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Optimization and validation of nevirapine related substances in formulation by RP-HPLC method

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Abstract
A simple RP- HPLC method was developed and validated for Nevirapine and its related substances in bulk drug. The developed method was found to be reliable, precise, accurate, cost effective and reproducible. Nevirapine related impurities are spiked and quantified at 0.2% specification level of test concentration and these impurities are found to be within the acceptable levels in the bulk drug substance. Hence the developed method was found to be suitable for the detection as well as quantification of impurities of Nevirapine. Developed method was validated according to ICH guidelines. Specificity was established by spiking impurities at 0.2% specification level and it was observed there is no interference from blank as well as impurities. This method was found to be accurate over the range of 85 – 115% and linear up to 120% with an acceptable correlation coefficient i.e. 0.999. The sensitivity of the method was established by LOD & LOQ which was within the acceptable range. This method was found to be precise over the suitable range with a % RSD of less than 15. The method was found to be sufficiently robust and rugged with an acceptable system suitability factors and % RSD of below 15. Hence the developed and validated RP-HPLC method can be successfully employed for the detection and quantification of Nevirapine and its related impurities in bulk drug as well as in formulation.

Keywords: RP- HPLC, nevirapine, reliable, precise, accurate

1. Introduction
Pharmaceutical Analysis may be defined as the application of analytical procedures used to determine the purity, safety and quality of drugs and chemicals. Pharmaceutical Analysis includes both qualitative and quantitative analysis of drugs and pharmaceutical substances starts from bulk drugs to the finished dosage forms [1].
Chromatography has been defined as a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in the order of their increasing distribution coefficients with respect to the stationary phase. Chromatographic separation of two components depends on the fact that they have different partition or distribution coefficients between the stationary and mobile phases [2- 3].

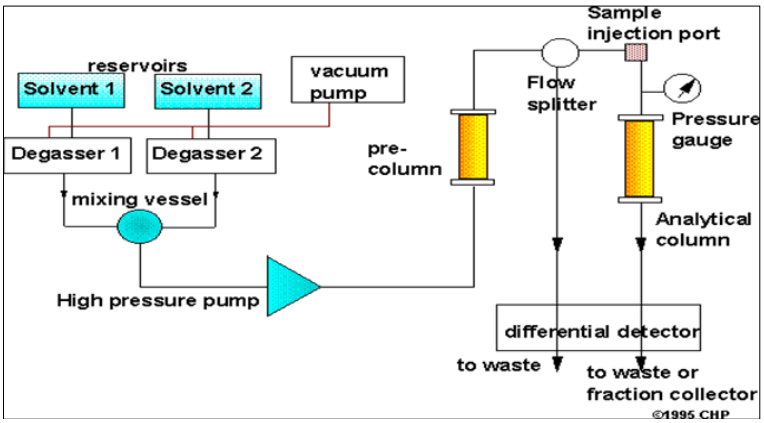
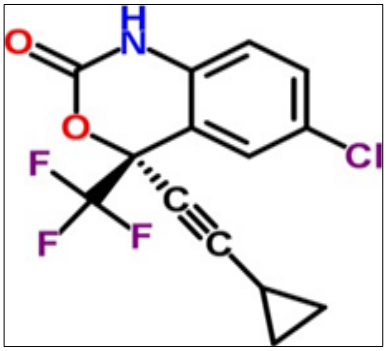


Fig 1: Instrumentation of HPLC

Nevirapine
Structure



Nevirapine is a non-nucleoside reverse transcriptase inhibitor (nNRTI) with activity against Human Immunodeficiency Virus Type 1 (HIV-1). 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 have become evident. It is always taken with at least one other HIV medication 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.

Nevirapine is readily absorbed (greater than 90%) after oral administration in healthy subjects and adults with HIV-1 infection. The absolute bioavailability in healthy adults following a single dose administration is 93 ± 9% (mean ± SD) for a 50 mg tablet and 91 ± 8% for an oral solution. Peak plasma nevirapine concentrations of 2 ± 0.4 mcg/mL (7.5 micromolar) were attained by 4 hours following a single 200 mg dose. Nevirapine tablets and suspension have been shown to be comparably bioavailable and interchangeable at doses up to 200 mg. When the oral tablet is given with a high-fat meal, the extent of absorption is compared to that of the fasted-state.

