

Determination of Nevirapine in Human Plasma by High Performance Liquid Chromatography with Ultraviolet Detection

Purnima Hamrapurkar*, Mitesh Phale, Priti Patil, Nitul Shah

Department of Pharmaceutical Analysis, Prin. K. M. Kundnani College of Pharmacy,
Jote Joy Building, Rambhau Salgaonkar Marg, Cuffe Parade, Colaba,
Mumbai- 400 005, India

*Corres. Author: phamrapurkar@gmail.com
Tel no: +91 22 2216 4368, Fax no: + 91 22 22165282

ABSTRACT: A simple, specific, accurate, and precise reverse phase liquid chromatographic method (RP-HPLC) with ultraviolet detection has been developed and validated for the estimation of nevirapine from human plasma. Chromatographic separation was achieved on Waters RP-C-18 (Waters) 10 μ m column having 250 \times 4.6 mm ID with a mobile phase containing 15 mM aqueous phosphate buffer: acetonitrile (65:35 % v/v) in isocratic mode. The flow rate was 1.0 ml / min and effluents were monitored at 283 nm. The retention time of nevirapine and the internal standard was 5.1 min and 6.2 min respectively. The linearity of the method was good ($r^2 > 0.9995$), as also were intra-day and inter-day precision.

The method was validated for accuracy, specificity, limit of quantification, limit of detection, robustness and stability. The results showed that proposed method can be successfully applied for the quantitative determination of nevirapine in human plasma.

Keywords: Nevirapine, RP-HPLC, validation, precise, accurate

INTRODUCTION

Nevirapine (Fig.1), 11-cyclopropyl-4-methyl-5, 11-dihydro-6H-dipyrido [3, 2-b: 2', 3'-e] [1, 4] diazepin-6-one is a reverse transcriptase (RT) inhibitor of human immunodeficiency virus type 1 (HIV-1)¹⁻². Nevirapine inhibits replication of HIV-1 by interfering with viral RNA-directed DNA polymerase (reverse transcriptase). It binds directly to herodimeric HIV-1 reverse transcriptase and exerts a virustatic effect by acting as a specific, noncompetitive HIV-1 reverse transcriptase inhibitor; it appears to inhibit viral RNA- and DNA-dependent DNA polymerase activities by disrupting the catalytic site of the enzyme.³

Literature survey reveals that there are several analytical methods available for determination of nevirapine from biological matrices⁴⁻¹⁴, bulk drug and dosage forms¹⁵⁻¹⁷, and analytical methods for determination of nevirapine with combination of other antiviral drugs.¹⁸⁻⁵⁰

Literature survey further revealed that there are very few reported HPLC method for the analysis of nevirapine alone in human plasma. The methods reported include HPLC with fluorescence detection or with ion pairing, HPLC with mass detection or with LCMS-MS analysis, although such detector systems may not be available in most laboratories.



Fig: 1 Structure of nevirapine

The reported methods are either costlier or include cumbersome and expensive extraction procedures. In this paper, we used an HPLC method with UV detection and the results were satisfactory. UV detection is simple and available to most analytical laboratories. In addition, this method involves a simple liquid-liquid extraction with excellent reproducibility, which makes it suitable for pharmacokinetic and bioequivalence studies. Thus, an appropriate and simple analytical procedure for the quantitative determination of nevirapine from human plasma is of considerable importance.

Keeping this objective in mind an attempt has been made to develop and validate a simple HPLC method for the analysis of nevirapine which would be highly sensitive, having good resolution and reproducible. Various validation aspects of the analysis, accuracy, precision, recovery and the limits of detection and quantification etc., have been measured.

EXPERIMENTAL

Chemicals and Reagents

The working standard of nevirapine was procured from Cipla Ltd., India. Carbamazepine as internal standard was procured from Cipla Ltd., India. HPLC grade acetonitrile, methanol (purity 99.9 %) and ethyl acetate was procured from Merck (Darmstadt, Germany). The HPLC grade water was obtained by double distillation and purification through Milli-Q water purification system. Potassium dihydrogen phosphate buffer (AR grade), sodium hydroxide and ortho phosphoric acid (AR grade) was procured from Qualigens Fine Chemicals (Mumbai, India).

Equipment

The Liquid chromatographic system consisted of following components: Jasco HPLC model containing PU 1580 Intelligent HPLC pump, Jasco UV 1520 Intelligent UV-Vis Detector and Rheodyne injector (7725i) with 20 μ l fixed loop. Chromatographic analysis was performed using Borwin Chromatography software on a Waters RP - C-18 (Waters) column having 250 \times 4.6 mm ID and 10 μ m particle size. The Mettler electronic micro balance (MT5) was used for weighing purpose.

