High Resolution RP-HPLC Method for the Determination of Nevirapine and Associated Impurities

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ABSTRACT

Objective of the present research work is to develop a sensitive, selective and accurate new RP-HPLC method with UV detection and determination for estimation of Nevirapine (NVP) and its impurities in bulk drug. The separation and quantification was achieved with Kromosil C18 isocratic column, (150 mm × 4.6 mm i.d., particle size 3.5 µm, maintained at ambient temperature), HPLC system (Peak LC P7000), a mixture of 20% acetonitrile, 80% buffer (sodium per chlorate) (v/v), at pH of 4.8 and the flow rate was set at 1.0 ml/min. and UV detection at 220 nm. The retention time for NVP, Impurity-A and Impurity-B were found to be 5.5, 7.8, 3.4 min respectively. The method was validated for Linearity, Accuracy, and Precision. The Limit of detection of NVP, Impurity-A and Impurity-B were found to be 0.03, 0.03, 0.03(µg/ml) respectively.

Keywords: Nevirapine, ICH guidelines, RP-HPLC, RS Method, Validation.

INTRODUCTION

NVP is a non-nucleoside reverse transcriptase inhibitor used to treat HIV-1 and AIDS. Possible side effects of NVP are Diarrhea, headache, mild nausea or stomach pain, tiredness, vomiting. Some severe allergic reactions like rash, tightness in the chest, swelling of the mouth, face, lips, or tongue, itching, hives, difficulty breathing.

MATERIALS AND METHODS

Pure forms (Above 99%) of NVP and Impurity-A, Impurity-B were obtained as gift samples from Hetero Labs, Hyderabad, India. HPLC grade solvents (Methanol, Acetonitrile, water) were procured from Merck, Mumbai, India. The mobile phase and all the solutions were filtered through a 0.45mm membranes prior (Merckmillipore) to use. Per chloric acid was purchased from S.D. Fine Chem Ltd., Mumbai, India.

Preparation of Mobile phase

Acetonitrile and Sodium per chlorate (pH: 4.8) were taken in20:80 ratio and mixed well. The pH of the solution was adjusted to 4.8 with Perchloric acid. The prepared mobile phase was filtered through 0.45mm filter membrane.
Preparation of stock solutions

1. NVP stock solution: 10 mg of NVP drug was dissolved in 100 ml Acetonitrile to obtain 100 µg/ml.
2. Impurity-A stock solution: 20 mg of standard Impurity-A was dissolved in 100 ml Acetonitrile to obtain 200 µg/ml.
3. Impurity-B stock solution: 20 mg of standard Impurity-B was dissolved in 100 ml Acetonitrile to obtain 200 µg/ml.

Preparation of standard solutions

0.5 ml of standard stock and 0.5 ml of impurities stock solutions are taken in to 100 ml volumetric flask and make up to 100 ml with Acetonitrile to obtain 0.5 ppm of NVP and 1 ppm of impurity-A and impurity-B. The standard concentration equal to unknown impurity spec (0.1%) and impurity-A and B concentration equal to 0.2% (as per USP limit).

Preparation of sample solution

50 mg of API sample taken in to 100 ml of Acetonitrile to obtain 500 µg/ml concentration sample solution.

Apparatus and chromatographic condition

The method was developed and validated with HPLC system (Peak LC P7000) with isocratic pump, manual rhodyne injector with 20 µL volume loop and UV-VIS detector (UV7000) and PEAK Chromatographic version 1.06. The API and impurity were scanned with UV-Visible spectrophotometer Tech comp UV2301 with Hitachi software. Kromasil C18 column (100 mm x 4.6 mm x 3.5 µ) RP-18 HPLC column (150 x 4.6 mm, 3.5 micron) was used for separation. HPLC detector wavelength was fixed at 220 nm. Analysis was performed at ambient temperature.

