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ANALYTICAL RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF NEVIRAPINE IN API AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

An accurate, precise, specific, simple and rapid RP-HPLC method was developed and subsequently validated for the determination of Nevirapine in API and Pharmaceutical dosage form. Better separation of the drug was achieved on Waters ODS (C_{18}) RP Column, 250 mm x 4.6 mm. with a mobile phase consisting of a mixture of Methanol: Phosphate buffer in the ratio of 75:25 v/v at a flow rate of 1ml/min, with detection at 279nm using UV-Visible Detector. The developed method was validated for different parameters such as linearity, accuracy, precision, limit of detection (LOD), limit of Quantization (LOQ), robustness and the results were found to be within the limits according to ICH (International Conference on Harmonization) guidelines. The retention time of Nevirapine was found to be 3.462 min. The method was found to be linear in the range of 0- 14µg/ml with a correlation coefficient (r2) of 0.999. The LOD and LOQ of the method were calculated to be 0.003 and 0.009µg/ml respectively. The Precision was estimated by employing repeatability; intra-day and inter-day studies and the results were calculated as %RSD values and were found to be within the limits. The average recovery of the analyte was found to be within the limit which confirms the accuracy of the method.

KEYWORDS: Nevirapine, RP-HPLC, Method Development, ICH Guidelines.

1. INTRODUCTION

A potent, non-nucleoside reverse transcriptase inhibitor,[1,2,3] (NNRTI) used in combination with nucleoside analogues for treatment of Human Immunodeficiency Virus Type 1 (HIV-1) infection and AIDS. Structurally, nevirapine belongs to the dipyridodiazepinone chemical class. For use in combination with other antiretroviral drugs in the ongoing treatment of HIV-1 infection. Nevirapine is a non-nucleoside reverse transcriptase inhibitor (nNRTI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1),[4,5,6] HIV-2 RT and eukaryotic DNA polymerases (such as human DNA polymerases alpha, beta, or sigma) are not inhibited by nevirapine. Nevirapine is, in general, only prescribed after the immune system has declined and infections,[7,8] have become evident. It is always taken with at least one other HIV medication,[9,10] such as Retrovir or Videx. The virus can develop resistance to nevirapine if the drug is taken alone, although even if used properly, nevirapine is effective for only a limited time. Nevirapine binds directly to reverse transcriptase (RT) and blocks the RNA-dependent and DNA-dependent DNA polymerase activities by causing a disruption of the enzyme's catalytic site. The activity of nevirapine does not compete with template or nucleoside triphosphates.[11,12] The IUPAC,[13] Name for Nevirapine is 2-cyclo propyl-7-methyl-2,4,9,15-tetra azatricycle [9.4.0.0^{3},^{8}] penta deca-1(11),3,5,7,12,14-hexaen-10-one. The molecular formula,[14] of Nevirapine is $C_{15}H_{14}N_4O$. The molecular weight is 266.2979g/mol. The chemical structure,[15,16] of Nevirapine shown in following Fig-1.

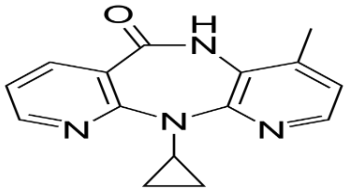

Fig. 1: Structure of Nevirapine.

2. METHODOLOGY

2.1. Instruments

Table 1: List of Instruments.

S. No.	Instruments/Equipments/Apparatus
1.	**HPLC** with Empower2 Software with Isocratic with UV-Visible Detector.
2.	**ELICO SL-159** UV – Vis spectrophotometer
3.	Electronic Balance **(SHIMADZU ATY224)**
4.	Ultra Sonicator **(Wensar wuc-2L)**
5.	Vacuum filtration unit
6.	**Waters ODS (C_8) RP Column, 250 mm x 4.6 mm.**
7.	P^H Analyzer **(ELICO)**

2.2. Chemicals / Reagents

Table 2: List of Chemicals.

S. No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
3.	Dipotassium hydrogen orthophosphate	96%	L.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
5.	Potassium dihydrogen orthophosphate	99.9	L.R.	Sd fine-Chem ltd; Mumbai
6.	Orthophosphoric acid	99.9	L.R.	Sd fine-Chem ltd; Mumbai
7.	Glacial acetic acid	99.99	L.R	Sd fine-Chem ltd; Mumbai

3. METHOD DEVELOPMENT

3.1 HPLC Instrumentation & Conditions

The HPLC system employed was **WATERS** with Empower2 Software with Isocratic with UV-Visible Detector.

