STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT FOR THE SIMULTANEOUS ESTIMATION OF NEBIVOLOL AND VALSARTAN IN PHARMACEUTICAL DOSAGE FORM.

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ABSTRACT

The present study deals with development and validation of a simple, precise, accurate, sensitive, specific and reliable stability indicating RP-HPLC method for simultaneous estimation of Nebivolol (NE Biv) and Valsartan (VAL) in pharmaceutical dosage form. This method was developed with mobile phase containing ACN, Potassium Dihydrogen orthophosphate and buffer (0.1% v/v ortho phosphoric acid (OPA) in water, pH = 3) in the ratio of (50:50), C_{18} (250 x 4.6mm, 5µm) as a stationary phase and flow rate (1 ml/min). Detection was carried out at 282 nm in UV-2000 detector. The selected chromatographic conditions were found effectively to separate Valsartan and Nebivolol at 4.27 and 6.96 min respectively. The proposed method has been validated for precision, accuracy, robustness. Thus, the statistical analysis confirms that developed methods were successfully used for analysis of formulation and routine analysis of drugs in Quality Control laboratories.

KEYWORDS: Valsartan (VAL), Nebivolol (NE Biv), Acetonitrile (ACN), ortho phosphoric acid (OPA), RP-HPLC (Reversed phase High-performance liquid chromatography) method.

INTRODUCTION

Valsartan (VAL) chemically is N– [*p*-(*o*-1*H*-Tetrazol- 5*yl*phenyl) benzyl]-N-valeryl-L-valine, is an angiotensin II receptor blocker used to manage hypertension alone or in combination with other antihypertensive agents and to manage heart failure in patients who are intolerant to ACE inhibitors.

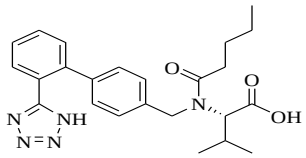

Fig.1 Structure of Valsartan

Nebivolol (NE Biv) chemically is 1-(6-fluoro-3,4-dihydro-2*H*-1-benzopyran-2-*yl*)-2-{[2-(6-fluoro-3,4-dihydro-2*H*-1-benzopyran-2-*yl*)-2-hydroxyethyl] amino} ethan-1-ol is a β- blocker used to treat hypertension and aid in the management of heart failure.

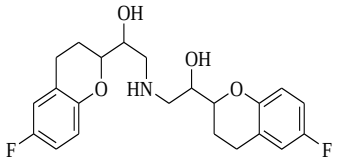

Fig.2 Structure of Nebivolol (NE Biv)

With the advent of International Conference on Harmonization (ICH) guidelines, the requirement of establishment of dissolution method development with stability -indicating assay method (SIAM) has become more clearly mandated. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, etc. and separation of drug from degradation products. Thus, the objective of this work is to develop a new sensitive stability indicating RP-HPLC method for simultaneous determination of valsartan and nebivolol in mixture. Also, it is validated for market product containing valsartan and nebivolol in tablet dosage form.

MATERIALS AND METHODS

Instruments Used

Standard Valsartan and Nebivolol were obtained as gift samples from Shanku Pharma; gradient method chromatography with PDA detector was used with Open lab Software.

Materials

Methanol (CH_3OH) and Acetonitrile (ACN) - HPLC grade, Water (H_20)- HPLC grade, Finar limited, Gujarat was used. Potassium Dihydrogen Phosphate and Orthophosphoric Acid- AR grade, Ranbaxy chemical was used. A commercial tablet formulation Nebicard-V was purchased from local market.

Methods

Selection and Detection of wavelength

Solution of 50 and 800 ppm of each Nebivolol and Valsartan were prepared and scanned over the range 200-400 nm and the spectra were recorded. Wavelength at 282 nm (at which both the drugs showed good absorbance) was selected as a detection wavelength (figure 3).

