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High Resolution RP-HPLC Method for the Determination of Nevirapine and Associated Impurities

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ABSTRACT

Objective of the present research work is to develop a sensitive, selective and accurate new RPHPLC method with UV detection and determination for estimation of Nevirapine (NVP)and its impurities in bulk drug. The separation and quantification was achieved with Kromosil C18 isocratic column, (150 mm × 4.6 mm i.d., particle size 3.5 µm, maintained at ambient temperature), HPLC system (Peak LC P7000), a mixture of 20% acetonitrile, 80% buffer (sodium per chlorate) (v/v), at *p*H of 4.8 and the flow rate was set at 1.0 ml/min. and UV detection at 220 nm. The retention time for NVP, Impurity-A and Impurity-B were found to be 5.5, 7.8, 3.4 min respectively. The method was validated for Linearity, Accuracy, and Precision. The Limit of detection of NVP, Impurity-A and Impurity-B were found to be 0.03, 0.03, 0.03(µg/ml) respectively.

Keywords: Nevirapine, ICH guidelines, RP-HPLC, RS Method, Validation.

INTRODUCTION

NVP is a non-nucleoside reverse transcriptase inhibitor used to treat HIV-1 and AIDS. Possible side effects of NVP are Diarrhea, headache, mild nausea or stomach pain, tiredness, vomiting. Some severe allergic reactions like rash, tightness in the chest, swelling of the mouth, face, lips, or tongue, itching, hives, difficulty breathing.

MATERIALS AND METHODS

Pure forms (Above 99%) of NVP and Impurity-A, Impurity-B were obtained as gift samples from Hetero Labs, Hyderabad, India. HPLC grade solvents (Methanol, Acetonitrile, water) were procured from Merck, Mumbai, India. The mobile phase and all the solutions were filtered through a 0.45mm membranes prior (Merckmillipore) to use. Per chloric acid was purchased from S.D. Fine Chem Ltd., Mumbai, India.

Preparation of Mobile phase

Acetonitrile and Sodium per chlorate (*p*H: 4.8) were taken in20:80 ratio and mixed well. The *p*H of the solution was adjusted to 4.8 with Perchloric acid. The prepared mobile phase was filtered through 0.45mm filter membrane.

Preparation of stock solutions

1. NVP stock solution: 10 mg of NVP drug was dissolved in 100 ml Acetonitrile to obtain 100 µg/ml.
2. Impurity-A stock solution: 20 mg of standard Impurity-A was dissolved in 100 ml Acetonitrile to obtain 200 µg/ml
3. Impurity-B stock solution: 20 mg of standard Impurity-B was dissolved in 100 ml Acetonitrile to obtain 200 µg/ml

Preparation of standard solutions

0.5ml of standard stock and 0.5ml of impurities stock solutions are taken in to 100ml volumetric flask and make up to 100 ml with Acetonitrile to obtain 0.5ppm of NVP and 1ppm of impurity-A and impurity-B. The standard concentration equal to unknown impurity spec (0.1%) and impurity-A and B concentration equal to 0.2% (as per USP limit)

Preparation of sample solution

50mg of API sample taken in to 100ml of Acetonitrile to obtain500 µg/ml concentration sample solution**.**

