the quantification of NVP in both bulk and dosage forms. This assay demonstrates a linear response over a broad concentration range and employs a mobile phase that is straightforward to prepare, with an economical and readily accessible diluent. The HPLC method developed presents numerous benefits, including rapid analysis, excellent peak symmetry, outstanding linearity, high sensitivity, simplicity, precision, accuracy, and robustness. These characteristics contribute to the method's high quality, making it suitable for analyzing NVP samples in a Quality Control laboratory.

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