Table 1: Drug profile of Nevirapine

Name	Nevirapine
IUPAC name	2-cyclopropyl-7-methyl-2,4,9,15-tetraazatricyclo [9.4.0.0^{3,8}] pentadeca-1(11),3,5,7,12,14-hexaen-10-one
Molecular formula	$C_{15}H_{14}N_4O$
Molecular weight	266.2979
PKa	P^{Ka} (strongest acidic) – 12.52 P^{Ka} (strongest basic) – (-1.5).
Melting point	139-141 °C
Category	Anti-HIV Agents Non-nucleoside Reverse Transcriptase Inhibitors
Appearance	White to slight pink powder.
Solubility	Freely soluble in methanol. Practically insoluble in water.
Brand formulations	Stocrin, Sustiva, Efavir.

Impurity Profile

Table 2: Impurity profile

S. No	Name of the impurity	Pharmacopoeia	Category of related substances	Impurity specification level
1	IMP-1	International pharmacopoeia & USP	Degradation by hydrolysis	0.2%
2	IMP-2	International pharmacopoeia & USP	Process related and degradation by hydrolysis	0.2%

Nevirapine is a non-nucleoside reverse transcriptase inhibitor used as part of a management regimen for HIV-1 virus infection.

The proposed HPLC methods are found to have complicated procedure and longer run time so a study was proposed to develop a simple method with lesser run time. The developed method was validated according to ICH guidelines. The different objectives that are to be achieved during development of new analytical and validated method for related substances of Nevirapine are:

- To develop new analytical method
- To validate the developed analytical method according to ICH guidelines
- To achieve reproducibility and minimize the error
- To achieve quantification of impurities at their specification level [4-9].

2. Materials and Methods

Table 3: Standards and samples

S. No.	Material name	Potency
1	Nevirapine drug substance	NA
2	IMP-1	99.8%
3	IMP-2	99.5%

Table 4: Instruments

S. No.	Instrument	Make and model
1	HPLC	Water's HPLC
2	Analytical balance	Metler Toledo

3	P^H meter	Metler Toledo
4	Sonicator	Spectrochem
5	U.V. detector	Waters model
6	P.D.A detector	Waters model

Table 5: Reagents and chemicals

S. No	Name of the material	Grade	Make
1	Potassium di hydrogen orthophosphate	HPLC grade	Merck
2	Ortho phosphoric acid	AR	Merck
3	Acetonitrile	HPLC	Merck
4	Methanol	HPLC	Merck
5	Water	Milli Q	Merck

Method development parameters

Selection of the following parameters is very important in method development.

- Mode of Chromatography
- Column
- Mobile phase composition and buffer p^H
- Wavelength
- Flow rate
- Injection volume
- Column temperature
- Solvent delivery system

Mode of chromatography

Selection of mode of chromatography is an important step in the method development. The basis for the selection of mode of chromatography is the nature of the molecule as well as its polarity. In this experiment reverse phase chromatographic mode was selected.

Selection of the column

Column plays an important role in the chromatographic separation. The following parameters should be considered while selecting a column.

- Length and diameter of the column
- Packing material
- Pore size, surface area and end capping
- Percentage of carbon loading

In this experiment Inertsil ODS 3V (250 X 4.6mm X 5µm) column was selected. It's a validated column and is having low surface silanol activity which is helpful in obtaining a good peak shape. This column is end capped and also shows high inertness to both acidic as well as basic components. It is having high durability over the P^H range of 2 to 9. It is also stable at high temperatures.

Selection of buffer p^H and mobile phase composition

Buffer and its strength play a major role in deciding the peak symmetry and separation. The strength of the buffer can be altered if necessary to achieve the required separation.

P^H of the buffer also plays an important role in achieving the chromatographic separation as it controls the elution properties by controlling the ionization characteristics. Buffer p^H is selected based on the P^{Ka} of the analyte molecule.

In this method initial trials were conducted by using phosphate buffer of p^H

Acidic p^H was used because Nevirapine has P^{Ka} of -1.5 which indicates that it is strongly acidic in nature & hence to maintain the analyte in unionized state acidic p^H (2.5) was selected. Acetonitrile is selected as an organic solvent because of its favorable UV transmittance, low viscosity and low back pressure. The peak shape and resolution was good with phosphate buffer of p^H 3: Acetonitrile but the run time was not satisfactory. Hence 0.1%Orthophoshoric acid in water: 0.1%Orthophoshoric acid in Acetonitrile which is having a p^H around 2.5 was employed as mobile phase with which good peak shape, peak symmetry, and reasonable run time was achieved.

