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DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF VALSARTAN AND NEBIVOLOL IN DOSAGE FORM

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ABSTRACT

RP-HPLC method have been developed for the simultaneous estimation of Valsartan and Nebivolol in pharmaceutical dosage form. RP-HPLC method was carried on Phenomenex C-18 column (150 mm × 4.6 mm, 5 µ) with Phenomenex Security Guard Cartridges C18(4x3mm) using a mobile phase acetonitrile:methanol:phosphate buffer with pH 3.0 (40:30:30) as a mobile phase at 1.0ml/min flow rate at 228 nm. The linearity was found to be in the range of 32-160 µg/ml and 2-10 µg/ml with regression coefficient (r^2= 0.980, and r^2=0.983) for Valsartan and Nebivolol respectively. The peaks obtained were sharp having clear baseline separation with a retention time 3.9min and 2.2 min for Valsartan and Nebivolol. This method is accurate and precise and can be employed for routine analysis of valsartan and nebivolol in different pharmaceutical dosage forms.

Keywords – Valsartan, Nebivolol, RP-HPLC and ICH Guideline.

1. INTRODUCTION

Cardiovascular disease is the leading cause of mortality worldwide. Arterial hypertension is a major cardiovascular risk factor, affects almost one third of the adult population and is associated with approximately 7.6 million deaths annually. High blood pressure affects the human vasculature and results in functional and structural alterations of the vessels all over the body.[1]

Valsartan is antihypertensive agent (N-valeryl-N[[2-(1H-tetrazol-5-yl)biphenyl-4 yl]methyl]. It specifically blocks the action of angiotensin II, such as aldosterone, vasopressin and endothelin secretion, vasoconstriction, dieresis, endothelial cell hyperplasia, mitogenesis and induction of growth factors and production of collagen. Valsartan has a simple pharmacokinetic profile and requires no metabolism to become active.[2]

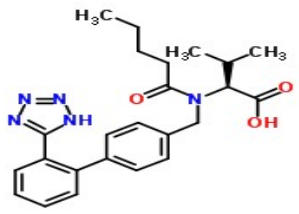

Fig.1. Chemical Structure of Valsartan[3]

Nebivolol α,α1-[imino bis (methy-lene)]bis[6-fluoro-3,4-dihydro-2 *H* -1-benzopyran-2-methanol] is a highly selectiveβ1-blocker with nitric oxide-mediated vasodilatory actions and beneficial effects on vascular endothelial function. It has been clinically used for the treatment of hypertension and chronic heart failure. Nebivolol is endowed with peripheral vasodilating properties mediated by the modulation of the endogenous production of nitric oxide and thus lowers peripheral resistance.[4]

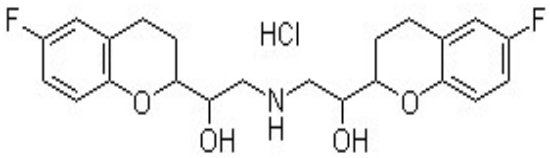

Fig.2. Chemical Structure of Nebivolol[3]

Analysis of fixed dose combination (FDC) is quite exigent job for pharmaceutical manufacturers in adherence with strict rules and regulations about the safety and quality of manufactured products in the pharmaceutical industry. Analytical research and development for estimation of Valsartan and Nebivolol was found to be interesting and challenging job. As there is few methods reported for the determination of these drugs in combination. Hence, the present work is to develop reverse phase high performance liquid chromatographic (RP-HPLC) method for determination of Valsartan and Nebivolol combined in tablet dosage form.

2. MATERIALS AND METHODS

2.1 Materials

Valsartan and Nebivolol was obtained as gift sample from Mylan Pharma. Pvt. Ltd. Aurangabad Maharashtra and Torrent Pharma. Ltd. Gandhinagar, Bhat, Gujarat. The tablet sample of Valsartan and Nebivolol combination Nebicard V were obtained from Torrent Pharmaceuticals Ltd. Vill. Bhud & Makhnu Majra, Solan, Himachal Pradesh. HPLC grade methanol, acetonitrile, water were purchased from Merck Specialities Pvt. Ltd. Mumbai. Potassium dihydrogen o-phosphate and o-phosphoric acid were purchased from SDFCL Fine Chem Ltd. Worli road, Mumbai.