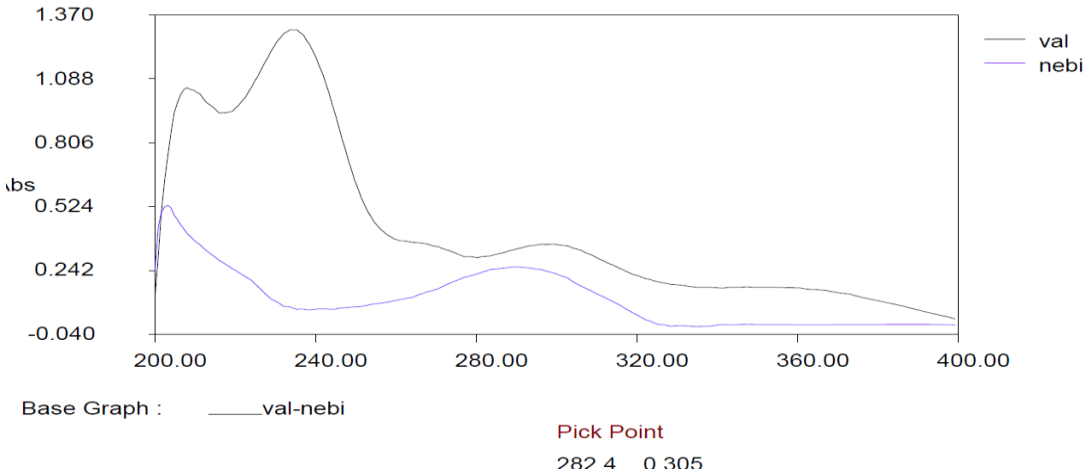

Figure 3: Selection of analytical wavelength

Selection of Mobile phase

After trials of various mobile phase compositions Acetonitrile (ACN), Potassium Dihydrogen orthophosphate and buffer (0.1% v/v ortho phosphoric acid (OPA) in water (50:50), pH=3), is selected for the estimation. Chromatogram in optimized mobile phase is shown in Figure 4.

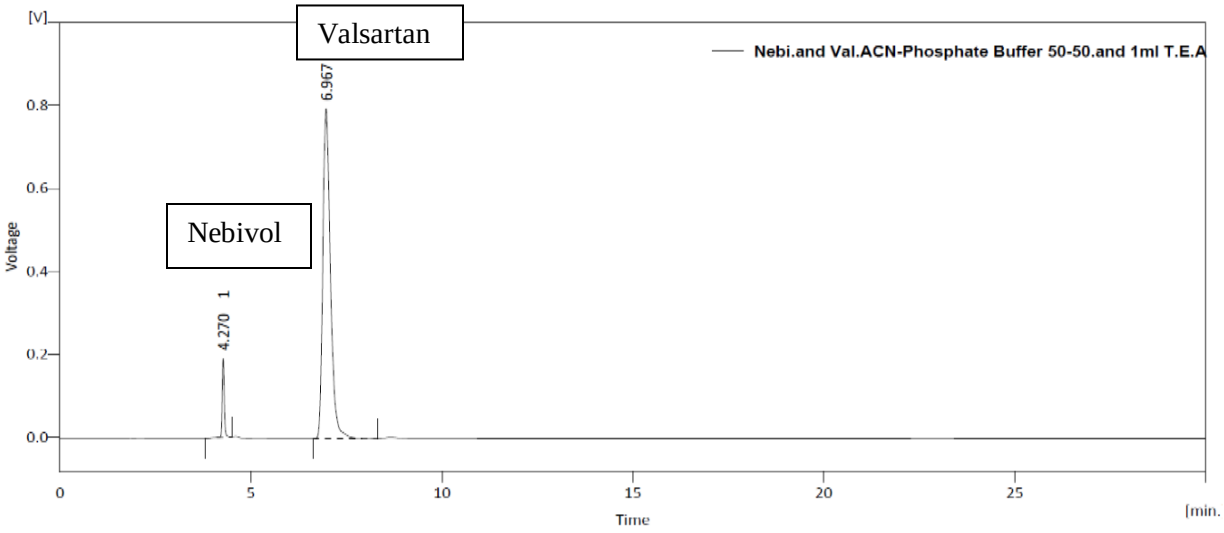

Figure 4: Chromatogram in optimized mobile phase

Preparation of working standard and sample stock solutions
From Nebivolol Standard Stock solution 1 ml was taken into 10 ml volumetric flask and was made up to the mark with the mobile phase to get 50 µg/ml of Nebivolol Working Standard Solution.