RESULTS AND DISCUSSION

The aim of this work is to develop a RPHPLC method to quantify NVP and Impurity-A, Impurity-B in Bulk drug. Previously few methods[5-19] are available for analysis of NVP in formulations and bulk drug. Ch Venkata Reddiah et al[20] reported one HPLC method for analysis of NVP and its impurities. While developing method at initial stage of the method development trials done with NH₄H₂PO₄, Sodium per chlorate buffer solution at different pH and acetonitrile as solvent and C18 column but the separation of NVP and Impurity-A is not good. Finally the best separation with good elution was achieved with Sodium per chlorate and acetonitrile at pH 4.8. Diluent and standard solution represented in figure-2 and 3. NVP and Impurity-A, Impurity-B are well separated and the peak shape, tailing factor (less than 2.0) and resolution also within the limit.

System suitability is an important test of method development of analytical procedures. System suitability test parameters are established for the developed method. Freshly prepared standard solution in to the system for three replicate injections (at 10 µg/ml, 20 µg/ml, 30 µg/ml) and calculated the percentage relative standard deviation (RSD) for area and retention time and the results found to be satisfactory. Three replicate standard solution results tabulated the results in table.1.

Fig.1: Chemical structure of NVP and its Impurities
Method validation

Once the HPLC method development was over, validated the developed method as per ICH and FDA \[^{[1-5]}\] guidelines with parameters like, linearity, precision, accuracy and range, ruggedness, robustness etc.

Precision of the developed method was evaluated by carrying out six different sample preparations for all individual and combination products. Percentage relative standard deviation (% RSD) was found to be less than 1% for within a day and day to day variations, which proves that that method is precise. Results were shown in Table-2.

For linearity test the standard solution was taken as 100% concentration and linearity range was fixed 25%, 50%, 75%, 100%, 125%, 150%, 200% Linearity solutions are prepared from stock solutions and standard solution by serial dilution. The linearity results were given in Table.3. The linearity graph was shown in Graph.1, Graph.2 and Graph.3.

![Representative chromatogram of blank](image1)

Fig. 2: Representative chromatogram of blank

![Representative Standard chromatogram with NVP, Impurity-A, Impurity-B](image2)

Fig. 3: Representative Standard chromatogram with NVP, Impurity-A, Impurity-B
The ruggedness of the method was determined by carrying out the experiment on other HPLC by different Analysts using different columns of similar types. The percentage RSD of six different preparations assay values with two different instruments, analysts and columns were given in table 4. Robustness of the method was determined by making small changes in the chromatographic conditions and found to be unaffected by small changes like pH changes ± 0.1, flow rate ± 1%, wavelength ±2 nm, temperature ± 2°C, and ± 2% change in organic solvent in the mobile phase. The Robustness results are shown in the Table 5. The LOQ and LOD concentrations of developed method are given in Table 4. Analysis of NVP, Impurity-A, Impurity-B in analyzed in NVP Bulk drug by using developed HPLC method. The chromatogram was given shown in Figure 4.

### Forced degradation studies

50 mg of NVP was diluted in 100 ml of 0.1 N HCl and heated up to 400°C. 50 mg of NVP was diluted in 100 ml of 0.1 NaOH and heated up to 400°C. 50 mg of NVP was taken in 50 ml of H2O2 and make up to 100 ml with diluents. The three prepared solutions are injected and calculated percentage of

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**Table 1: System suitability test results of NVP, Impurity-A, Impurity-B**

| No of Injections | Concentration (µg/ml) | System suitability of NVP | R.T | Resolution | T. Plates | Tailing Factor | Peak Area |
|------------------|-----------------------|---------------------------|-----|------------|-----------|----------------|-----------|
| 1                | 10                    | 5.509                     | 16.24 | 26581     | 1.22      | 69812          |
| 2                | 10                    | 5.505                     | 16.31 | 27005     | 1.18      | 67781          |
| 3                | 10                    | 5.490                     | 15.83 | 27055     | 1.18      | 67781          |
| 1                | 20                    | 5.492                     | 15.78 | 26585     | 1.22      | 91533          |
| 2                | 20                    | 5.497                     | 16.01 | 27279     | 1.20      | 76300          |
| 3                | 20                    | 5.517                     | 16.27 | 26586     | 1.20      | 76609          |
| 1                | 30                    | 5.525                     | 16.08 | 26723     | 1.18      | 98420          |
| 2                | 30                    | 5.515                     | 15.89 | 26609     | 1.12      | 96883          |
| 3                | 30                    | 5.503                     | 16.00 | 27055     | 1.15      | 95925          |

