

DEVELOPMENT OF A NEW RP-HPLC METHOD FOR THE ESTIMATION OF NEVIRAPINE IN TABLET DOSAGE FORM

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ABSTRACT

Objective: To develop a selective, sensitive and accurate new reverse phase high performance liquid chromatography method with UV detection and determination for estimation of Nevirapine in tablet formulation.

Experimental: The separation and quantification was achieved by Welchrom C₁₈ isocratic column, (250 mm × 4.6 mm i.d., particle size 5 μm, maintained at ambient temperature), Shimadzu LC-20AT Prominence Liquid Chromatograph, a mixture of Methanol: Acetonitrile: Water (50:30:20 v/v), with apparent pH of 4.6 and the flow rate was set at 1.0 ml/min and UV detection at 225 nm.

Results and discussion: The retention time for nevirapine was found to be 3.660 min. The method was validated for Linearity, Accuracy, and Precision. Linearity for nevirapine was in the range of 2-10 μg/ml with correlation coefficient values 0.9999. The %RSD reported was found to be less than 2% indicate that method is precise and accurate. The mean recoveries were found in the range of 99.58 -100.40%. The Limit of Detection and Limit of Quantitation were found to be 0.0815 μg/ml and 0.2470 μg/ml respectively, which indicate sensitivity of the method. No interfering peaks were found in the chromatogram indicating that excipients used in tablet formulations didn't interfere with the estimation of the drug by proposed HPLC method.

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

INTRODUCTION

Nevirapine (NVP) is 11-cyclopropyl-4-methyl-5,11-dihydro-6H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one. Nevirapine[1] belongs to a class of drugs known as Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) with activity against HIV-I. It binds directly to Reverse Transcriptase and blocks the RNA dependent and DNA dependent DNA polymerase activities by causing a disruption of the enzyme's catalytic site. The HIV-II Reverse Transcriptase and human DNA polymerases are not inhibited by NVP. The enzyme reverse transcriptase transforms single-stranded viral RNA into DNA. Drugs in the NNRTI class stop HIV[2] from replicating within cells by binding near reverse transcriptase's active site and inhibiting polymerase activity. Nevirapine is an anti-HIV drug that reduces the amount of virus in the body. Anti-HIV drugs such as nevirapine slow down damage to the immune system and prevent the occurrence of AIDS-defining illnesses.

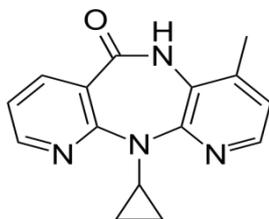


Fig. 1: Structure of Nevirapine

Literature survey reveals few analytical methods were reported for the determination of NVP in bulk and pharmaceutical preparations and in biological fluids by UV spectrophotometry[3-4], High Performance Liquid Chromatography[5-10], Ion pair HPLC[11-13]. However most of the available methods have limitations such as a long runtimes, low sensitivity, uneconomical and have poor symmetry. Keeping in view of these, we have decided to develop a simple, accurate, precise and reliable RP-HPLC method for the estimation of NVP in pharmaceutical dosage forms. Indeed the established method was validated with respect to specificity, linearity, precision, accuracy, robustness, LOD and LOQ according to ICH guidelines (ICH, 1997)[14].

MATERIALS AND METHODS

Chemicals and Reagents

An analytically pure sample of NVP Active Pharmaceutical Ingredient (API) was procured as gift sample from Cipla Ltd., Mumbai, India. All the chemicals were analytical grade. HPLC grade acetonitrile and triethylamine were obtained from Merck Pharmaceuticals Private Ltd., Mumbai, India. Methanol and water used were of HPLC grade and procured from Merck Specialties Private Ltd., Mumbai, India. Commercial tablets of NVP formulation was procured from local pharmacy. NEVIMUNE tablets containing NVP with labeled amount of 200 mg per tablet are manufactured by Cipla Ltd., Mumbai, India.

Instruments and Chromatographic conditions

The HPLC analysis was performed on Shimadzu LC-20AT Prominence Liquid Chromatograph comprising a LC-20AT VP pump, Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom C₁₈ column (4.6 mm i.d. X 250 mm, 5 micron particle size). A manually operating Rheodyne injector with 20 μL sample loop equipped with the HPLC system was used for the development and validation of the this method. The HPLC system was provided with "Spinchrom" data acquisition software. The mobile phase composed of a mixture of methanol, acetonitrile and water in ratio of 50:30:20, v/v with pH adjusted to 4.6 with o-phosphoric acid. Triethylamine was used as column modifier. The mobile phase was set at a flow rate of 1 mL/min. Eluate was monitored at 225 nm. In addition, an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), a sonicator (spectra lab, model UCB 40), UV-Visible Spectrophotometer (Systronics model 2203) were used in this study.