From Valsartan Standard Stock solution 1 ml was taken in to 10 ml volumetric flask and was made up to the mark with the mobile phase to get 80 µg/ml of Valsartan Working Standard Solution.

Optimized Chromatographic Conditions

Column	C_{18} (25 cm × 0.46 cm) Hypersil BDS
Mobile Phase	Acetonitrile (ACN), Potassium Dihydrogen orthophosphate and buffer (0.1% v/v ortho phosphoric acid (OPA) in water, (50:50), pH=3)
Flow rate	1 ml/min
Detection	282 nm
Column Temperature	30ºC
Run Time	15 min
Injection volume (loop)	20 µl

Calibration of standards
Calibration curve of Valsartan and Nebivolol were prepared for concentration range (400-1200 µg/ml and 25-75 µg/ml) was prepared by pipette out different volumes from each stock solution and dilute up to the marks with mobile phase.

Method validation
The method was validated by the different parameters such as linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ), robustness.

Linearity
Linearity was established by least squares linear regression analysis of the calibration curve. Calibration curve of Valsartan and Nebivolol were chromatographed over the range of 400-1200 µg/ml and 25-75 µg/ml respectively. Linearity plots were shown in Fig.5 and Fig.6. Results for linearity are shown in table.1.

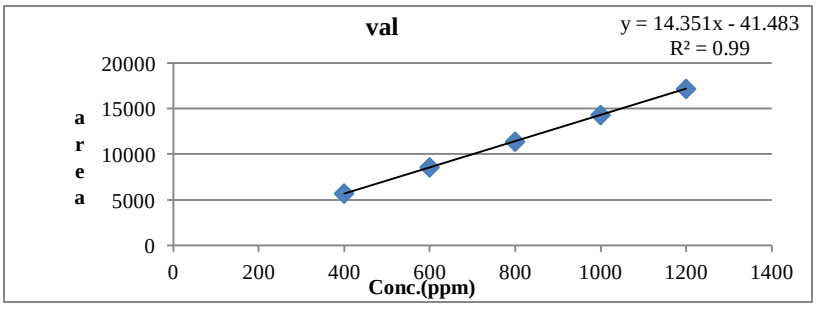

Fig 5. Calibration curve of Valsartan

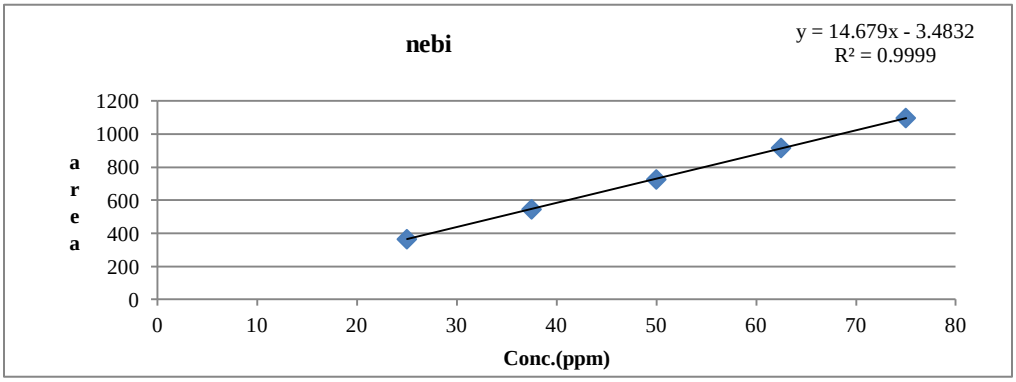

Fig 6. Calibration curve of Nebivolol

Table 1. Statistical analysis data of calibration curve

Sr. no.	Valsartan	Area Mean ± S.D. (n=3)	% R.S. D	Nebivolol	Area Mean ± S.D. (n=3)	% R.S. D
1	400	5708.907±0.999	0.017	25	365.551±1.000	0.273
2	600	8579.03±1.000	0.011	37.5	545.961±0.999	0.182
3	800	11394.852±0.999	0.008	50	726.589±1.154	0.158
4	1000	14326.282±1.000	0.005	62.5	916.714±1.000	0.109
5	1200	17185.87±1.524	0.008	75	1097.643±0.999	0.091