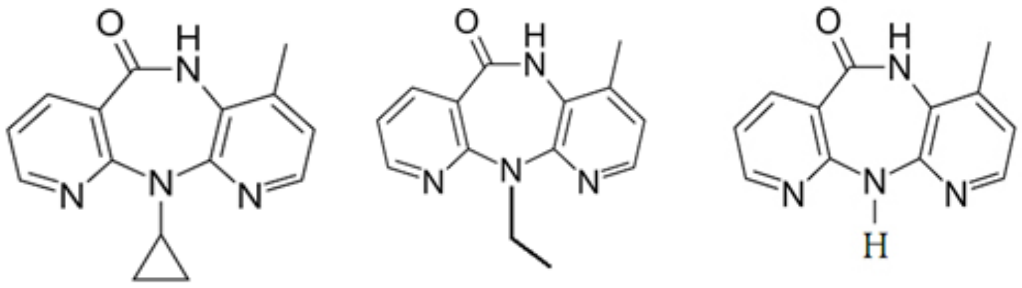
Apparatus and chromatographic condition

The method was developed and validated with HPLC system (Peak LC P7000) with isocratic pump, manual rheodyne injector with 20 µL volume loop and UV- VIS detector(UV7000) and PEAK Chromatographic version 1.06 . The API and impurity were scanned with UV-Visible spectrophotometer Tech comp UV2301 with Hitachi software. Kromasil C18 column (100mm ×4.6mm×3.5µ) RP-18 HPLC column (150 × 4.6 mm, 3.5micron) was used for separation. HPLC detector wavelength was fixed at 220 nm. Analysis was performed at ambient temperature.

RESULTS AND DISCUSSION

The aim of this work is to develop a RPHPLC method to quantify NVP and Impurity-A, Impurity-Bin Bulk drug. Previously few methods[5-19] are available for analysis of NVP in formulations and bulk drug.Ch Venkata Reddiah *et al*[20] reported one HPLC method for analysis of NVP and its impurities. While developing method at initial stage of the methoddevelopment trials done with $NH_4H_2PO_4$, Sodium per chlorate buffer solution at different *p*H and acetonitrile as solvent and C18 column but the separation of NVP and Impurity-Ais not good. Finally the best separation with good elution was achieved with Sodium per chlorate and acetonitrile at *p*H 4.8.Diluent and standard solution represented infigure-2 and 3. NVP and Impurity-A, Impurity-B are well separated and the peak shape, tailing factor (less than 2.0) and resolution also within the limit.

System suitability is an important test of method development of analytical procedures. System suitability test parameters are established for the developed method. Freshly prepared standard solution in to the system for three replicate injections (at 10 µg/ml, 20 µg/ml, 30 µg/ml) and calculated the percentage relative standard deviation(RSD) for area and retention time and the results found to be satisfactory. Three replicate standard solution results tabulated the results in table.1.



Nevirapine(NVP) **Impurity-1 (Compound-A)** **Impurity-2 (Compound-B)**

Fig.1: Chemical structure of NVP and its Impurities

Method validation

Once the HPLC method development was over, validated the developed method as perICH and FDA [1-5] guidelines with parameters like, linearity, precision, accuracy and range, ruggedness, robustness etc.

Precision of the developed method was evaluated by carrying out six different sample preparations for all individual and combination products. Percentage relative standard deviation (% RSD) was found to be less than 1% for within a day and day to day variations, which proves that that method is precise. Results were shown in Table-2.

For linearity test the standard solution was taken as 100% concentration and linearity range was fixed 25%, 50%, 75%, 100%, 125%, 150%. 200% Linearity solutions are prepared from stock solutions and standard solution by serial dilution. The linearity results were given in Table.3. The linearity graph was shown in Graph.1, Graph.2 and Graph.3.