Preparation of Reagents and Standards

a. Mobile phase

The mobile phase was prepared by mixing of methanol, acetonitrile and water (all of HPLC grade) in the ratio of 50:30:20, v/v. Then pH is adjusted to 4.6 with 0.1N o-phosphoric acid and 0.5ml triethylamine is added as column modifier. It is filtered through 0.45 μm nylon membrane filtered and degassed by sonication.

b. Stock and Working Standard Solutions

Accurately 10 mg of NVP was weighed and transferred to a 10 mL clean, dry volumetric flask and mobile phase was added and sonicate to dissolve. The volume was made upto the mark with mobile phase. This is stock standard solution of NVP with concentration of 1mg/mL. Prepare five working standard solutions for calibration by adding defined volumes of the stock standard solution and diluting with mobile phase. The concentrations of NVP are 2.0, 4.0, 6.0, 8.0, 10.0 µg/mL, respectively in mobile phase.

Tablet Sample preparation

Weigh 20 tablets of NVP (NEVIMUNE), determine the average weight and ground into a fine powder. A quantity of powder equivalent to 100 mg of NVP was weighed accurately and transferred into a 100 mL volumetric flask. Add 80 mL mobile phase and place in an ultrasonication bath until dissolution is complete. The volume was made upto the mark with the same. Pipette out 1.0 mL of the sample solution into a 10 mL volumetric flask and dilute with mobile phase upto the mark. Mix well. The resulting solution was filtered using 0.45 µm filter and degassed by sonication. The resulting solution is further diluted to give a concentration of approximately 10µg/mL.

Selection of detection wavelength

The UV spectrum of diluted solutions of various concentrations of NVP in mobile phase was recorded using UV spectrophotometer. The spectra of the standard drug showed that the wavelength of maximum absorbance was found to be 225 nm. This wavelength was used for detection of NVP.

Linearity curve for Nevirapine

Linearity of the proposed method was checked by analyzing each calibration standard solutions (2, 4, 6, 8, 10 µg/mL) for NVP. The average peak area and retention time were recorded. Linearity curve was constructed by plotting concentration of NVP on X-axis and average peak areas of standard NVP on Y-axis and linearity equation was computed for NVP. The linearity range was found to be 2-10 µg/mL. The results were tabulated in Table 1. The standard chromatograms of NVP calibration standards have been depicted in Figure 2 to Figure 6. Results show that a good correlation exists between peak area and concentration of drug within the linearity range. The regression graph for NVP is presented in Figure 7. The data of regression analysis is presented in Table 2.

Table 1: Calibration data of the proposed HPLC method for estimation of Nevirapine

S. No.	Concentration, µg/mL.	Retention time, (t _r) min.	Peak area, mV.s.
1.	0	-	0
2.	2	3.657	449.165
3.	4	3.660	906.357
4.	6	3.663	1357.704
5.	8	3.657	1814.034
6.	10	3.663	2288.329
Slope		228.3942714	
Intercept		-6.039857143	
Correlation Coefficient [CC] (r)		0.999966719	
Squared CC (R ²)		0.999933438	
Residual sum of squares		243.0640363	

Table 2: Linear regression data of the proposed HPLC method of nevirapine

Parameter	Method
Detection wavelength(λ _{max})	UV at 225 nm
Linearity range (µg/mL)	2-10 µg/mL
Regression equation (Y = a + bX)	Y = - 6.0399 + 228.39X
Slope(b)	228.39
Intercept(a)	- 6.0399
Standard error of slope (S _b)	0.931711245
Standard error of intercept (S _a)	5.641791762
Standard error of estimation (S _e)	7.79525555
Regression coefficient (R ²)	0.999933438
% Relative standard deviation* i.e., Coefficient of variation(CV)	0.378322875
Percentage range of errors* (Confidence limits)	
0.005significance level	1.162234313
0.001 significance level	1.823048333

*Average of 6 determinations; Acceptance criteria < 2.0.

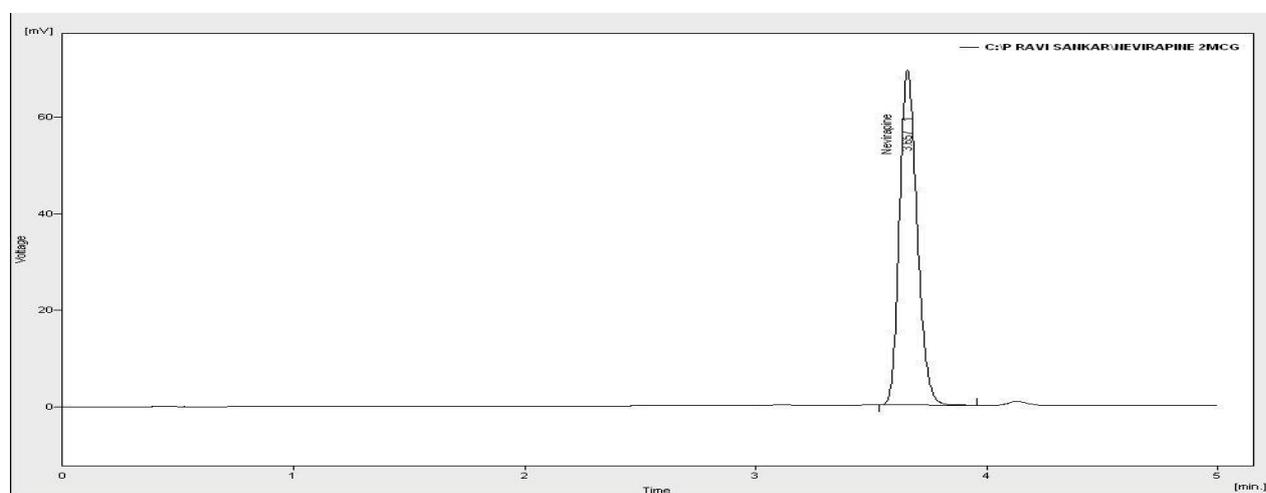


Fig. 2: Standard chromatogram of Nevirapine (2 µg/ml)