Accuracy (Recovery study)

Accuracy of an analysis is determined by calculating systemic error involved. Recovery of Valsartan & Nebivolol were calculated by standard addition method at 3 different concentration levels 80 %, 100% and 120 % of the target concentration 400-1200 µg/ml of Valsartan and 25-75 µg/ml of Nebivolol in triplicate and calculating % recovery. The mean % recovery was found to be 98.90 % - 99.80 % for Valsartan and 99.10 %- 100.30% for Nebivolol respectively. Results are shown in table 2.

Table 2: Accuracy data for Valsartan

Sr. no.	Conc. Level (%)	Sample amount (µg/ml)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S. D	% R.S. D
1	80%	400	320	320.9565703	100.2989282	99.65856202 ± 0.58	0.583
2		400	320	317.3267858	99.16462056		
3		400	320	318.4388393	99.51213727		
4	100%	400	400	401.8527129	100.4631782	100.9071978 ± 0.76	0.762
5		400	400	401.8527129	100.4631782		
6		400	400	407.1809476	101.7952369		

7	120%	400	480	482.1756568	100.4532618	100.5322272 ± 188	0.187
8		400	480	481.9032491	100.3965102		
9		400	480	483.5851662	100.7469096		

Accuracy data for Nebivolol

Sr. no.	Conc. Level (%)	Sample amount (µg/ml)	Amount Added (µg/ml)	Amount recovered (µg/ml)	% Recovery	% Mean Recovery ± S. D	% R.S. D
1	80%	25	20	19.65026357	98.25131786	98.70358522 ± 0.85	0.869143992
2		25	20	19.63329485	98.16647426		
3		25	20	19.93859271	99.69296355		
4	100%	25	25	24.9518756	99.8075024	99.6172302 ± 0.22	0.229944991
5		25	25	24.8407444	99.36297759		
6		25	25	24.92030266	99.68121062		
7	120%	25	30	29.75784803	99.19282678	100.053317 ± 0.92	0.927407758
8		25	30	30.31093092	101.0364364		
9		25	30	29.97920636	99.93068788		

Precision

Repeatability of the method was assessed by analysing six injections of a homogeneous sample of 50 µg/ml of Nebivolol and 800 µg/ml of Valsartan performed at two levels (intraday and inter day). Intraday precision was performed using three different concentration 400, 800, 1200 µg /ml for Valsartan and 25, 50, 75 µg/ml for Nebivolol in triplicate at three different time intervals in a day. Interday precision was performed using three different concentration 400, 800,1200 µg /ml for Valsartan and 25,50,75 µg/ml for Nebivolol in triplicate for three consecutive days. (Table 3).

Table 3: Intraday Precision for Nebivolol and Valsartan

Nebivolol				Valsartan			
Sr. No	Conc.(µg/ml)	Area Mean ± S.D. (n=3)	% R.S. D	Sr. No	Conc(µg/ml)	Area Mean ± S.D. (n=3)	% R.S. D
1	25	370.149±0.577	0.155	1	400	5796.044±0.577	0.009
2	50	750.508±1.0005	0.133	2	800	11750.099±0.999	0.008
3	75	1096.106±1.001	0.091	3	1200	17129.852±0.577	0.003