2.2 Instrumentation

The analysis was performed using Younglins Acme 9000 series quaternary gradient pump SP930D. HPLC system accomplished with UV 730D UV- Visible detector with 20µl Rheodyne injector. The data was proccessed using Autochro-3000 software. Column C18 (150×45,5µ) Phenomenex with UV method analysis was performed on a UV- Visible Double Beam Spectrophotometer Shimadzu 1800. All chemicals were weighed using Electronic balance AY220 (Shimadzu, Japan). Measurement of pH of buffer solutions was made by using Electrolab pH meter with magnetic stirrer. Mobile phase filtered through a nylon 6,6 membrane 0.45 µm 47 mm filters (pall India Pvt. Ltd., Mumbai) using vaccum pump. Ultra Sonicator (Microlean- 103) was used for degassing the mobile phase. The solution were filtered through 0.45µ syringe filter (Phenomenex).

2.3 Chromatographic Conditions

The chromatographic separation was performed in Analytical Column: Phenomenex C 18 column (150 mm × 4.6 mm, 5µm) using mobile phase comprised mixture of acetonitrile: methanol: phosphate buffer (pH adjusted to 3.0) in ratio (40:30:30v/v) at flow rate 1ml/min with isocratic elution. The injection volume was 20µl and run time was 3.9min and 2.2min for Valsartan and Nebivolol. Detection was carried out at 228nm.

2.4 Preparation of Standard Stock Solution

2.4.1 Standard Stock Solution of VAL

Accurately about 16mg of standard VAL was weighed and transferred to a 10ml volumetric flask then dissolved in the methanol and volume was made up to the mark with methanol to obtained conc. of 1600µg/ml of VAL and labelled as 'Std Stock VAL'.

2.4.2 Standard Stock Solution of Nebivolol

Accurately about 10mg of standard NEB was weighed and transferred to a 10ml volumetric flask then dissolved in the methanol and the volume was made up to the mark with methanol to obtained conc. of 1000µg/ml of NEB. From above solution 1ml was diluted to 10ml with methanol solvent to obtain conc. of 100µg/ml of NEB and labelled as 'Std Stock NEB'.

2.4.3 Combined Standard Stock Solution of VAL and NEB

1ml of 'Std Stock VAL' and 1ml of 'Std Stock NEB' was transferred to 10ml volumetric flask and diluted to 10ml with methanol to get 'Std Stock MIX VN' (160µg/ml VAL and 10µg/ml NEB).

2.5 Method validation

The method was developed and validated as per ICH guidelines it is suitable for the intended purpose with respect to various parameters such as specificity, linearity, accuracy, precision, limit of detection, limit of quantification and robustness, system suitability.[12]

2.6 Specificity

The chromatogram of standard solution of mixture of Valsartan and Nebivolol was compared with formulation to observe the interference of excipient.

2.7 Linearity

2, 4, 6, 8, and 10ml of 'Std Stock MIX VN' were transferred in a series of 10ml volumetric flasks. The volume was made up to the mark with mobile phase to obtain the concentration of 32, 64, 96, 128 and 160µg/ml of Valsartan and 2, 4, 6, 8 and 10µg/ml of Nebivolol. The solutions were filtered through syringe filter and 20µl injected into the HPLC system and their chromatogram were recorded for 10mins. Under the chromatographic conditions as described above after getting a stable baseline. Peak areas were recorded for all the peaks. Calibration curves of Valsartan and Nebivolol were constructed by plotting the peak area of VAL *v/s* conc. of VAL and peak area of NEB *v/s* conc. of NEB, respectively. The correlation coefficient (r^2) of least square linear regression for VAL and NEB was calculated.

2.8 Range

The range of analytical method was decided from the interval between upper and lower level of calibration curves.