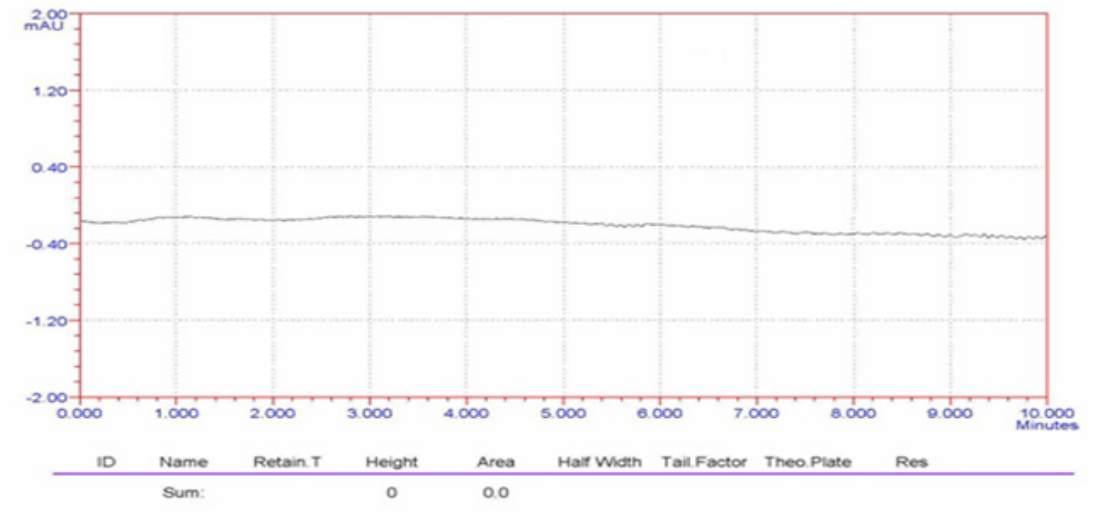


Fig. 2: Representative chromatogram of blank

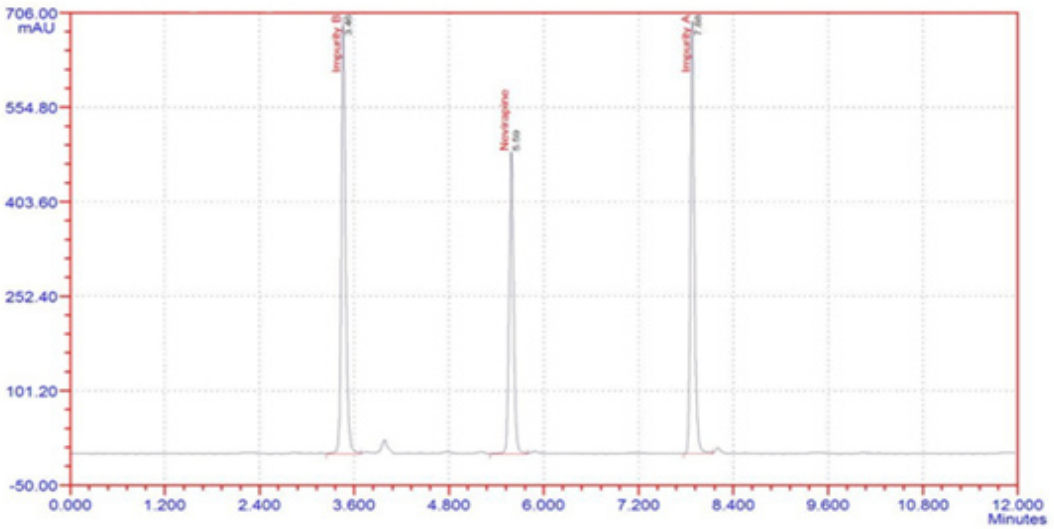


Fig. 3: Representative Standard chromatogram with NVP, Impurity-A, Impurity-B

The ruggedness of the method was determined by carrying out the experiment on other HPLC by different Analysts using different columns of similar types. The percentage RSD of six different preparations assay values with two different instruments, analysts and columns were given in table.4. Robustness of the method was determined by making small changes in the chromatographic conditions and found to be unaffected by small changes like*p*H changes ± 0.1, flow rate ± 1%, wavelength ±2 nm, temperature ± 2° C, and ± 2% change in organic solvent in the mobile phase. The Robustness results are shown in the Table 5.The LOQ and LOD concentrations of developed method are given in Table.4Analysis of NVP, Impurity-A, Impurity-B in analyzed in NVP Bulk drug by using developed HPLC method**.** The chromatogram was given shown in Figure.4

Forced degradation studies

50 mg of NVP was diluted in 100ml of 0.1 N HCl. and heated up to 400°C. 50 mg of NVP was diluted in 100 ml of 0.1 NaOH and heated up to 400°C. 50 mg of NVP was taken in 50 ml of H_2O_2 and make up to 100 ml with diluents. The three prepared solutions are injected and calculated percentage of