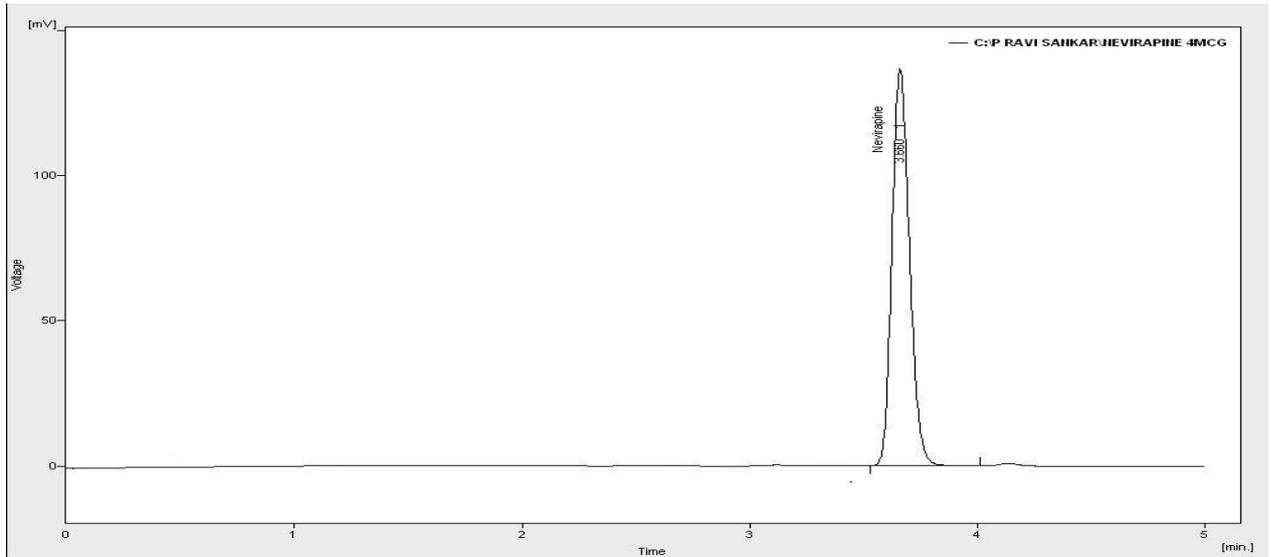


Fig. 3: Standard chromatogram of Nevirapine (4 µg/ml)

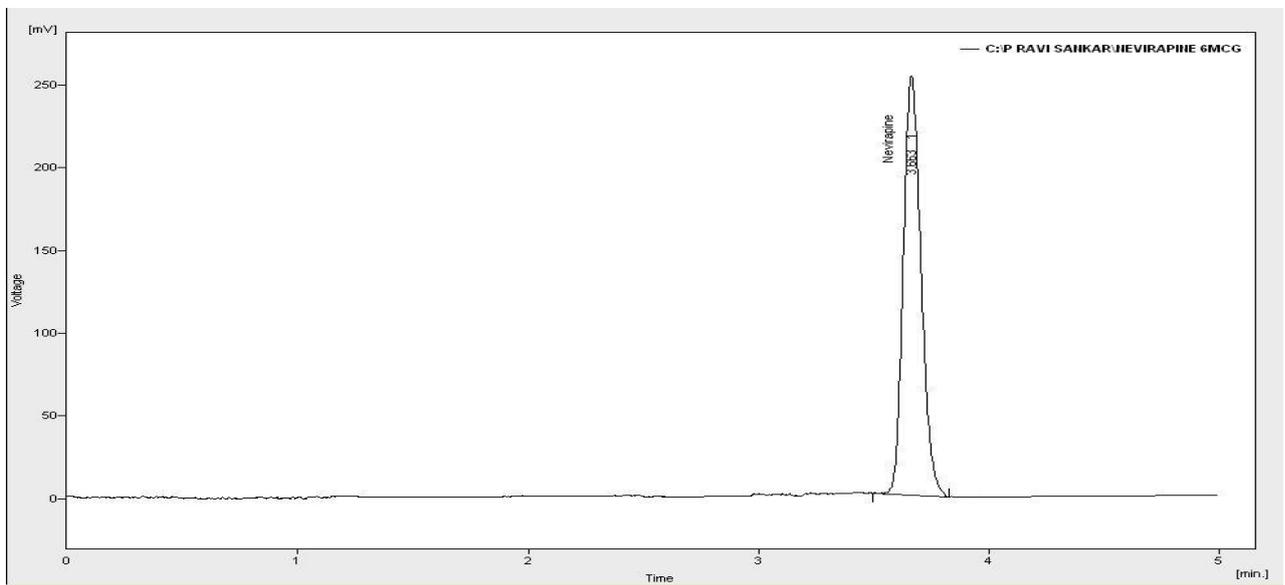


Fig. 4: Standard chromatogram of Nevirapine (6 µg/ml)