3.2 Initialization of the instrument

The HPLC instrument was switched on. The column,[17] was washed with HPLC water for 45 minutes. The column was then saturated with mobile phase for 45 minutes. The mobile phase was run to find the peaks. After 20 minutes the standard drug solution was injected in HPLC.[18,19,20]

3.3 Mobile Phase Preparation

Preparation of Phosphate buffer

Accurately weigh about 6.8 grams of Potassium dihydrogen orthophosphate was and transfer into a 1000ml (1 litre) beaker, dissolved and diluted to 1000ml with HPLC water (0.01M). The Ph[21] was adjusted to 3.60 with ortho-phosphoric acid.

Mobile phase preparation

The mobile phase used in this analysis consists of a mixture of Buffer (0.01M potassium dihydrogen phosphate & pH adjusted to 2.2 with ortho phosphoric acid) and Methanol in a ratio of 40:60

3.4 Preparation of solutions

Preparation of Standard solution

Working concentration should be around 10µg/ml.

Accurately weighed around 25mg of Nevirapine working,[22] standard, taken into a 25 ml volumetric flask, then dissolved and diluted to volume with the mobile phase to obtain a solution having a known concentration of about 1000 mcg/ml.

Further dilutions have been made to get the final concentration of 10µg/ml.

Preparation of Test solution

Diluted quantitatively an accurately measured volume of label claim solution with diluents to obtain a solution containing about a linear range.

3.5 Diluent

Mobile phase,[23] can be used as diluent.

4. METHOD VALIDATION

1. Accuracy

Recovery study

To determine the accuracy,[25] of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of Nevirapine were taken and added to the pre-analyzed formulation of concentration 10µg/ml. From that percentage recovery,[24] values were calculated. The results were shown in Table-3.

Table 3: Accuracy Readings.

Sample ID	Concentration (µg/ml)		Peak Area	% Recovery of Pure drug	Statistical Analysis
	Amount Added	Amount Found			
S_1 : 80 %	8	8.079	595625	100.987	Mean= 100.7453% S.D. = 0.670976 % R.S.D.= 0.66601
S_2 : 80 %	8	8.021	591457	101.262	
S_3 : 80 %	8	7.999	589875	99.987	
S_4 : 100 %	10	9.998	734587	99.98	Mean= 99.82667% S.D. = 0.517333 % R.S.D.= 0.51823
S_5 : 100 %	10	9.925	729268	99.25	
S_6 : 100 %	10	10.025	736524	100.25	
S_7 : 120 %	12	11.910	872949	99.25	Mean= 100.0357% S.D. = 0.837025 % R.S.D.= 0.83672
S_8 : 120 %	12	12.110	887456	100.916	
S_9 : 120 %	12	11.993	878975	99.941	

2. Precision

2.1 Repeatability

The precision,[26] of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug. Nevirapine

(API) the percent relative standard deviations were calculated for Nevirapine is presented in the Table-4.

Table 4: Repeatability Results of Precision.

HPLC Injection Replicates of Nevirapine	Retention Time	Peak Area
Replicate – 1	3.459	729874
Replicate – 2	3.462	728957
Replicate – 3	3.462	729268
Replicate – 4	3.463	729842
Replicate – 5	3.464	728714
Replicate – 6	3.463	729863
Average	**3.462167**	**729419.7**
Standard Deviation	**0.001722**	**513.0999**
% RSD	**0.049749**	**0.070344**

2.2 Intermediate Precision

2.2.1 Intra-assay & inter-assay

The intra & inter day variation,[27] of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Nevirapine revealed that the proposed method is precise.[28]

Table 5: Results of intra-assay & inter-assay.

Conc. Of Nevirapine (API) (µg/ml)	Observed Conc. Of Nevirapine (µg/ml) by the proposed method			
	Intra day		Inter day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	8.03	1.03	7.98	1.02
10	10.05	0.85	10.03	0.53
12	12.01	0.97	11.02	0.98

3. Linearity and Range

The calibration curve showed good linearity in the range of 0-14µg/ml, for Nevirapine (API) with correlation coefficient[29] (r^2) of 0.999 (Fig-2). A typical calibration curve has the regression equation of y = 72394x + 10725 for Nevirapine.

To evaluate the linearity, serial dilution of analyte were prepared from the stock solution was diluted with mobile phase to get a series of concentration ranging from 6, 8, 10, 12 and 14µg/ml. The prepared solutions were filtered through whatmann filter paper (No.41). From these solutions, 20µl injections of each concentration were injected into the HPLC system and chromatographed,[28] under the optimized conditions. Calibration curve,[30] was

constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis). The results which are given in Table below were within acceptable limits.