Table 1: System suitability test results of NVP, Impurity-A, Impurity-B

No of Injections	Concentraion (µg/ml)	R.T	Resolution	T. Plates	Tailing Factor	Peak Area
		System suitability of NVP				
1	10	5.509	16.24	26581	1.22	69812
2	10	5.505	16.31	27371	1.16	65957
3	10	5.490	15.83	27005	1.18	67781
1	20	5.492	15.78	26585	1.22	91533
2	20	5.497	16.01	27279	1.20	76300
3	20	5.517	16.27	26586	1.20	76609
1	30	5.525	16.08	26723	1.18	98420
2	30	5.515	15.89	26609	1.12	96883
3	30	5.503	16.00	27055	1.15	95925
		System suitability of Impurity-A				
1	10	7.847	17.75	59757	1.73	67747
2	10	7.852	17.55	54625	1.64	67779
3	10	7.852	17.72	55822	1.59	70602
1	20	7.825	17.63	57987	1.60	84307
2	20	7.857	17.96	58670	1.61	81767
3	20	7.839	17.52	58453	1.62	84501
1	30	7.857	17.70	60075	1.56	101203
2	30	7.850	17.26	53905	1.67	103745
3	30	7.850	17.78	58217	1.60	103959
		System suitability of Impurity-B				
1	10	3.305	0.0	9423	1.35	83795
2	10	3.293	0.0	9106	1.40	80442
3	10	3.307	0.0	8639	1.37	90287
1	20	3.302	0.0	8533	1.37	83795
2	20	3.303	0.0	8789	1.39	80442
3	20	3.274	0.0	8689	1.35	90287
1	30	3.302	0.0	8943	1.39	114618
2	30	3.303	0.0	8897	1.38	103693
3	30	3.274	0.0	8962	1.33	108958

Table 2: Precision results of NVP, Impurity-A, Impurity-B

Injections	NVP	Impurity-A	Impurity-B
1	13996	17124	16511
2	14167	17306	16553
3	13845	17098	16498
4	14128	17185	16435
5	13855	17254	16584
6	13871	17068	16472
% RSD	0.936	0.495	0.298

degradation. Degradation peaks of NVP at different conditions are shown in Figure.7 to Figure.9.

CONCLUSION

The method was developed at 220 nm UV-Wave length. The mobile phase was fixed as Aceteonitrile and Buffer on the basis of drug solubility. The ratio of organic solvent and buffer was confirmed on trail and error basis. $NH_4H_2PO_4$ and Perchloric acid are used as buffer. Method was finally developed with Perchloric acid at *p*H 4.8. The method

Table 3: Linearity results of NVP, Impurity-A, Impurity-B

Percentage of Concentration	NVP	Impurity-A	Impurity-B
25 %	9587	11334	9668
50%	10851	13559	12568
75%	12574	15451	14689
100%	13841	17239	16626
125%	15345	18885	19224
150%	16629	20975	21277
200%	18316	23140	23974
y=mx+c	y=11750x+8039	y=7800x+9458	y=9394x+7498
Co-relation Coefficient	0.998	r2=0.998	0.998