2.9 Precision

The precision of an analytical method was studied by performing Repeatability and intermediate precision.

a) Repeatability

6µg/ml of Nebivolol and 96µg/ml of Valsartan solution was filtered through syringe filter and 20µl injected into the HPLC system and its chromatogram was recorded under the same chromatographic conditions after getting a stable baseline. Peak area was recorded. The procedure was repeated for six times and calculate the RSD.

b) Intermediate Precision

Intra-day Precision: Intra-day precision was determined by analyzing the standard solution of NEB (6µg/ml) and VAL (96µg/ml) at 8.00am and 4.00pm on same day following the procedure of repeatability and calculated the RSD.

2.10 Accuracy

20 Tablets (Nebicard V) were weighed and finely powdered, an accurately weighed tablet powder (368mg) equivalent to 5mg Nebivolol and 80mg Valsartan was dissolved to 100 ml volumetric flask with methanol and 1.5ml diluted 10ml with HPLC methanol. 0.5 ml of above solution was transferred in a different 10ml volumetric flask labelled as 0, 80%, 100%, 120%. Then 0, 2, 2.5, 3ml of 'Std Stock Mix VN' was added and was made up to the mark with mobile phase. All the solutions were filtered through syringe filter and injected into the HPLC system and their chromatograms were recorded under the same chromatographic conditions after getting a stable baseline. Peak areas were recorded and percent recoveries were calculated.

2.11 Limit of Detection

LOD calculated by the following formulae.

LOD = 3.3(SD/S)

Where, SD- Standard deviation, S- slope of curve

2.12 Lmit of Quantification

LOQ calculated by the following formulae.

LOQ = 10(SD/S)

Where, SD- Standard deviation, S- slope of curve

2.13 Robustness

Combined standard solution of VAL (160µg/ml), NEB (10µg/ml) was prepared and analyzed at different flow rates (0.9, 1.0, 1.1 ml/min) separately.

2.14 System Suitability

Chromatograms were studied for different parameters such as tailing factor, resolution and theoretical plates to see that whether they comply with the recommended limit or not.

2.15 Assay of Tablet Formulation

Twenty marketed tablets of combination of Valsartan 80mg and Nebivolol 5mg (Nebicard V) were accurately weighed and triturated. The average weight of per tablet was calculated and tablet powder equivalent to 80mg Valsartan and 5mg Nebivolol was weighed and transferred to 100 ml volumetric flask dissolved with methanol. 1.5 ml of above solution was transferred in 10ml volumetric flask and diluted with HPLC methanol. The solution was filtered through syringe filter and injected into the HPLC system and their chromatogram was recorded under the same chromatographic conditions after getting a stable baseline. Peak area was recorded and percentage of label claim calculated.

3. RESULTS AND DISCUSSION

In order to develop RP-HPLC method for combination of valsartan and nebivolol in bulk and pharmaceutical formulation. The chromatographic conditions were optimized in order to find the best conditions for the separation of valsartan and nebivolol. Different mobile phases like acetonitrile, methanol, water and different ranges of buffers in varying proportions of mobile phases, varying pH and temperature conditions were tried for better resolution.

After several combinations of mobile solvents with stationary phase C18, the above method has been optimized i.e. acetonitrile: methanol: buffer (pH 3.0) in ratio of (40:30:30 v/v) respectively using C18 column which has given good resolution (r^2 for VAL 0.980 and NEB 0.983) and capacity factor, acceptable system suitability. Chromatographic peaks of both the drugs were identified by overlaying individual drug with chromatograph of mixture is shown in Fig. 3 and 4. Both drugs eluted within 10 min which will reduce the analysis time and cost. The optimized chromatographic conditions are given in Table 1. The representative chromatogram of standard Valsartan and Nebivolol is shown in Fig.5.

Table 1: Optimization of Chromatographic Conditions

Parameter	Optimized conditions
HPLC system	Younglins Acme 9000 series quaternary gradient pump system with autochrom-3000 software
Column	C-18 column (150mm × 4.6mm, 5µm) with Phenomenex Security Guard Cartridges C18 (4×3mm)
Mobile phase	acetonitrile: methanol: buffer (pH 3.0) (40:30:30 v/v/v)
Flow rate	1ml/min
Detection wavelength	228nm
Injection volume	20µl
Concentration of standard Valsartan and Nebivolol	160µg/ml and 10µg/ml