Selection of wavelength

A solution containing 0.25mg/ml of Nevirapine working standard and IMP-1 and IMP-2 at their specification level was prepared, injected and scanned by using PDA detector from 200-400 nm.

In this method PDA detector has been used in full scan mode in order to scan all relevant wavelengths of sample. Then determine the lambda max of the sample's spectra using the software. Once the analysis is completed review the spectral data to determine which prominent peak wavelengths have the maximum signal to noise (S/N) ratio. These "peaks" can be used as the individual wavelengths for integration and purity determination. Edit the method to use the discreet wavelengths found in the spectra of sample as shown in the Fig.no.3. Using full scan mode will enable to know about any other components which absorb at wavelength far away from or near the analyte peak. These compounds can add or subtract signal from the main peak making it appear to be more or less concentrated (or more or less pure) than it actually is. In this experiment the maximum absorbance selected for Nevirapine and its related substances was 245nm [10-15].

3. Results and Discussion

Chromatograms of initial trials

Criteria for trial 1:

The main basis of the separation in trial 1 is p^H of the mobile phase. It is generally recommended to maintain the P^H of the mobile phase 2 units above or below the P^{Ka} of the drug molecule. P^{Ka} will depend on the structure of the molecule. The literature survey revealed that the Nevirapine is very strong acid (p^{Ka} of -1.50). Hence to maintain the P^H in acidic range the initial trails are conducted by using phosphate buffer of P^H 3.The mobile phase employed in trial -1 is Phosphate Buffer: Acetonitrile in the ratio of 50:50. Chromatogram for trial 1 is shown in Fig no: 4. Results of trial 1 are tabulated in table no: 13.

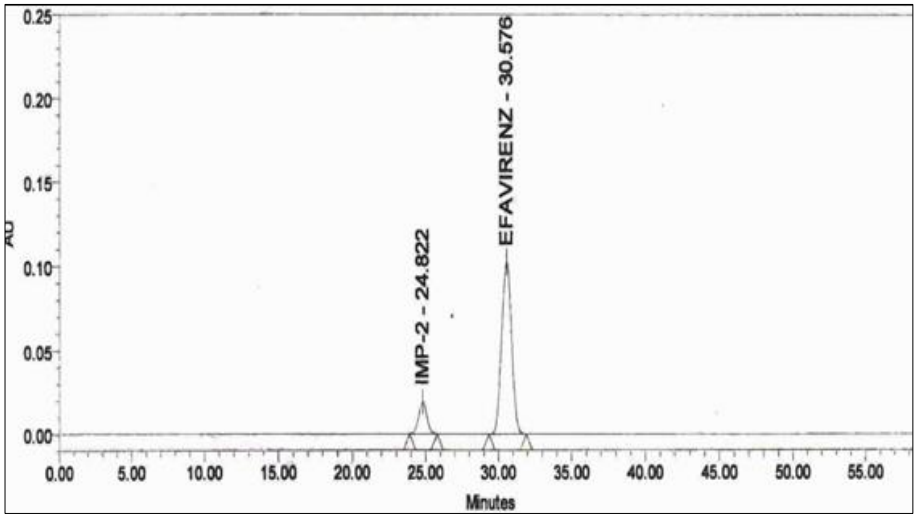


Fig 1: Chromatogram of trial 1

Table 6: Trial 1 results

Peak name	RT	Area	USP Resolution	USP Plate count	USP Tailing
IMP-2	24.822	747853		8861.63	1.00
Nevirapine	30.576	4929891	4.85	8928.01	1.00

Observation

Peak was eluted with good symmetry and resolution but run time was more.

Criteria for trial 2

The basic criterion selected in trial 2 is effect of polarity on separation. A molecule's structure, activity, and physicochemical characteristics are determined by the arrangement of its constituent atoms and the bonds between them. Within a molecule, a specific arrangement of certain atoms that is responsible for special properties and predictable chemical reactions is called a functional group. This structure often determines whether the molecule is polar or non-polar. Organic molecules are sorted into classes according to the principal functional groups each contains. Using a separation mode based on polarity, the relative chromatographic retention of different kinds of molecules is largely determined by the nature and location of these functional groups [35]. Hence in the trial 2 development was done mainly by focusing on the polarity.