Interday Precision for Nebivolol and Valsartan

Nebivolol				Valsartan			
Sr. No	**Conc(µg/ml)**	**Area Mean ± S.D. (n=3)**	**% R.S. D**	**Sr. No**	**Conc(µg /ml)**	**Area Mean ± S.D. (n=3)**	**% R.S. D**
1	25	376.186±0.999	0.265	1	400	5912.753±0.577	0.009
2	50	740.672± 0.578	0.078	2	800	11580.147±1.001	0.008
3	75	1097.725±0.999	0.091	3	1200	17264.103±1.001	0.005

n=Three determination

Repeatability study for Nebivolol and Valsartan

Nebivolol					Valsartan				
Sr. no	**Conc.(µg/ml)**	**Area Mean ± S.D. (n=3)**	**Mean ± S.D. (n=3)**	**% R.S. D**	**Sr.no**	**Conc(µg/ml)**	**Area Mean ± S.D. (n=3)**	**Mean ± S.D. (n=3)**	**% R.S. D**
1	50	718.180	726.404 ± 6.07	0.83	1	800	11296.759	11421.572 ± 73.541	0.64
2		724.674			2		11444.449		
3		723.597			3		11422.446		
4		735.613			4		11497.008		
5		725.428			5		11384.782		
6		730.934			6		11483.985		

LOD and LOQ

LOD and LOQ of the drug were calculated from signal-to-noise (S/N) ratio (i.e., 3.3 for LOD and 10 for LOQ) taking from the samples of 400, 800, 1200 µg/ml of Valsartan and 25, 50, 75 µg/ml of Nebivolol. The results were shown in table 4.

Table 4: LOD and LOQ of Nebivolol and Valsartan

Drug	**LOD [µg /ml]**	**LOQ [µg /ml]**
Nebivolol	0.017	0.051
Valsartan	3.240	9.820

Robustness

Small variation in the flow rate (± 0.2 ml/min.), organic phase ratio (±2%), by using 50 µg/ml and 800 µg/ml of Nebivolol and Valsartan were made. The results were shown in table 5.

Table 5: Robustness study for Nebivolol

Sr no.	Area at Flow rate (- 0.2 ml/min)	Area at Flow rate (+ 0.2 ml/min)	Area at M.P(-0.2)	Area at M.P(+0.2)	Area at PH (-2)	Area at PH (+2)
1	584.342	874.715	727.299	743.700	710.767	742.871
2	593.183	876.371	719.683	746.822	704.921	744.254
3	596.623	884.801	725.297	744.254	709.038	745.582
%RSD	1.071	0.616	0.545	0.224	0.424	0.182

Robustness study for Valsartan

Sr no.	Area at Flow rate (- 0.2 ml/min)	Area at Flow rate (+ 0.2 ml/min)	Area at M.P(-0.2)	Area at M.P(+0.2)	Area at PH (-2)	Area at PH (+2)
1	9140.327	13718.654	11331.528	11617.999	11109.866	11632.316
2	9288.420	13769.117	11327.246	11670.714	11121.373	11616.862
3	9297.116	13873.699	11329.446	11595.063	11118.583	11634.555
%RSD	0.953	0.574	0.019	0.334	0.054	0.083

System suitability

It is defined as tests to measure the method that can generate result of acceptable accuracy and precision. The system suitability was carried out after the method development and validation have been completed. For this, parameters like Plate number (N), Resolution (R), tailing factor, Capacity factor, HETP, Peak symmetry of samples were measured. The results were shown in table 6.

Table 6. System suitability data for the developed method

Parameters	Nebivolol	Valsartan
Retention Time	4.270	6.967
Theoretical plates per column	28058	5908
Tailing factor	1.286	1.542

Specificity

Commonly used excipients in tablet preparation were spiked in a pre-weighed quantity of drugs and then area was measured and calculations carried out to determine the quantity of the drugs.

Assay of marketed formulation

Average weight of 20 tablets were calculated and ground to fine powder. A quantity of powder equivalent to 80 mg of Valsartan and 50 mg of Nebivolol was transferred into 100 ml volumetric flask containing 60 ml of Mobile phase, sonicated for 10 min and diluted to mark with same solvent to obtain 50 µg/ml of Nebivolol and 800 µg/ml of Valsartan. The resulting solution was filtered using 0.45 µm filter (Milli filter, MA). In which 20 µl of the test solution was injected and chromatogram was recorded under optimized chromatographic condition and peak area was

measured. The assay procedure was made in triplicate and % drug was calculated. Results are shown in table.7