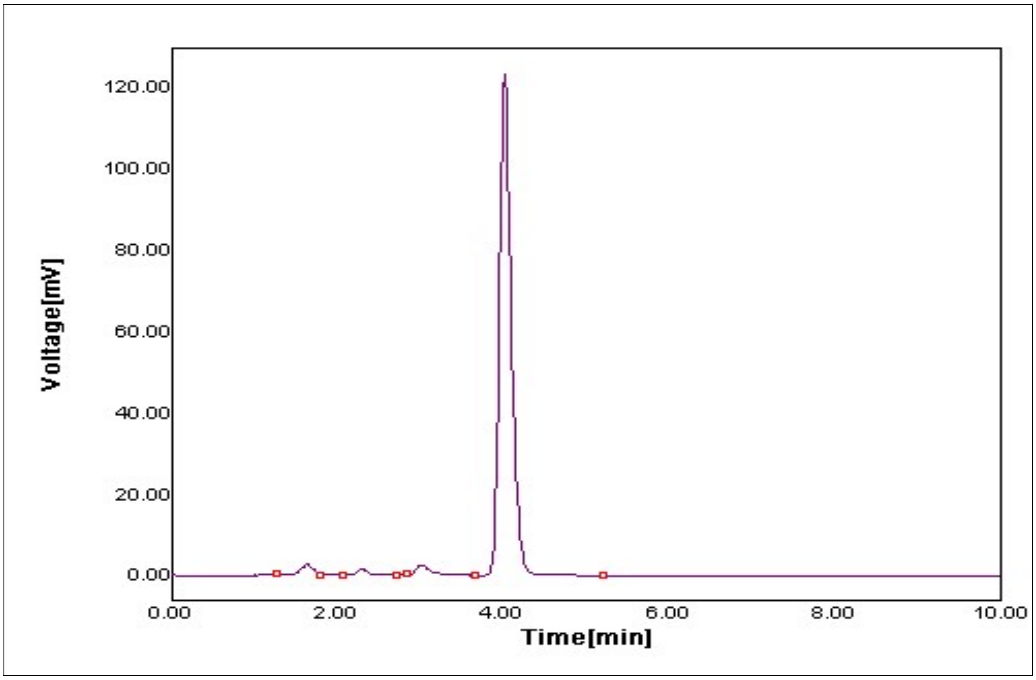

Fig.3 Chromatogram of Valsartan (160µg/ml) in optimized chromatographic conditions

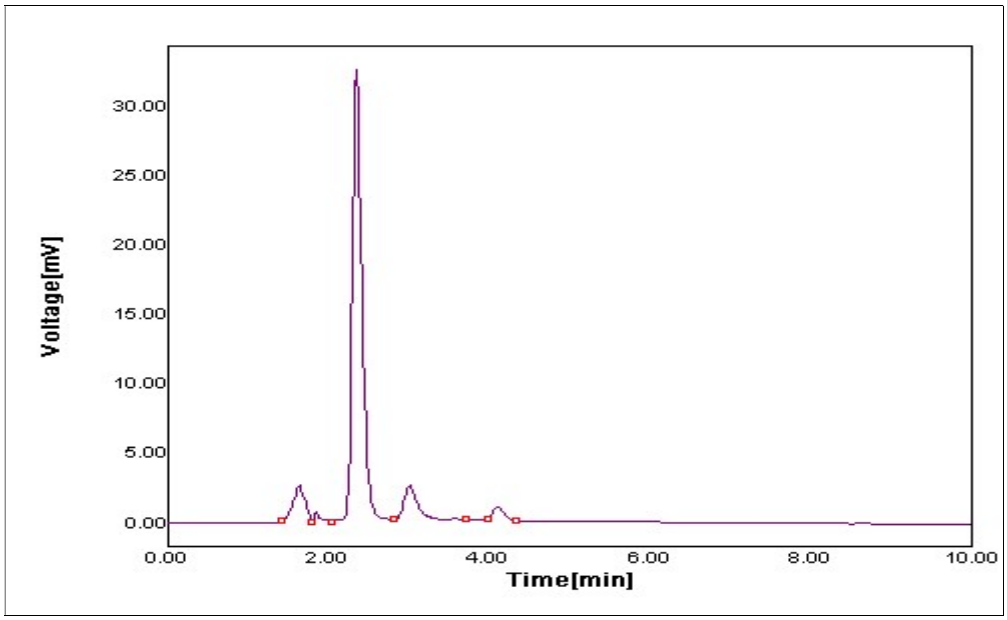

Fig.4 Chromatogram of Nebivolol (10µg/ml) in optimized chromatographic conditions