Preparation of Standard Solution

A stock solution of nevirapine (1 mg / ml) was prepared in methanol. Standard solution was prepared by dilution of the stock solution with methanol to give solution in of 10 μ g / ml. Resultant solution was filtered through Whatman filter Paper No.1. Similarly, dilutions were made for Carbamazepine, which was used as internal standard (I.S)

Preparation of Calibrators and Quality Control samples in human plasma

Calibrators ranging from 0.010 – 3.500 μ g / ml were prepared by subsequent dilution of the above stock solution with methanol. The quality control samples (QC samples) were prepared by spiking drug free plasma to give final concentration of low (LQC – 0.030 μ g / ml), medium (MQC – 1.500 μ g / ml) and high (HQC – 3.000 μ g / ml). These QC samples were stored at -20 $^{\circ}$ C.

Preparation of Mobile Phase

Potassium dihydrogen phosphate buffer was weighed (136.1g) and dissolved in 1000 ml of water to get 1 M solution. From this solution 15 ml was taken and volume was made up to 1000 ml to get a solution of 15 mM. The pH of the solution was adjusted to 3.2 with ortho phosphoric acid. The final volume was adjusted by adding this 650 ml of buffer to 350 ml of acetonitrile, which resulted in pH 3.5 for final mobile phase. The final solution was mixed well, was sonicated for 10 min and filtered using Whatman filter Paper No.1.

Extraction Procedure from plasma

To 1.0 ml of drug spiked plasma 50 μ l of I.S (internal standard – Carbamazepine, 10 μ g / ml) was added, vortex for 1 min. To this add 0.5 ml of 0.5 N sodium hydroxide, vortex for 1 min. The drug was then extracted using 5.0 ml of ethyl acetate by vortexing for 10 mins. Organic layer was separated and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 200 μ l of mobile phase and 100 μ l was injected on to the HPLC.

Chromatographic Condition

A Waters reverse phase C-18 column, equilibrated with mobile phase 15 mM aqueous phosphate buffer: acetonitrile (65:35 % v/v & pH 3.5) was used. The active principle was eluted isocratically and the mobile phase flow rate was maintained at 1.0 ml / min. The effluents were monitored at 283 nm with the detector. The sample was injected using a 20 μ l fixed loop, and the total run time was 10 min.

VALIDATION OF HPLC METHOD

The proposed RP-HPLC method was validated as per ICH guidelines.⁵¹

System suitability and specificity

The specificity of the RP-HPLC method was determined by comparison of the chromatogram of standard and sample solution. The parameters like retention time (Rt), resolution (Rs), tailing factor (Tf)

and theoretical plates were calculated. Results of system suitability are recorded in Table 1.

Precision and accuracy

Precision study was performed to find out intra-day and inter-day variation. It was carried out by estimating the corresponding responses 3 times on the same day and on 3 different day (first, second and fifth day) for 3 different concentration of nevirapine (0.030, 1.500, 3.000 $\mu\text{g} / \text{ml}$) and the results are reported in terms of relative standard deviation (RSD). The repeatability studies were carried out by estimating response of 3 different concentration of nevirapine (0.030, 1.500, 3.000 $\mu\text{g}/\text{ml}$) in triplicate.

Recovery studies

Recovery studies were performed by standard addition method at three levels i.e. 80 %, 100 % and 120 %. A known amount of standard nevirapine was added to preanalyzed sample and was subjected to proposed HPLC method.

Detection limit and quantitation limit

A calibration curve was prepared using concentrations in the range of 0.010 – 3.5 $\mu\text{g} / \text{ml}$ (expected detection limit range). Detection limit for nevirapine was 0.005 $\mu\text{g} / \text{ml}$ and quantitation limit was 0.010 $\mu\text{g} / \text{ml}$. The standard deviation of y-intercepts of regression line was determined and kept in following equation for the determination of detection limit and quantitation limit. Detection limit = $3.3 \sigma / s$; Quantitation limit = $10 \sigma / s$, where σ is the standard deviation of y-intercepts of regression lines and s is the slope of the calibration curve.