Table 7. Assay of marketed formulation

Tablet	**Nebicard-V**	
Label claim	**Nebivolol (5 mg)**	**Valsartan (80 mg)**
Assay (% of label claim*) Mean ± S. D.	100.669 ± 0.828	99.995 ± 0.073

Forced degradation

1) Acid degradation

1 ml of stock solution was transferred into 10 ml of volumetric flask and to that 2 ml of 0.1 N HCl solution was added and mixed well. This solution was kept at room temperature (RT) for 4 hrs. The resulting solution is then neutralized using 0.1N NaOH solution. The volume is then made up using the diluent to get 5 µg/ml for Nebivolol and 80 µg/ml for Valsartan. The above solution thus obtained is filtered and then injected.

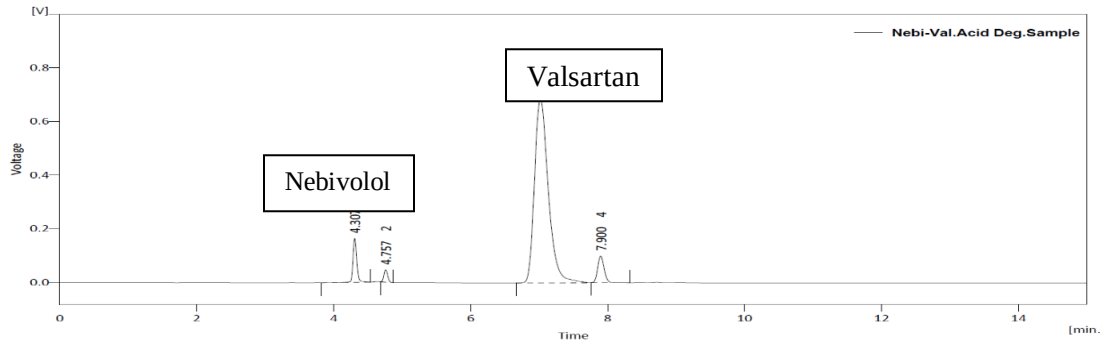

Fig 7. Chromatograph of acid degradation study

2) Base degradation

1 ml of stock solution was transferred into 10 ml of volumetric flask and to that 2 ml of 0.1 N NaOH solution was added and mixed well. This solution was kept at room temperature (RT) for 4 hrs. The resulting solution is then neutralized using 0.1N HCl solution. The volume is then made up using the diluent to get 5 µg/ml for Nebivolol and 80 µg/ml for Valsartan. The above solution thus obtained is filtered and then injected.

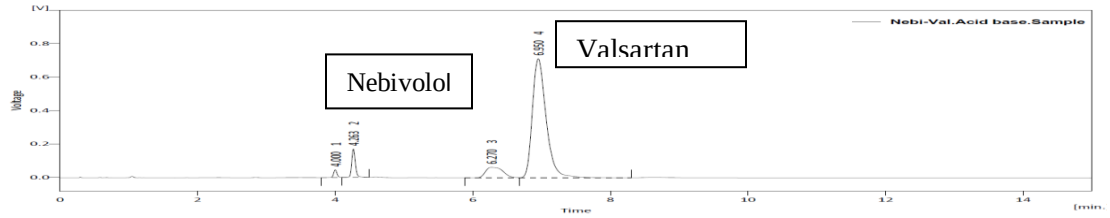

Fig. 8. Chromatograph of base degradation study

3) Thermal Degradation

Thermal decomposition studies were performed by transferring 1 ml of stock solution in to 10 ml of volumetric flask and were kept in an Oven at 105°C for 24 hrs. After time period volume was adjusted with diluent to get 5 µg/ml for Nebivolol and 80 µg/ml for Valsartan.

