Estimation of Nevirapine from Human Plasma by LC-ESI-MS/MS: a Pharmacokinetic Application

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Abstract

A selective, sensitive, and fast High Performance Liquid Chromatography with mass spectrometric (MS) detection method was developed and validated in human plasma. Nevirapine and Enalapril (internal standard, IS) were extracted from human plasma via solid phase extraction (SPE) technique. After the elution through SPE cartridge, samples were directly analyzed using LC-ESI-MS/MS system. An isocratic mode is used to separate interference peaks using a hypurpur advanced C-18, 50 X 4.6 mm ID, 5µ, column. The mobile phase composition was 0.1% formic acid in Milli-Q water (v/v) to acetonitrile at a ratio of 15:85, v/v. The m/z of nevirapine and IS were 267.00 and 377.10, respectively. Linearity ranges were 10.00 to 5000.00 ng/mL. Calibration functions, lower limit of quantitation (LLOQ), stability, intra- and inter-day reproducibility, accuracy, and recovery are estimated. This method was free from matrix effects and any abnormal ionization. This method was successfully applied to a pharmacokinetic study of nevirapine.

Keywords: Nevirapine; Solid phase extraction; Matrix effects; Method validation; Liquid-liquid extraction; Protein precipitation; Pharmacokinetic

Introduction

Nevirapine (NVP) (Figure 1) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) that binds to the membrane of the HIV virus inhibiting viral replication. It also probably inactivates cell-free viroins present in the genital tract and in human milk. It is best used synergistically with at least one nucleoside reverse transcriptase inhibitor (NRTI) drug – the most widely studied of which is zidovudine (q.v.). Nevirapine is well absorbed by mouth, widely distributed (VD ~ 1-2 l/kg), penetrates the CSF well and, because it is lipophilic, rapidly transferred across the placenta. A substantial quantity appears in breast milk. There is no evidence of teratogenicity. It is extensively metabolised by the cytochrome P450 isoenzyme system in the liver with a half life of 40–60 hours when treatment is first started – a half life that is almost halved by enzyme autoinduction after 1–2 weeks. It is also reduced in patients on rifampicin, but extended in patients taking a range of other drugs including cimetidine, erythromycin, and fluconazole. The most important adverse effects occasioned with sustained use are a skin rash (which is sometimes severe) and liver dysfunction (which is reversible if treatment is stopped). Nevirapine is used to prevent the babies of human immunodeficiency virus (HIV) infected women becoming infected during delivery. Some babies born to women whose infectious status is unknown may also merit treatment. Twice weekly use may reduce the risk of infection during lactation, but resistance soon develops during monotherapy. Combined treatment with zidovudine (q.v.) costs more, but further reduces viral transmission, and may make later drug resistant less likely [1-6].

Several HPLC [7-14] and LC-MS/MS [15-18] methods are available for estimation of nevirapine from human plasma. Among all these reported methods only one method i.e Laurito et. al. [18] has a LLOQ value of 10 ng/mL, and other methods have LLOQ values ranging from 25-250 ng/mL. The analysis time of each single run for the method published by Laurito et. al. was 5 mins, whereas, in this present manuscript the analysis time is only 3 mins. So, Laurito et. al. has achieved the sensitivity by compromising with analysis time. The injection volume in the present manuscript is 5µL only, so by increasing the injection volume more sensitivity can also be achieved. Simple solid phase extraction (SPE) technique is used to extract nevirapine from human plasma which gives cleaner sample than liquid-liquid extraction or protein precipitation. After elution the sample was directly injected into LC-MS/MS without any evaporation step, which is an easy and fast sample preparation technique. So, in comparison with all other published methods, the present manuscript describes a method with highest sensitivity and maximum throughput.

Experiment

Apparatus and software

The HPLC system with an auto sampler was a Shimadzu LC-

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10ADvP (Shimadzu, Japan) coupled with Applied Biosystem Sciex (MDS Sciex, Canada) API 3000 Tandem mass spectrometer. The auto sampler was SIL-HTC from Shimadzu, Japan. The solvent delivery module was LC-20AD from Shimadzu, Japan. The chromatographic integration was performed by Analyst software (version: 1.4.2; Applied Biosystems). Positive pressure unit used for SPE was from Orocchem technologies Inc (Lombard, IL, USA).