**System suitability of Impurity-A**

| No of Injections | Concentration (µg/ml) | System suitability of Impurity-A | R.T | Resolution | T. Plates | Tailing Factor | Peak Area |
|------------------|-----------------------|----------------------------------|-----|------------|-----------|----------------|-----------|
| 1                | 10                    | 7.847                            | 17.75 | 59757     | 1.73      | 67747          |
| 2                | 10                    | 7.852                            | 17.55 | 54625     | 1.64      | 67779          |
| 3                | 10                    | 7.852                            | 17.72 | 55822     | 1.59      | 70602          |
| 1                | 20                    | 7.825                            | 17.63 | 57987     | 1.60      | 84307          |
| 2                | 20                    | 7.857                            | 17.96 | 58670     | 1.61      | 81767          |
| 3                | 20                    | 7.839                            | 17.52 | 58453     | 1.62      | 84501          |
| 1                | 30                    | 7.857                            | 17.70 | 60075     | 1.56      | 101203         |
| 2                | 30                    | 7.850                            | 17.26 | 53905     | 1.67      | 103745         |
| 3                | 30                    | 7.850                            | 17.78 | 58217     | 1.60      | 103959         |

**System suitability of Impurity-B**

| No of Injections | Concentration (µg/ml) | System suitability of Impurity-B | R.T | Resolution | T. Plates | Tailing Factor | Peak Area |
|------------------|-----------------------|----------------------------------|-----|------------|-----------|----------------|-----------|
| 1                | 10                    | 3.305                            | 0.0  | 9423       | 1.35      | 83795          |
| 2                | 10                    | 3.293                            | 0.0  | 9106       | 1.40      | 80442          |
| 3                | 10                    | 3.307                            | 0.0  | 8639       | 1.37      | 90287          |
| 1                | 20                    | 3.302                            | 0.0  | 8533       | 1.37      | 83795          |
| 2                | 20                    | 3.303                            | 0.0  | 8789       | 1.39      | 80442          |
| 3                | 20                    | 3.274                            | 0.0  | 8689       | 1.35      | 90287          |
| 1                | 30                    | 3.302                            | 0.0  | 8943       | 1.39      | 114618         |
| 2                | 30                    | 3.303                            | 0.0  | 8897       | 1.38      | 103693         |
| 3                | 30                    | 3.274                            | 0.0  | 8962       | 1.33      | 108958         |
Table 2: Precision results of NVP, Impurity-A, Impurity-B

| Injections | NVP  | Impurity-A | Impurity-B |
|------------|------|------------|------------|
| 1          | 13996| 17124      | 16511      |
| 2          | 14167| 17306      | 16553      |
| 3          | 13845| 17098      | 16498      |
| 4          | 14128| 17185      | 16435      |
| 5          | 13855| 17254      | 16584      |
| 6          | 13871| 17068      | 16472      |
| % RSD      | 0.936 | 0.495      | 0.298      |

degradation. Degradation peaks of NVP at different conditions are shown in Figure.7 to Figure.9.

Table 3: Linearity results of NVP, Impurity-A, Impurity-B

| Percentage of Concentration | NVP  | Impurity-A | Impurity-B |
|-----------------------------|------|------------|------------|
| 25 %                        | 9587 | 11334      | 9668       |
| 50%                         | 10851| 13559      | 12568      |
| 75%                         | 12574| 15451      | 14689      |
| 100%                        | 13841| 17239      | 16626      |
| 125%                        | 15345| 18885      | 19224      |
| 150%                        | 16629| 20975      | 21277      |
| 200%                        | 18316| 23140      | 23974      |
| y=mx+c                      | y=11750x+8039 | y=7800x+9458 | y=9394x+7498 |
| Co-relation Coefficient     | 0.998 | r2=0.998   | 0.998      |

CONCLUSION

The method was developed at 220 nm UV-Wave length. The mobile phase was fixed as Acetonitrile and Buffer on the basis of drug solubility. The ratio of organic solvent and buffer was confirmed on trial and error basis. NH$_4$H$_2$PO$_4$ and Perchloric acid are used as buffer. Method was finally developed with Perchloric acid at pH 4.8. The method...
Graph 1: linearity graph of NVP