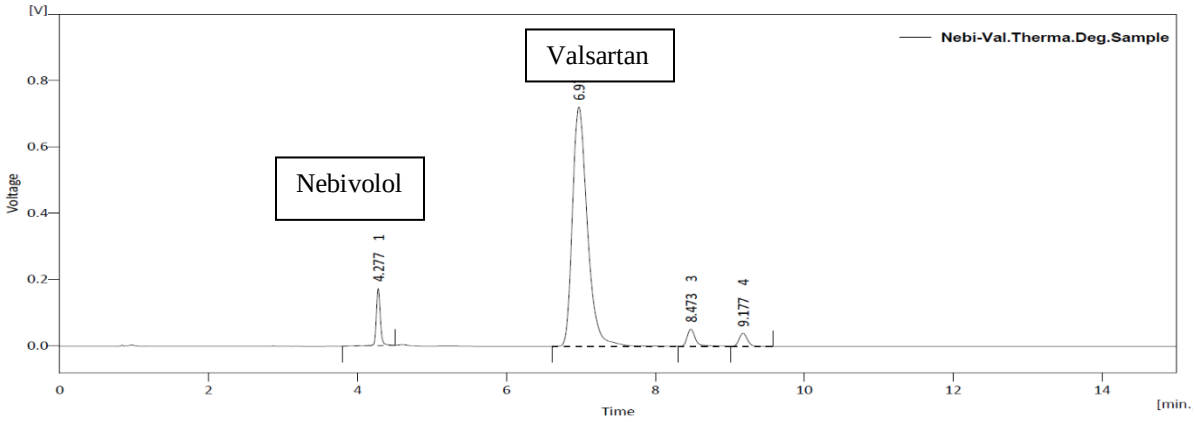

Fig 9. Chromatograph of Thermal Degradation

4) Oxidative degradation

Oxidative decomposition studies were performed by transferring 1 ml of stock solution in to 10 ml of volumetric flask and 2 ml of 30% (Hydrogen peroxide) H_2O_2 solution was added and mixed well and put for 4 hrs at Room temperature (RT). After time period volume was adjusted with diluent to get 5 µg/ml for Nebivolol and 80 µg/ml for Valsartan.

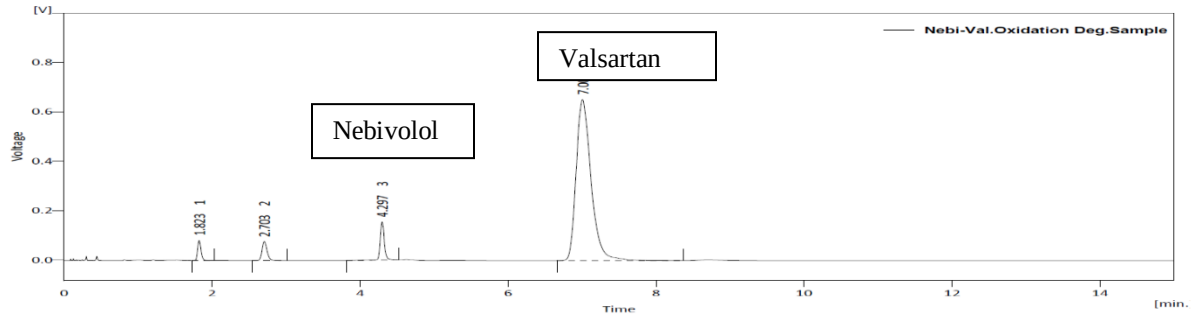

Fig 10. Chromatograph of Oxidative Degradation

5) Photo degradation

Photo decomposition studies were performed by transferring 1 ml of stock solution in to 10 ml of volumetric flask and it was kept in UV Chamber for 10 hrs. After time period volume was adjusted with diluent to get 5 µg/ml for Nebivolol and 80 µg/ml for Valsartan.