Chemicals and reagents

Nevirapine and enalapril (IS) were procured from Cadila Pharmaceutical Ltd., Dholka, Ahmedabad, India. Formic acid was procured from Merck Specialties Pvt. Ltd. Mumbai, India. Water used was collected from water purification systems (Milli Q, Milli Pore, USA) installed in laboratory. Methanol and acetonitrile were of HPLC grade and were supplied by J. T. Baker, USA. Fresh frozen human plasma (K$_2$-EDTA as anticoagulant) was used during validation and study sample analysis, and was supplied by Clinical department of Cadila Pharmaceuticals Limited, 1389-Trasad road, Dholka, Gujarat, India. Plasma was stored into -70±5ºC.

Standards and working solutions

Individual stock standard solution of NVP and IS containing 1mg/mL was prepared by dissolving pure compound in methanol. The intermediate and working solutions of NVP were prepared from corresponding stock solutions by diluting with diluent (Water: Methanol 30:70 v/v). Calibration standards were established between 10.00 to 5000.50 ng/mL of using nine concentration levels. Quality control (QC) standards of three different levels low (LQC) (30.00 ng/mL), medium (MQC) (2400.25 ng/mL) and high (HQC) (3900.40 ng/mL) were also prepared. All these stock solutions, calibration standards and QC samples were stored at 4±2ºC. These solutions were found to be stable and used for the complete method validation.

Chromatographic conditions

Chromatographic separation was performed on a Hypurity advance C18, 50 x 4.6 mm ID, 5µ, analytical column and the mobile phase was a mixture of 0.10% (v/v) formic acid in water to acetonitrile at a ratio of 15:85, v/v. Injection volume was 5µL. The flow rate was 0.500 mL/min. Total analysis time of single injection was 3.00 minutes. Column oven temperature and auto sampler temperature was set to 40°C and 5°C, respectively.

Mass Spectrometric conditions

The plasma NVP concentrations were quantified using SCIEX API 3000 LC-MS/MS system (MDS Sciex, Canada), equipped with an ESI interface used to generate positive ions [M+H]$^+$. The optimized ion spray voltage and temperature were set at 4000 V and 400°C. The typical ion source parameters, viz., declustering potential, collision energy, entrance potential, focusing potential and collision cell exit potential were 50, 39, 15, 400 and 16 V for NVP and 50, 25, 10, 300 and 10 V for the IS, respectively. Nitrogen gas was used as gas 1, gas 2, curtain gas and collision-activated dissociation gas, which were set at 12, 8000, 6 and 12 psi, respectively. Quantification was performed by multiple reaction monitoring of the protonated precursor ion and the related product ion for NVP using the IS method with a peak area ratios and a linear least-squares regression curve with weighting factor of 1/x². The mass transitions used for NVP and the IS were m/z 267.00> 226.20 and m/z 377.10> 234.10 respectively, with a dwell time of 300 ms per transition. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (Version 1.4.2; Applied Biosystems).

Preparation of extracted samples

Solid phase extraction technique was used to extract the NVP from human plasma samples. 20µL of IS (5µg/mL) sample was added to each sample and the sample was vortexed for 15 seconds, LiChrosep Sequence 30mg/1mL SPE cartridge (Merck, India) was conditioned with 1 mL of methanol, followed by equilibration with 1 mL of Milli-Q water. Then the sample was loaded and washed two times with 1 mL of Milli-Q water. Then the cartridge was dried under nitrogen for 1 min. and the sample was eluted with 1 mL of mobile phase and injected directly into LC-MS/MS system.

Clinical protocol

The bioequivalence study protocol presented in this manuscript was approved by the independent medical ethics committee of Cadila Contract Research Organization, Ahmedabad, Gujarat, India. A nonrandomized, single-treatment, single -period, single –sequence, single- dose pharmacokinetic study was conducted, during which subjects were administered a nevirapine 140 mg capsule along with 200 mL of drinking water. Doses were administered after an over-night fasting of at least 10 h. Volunteers were healthy, adult, male, human Indian subjects. A total of 20 blood samples from each volunteer were collected including a pre-dose sample prior to drug administration and after drug administration at 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50, 5.00, 5.50, 6.00, 8.00, 10.00, 12.00, 16.00, 24.00, 48.00, and 72.00 h.