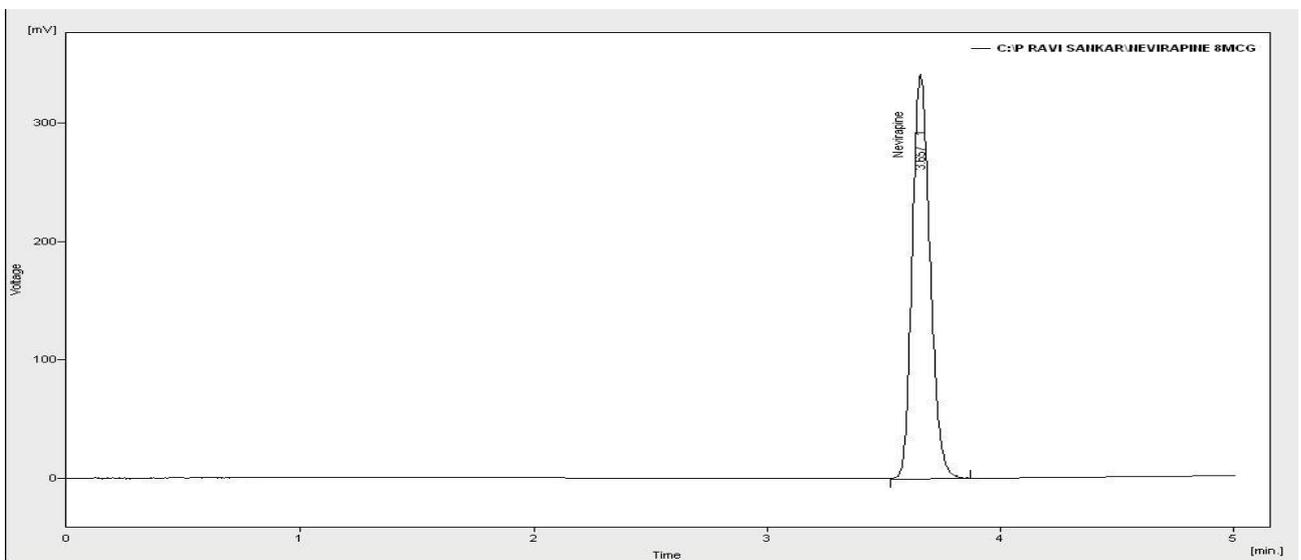


Fig. 5: Standard chromatogram of Nevirapine (8 µg/ml)

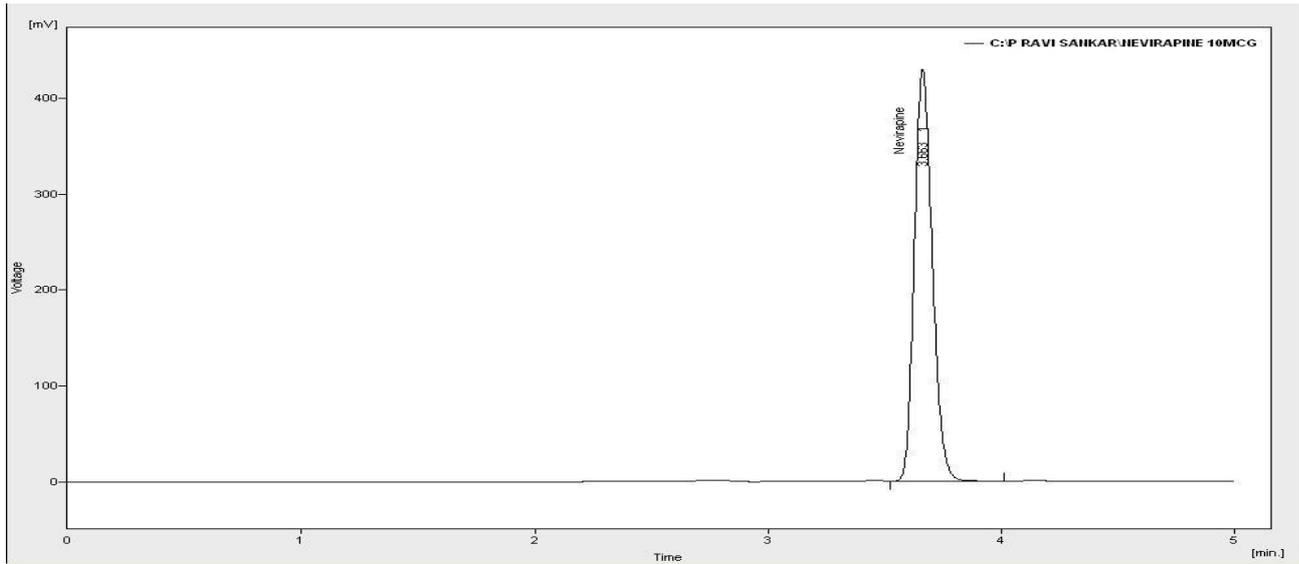


Fig. 6: Standard chromatogram of Nevirapine (10 µg/ml)