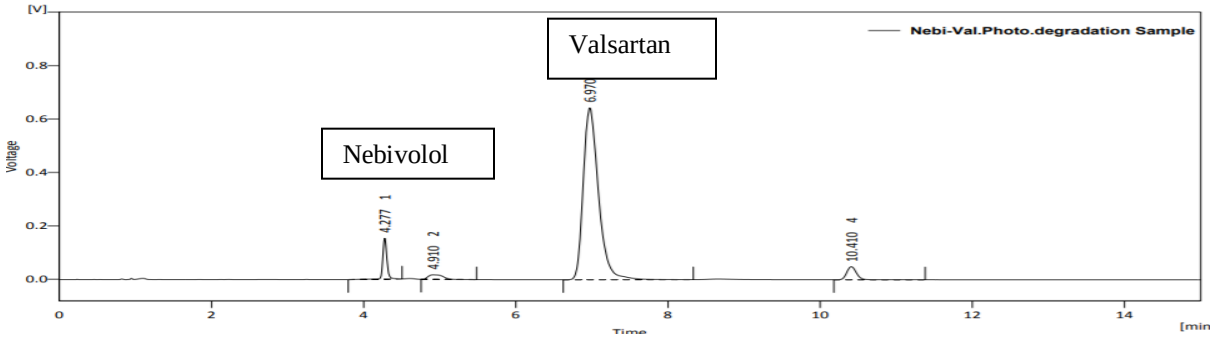

Fig 11. Chromatograph of Photo Degradation

RESULT AND DISCUSSION

The present work aimed development and validation of stability indicating RP-HPLC method for simultaneous estimation of Valsartan and Nebivolol. Method was developed in mobile phase containing Acetonitrile (ACN), Potassium Dihydrogen orthophosphate and buffer (0.1% v/v ortho phosphoric acid (OPA) in water (50:50), pH=3). Detection was carried out at 282 nm. Method was validated as per ICH guidelines. Linearity and regression data were shown in table 1 and Fig.4, 5. % recovery for Valsartan and Nebivolol were within the range (98%-102%). Results were shown in table 2. Hence, it is found that the developed method is accurate. % RSD values were < 2 for repeatability, intra-day and inter-day precision. Results were shown in table 3. So, the developed method was found to be precise. LOD and LOQ values were shown in table 4. LOD & LOQ confirms the method to be sensitive. Small changes were carried out in mobile phase and flow rate for robustness study, in that % RSD of area was found to be <2. Results were shown in table 5. So, the developed method was found to be robust. Various forced degradation conditions were performed in proposed method and it can efficiently separate all the degradation products from the drugs. % Degradation values are 5% to 20% degradation of the drug substance, have been considered as reasonable and acceptable for validation of chromatographic assays. Results were shown in table 8 and 9. So, the developed method is stability indicating.

Table 8: Stability data

Stress condition	**Nebivolol**			**Valsartan**		
	Area of standard	**Area of Degradation**	**% Degradation**	**Area of standard**	**Area of Degradation**	**% Degradation**
Acid degradation	732.31	632.833	13.78	11221.96	9671.302	13.82
Base Degradation	732.31	637.239	12.98	11221.96	9913.907	11.66
Thermal Degradation	732.31	659.662	9.92	11221.96	10102.281	9.98
Oxidative Degradation	732.31	596.251	18.58	11221.96	9138.053	18.57
Photo Degradation	732.31	596.251	20.10	11221.96	9854.14	9.98

Table 9: Summary of validation parameters

Parameters	**Valsartan**	**Nebivolol**
Linear Range	400-1200 µg/ml	25-75 µg/ml
% Recovery	98.90-99.80 %	99.10-100.30%
Repeatability (% RSD, n=6)	0.644	0.837
Precision (RSD) Intra - day (n=3) Inter - day (n=3)	 0.918-0.391 0.380-0.034	 0.732-0.241 0.688-0.298
Limit of Detection (µg/ml)	3.240	0.017
Limit of Quantitation (µg/ml)	9.820	0.051
Robustness	Robust	Robust
Specificity	Specific	Specific
Peak Purity data	0.999	0.998

CONCLUSION

Stability indicating RP-HPLC method for simultaneous estimation of Valsartan and Nebivolol was developed and validated as per ICH guidelines. The developed method was found to be accurate and precise with % RSD <2%. So, the developed method is simple, accurate, precise, sensitive and robust. As the % degradation of drug substance was between 5%-20%, the developed method was found to be stability indicating. Also, this method can be used for routine drug analysis in Quality control department.

CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this investigation.

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