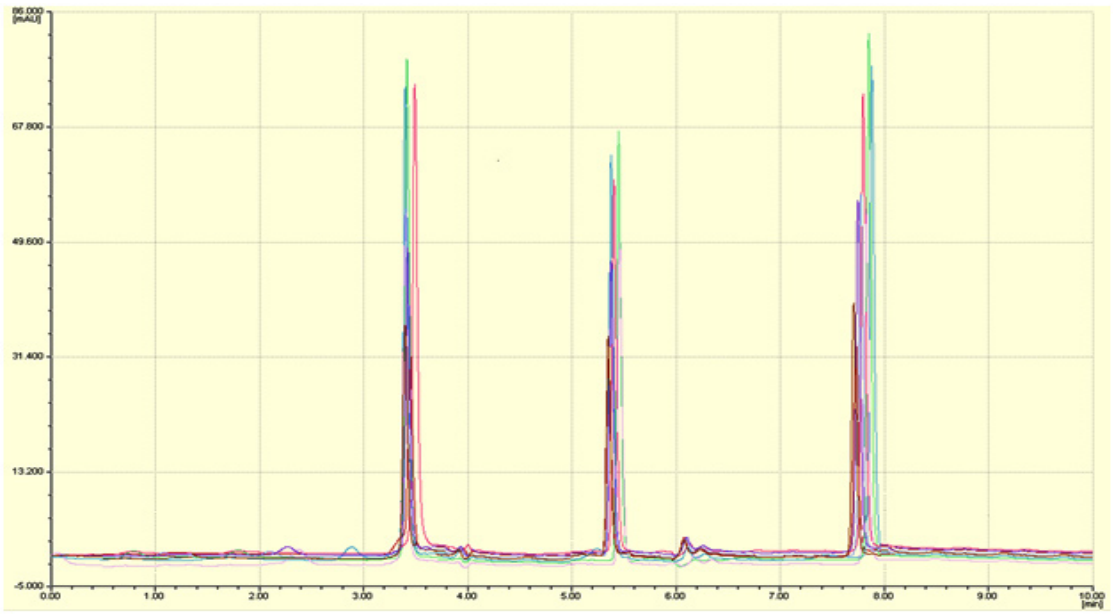
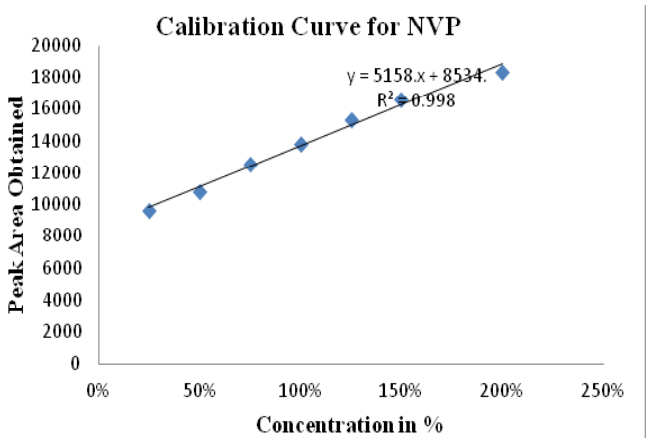
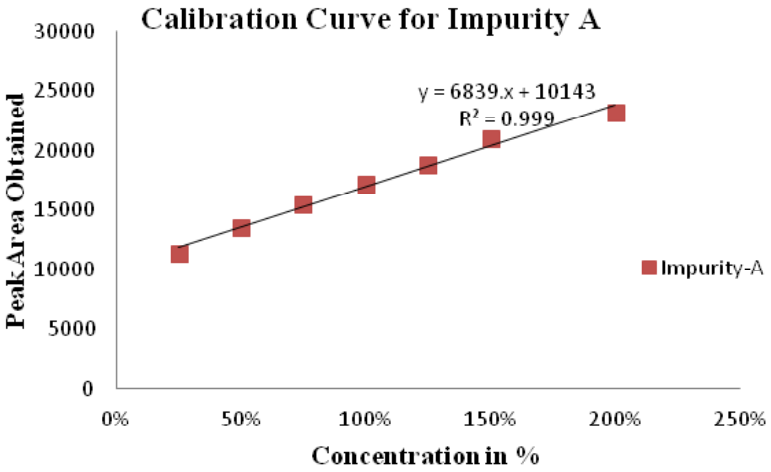