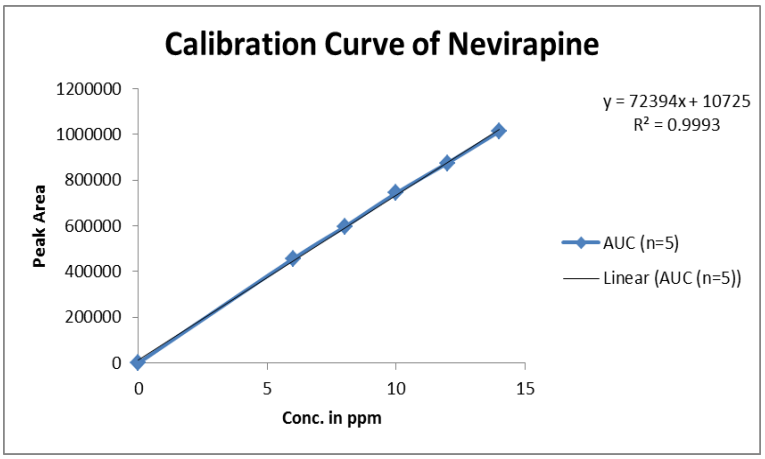

Fig. 2: Calibration curve of Nevirapine (API).

Table 6: Linearity results of Nevirapine.

CONC.	AUC (n=5)
0	0
6	455874
8	595872
10	743542
12	875632
14	1013121

4. Method Robustness

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & Acetonitrile content in mobile phase (±2%) studied to determine the robustness,[31,32] of the method are also in favour of (Table-7, % RSD < 2%,[33]) the developed RP-HPLC method for the analysis of Nevirapine (API).

Table 7: Result of Method Robustness Test.

Change in parameter	% RSD
Flow (1.1 ml/min)	0.08
Flow (0.9 ml/min)	0.49
Temperature (27^{0}C)	0.32
Temperature (23^{0}C)	0.19
Wavelength of Detection (281 nm)	0.41
Wavelength of detection (277 nm)	0.38

5. LOD & LOQ

The Minimum concentration level at which the analyte can be reliable detected (LOD[34]) & quantified (LOQ,[35]) were found to be 0.8 & 2.5 µg/ml respectively.

6. System Suitability Parameter

System suitability parameter test is an integral part of many analytical procedures. This test is based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters,[36] were established. The data are shown in Table-8.

Table 8: Data of System Suitability Parameter.

S. No.	Parameter	Limit	Result
1	Resolution	Rs > 2	9.15
2	Asymmetry	T ≤ 2	Nevirapine=0.12
3	Theoretical plate	N > 2000	Nevirapine=3246

7. Assay of Nevirapine in Dosage Form

Nevirapine 200mg

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 100 mg of drugs were transferred to 100 ml volumetric flask, and 70 ml of HPLC grade methanol was added and solution was sonicated for 15 minutes, there after volume was made up to 100 ml with same solvent. Then 10 ml of the above solution was diluted to 100 ml with HPLC grade methanol. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. From this stock solution (3.5 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system.

The solution prepared was injected in five replicates into the HPLC system and the observations were recorded.

A duplicate injection of the standard solution was also injected into the HPLC system and the peak areas were recorded. The data are shown in Table-9.

Assay

$$\% \text{ Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{AW}{LC} \times 100$$

Where:

AT = Peak Area of Test obtained with test preparation

AS = Peak Area of Standard obtained with standard preparation

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity of working standard

Assay,[37] was performed as described in previous chapter. Results obtained are tabulated below:

Table 9: Assay of Nevirapine Tablets.

Brand name of tablets	Labelled amount of Drug (mg)	Mean (±SD) amount (mg) found by the proposed method (n=6)	% Purity
Nevirex Tab (Imunus (Aurobindo)	200	199.65 (±0.09)	99.38 % (±0.48)

RESULT AND DISCUSSION

The amount of drug in Nevirex tablets was found to be 199.65 (±0.09) mg/tab for Nevirapine and 99.38 (±0.48) mg/tab for Nevirapine.

Results and Discussion

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Nevirapine different chromatographic conditions were applied & the results observed are presented in previous chapters.

Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution.

In case of RP-HPLC various columns are available, but here Waters ODS C_{18}, 5µm, 25cmx4.6mm i.d. column was preferred because using this column peak shape, resolution and absorbance were good.

Mobile phase & diluent for preparation of various samples were finalized after studying the solubility of API in different solvents of our disposal (methanol, Acetonitrile, dichloromethane, water, 0.1N NaOH, 0.1NHCl).

The drug was found to be Nevirapine is soluble in organic solvents such as DMSO and dimethyl formamide (DMF), which should be purged with an inert gas. Using these solvents with appropriate composition newer methods can be developed and validated.

Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Nevirapine it is evident that most of the HPLC work can be accomplished in the wavelength range of 240-300 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 20 µl were found to be the best analysis.

The result shows the developed method is yet another suitable method for assay & stability which can help in the analysis of Nevirapine in different formulations.

CONCLUSION

A sensitive & selective RP-HPLC method has been developed & validated for the analysis of Nevirapine API and tablet dosage form.

Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

The result shows the developed method is yet another suitable method for assay, purity which can help in the analysis of Nevirapine in different formulations.

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