Determination of nevirapine in human plasma by HPLC

Pranjali S. Ranaware¹, Anita M. Ingle¹, Abhijeet Ladke¹, Ashwini R. Madgulkar² and Mrinalini C. Damle²

¹Department of Quality Assurance, AISSMS College of Pharmacy, Pune, India
²Department of Pharmaceutical Chemistry, AISSMS College of Pharmacy, Pune, India

ABSTRACT

Nevirapine is a non–nucleoside Reverse Transcriptase inhibitor, antiviral agent. A simple, selective, and sensitive high performance liquid chromatography method for the determination of Nevirapine in human plasma was developed. Dicloxacillin was used as an internal standard. The method utilizes simple protein precipitation as the sample preparation technique using acetonitrile as precipitating agent. The samples were analyzed on HiQ sil C18 HS column with the mobile phase containing mixture of methanol: phosphate buffer 0.01M (pH 3.0, adjusted with glacial acetic acid) (70: 30% v/v). The calibration curve was linear ($r^2>0.99$) through the range of 0.2-1.2 µg/ml. The lower limit of quantification was found to be 0.2 µg/ml. % R.S.D. was less than 4% for intra- and inter-day precision. The mean recovery was found to be 96.50% for Nevirapine. Nevirapine in plasma samples was stable when tested for parameters as per EMEA and US CDER guidelines. The method showed acceptable values for accuracy, precision, recovery, sensitivity and stability. The method is well suited for routine analysis of Nevirapine in human plasma and can further be extended for pharmacokinetic studies.

Keywords: HPLC, human plasma, Nevirapine, protein precipitation.

INTRODUCTION

Nevirapine is 11-Cyclopropyl–5, 11–dihydro–4–methyl–6H-dipyrido[3,2–b:2’,3’-e]-[1,4]diazepin–6–one is a non-nucleoside reverse transcriptase inhibitor (NNRTI) used to treat HIV-1 infection and AIDS. A single dose of Nevirapine given to both mother and child reduced the rate of HIV transmission by almost 50%. Mechanism of action is that, NNRTIs exhibit a classical noncompetitive inhibition pattern with the enzyme. Nevirapine is readily absorbed after oral administration with a peak plasma concentration at 4 hr. Cmax for Nevirapine is 1-2 µg/ml. The concentration of the drug in the CNS is 45% of that in plasma. It crosses the placenta and has been detected in breast milk. Nevirapine undergoes extensive metabolism in the liver mainly by the cytochrome P450 isoenzymes of the CYP3A family. It is excreted via urine as the glucuronide conjugates of the hydroxylated metabolites. The drug is widely distributed in body tissues and the CNS. [1, 2]

Several methods are reported to determine the Nevirapine in biological fluids by using LC-ESI-MS/MS [3], two-dimensional LC–MS/MS [4], tandem mass spectrometry method [5], ion-pair reversed-phase high-performance liquid chromatography with ultraviolet detection [6], HPLC with UV detection [7-10]. Few methods have also been reported to determine the levels of Nevirapine in biological fluids in combination with lopinavir, zidovudine, amivudine [11, 12].

LC-ESI-MS/MS, tandem mass spectrometry methods are developed for the determination of Nevirapine in human plasma. However, the methods are expensive and therefore may not be employed for routine analysis. There are many HPLC UV methods available for the determination of Nevirapine in human plasma but methods developed but suffer from drawbacks viz.
i) Complex mobile phase
ii) Tedious, complicated extraction procedure which includes evaporation to dryness under helium at 30 °C
iii) Many additional washing steps are required so more time is required for plasma sample preparation.

The present method describes a simple, selective, and sensitive HPLC method with UV detection with a calibration range of 0.2-1.2 µg/ml for Nevirapine in human plasma. The method utilizes simple, rapid protein precipitation with acetonitrile as the sample preparation technique. Dicloxacillin was used as an internal standard. The structures for Nevirapine and IS are described in Figure 1. The mobile phase consists of Methanol: Phosphate buffer (0.01M) pH 3 (70:30 v/v) which is easy to prepare. The method has been validated as per the Guidelines for EMEA and US CDER [13, 14].