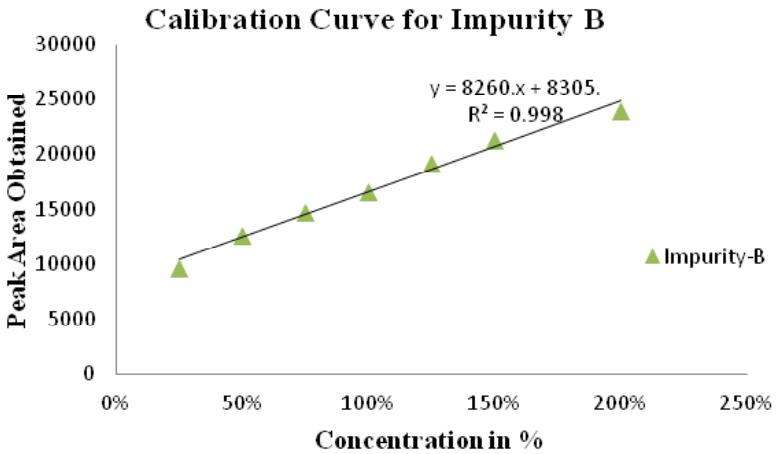
Fig.4: Linearity overlaid Chromatograms



Graph 1: linearity graph of NVP



Graph 2: linearity graph of Impurity-A



Graph 3: linearity graph of Impurity-B

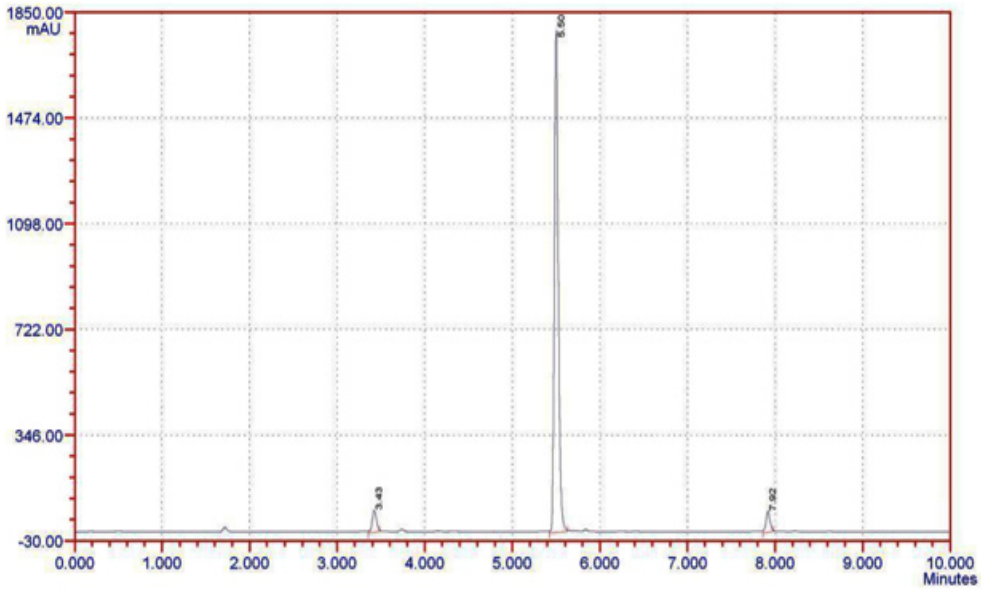


Fig. 5: Representative Sample chromatogram of NVP, Impurity-A, Impurity-B

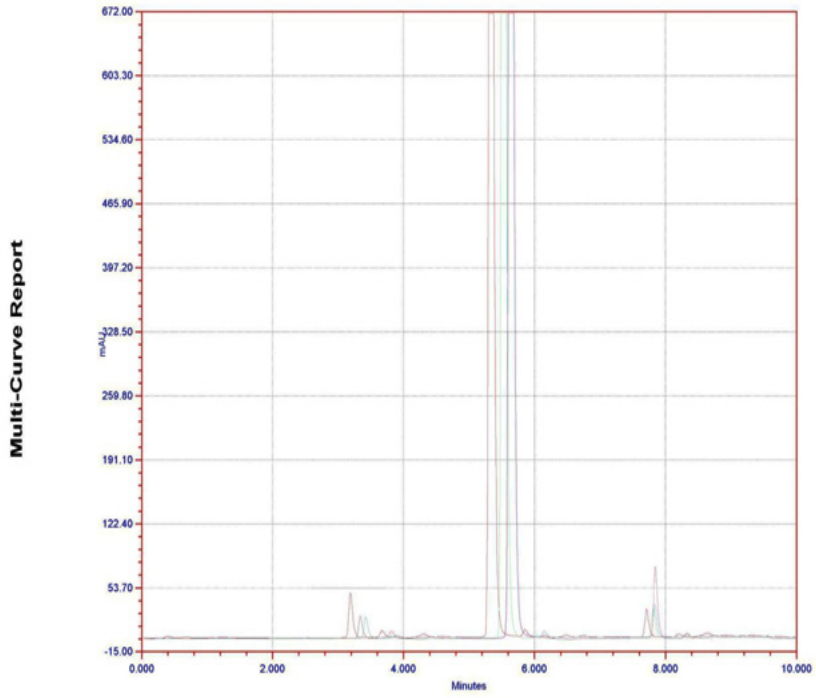


Fig. 6: Representative Degradation overlaid chromatogram of NVP

Table 4: Ruggedness results

Test	Ruggedness NVP	Imp-A	Imp-B
Standard solution Area	13996	17124	16511
Mean of Ruggedness Six injections peak area	13856	16971	16418
Percentage of Change in peak area	1.000%	0.893%	0.563%