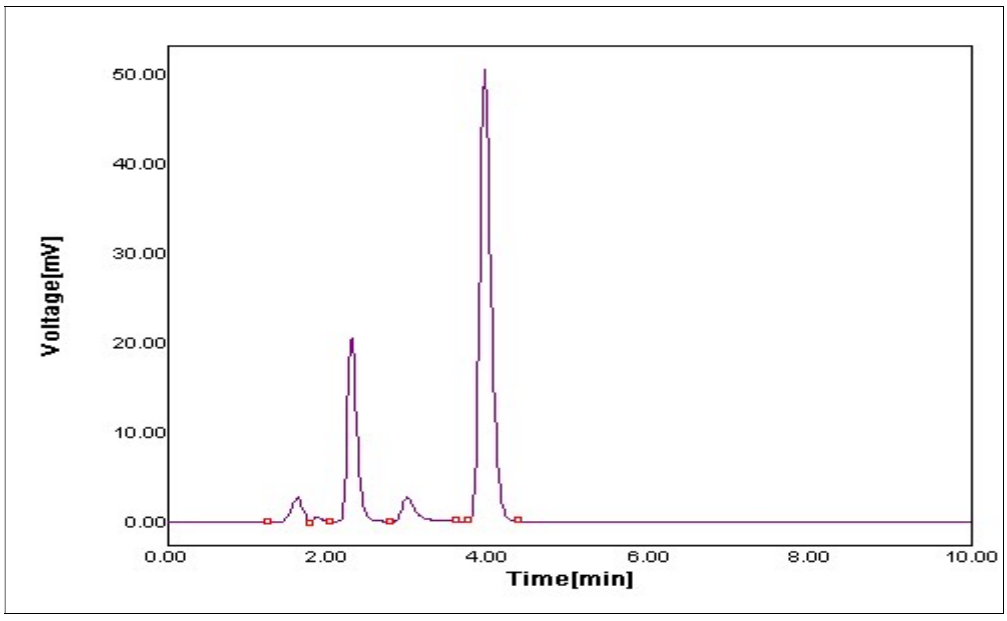

Fig.5 Representative Chromatogram of Standard NEB (10µg/ml) & VAL (160µg/ml) in optimized chromatographic conditions

The chromatogram for the specificity of Nebivolol and Valsartan combination marketed tablet dosage form showed peak at a retention time 2.5 min and 4.2 min. Retention time of both the drugs in standard mixture shows peak at 2.6 min (NEB) and 4.1 min (VAL) in Fig 6.