Extraction recovery

The extraction recovery of analyte was determined by measuring the peak areas of the drug from the prepared plasma quality control samples. 0.030 $\mu\text{g} / \text{ml}$, 1.500 $\mu\text{g} / \text{ml}$ and 3.000 $\mu\text{g} / \text{ml}$ plasma samples were taken as LQC (low quality control), MQC (medium quality control) and HQC (high quality control) samples respectively. The peak areas of extracted LQC, MQC and HQC were compared to the absolute peak area of the unextracted samples containing the same concentration of the drug as 100 %. To obtain good extraction efficiency the extraction recovery of nevirapine was determined using five replicates of each QC samples. The extraction of IS was also determined.

Stability study

The stability of nevirapine in plasma was evaluated with four studies; a short-term stability study, a long-term stability study, a freeze thaw study and stability in processed sample. Plasma blank samples were spiked with nevirapine at concentration of 0.030 $\mu\text{g} /$

ml (LQC), 1.500 $\mu\text{g} / \text{ml}$ (MQC) and 3.000 $\mu\text{g} / \text{ml}$ (HQC) and each concentration was carried out for five times. Plasma samples were extracted and subsequent HPLC analysis was carried out as described previously. Short-term stability test was performed at room temperature. Plasma samples spiked with nevirapine were kept at room temperature for 12 hr, extracted and then analyzed. The long term stability study was carried out with plasma blank samples spiked with nevirapine, which were stored -20° and they were analyzed periodically 1 months against a standard curve prepared on the analysis day. For freeze thaw stability spiked samples were analyzed immediately after preparation and on a daily basis after repeated freeze thaw cycles at -20°C on three consecutive days. Finally the stability in the processed sample ready for injection was determined at three levels of concentration, 0.030 $\mu\text{g} / \text{ml}$ (LQC), 1.500 $\mu\text{g} / \text{ml}$ (MQC) and 3.000 $\mu\text{g} / \text{ml}$ (HQC). The processed QC samples ready for injection were kept for 8 hr before HPLC analysis.

Ruggedness and Robustness

Robustness of the method was studied by changing the composition of organic phase by $\pm 5\%$ and pH by 0.2, and also by observing the stability of the drug for 24 hr at ambient temperature in mobile phase. Ruggedness was carried out by changing the instrument and analyst.

RESULTS AND DISCUSSION

The development of the RP-HPLC method for the determination of drugs from human plasma has received considerable attention in recent years because of its importance in bioavailability and bioequivalence.

HPLC Method Development:

Nevirapine is practically insoluble in water, soluble in dichloromethane, in dimethylsulphoxide and in dimethylformamide, slightly soluble in methanol. Calibrator solutions were prepared in methanol. Solubility increases at pH < 3 . During the HPLC method developments following considerations were made:

Selection of column:

Since nevirapine is weakly basic in nature (ionizes in acidic medium) so reverse – phase chromatography was thought to be the best choice. The efficiency of two different reverse – phase column C18 and C8 was evaluated. C18 column was preferred for the separation of drug because C8 column was showing decreased retention of drug (2.3 min.) and plasma impurities were getting coeluted with drug peak. However the above-mentioned problems were solved using C18 column and retention time was adjusted (5.2

min.) using C18 column. There was no other interfering peak around the retention time of nevirapine. Blank sample did not show any significant peak at the retention time of the drug. Sufficient resolution between the drug (Nevirapine) and internal standard (Carbamazepine) peak was also obtained.

Selection of mobile phase:

The mobile phase was chosen after several trials with other solvent combinations. Mobile phase selection was based on peak parameters (symmetry, tailing), run time, ease of preparation and cost. Acetonitrile: water (80:20 % v/v) was optimized after several trials as the mobile phase; but with this combination resolution could not be achieved between the solvent and drug peaks, plasma impurities and drug peaks, drug and internal standard peak. The problem of resolution could not be solved even with mobile phases of combinations acetonitrile: water (75:25 % v/v) and acetonitrile: water (70:30 % v/v). However good resolution was obtained using acetonitrile: water (40:60 % v/v), but the peak were broad, showed tailing and the response was not reproducible. These problems were solved when water in mobile phase was replaced with 25 mM potassium dihydrogen phosphate buffer, as buffer forms ion pair with free silanol groups and thus reduces the tailing. Buffer pH was adjusted to 3.2 with ortho phosphoric acid, and after addition to acetonitrile, final mobile phase pH was 3.5. However no significant change in peak shape and response was observed in pH range of 3.0 to 3.8. Acetonitrile has low viscosity, low vapor pressure (low back pressure, high efficiency separation) and is transparent in UV region therefore it was used as organic modifier. Proportion of acetonitrile in mobile phase was altered to get good resolution and desired retention time. Since pKa of nevirapine is 2.8, so in the acidic pH probability of drug remaining in ionized form is more, which in turn has an effect on peak shape and retention time. This statement was supported when improved peak shape, tremendous decrease in tailing and reproducible response was observed between the pH ranges of 3.0 to 3.8.