Results and Discussions

Optimization of chromatographic condition and sample cleanup

The successful analysis of the analyte in biological fluids using LC-MS/MS relies on the optimization of chromatographic conditions, sample preparation techniques, chromatographic separation and post column detection, etc [19,20]. Thus, for better selectivity and sensitivity different types of columns and mobile phases were used. The length of the column varied from 50 mm to 150 mm, and the particle size varied from 3.5µ to 5µ. Columns of different stationary phases like C8, C18, cyan etc were used which demonstrated significant effects on peak shape. Both the peaks were eluted early in cyano columns, whereas the use of C8 resulted in poor peak shape. Finally, a Hypurity advanced C18, 50 X 4.6mm ID, analytical column of 5µ particle sizes was selected for analysis.

The influences of strength of the buffer, pH and different organic modifier on the signal intensities were also studied. Based on the peak intensity and matrix effects NVP and IS, 0.1% formic acid (v/v) and acetonitrile (15:85, v/v) as mobile phase at a flow rate of 0.500 mL/min were selected for further studies. Initially, 70% acetonitrile: 30% of 0.1% formic acid (v/v) at a flow rate of 0.500 mL/min was tried. However, this proportion of organic phase led to peak deformation. Therefore, the 15:85 (v/v) buffer to organic phase were selected as optimum.

Different extraction techniques were tried to extract NVP from plasma samples. NVP was extracted by using protein precipitation, liquid-liquid extraction and solid phase extraction. Methanol and acetonitrile were used as precipitating agent separately, whereas, ethyl acetate was used as liquid-liquid extraction solvent. It showed matrix effects in precipitation and liquid-liquid extraction technique, whereas, no matrix effects were observed in SPE technique. Finally, solid phase extraction technique was adopted. In this method different cartridges were tested, but except LiChrosep Sequence 30mg/1mL, other cartridges showed inconsistent recovery. So to get the optimum
and consistent recovery the above mentioned extraction technique was finalized for further sample analysis.

**Method validation**

A full validation according to the USFDA guidelines [22] was performed for the assay in human plasma.

### Aqueous solution linearity: Aqueous solution linearity of calibration standards i.e. spiking solution checking was assessed by subjecting the spiked concentrations and the respective peak areas using $1/X^2$ (X – concentration) linear least-squares regression analysis. The calibration curves had a correlation coefficient (r) of 0.9900 or better. In aqueous solution linearity test all calibration standards accuracy were within 85 – 115%, except LLOQ where it was 80 – 120%.

<table>
<thead>
<tr>
<th>Analyte name</th>
<th>QC Levels</th>
<th>Nominal conc. (ng/mL)</th>
<th>N</th>
<th>Mean conc. (ng/mL)</th>
<th>SD(±)</th>
<th>%RSD</th>
<th>% Accuracy</th>
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</thead>
<tbody>
<tr>
<td>Nevirapine</td>
<td>LQC</td>
<td>30.00</td>
<td>18</td>
<td>30.60</td>
<td>2.203</td>
<td>7.20</td>
<td>102.01</td>
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<td></td>
<td>MQC</td>
<td>2400.25</td>
<td>18</td>
<td>2390.03</td>
<td>240.097</td>
<td>10.05</td>
<td>99.57</td>
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<tr>
<td></td>
<td>HQC</td>
<td>3900.40</td>
<td>18</td>
<td>3904.75</td>
<td>323.009</td>
<td>8.27</td>
<td>100.11</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment name</th>
<th>QC level</th>
<th>Mean accuracy</th>
<th>Mean Precision (%CV)</th>
<th>% change</th>
<th>Stability Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bench top</td>
<td>LQC</td>
<td>102.89</td>
<td>3.51</td>
<td>-6.28</td>
<td>07 h</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>92.72</td>
<td>2.90</td>
<td>-1.07</td>
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<tr>
<td>Freeze thaw</td>
<td>LQC</td>
<td>97.32</td>
<td>6.37</td>
<td>-1.10</td>
<td>4 cycles</td>
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<tr>
<td></td>
<td>HQC</td>
<td>95.11</td>
<td>3.30</td>
<td>3.98</td>
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<tr>
<td>Auto sampler</td>
<td>LQC</td>
<td>107.75</td>
<td>7.29</td>
<td>9.49</td>
<td>32 h</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>97.48</td>
<td>2.09</td>
<td>6.57</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Inter and intra-day accuracy and precision of nevirapine.