Table 5: Robustness results

Change in Parameter	Robustness NVP	Percentage of Change in peak area	Imp-A	Percentage of Change in peak area	Imp-B	Percentage of Change in peak area
Standard solution Area	13996	0.000	17124	0.000	16511	0.000
*p*H at 4.6	13745	1.793	16954	0.992	16452	0.537
*p*H at 4.9	13895	0.721	16977	0.858	16398	0.684
flow rate at 1.1 ml/min	13915	0.578	17049	0.437	16477	0.205
flow rate at 0.9 ml/min	13887	0.778	17138	0.081	16402	0.660
wavelength 222 nm	13752	1.743	17089	0.204	16582	0.430
wavelength 218 nm	13790	1.471	17055	0.402	16601	0.545

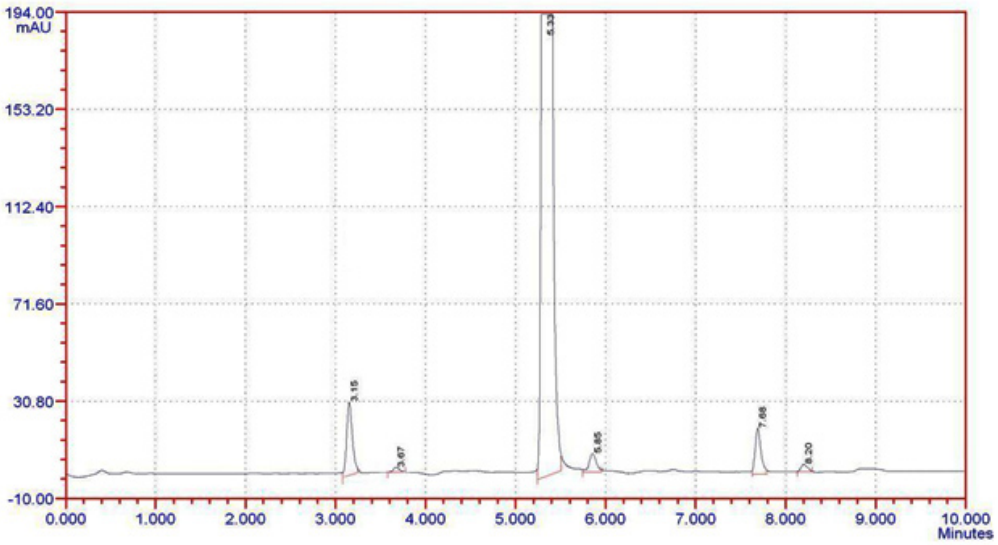


Fig. 7: Representative Acid degradation chromatogram of NVP

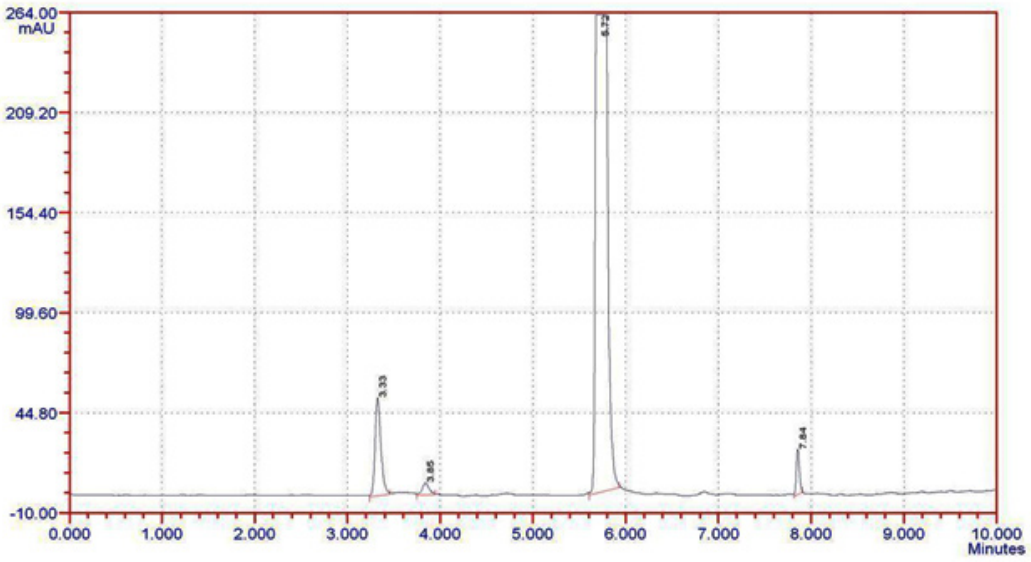


Fig. 8: Representative Basic degradation chromatogram of NVP

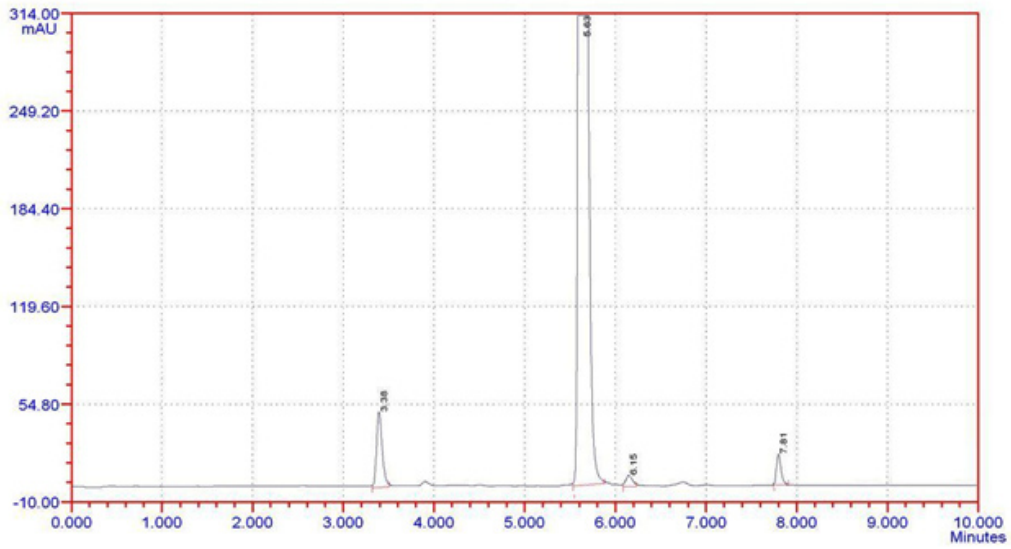


Fig. 9: Representative Peroxide degradation chromatogram of NVP

Table.6: The LOQ and LOD concentrations of NVP, Impurity-A, Impurity-B

S.No	Parameter	NVP	Imp-A	Imp-B
1	LOQ (µg/ml)	0.1	0.1	0.1
2	LOD(µg/ml)	0.03	0.03	0.03