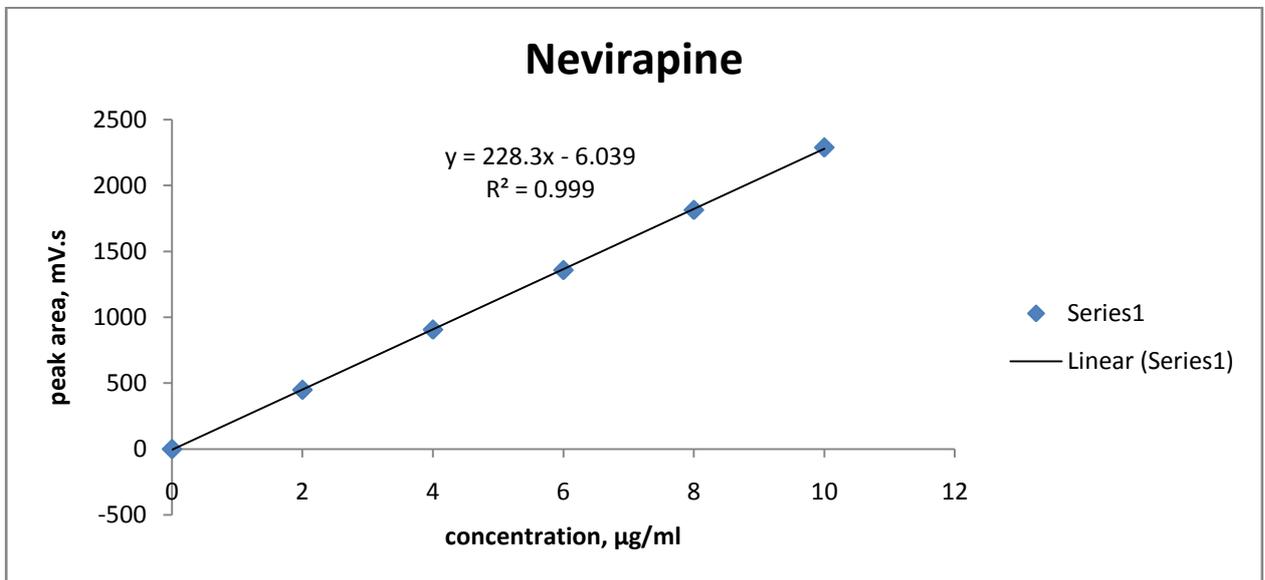


Fig. 7: Calibration plot of Nevirapine

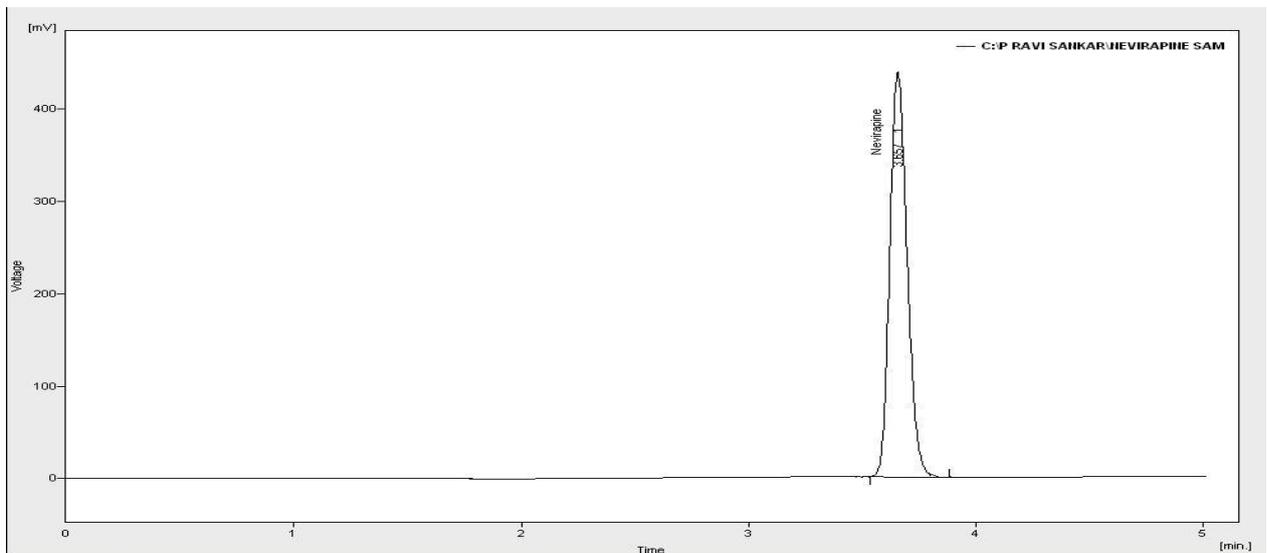


Fig. 8: Chromatogram of market formulation (NEVIMUNE 200 mg tablets) of Nevirapine

Table 3: Assay results of nevirapine formulation

S. No.	Formulations	Labelled amount	Amount found*	% Assay \pm SD*
1	NEVIMUNE tablets (Cipla Ltd., Mumbai, India)	200 mg/tablet	204.81 mg/tablet	102.40 \pm 0.377 %

*Average of 6 determinations; SD is standard deviation.