Table 2: Overall statistics of QC samples of precision and accuracy batches during.

Table 3: Summary of stability data of nevirapine.
Specificity and selectivity: Six different lots of plasma along with one lipemic plasma and one haemolyzed plasma were analyzed to ensure that no endogenous interferences were present at the retention time of NVP and IS. Six LLOQ level samples along with plasma blank from the respective plasma lots were prepared and analyzed. In all plasma blanks, the response at the retention time of NVP was less than 20% of LLOQ response and at the retention time of IS, the response was less than 5% of mean IS response in LLOQ. (Figure 2) shows a typical chromatogram of plasma blank; (Figure 3) represents the chromatogram of LLOQ.

Accuracy and precision: For the validation of the assay, QC samples were prepared from normal plasma at three concentration levels (low, medium and high) according to the procedure mentioned in section 2.6. The respective concentrations were 30.00, 2400.25 and 3900.40 ng/mL. Six replicates of each QC sample were analyzed together with a set of calibration standards. Three such precision and accuracy batches were analyzed in three different days. The obtained accuracy and precision (inter- and intra-day) data are presented in (Table 1 and Table 2). The result showed that the analytical method was accurate, as the accuracy of QCs were within the acceptance limits of 100±15% at their respective concentration levels. The precision around the mean value was never greater than 15% at any of the concentrations studied. (Figure 4) represents the chromatogram of upper limit of quantization (ULOQ).

Recovery study: Recovery was evaluated by comparing extracted QC samples of three different levels in six replicates with aqueous samples of same level. The mean recovery at LQC level was 111.43 %, for MQC level was 90.06%, and for HQC level was 93.81%. The mean recovery of all three QC levels was 98.43% and the %CV of mean recovery of all three QC levels was 11.59, where as the mean recovery of IS was 88.74% and the %CV was 4.51.

Haemolysis effects: To determine the haemolysis effects, QC samples at all three concentration levels i.e. LQC (30.00 ng/mL), MQC (2400.25 ng/mL) and HQC (3900.40 ng/mL) were prepared from haemolyzed plasma. Six replicates of each QC sample were analyzed together with a set of calibration standard prepared in normal plasma. The average % accuracy of LQC level was 109.68, for MQC level was 111.84 and for HQC level was 110.03. The %CV of LQC was 1.89 and for HQC was 2.11. Matrix effects: The effect of human plasma constituents over the ionization of NVP and IS were determined by comparing the responses of the post-extracted QC samples (n = 18) with the response of analytes from aqueous samples at Low & High QC concentrations. The % accuracy for LQC was 96.96 and for HQC was 94.65, % CV LQC and HQC was 6.38 and 2.83 respectively.

Dilution integrity: First, a dilution quality control sample (15001.50 ng/mL), which was three times of ULOQ, was prepared in plasma. Then six samples each of 1/5th (3000.30 ng/mL) and 1/10th (1500.15 ng/mL) dilution from the above prepared sample were processed in plasma and analyzed with freshly processed calibration standards as per the extraction method. The % CV were found 9.01 and 0.72 for 1/5th and 1/10th diluted samples, respectively, and % nominal were found 100.39 and 101.62 for 1/5th and 1/10th diluted samples respectively.

Figure 3: Representative chromatogram of lower limit of quantization (LLOQ, 10.00 ng/mL).
Stability studies: The stability of NVP and IS were investigated in the stock and working solutions, in plasma during storage, during processing, after four freeze-thaw cycles, and in the final extract. Stability samples were compared with freshly processed calibration standards and QC samples. Analyte and IS were considered stable when the percent change of concentration was ±10 with respect to initial concentration. Summary of stability data is presented in (Table 3).

Calibration curve

The plasma calibration curve was prepared using nine calibration...
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References