Order of polarity

IMP-2 > Nevirapine > IMP-1

In the trial one the elution time was more. Hence the organic phase concentration has been increased in mobile phase so as to decrease the run time and to elute the IMP-1 which is relatively non polar when compared to IMP-2 and Nevirapine and also the effect of flow rate and temperature on the separation was observed. Chromatogram for trial 2 is shown in Fig no: 5 & results of trail 2 are shown in table no: 14.

Table 7: Trial 2 results

Peak name	RT	Area	USP Resolution	USP Plate count	USP Tailing
IMP-2	5.326	573101		5336.07	1.03
Nevirapine	6.233	5093251	2.93	5728.85	1.02
IMP-1	22.448	3111578	24.96	8548.36	0.97

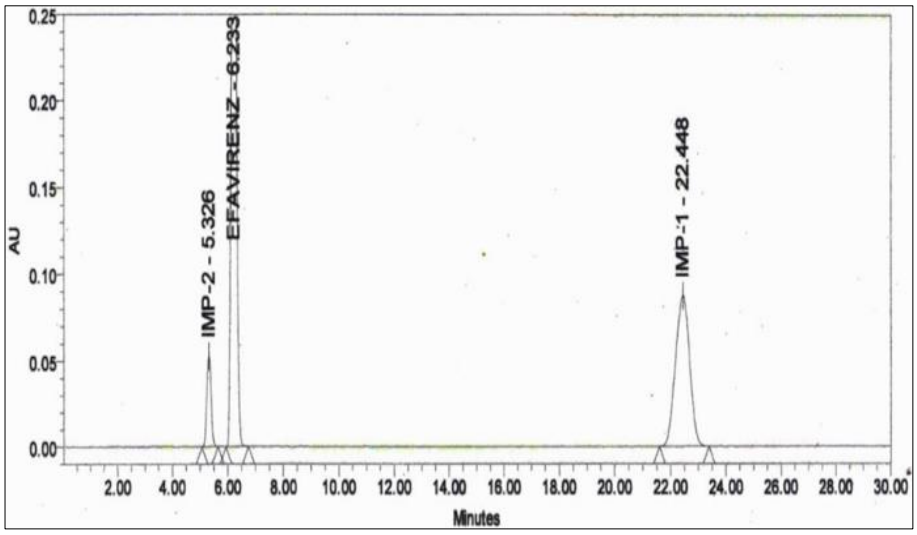


Fig 2: Chromatogram for trial 2

Observation

- Run time was decreased.
- Peak was eluted with good symmetry and resolution.

Criteria for trial 3

In trial 2 the organic phase concentration was very high in the mobile phase. It is generally recommended not to use the higher organic concentrations when we use salt buffers as it causes precipitation of buffer. Hence trial 3 was conducted by changing the mobile phase. The mobile phase used in trial 3 was 0.1% OPA in water: 0.1% OPA in acetonitrile in the ratio of 20:80. When this mobile phase was employed run time was decreased to 13.32 min from 22 min in the trial 2. By changing the temperature and flow rate the run time was still decreased to 9.42 min from 13.32 min. In trial 2 when higher concentration (0.5mg/ml) was used peak was distorted and peak purity is failed. Hence trial 3 was observed by decreasing the concentration to 0.25mg/ml. in optimized method injection volume is decreased to 10µl instead of 20µl because peak distortion was observed due to increased injection volume. Chromatogram for trial 3 is shown in Fig no: 6 & 7. And results are tabulated in table no: 15 & 16.