Quantitative determination of Nevirapine in pharmaceutical tablet dosage form:

The proposed method was applied to the assay of NVP tablets. The drug content was calculated as an average of six determinations and assay data is shown in Table 3. The results were very close to the labeled value of commercial tablets. Figure 8 shows the representative sample chromatogram of NVP.

Validation of the proposed method

The developed method of analysis was validated with respect to various parameters such as like system suitability, specificity, linearity, precision, accuracy, robustness, limit of detection (LOD) and limit of quantitation (LOQ) as per the ICH guidelines.

System suitability

The chromatographic systems used for analysis must pass the system suitability limits before sample analysis can commence. Set up the chromatographic system, allow the HPLC system to

stabilize for 40 min. the system suitability was assessed by injecting blank preparation (single injection) and standard preparation (six replicates) and recording the chromatograms to evaluate the system suitability parameters like resolution (NLT 2.0), tailing factor (NMT 1.5), theoretical plate count (NLT 3000) and % RSD for peak area of six replicate injections of LMS standard (%RSD NMT 2.0). The system suitability data is reported in Table 4.

Specificity

The specificity of the method was performed by observing interference of any encountered ingredients present in the formulations. The test results obtained were compared with the results of those obtained for standard drug. It was shown that those ingredients did not interfering with the elution of nevirapine. Furthermore the well-shaped peaks also indicate the specificity of the method. The results for specificity are presented in Table 5. The chromatogram for placebo indicating the specificity of developed method is shown in Figure 9.

Table 4: Optimized chromatographic conditions and system suitability parameters of proposed RP-HPLC method for nevirapine

Parameter	Chromatographic conditions
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph
Column	WELCHROM C ₁₈ Column (4.6 mm i.d. X 250 mm, 5 μ m particle size)
Detector	SHIMADZU SPD-20A prominence UV-Vis detector
Diluents	Methanol: Acetonitrile: Water (50:30:20, v/v, pH-4.6 using o-phosphoric acid)
Mobile phase	Methanol: Acetonitrile: Water (50:30:20, v/v, pH-4.6 using o-phosphoric acid)
Column modifier	Triethylamine (0.5 mL)
Flow rate	1 mL/min.
Detection wave length	UV at 225 nm.
Run time	5 minutes
Column back pressure	156 kgf
Temperature	Ambient temperature(25°C)
Volume of injection loop	20 μ L
Retention time (t _R)	3.660 min
Theoretical plates [th.pl] (Efficiency)	10,706
Theoretical plates per meter [t.p/m]	214,119
Tailing factor (asymmetry)	1.038

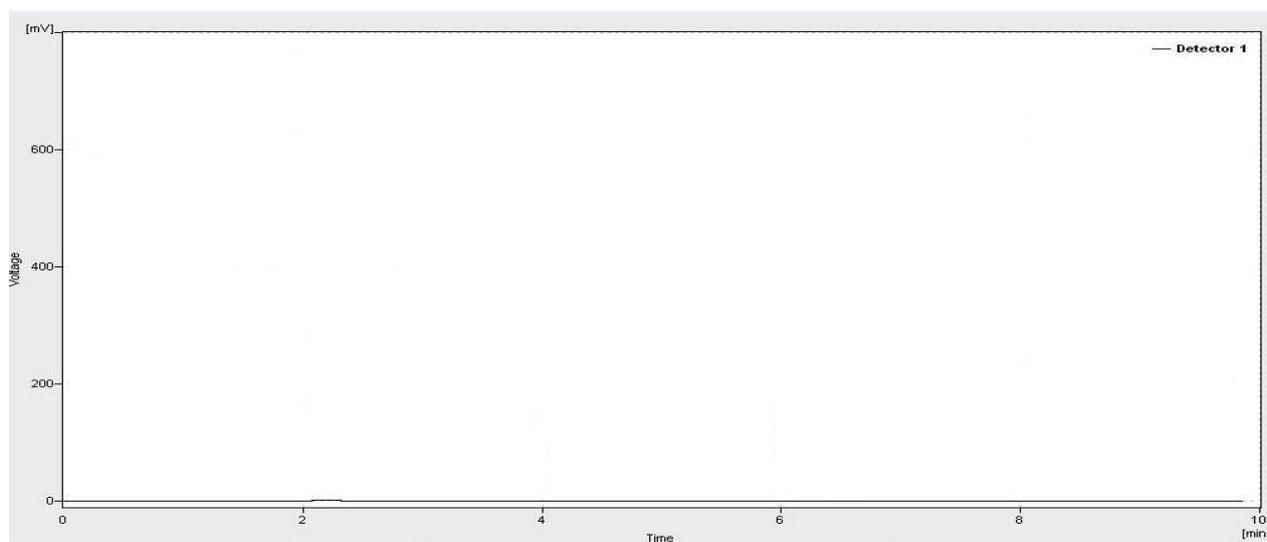


Fig. 9: Chromatogram of placebo

Table 5: Specificity study for nevirapine

Name of the solution	Retention time, (t _R) min.
Mobile phase	No peaks
Placebo	No peaks
Nevirapine, 10 µg/mL	3.663 min.