EXPERIMENTAL SECTION

Instruments:
Chromatographic separation was performed on a Jasco chromatographic system equipped with a Jasco HPLC pump Model PU2080 plus, Jasco UV-2075 plus detector, Rheodyne injector with 50 µl loop volume and Elga water system for HPLC grade water.

Chemicals and Reagent:
Nevirapine working standard was kindly supplied by Emcure Pharmaceuticals Ltd, Bhosari, Pune and Dicloxacillin (IS) by Maxim Pharmaceuticals, Pune. The drugs were used as such without further purification. AR grade methanol, dihydrogen orthophosphate, acetonitrile, glacial acetic acid were purchased from S. D. fine-chemical Laboratories, Mumbai, India.

General procedure:
The chromatographic parameters were given below
Chromatographic conditions-

Mobile Phase- Methanol: Phosphate buffer 0.01M (pH-3.0 adjusted with glacial acetic acid) (70:30 v/v)
Flow rate -1.0 mL min⁻¹
Injection volume- 50 µl
Detection wavelength -227 nm
Column- HiQ sil C18 HS column
Internal Standard- Dicloxacillin

Sample preparation:
Stock solutions for Nevirapine and Internal standard (IS) were prepared by separately dissolving in methanol, to obtain a concentration of 1 mg/ml. Working solution for Nevirapine was prepared by diluting suitably with methanol to get the concentration of 0.2-1.2 µg/ml. The stock solutions were stored at 4° C. Working solution for IS was prepared by diluting suitably stock solution of IS with methanol to get the concentration of 50 µg/ml.

Q.C. (Quality Control) samples for Nevirapine were prepared at concentration levels so as to get 0.4, 0.6, 0.8 µg/ml. Spiked plasma was prepared by taking 4.5ml plasma, 0.25ml solution of Nevirapine (4, 6, 8 µg/ml) and 0.25 ml stock solution of IS (50 µg/ml) were added. The contents of the tubes were vortexed for 1 min. 1ml of this spiked plasma
was taken, 1ml of Acetonitrile as precipitating agent were added, vortexed for 2min. It was centrifuged for 10 minutes at 2500 rpm. After centrifugation, 50 µl aliquots of each concentration were injected into the HPLC system. The calibration curve for Nevirapine was obtained using six calibration standard levels (0.2, 0.4, 0.6, 0.8, 1.0, 1.2 µg/ml). Linear regression analysis was done, considering the ratio of the peak area of analyte to internal standard versus concentration applied. A correlation coefficient of more than 0.99 was obtained for calibration curve.

VALIDATION
The method was validated as per EMEA and US CDER guidelines.

Selectivity:
Selectivity is the ability of an analytical method to differentiate and quantify the analytes in the presence of other components in the sample. The selectivity of the method was evaluated by analyzing pooled plasma samples obtained from different sources spiked at LLOQ (Lower Limit of Quantification – 0.2 µg/ml in plasma).

Calibration/standard curve:
Linearity was tested for the range of concentrations 0.2-1.2 µg/ml. Each sample in five replicates was analyzed and peak areas were recorded. The response factor for each concentration was calculated by taking ratio of peak area of Nevirapine and IS. The response factors were then plotted against the corresponding concentrations to obtain the calibration graphs.

Accuracy, precision and lower limit of quantification:
The accuracy and precision of the method were evaluated using the Q.C. samples. Intra-day accuracy and precision was measured by consecutively analyzing Q.C. samples in one single day. The procedure was repeated for three different days to test the inter-day accuracy and precision. Accuracy was calculated as percentage accuracy, whereas precision was measured in terms of relative standard precision (R.S.D.) of each calculated concentration. Lower limit of quantification (LLOQ) was found to be 0.2 µg/ml, since the response obtained was five times the response compared to blank.

Recovery:
Recovery for Nevirapine was evaluated at three concentration levels corresponding to three routine Q.C. samples (0.4, 0.6, 0.8 µg/ml) analyzed in triplicate. Recovery was determined by comparing the ratio of the peak area of Nevirapine obtained after the application of the processed plasma calibration samples with those achieved by working standard solution in the methanol.