Graph 2: linearity graph of Impurity-A

Graph 3: linearity graph of Impurity-B
Fig. 5: Representative Sample chromatogram of NVP, Impurity-A, Impurity-B

Fig. 6: Representative Degradation overlaid chromatogram of NVP
Table 4: Ruggedness results

| Test                          | NVP     | Imp-A   | Imp-B   |
|-------------------------------|---------|---------|---------|
| Standard solution Area Area  | 13996   | 17124   | 16511   |
| Mean of Ruggedness Six injections peak area | 13856   | 16971   | 16418   |
| Percentage of Change in peak area | 1.000% | 0.893%  | 0.563%  |

Table 5: Robustness results

| Change in Parameter          | NVP     | Percentage of Change in peak area | Imp-A     | Percentage of Change in peak area | Imp-B     | Percentage of Change in peak area |
|------------------------------|---------|-----------------------------------|-----------|-----------------------------------|-----------|-----------------------------------|
| Standard solution Area Area  | 13996   | 0.000                             | 17124     | 0.000                             | 16511     | 0.000                             |
| pH at 4.6                    | 13745   | 1.793                             | 16954     | 0.992                             | 16452     | 0.537                             |
| pH at 4.9                    | 13895   | 0.721                             | 16977     | 0.858                             | 16398     | 0.684                             |
| flow rate at 1.1 ml/min      | 13915   | 0.578                             | 17049     | 0.437                             | 16477     | 0.205                             |
| flow rate at 0.9 ml/min      | 13887   | 0.778                             | 17138     | 0.081                             | 16402     | 0.660                             |
| wavelength 222 nm            | 13752   | 1.743                             | 17089     | 0.204                             | 16582     | 0.430                             |
| wavelength 218 nm            | 13790   | 1.471                             | 17055     | 0.402                             | 16601     | 0.545                             |

Fig. 7: Representative Acid degradation chromatogram of NVP
was validated according to ICH guidelines. There was no interference in blank injection. The precision RSD of NVP, Impurity-A, Impurity-B are below 1.0% The linearity range was fixed between 25% to 200%. The correlation coefficient is 0.998. The ruggedness and robustness tests are passed. The L.O.Q ranges of NVP, Imp-A and Imp-B are 0.1 µg/ml and LOD ranges are 0.03 µg/ml. The NVP degradation study

**Table 6: The LOQ and LOD concentrations of NVP, Impurity-A, Impurity-B**

| S.No | Parameter  | NVP (µg/ml) | Imp-A (µg/ml) | Imp-B (µg/ml) |
|------|------------|-------------|---------------|---------------|
| 1    | LOQ (µg/ml)| 0.1         | 0.1           | 0.1           |
| 2    | LOD(µg/ml) | 0.03        | 0.03          | 0.03          |
Table 7: Degradation study results of NVP, Impurity-A, Impurity-B

| S. No. | Degrading Agent | Drug | Initial concentration of drug before degradation (µg/ml) | Final concentration of drug after degradation (µg/ml) | % of Degradation |
|--------|-----------------|------|----------------------------------------------------------|------------------------------------------------------|------------------|
| 1      | No degrading agent | NVP  | 1195565                                                  | 1195565                                              | 0.00             |
| 2      | 0.1 N HCL        | NVP  | 1195565                                                  | 1087081                                              | 9.07             |
| 3      | 0.1 M NaOH       | NVP  | 1195565                                                  | 1140772                                              | 4.58             |
| 4      | 50% H₂O₂        | NVP  | 1195565                                                  | 893682                                               | 25.25            |

was carried out at three conditions. NVP 25.25% degraded in peroxide condition. 9.07% degraded in Acidic condition. 4.58% degraded in Basic condition. The developed RPHPLC method was validated with precision, linearity, accuracy and proved to be sensitive and effective for the determination of NVP and its relative substances (Impurity-A, Impurity-B) during stability testing of the bulk drug. We can apply this method for routine quality control analysis in bulk drug manufacturing industries.

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