Selection of detection system:

The presence of Chromophores in the molecular structure, such as α - β -unsaturated ketone and pyridine ring makes UV-Visible method suitable for the determination of nevirapine. Selection of λ_{\max} was done based on spectrophotometric scan of compound. Nevirapine showed λ_{\max} at 222.0 and 283.0 nm. Less interference and more reproducibility in the analyte response was observed at 283.0 nm as compared to 222.0 nm during the HPLC run and therefore 283.0 nm was used as detection wavelength for HPLC analysis of nevirapine.

Selection of extraction method for Quality Control Samples:

As we know nevirapine is weak base having a pKa 2.8, hence it was extracted from an aqueous medium at high pH into organic solvent. Various solvents like ethyl acetate, methyl-tert-butyl ether, dichloromethane, chloroform, n-hexane and heptane were tried as extracting solvent, but gave poor recovery hence decreased response. However ethyl acetate gave 77.77 % recovery.

Thus the chromatographic conditions were optimized and good separation was achieved using a Waters RP - C-18 column and above mentioned mobile phase. The proposed method was suitable for the estimation of nevirapine in human plasma. The chromatographic conditions were optimized in order to get a sensitive and specific response during the analysis. The retention time was 5.1 min at a flow-rate of 1 ml/min. The total run time for an assay was approximately 10 min. The extraction procedure employed in this study produced clean and clear supernatants from plasma as there was no interference from endogenous compounds. The IS and analyte was completely resolved to baseline and samples could be injected at 12-min intervals. Figure 2 represents the chromatogram for blank plasma and Figure 3 is a representative chromatogram for spiked nevirapine and IS, using the proposed method. As shown in the figure 3, nevirapine was eluted forming symmetrical peak and well separated from the solvent front. The analyte and IS peak were well resolved, proving the specificity of the method. Observed retention time (5.1 min) allowed a rapid determination of the drug (Table 1).

The summary of the entire validation parameters is given in Table 2 while the detailed results of the assessments of precision, recovery and accuracy are given in Table 3. The results indicate that the method has a high degree of precision as the intraday and inter-day coefficients of variation were not greater than 4 % at low and high concentrations. The recovery was 79.59 % at 0.030 μg / ml, 76.74 % at 1.500 μg / ml and 76.98 % at 3.000 μg / ml which was constant at all levels of concentration of analyte and IS thus assuring that the sample preparation and extraction procedure was efficient for the compounds. Evidence of accuracy of the method is demonstrated in the results which ranged between 97.07 and 99.24 % for the compounds at low and high concentrations.

Nevirapine was stable for at least three freeze-thaw cycles. No significant decrease of nevirapine concentration in plasma was detected after exposing samples to three freeze/thaw cycles and % CV was found to be 2.65 at LQC, 3.47 at MQC and 0.66 at HQC. The short-term stability test performed at room temperature showed that three QC samples were stable

for 8 hr (%CV 3.09, 2.19 and 2.83 at LQC, MQC HQC, respectively). While the long term stability indicated that nevirapine samples were stable during 1 month, with a % CV of 3.34 at LQC, 2.57 at MQC and 2.02 at HQC. Finally, the stability in the processed sample ready for injection was also determined. Result showed that three QC samples were stable at least for 8 hr with loss not higher than 10%.

The method reported here is more sensitive and precise over other previous methods for the analysis of the drug and it also has the advantage of being simple, cost-effective and devoid of any cumbersome

extraction procedure. The composition of the mobile phase also proves the simplicity of the method. The mobile phase consisted mainly (65 %) of potassium dihydrogen phosphate solution and (35 %) of acetonitrile which are commonly available, thus, making the method cost-effective and affordable. The rapidity of the method is underlined by the relatively short analysis time. The maximum time required for a sample treatment prior to injection was 20 min, which is much shorter making the application of method easier and acceptable.