Stability:
As per EMEA and US CDER guidelines, stability was checked under different conditions viz.
1. Freeze-thaw stability
2. Short term stability
3. Long term stability
4. Stock solution stability
5. Post preparative stability

Freeze-thaw stability of Nevirapine was determined by assaying low and high Q.C. samples (0.4, 0.8 µg/ml) in triplicate over three freeze-thaw cycles. First freeze-thaw cycle consisted of 24 hrs freezing at -5°C followed by a complete thaw at a room temperature. The next two freeze-thaw cycles were of 12 hrs each frozen state at -5°C followed by a complete thaw at a room temperature. Short term stability consisted of two Q.C. samples stored for 4 hrs at room temperature and long term stability involved storage of two Q.C. samples for 14 days at 4°C. For stock solution stability, the stock solutions of the drug and IS were stored for period of 5 days in refrigerator at 4°C and then for 6 hrs at room temperature. Post preparative stability, where stability of the spiked samples for 0.6µg/ml of Nevirapine and 5µg/ml of IS were determined after the storage for 5 hrs at room temperature. All these Q.C. samples were then evaluated in triplicate and the results were compared with the freshly prepared samples of same concentrations.

RESULTS

Chromatographic characteristics:
Retention times for Nevirapine and IS were 5.1 and 6.5min, respectively. Representative chromatograms of blank human plasma, human plasma spiked with Nevirapine (0.8µg/ml) and spiked with Nevirapine (0.8µg/ml) with IS (5µg/ml) are shown in Figure 2, 3 and 4 respectively.
Figure 2: Typical chromatogram of blank human plasma

Figure 3: Typical chromatogram of blank human plasma spiked with Nevirapine (0.8µg/ml)
Selectivity:
The selectivity of the method was evaluated by analyzing pooled plasma samples obtained from different sources spiked at LLOQ (0.2 µg/ml) in which no interference by endogenous components was noted. % RSD (Relative standard deviation) for 6 replicates spiked at LLOQ was found to be 0.79%.

Calibration/standard curve:
The data for linearity studies was found to be best fitted by linear equation $y = mx + c$ in the range of concentration 0.2-1.2 µg/ml. With correlation coefficient 0.9988, a mean slope of 1.3136, mean y-intercept of 0.1787.

Accuracy, precision and lower limit of quantification:
The method showed good accuracy and precision in plasma samples. Table 1 shows the results for intra- and inter-day precision and accuracy for Nevirapine in plasma samples. Intra- and inter-day (%R.S.D.) precisions were $3.08 \pm 0.32$ and $2.78 \pm 0.40$ respectively. Intra- and inter-day accuracies were $98.79 \pm 0.088$ and $98.42 \pm 0.92$ respectively. LLOQ was found to be 0.2 µg/ml.

Table 1: Intra-day, Inter-day precision and Accuracy of in human plasma Q.C samples

<table>
<thead>
<tr>
<th>Theoretical (µg/ml)</th>
<th>Observed (mean µg/ml ± SD)</th>
<th>Precision (%R.S.D.)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>0.3967 ± 0.012</td>
<td>3.18</td>
<td>99.35</td>
</tr>
<tr>
<td>0.6</td>
<td>0.596 ± 0.019</td>
<td>3.34</td>
<td>99.56</td>
</tr>
<tr>
<td>0.8</td>
<td>0.7983 ± 0.021</td>
<td>2.71</td>
<td>97.45</td>
</tr>
<tr>
<td>Average</td>
<td>3.08 ± 0.32</td>
<td></td>
<td>98.79 ± 0.088</td>
</tr>
<tr>
<td>Inter-day</td>
<td>0.4</td>
<td>0.3916 ± 0.011</td>
<td>2.80</td>
</tr>
<tr>
<td>0.6</td>
<td>0.5873 ± 0.0186</td>
<td>3.18</td>
<td>97.88</td>
</tr>
<tr>
<td>0.8</td>
<td>0.7960 ± 0.0188</td>
<td>2.37</td>
<td>99.5</td>
</tr>
<tr>
<td>Average</td>
<td>2.78 ± 0.40</td>
<td>98.42 ± 0.92</td>
<td></td>
</tr>
</tbody>
</table>

% R.S.D. = SD/mean x 100, accuracy = observed/theoretical x 100
Recovery:
Table 2 shows the results of the recovery tests for the three Q.C. levels tested (0.4, 0.6, and 0.8 µg/ml). The extraction recovery in plasma samples ranged from 94.70 to 97.47 % for Nevirapine at three concentration levels. The mean recovery for Nevirapine was found to be 96.50 %