Precision

For evaluating the Intra-day (within-day) precision was investigated by replicate applications and measurements of peak area for LMS for six times on the same day under similar conditions. Inter-day (between-day) precision was calculated from % RSD values obtained from repeating the assay of same sample for six times on two different days. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0. Table 6 and Table 7 show the intra-day and inter-day precision results, respectively.

Accuracy/Recovery

Accuracy is the degree of agreement between a measured value and the accepted reference value. The accuracy studies of the proposed method was performed in triplicate samples at 3 different concentrations equivalent to 50%, 100% and 150% of the active ingredient, by spiking a known amount of NVP standard with placebo. The recovered amount of NVP, % RSD of recovery, % recovery of each concentration is calculated to determine the accuracy. Table 8 shows the recovery results for accuracy study of NVP.

Robustness

Robustness of the method was performed by intentionally modifying the chromatographic conditions such as the composition of mobile

phase, flow rate, detection wavelength. The chromatographic parameters of each analyte such as retention time, tailing factor and number of theoretical plates were measured at each changed conditions. Robustness is the ability to provide accurate and precise results under a variety of conditions. Table 9 shows the results for robustness study which indicated that the small change in the conditions did not significantly affect the determination of NVP.

Table 6: Results of precision study (intra-day) for nevirapine

Sample	Concentration (µg/mL)	Injection no.	Peak area (mV.s)	%RSD [#]
Nevirapine	10	1	2304.129	0.5512
		2	2297.824	
		3	2270.743	
		4	2292.296	
		5	2288.331	
		6	2276.712	

[#] Acceptance criteria < 2.0.

Table 7: Results of precision study (inter-day) for nevirapine

Sample	Concentration (µg/mL)	Injection no.	Peak area (mV.s)	%RSD [#]
Nevirapine	10	1	2315.354	0.9647
		2	2317.572	
		3	2285.628	
		4	2272.711	
		5	2308.372	
		6	2267.121	

[#] Acceptance criteria < 2.0

Table 8: Recovery data of for nevirapine

Recovery level	Amount added (mg)	Amount recovered (mg)	% recovery	Mean % Recovery ± SD	%RSD [#]
50%	12.31	12.12	98.45	99.45 ± 0.876	0.8807
	12.26	12.27	100.08		
	12.21	12.19	99.83		
100%	24.76	24.63	99.47	99.71 ± 0.384	0.3855
	24.87	24.75	99.51		
	24.84	24.88	100.16		
150%	36.81	36.93	100.32	100.00 ± 0.394	0.3942
	37.04	36.88	99.56		
	36.89	36.94	100.13		

[#] acceptance criteria < 2.0

Table 9: Robustness results of nevirapine

S. No.	Parameter ^a	Optimized	Used	Retention time (t _R), min	Plate count [§]	Peak asymmetry [#]	Remark
1.	Flow rate (±0.2 mL/min)	1.0 mL/min	0.8 mL/min	3.886	11185	1.062	*Robust
			1.2 mL/min	3.024	10384	1.073	*Robust
2.	Detection wavelength (±5 nm)	225 nm	230 nm	3.667	10682	1.047	Robust
			220 nm	3.657	10704	1.055	Robust
3.	Mobile phase composition (methanol content ±5 %)	50:50, v/v	55:45, v/v	3.976	11273	1.069	*Robust
			45:55, v/v	3.466	10629	1.055	*Robust

Acceptance criteria (Limits): [#]Peak Asymmetry < 1.5, [§]Plate count > 3000, * Significant change in Retention time

LOD and LOQ

Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. For calculating Limit of Detection and Limit of Quantitation, following formulae are used. LOD= 3.3σ/S and LOQ= 10σ/S, where SD=standard deviation of response (peak

area) and S= slope of the calibration curve. The LOD and LOQ values are presented in Table 10. The results of LOD and LOQ supported the sensitivity of the developed method.

Table 10: LOD and LOQ

Limit of Detection(LOD)	0.0815 µg/mL
Limit of Quantitation(LOQ)	0.2470 µg/mL

RESULTS AND DISCUSSION

In the present work, conditions were optimized for the development and validation of a precise, sensitive, rapid and accurate HPLC method for the determination of NVP in bulk drug and in pharmaceutical dosage forms. In order to achieve remarkable retention time and peak asymmetry, a C₁₈ stationary phase column (250mm X 4.6mm i.d, 5 µm particle size) and mobile phase composed of methanol, acetonitrile and HPLC grade water in a ratio of 50:30:20, v/v, with pH adjusted to 4.6 using o-phosphoric acid and triethylamine as column modifier at a flow rate of 1mL/min was selected. The retention time for NVP was found as 3.663 min. UV spectra of NVP showed that the drug absorbed maximum at 225 nm, so this wavelength was selected as the detection wavelength. A plot was drawn between concentration of the component and peak area. It was found to be linear in the range of 2-10µg/mL for NVP with good correlation coefficient (0.9999). On slight variation in the mobile phase ratio of upto ±5 %, the change in the peak asymmetry, plate count and retention time are within the limits which indicated that the method is robust. This shows that the method is having all system suitability parameters are found within the standard limits. The comparison of chromatograms of placebo, standard and sample, there was no interference observed from the peaks of placebo, standard and sample. It shows that the method is specific. Precision and accuracy of the developed methods are expressed in % RSD and % recovery of the API respectively. Low % RSD value and high % recovery indicate that the method is highly precise and accurate. The precision studies were performed and the % RSD of the determinations was found to be 0.5512 for intra-day precision and 0.9647 for inter-day precision which are within the limits. The accuracy of the method was found to be good with the overall % RSD for recovery at 50%, 100% and 150% levels were all within the limits. Method validation following ICH guidelines indicated that the developed method had high sensitivity with LOD of 0.0815 µg/mL and LOQ of 0.2470 µg/mL. The assay results of tablets by applying the HPLC method was found to be within the proposed pharmacopoeial limits and the assay values were found to be 102.40 ± 0.377 %.