was validated according to ICH guidelines. There us no interference in blank injection. The precision RSD of NVP, Impurity-A, Impurity-B are below 1.0 The linearity range was fixed between 25% to 200%. The correlation coefficient is 0.998.The ruggedness and robustness tests are passed. The L.O.Q ranges of NVP, Imp-A and IMP-B are 0.1 µg/ml and LOD ranges are 0.03 µg/ml. The NVP degradation study

Table 7: Degradation study results of NVP, Impurity-A, Impurity-B

S. No.	Degrading Agent	Drug	Initial concentration of drug before degradation (µg/ml)	Final concentration of drug after degradation (ig/ml)	% of Degradation
1	No degrading agent	NVP	1195565	1195565	0.00
2	0.1 N HCL	NVP	1195565	1087081	9.07
3	0.1 M NaOH	NVP	1195565	1140772	4.58
4	50 % H_2O_2	NVP	1195565	893682	25.25

was carried out at three conditons.NVP 25.25% degraded in peroxide condtion.9.07 % degraded in Acidic condition. 4.58% degraded in Basic condition. The developed RPHPLC method was validated with precision, linearity, accuracy and proved to be sensitive and effective for the determination of NVP and its relative substances (Impurity-A, Impurity-B) during stability testing of the bulk drug. We can apply this method for routine quality control analysis in bulk drug manufacturing industries.

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