Table 1: System suitability for the proposed method

Parameters	Values
Retention time	
Nevirapine	5.1 min
Carbamazepine (IS)	6.2 min
Resolution	8.37
Tailing factor	1.38
Theoretical plates	2674.75

Table 2: Summary of Validation Parameters for the proposed method

Parameters	Values
Detection limit ($\mu\text{g/ml}$)	0.005 $\mu\text{g} / \text{ml}$
Quantitation limit ($\mu\text{g/ml}$)	0.010 $\mu\text{g} / \text{ml}$
Calibration range ($\mu\text{g/ml}$)	0.010 – 3.5 $\mu\text{g} / \text{ml}$
Accuracy (%)	97.07 – 99.24
Precision (RSD ^a , %)	
Intraday (n=3)	1.26-2.89
Interday (n=3)	1.61-3.26
Slope	0.6153
Regression coefficient (r^2)	0.9995

^aRSD indicates relative standard deviation

Table 3: Precision and accuracy data

Precision			% Accuracy	
Actual Concentration (µg/ml)	Measured Concentration (µg/ml) ± S.D.; % R.S.D.			
	Intra-day	Inter-day	Intra-day	Inter-day
0.030	0.029 ± 0.001; 2.63	0.030 ± 0.001; 3.26	97.07	98.43
1.500	1.468 ± 0.042; 2.89	1.489 ± 0.046; 3.07	97.84	99.24
3.000	2.945 ± 0.037; 1.26	2.913 ± 0.047; 1.61	98.15	97.11

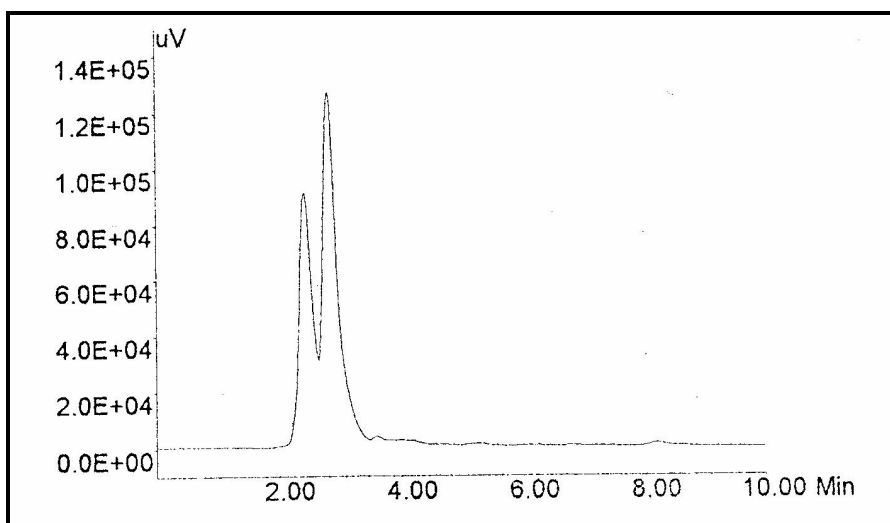


Fig: 2 Representative HPLC chromatogram of blank human plasma

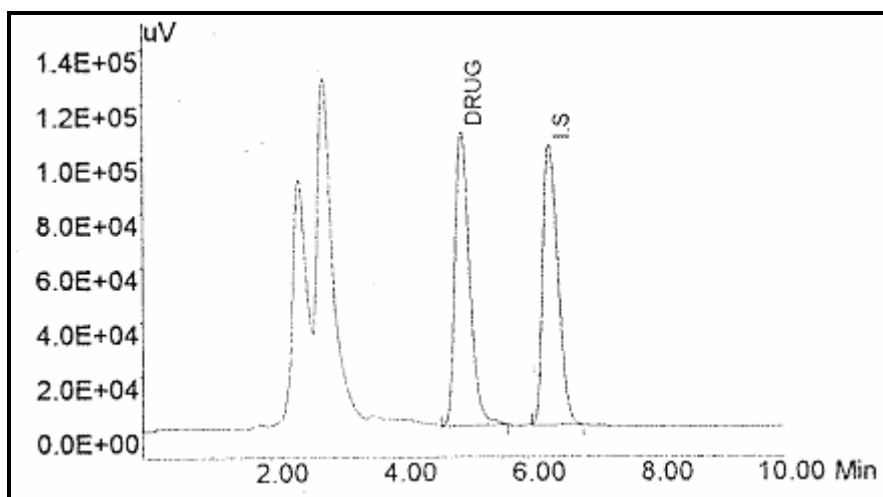


Fig: 3 Representative HPLC chromatogram of spiked nevirapine and IS

CONCLUSION

In conclusion, the HPLC method described is very simple, reproducible, sensitive and rapid. The method is also accurate, selective and cost-effective. It will facilitate the conducting of pharmacokinetic studies on nevirapine by easy quantification and Besides greater precision and sensitivity attained using this HPLC method, the specificity offered is undoubtedly another

advantage compared to the other costlier methods/techniques of analysis.

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