CONCLUSION

A HPLC method was developed for the quantification of NVP in bulk and dosage forms. The assay gives a linear response across a wide range of concentration and it utilizes a mobile phase which can be easily prepared and diluent is economical and readily available. The developed HPLC method offers various advantages like rapid, good peak symmetry and phenomenal linearity, highly sensitive, simple, precise, accurate and robust. The drug solutions employed in the study were stable upto 48 hours. These attribute the high quality of the method. So the developed method can be used for analysis of NVP samples in Quality Control laboratory.

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REFERENCES

1. Drug today medical journal, Lorina publication (India) Inc., Delhi-91, 2012, DT 78, Vol-1:578.

2. Vishal M, Tara Shankar B, Ishvarchandra Parmar, Virag Shah. Current treatment of hiv infection a review simplified: An understanding about hiv infection and anti hiv drugs mechanism of action, Int J Pharm Pharm Sci 2012; 4 Suppl 1:1-15.
3. Sarkar M, Khandavilli S, Panchagnula R. Development and validation of RP-HPLC and ultraviolet spectrophotometric methods of analysis for the quantitative estimation of antiretroviral drugs in pharmaceutical dosage forms. Journal of Chromatography B 2006; 830(2):349-354.
4. Anbazhagan S, Indumathy N, Shanmugapandiyan P, Sridhar SK. Simultaneous quantification of stavudine, lamivudine and nevirapine by UV spectroscopy, reverse phase HPLC and HPTLC in tablets. Journal of pharmaceutical and biomedical analysis 2005; 39(3):801-804.
5. Pav JW, Rowland LS, Korpalski DJ (1999). HPLC-UV method for the quantitation of nevirapine in biological matrices following solid phase extraction. Journal of pharmaceutical and biomedical analysis 1999; 20(1):91-98.
6. Rezk NL, Tidwell RR, Kashuba AD. Simultaneous determination of six HIV nucleoside analogue reverse transcriptase inhibitors and nevirapine by liquid chromatography with ultraviolet absorbance detection. Journal of Chromatography B 2003; 791(1):137-147.
7. Ramachandran G, Hemanthkumar AK, Kumaraswami V, Swaminathan S. A simple and rapid liquid chromatography method for simultaneous determination of zidovudine and nevirapine in plasma. Journal of Chromatography B 2006; 843(2):339-344.
8. Droste JAH, Verweij-van Wissen CPW GM, Burger DM. Simultaneous determination of the HIV drugs indinavir, amprenavir, saquinavir, ritonavir, lopinavir, nelfinavir, the nelfinavir hydroxymetabolite M8, and nevirapine in human plasma by reversed-phase high-performance liquid chromatography. Therapeutic drug monitoring 2003; 25(3):393-399.
9. Lopez RM, Pou L, Gomez MR, Ruiz I, Monterde J. Simple and rapid determination of nevirapine in human serum by reversed-phase high-performance liquid chromatography. Journal of Chromatography B: Biomedical Sciences and Applications 2001; 751(2):371-376.
10. Samee W, Srilamai P, Ongart S, Suwannaratana R, Sornchaitawatwong C, Vorarat S. Simultaneous Determination of Lamivudine, Stavudine and Nevirapine in the Presence of Their Acid-Induced Degradation Products by HPLC. Thai Pharm Health Sci J 2007; 2(1):39-45.
11. Fan B, Stewart JT. Determination of zidovudine/ lamivudine/ nevirapine in human plasma using ion-pair HPLC. Journal of pharmaceutical and biomedical analysis 2002; 28(5):903-908.
12. van Heeswijk RP, Hoetelmans RM, Meenhorst PL, Mulder JW, Beijnen JH. Rapid determination of nevirapine in human plasma by ion-pair reversed-phase high-performance liquid chromatography with ultraviolet detection. Journal of Chromatography B: Biomedical Sciences and Applications 1998; 713(2):395-399.
13. Fan B, Stewart JT. Determination of zidovudine/ zalcitabine/ nevirapine in human plasma by ion-pair HPLC. Journal of liquid chromatography & related technologies 2001; 24(19):3017-3026.
14. ICH, Harmonized Tripartite Guideline. Validation of Analytical Procedure: Methodology (Q2B). International Conference on Harmonization, 1997.