<table>
<thead>
<tr>
<th>QC Levels(µg/ml)</th>
<th>% R.S.D.</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>1.82</td>
<td>94.70</td>
</tr>
<tr>
<td>0.6</td>
<td>1.73</td>
<td>97.47</td>
</tr>
<tr>
<td>0.8</td>
<td>1.63</td>
<td>97.33</td>
</tr>
<tr>
<td>Average</td>
<td>1.72</td>
<td>96.50%</td>
</tr>
</tbody>
</table>

% R.S.D. = SD/mean x 100

Stability:
Plasma Q.C. at two concentrations (0.4 and 0.8 µg/ml) was used for freeze-thaw. Short term, long term stability studies and stock solution stability were performed at three concentrations (0.4, 0.6, 0.8µg/ml). Post preparative stability was performed for the drug (0.6 µg/ml) and IS (25 µg/ml). It was performed to evaluate the influence of storage conditions from the sample collection to analysis. Table 3 represents the results of stability studies. Results indicated that Nevirapine is stable in human plasma for the given stability conditions. The deviation of the mean test responses to the freshly prepared solutions was less than 5% at any of the stability conditions.

<table>
<thead>
<tr>
<th>Stability</th>
<th>Conc. (µg/ml)</th>
<th>Mean Stability (%)</th>
<th>% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze thaw stability (three cycles)</td>
<td>0.4</td>
<td>98.23</td>
<td>4.36</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>99.68</td>
<td>2.14</td>
</tr>
<tr>
<td>Short term stability (for 4h at RT)</td>
<td>0.4</td>
<td>98.49</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>98.45</td>
<td>1.68</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>98.72</td>
<td>0.95</td>
</tr>
<tr>
<td>Long term stability (for 12 days at 4°C)</td>
<td>0.4</td>
<td>96.88</td>
<td>2.02</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>98.84</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>99.74</td>
<td>1.13</td>
</tr>
<tr>
<td>Stock solution stability (for 11 days)</td>
<td>0.4</td>
<td>97.21</td>
<td>2.16</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>97.88</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>98.15</td>
<td>1.19</td>
</tr>
<tr>
<td>Post preparative stability (for 4hrs at RT)</td>
<td>0.6</td>
<td>98.10</td>
<td>1.90</td>
</tr>
<tr>
<td></td>
<td>5 (IS)</td>
<td>97.90</td>
<td>1.46</td>
</tr>
<tr>
<td>Acceptance Criteria</td>
<td>85-115%</td>
<td>≤ 15%</td>
<td></td>
</tr>
</tbody>
</table>

% R.S.D. = SD/mean x 100, RT (room temperature).

DISCUSSION

Most published methods to quantify Nevirapine in body fluids use tedious extraction, purification steps and sometimes evaporation to dryness under helium at 30 °C. In this study, rapid and sensitive HPLC method has been developed for the determination of Nevirapine in human plasma by simple protein precipitation extraction technique. Validation results proved that the developed method performs well with selectivity, precision, accuracy, stability and linearity for the concentration range of Nevirapine expected to be found in human plasma after oral administration of 200-400mg dose. The validated method covers the wide range of linearity over 0.2- 1.2 µg/ml and is therefore suitable for the determination of Nevirapine in human plasma at different therapeutic dose levels. The mean recovery of Nevirapine was found to be 96.50%. The resolution between Nevirapine and endogenous substances was satisfactory. The proposed method can be used for therapeutic drug monitoring in order to optimize drug dosage on an individual basis. The developed method is able to measure concentration of Nevirapine to monitor drug concentration in body fluid, determination of drug level in plasma for dose regulation and bioavailability.

Acknowledgement
Authors are thankful to Emcure Pharmaceuticals Ltd. Bhosari, Pune and Maxim Pharmaceuticals, Pune for providing gift samples of Nevirapine and Dicloxicillin respectively. Authors are also thankful to Akshay Blood Bank, Pune for providing pooled human plasma and the Management, AISSMS College of Pharmacy, Pune, India for providing required facilities to carry out research work.
REFERENCES