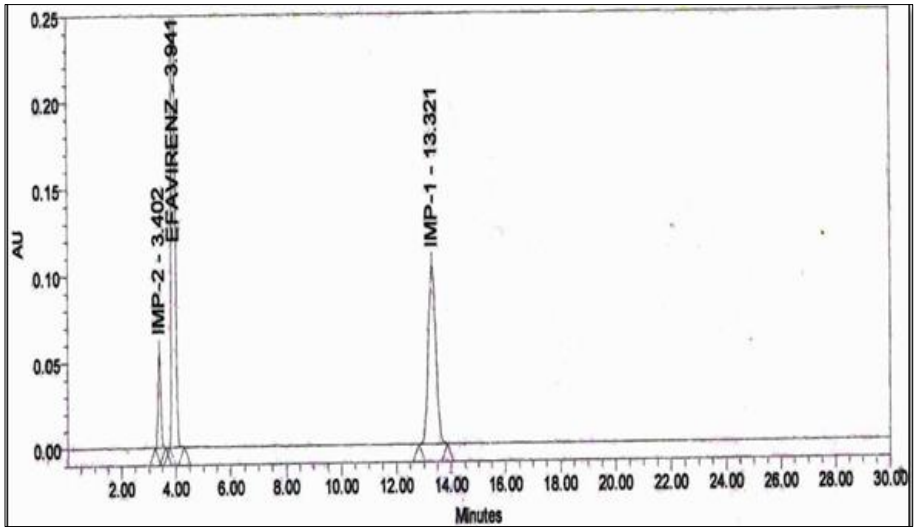


Fig 3: chromatogram for trial 3

Table 8: Trial 3 results

Peak name	RT	Area	USP Resolution	USP Plate count	USP Tailing
IMP-2	3.402	378851		5641.92	1.04
Nevirapine	3.941	3369949	2.74	6069.80	1.04
IMP-1	13.321	2050136	25.30	10148.59	1.01

Observation

Peak was eluted with a sufficient resolution and within a reasonable run time.

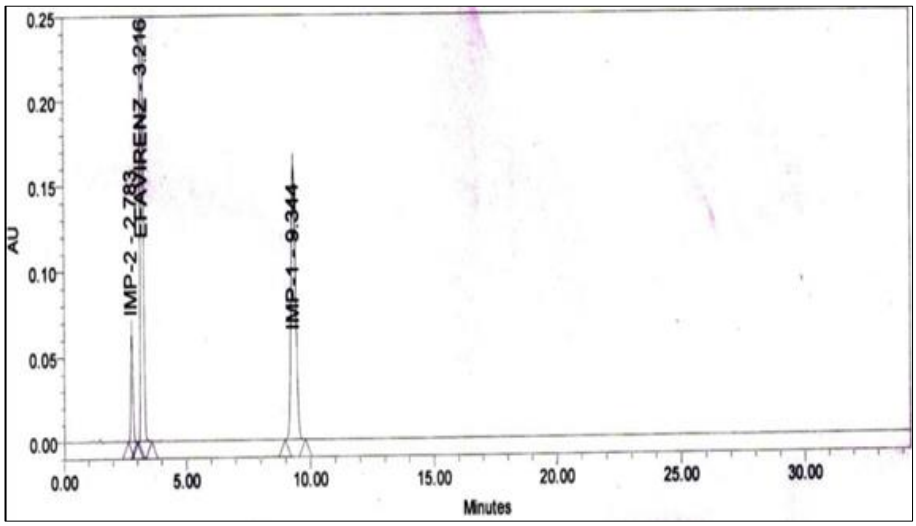


Fig 4: chromatogram for optimized method

Table 9: Results of optimized method

Peak name	RT	Area	USP Resolution	USP Plate count	USP Tailing
IMP-2	2.783	361876		5290.95	1.03
Nevirapine	3.216	3439364	2.71	6140.87	1.03
IMP-1	9.344	2087688	23.64	11601.56	1.02

Specificity

Blank Interference

Blank solution was prepared and injected. It was observed that no blank peaks were eluting at the retention time of Nevirapine peak and other impurities peaks.

Impurity Interferences

All impurities were spiked at their specification level and spiked and un-spiked sample solution and standard solution was analyzed. Peak purity of the Nevirapine in the spiked standard and spiked sample solution was found to be within the acceptable limit.

4. Conclusion

Developed method was validated according to ICH guidelines. Specificity was established by spiking impurities at 0.2% specification level and it was observed there is no interference from blank as well as impurities. This method was found to be accurate over the range of 85-115% and linear up to 120% with an acceptable correlation coefficient i.e. 0.999. The sensitivity of the method was established by LOD & LOQ which was within the acceptable range. This method was found to be precise over the suitable range with a % RSD of less than 15. The method was found to be sufficiently robust and rugged with an acceptable system suitability factors and % RSD of below 15. Hence the developed and validated RP-HPLC method can be successfully employed for the detection and quantification of Nevirapine and its related impurities in bulk drug as well as in formulation.

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