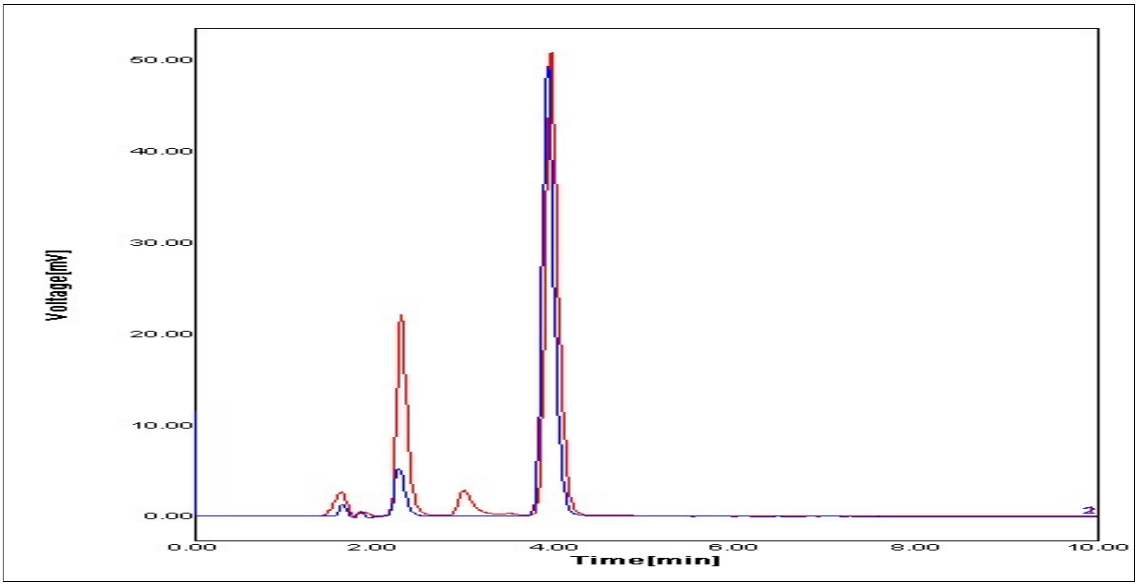

Fig.6. Overlain Chromatograms of sample and standard solution of similar concentration of drugs

In accuracy, the percentage recoveries of the results indicate that the recoveries are well within the acceptance range, therefore, method is accurate.

Table 2: Accuracy for HPLC method

Sr. No.	Level of % Recovery	Amount of 'Sample Stock-A' (ml)	Amount of Standard Drug Added (µg/ml)		Total Amount Found (µg/ml)		Amount Recovered (µg/ml)		% Recovery	
			VAL	NEB	VAL	NEB	VAL	NEB	VAL	NEB
1	0	0.5	0	0	22.10	2.5	-	-	-	-
2	80	0.5	32	2	53.23	4.4	31.13	1,9	98	95
3	100	0.5	40	2.5	63.07	4.9	40.97	2.4	102	96
4	120	0.5	48	3	71.01	5.4	49	2.9	102	96

The peak response is proportional to concentration and linear in the range of 32-160µg/ml and 2-10µg/ml, respectively for VAL and NEB (Fig.7,8,9). The correlation coefficient is 0.983 and 0.980 which is well within the acceptance criteria. (Table no.3)

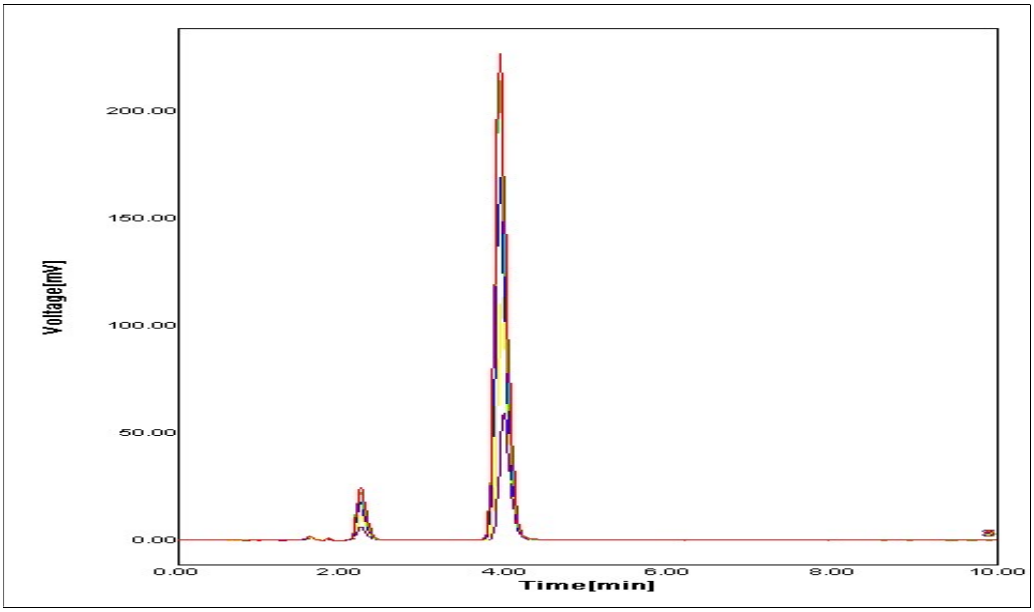

Fig.7. Overlain Chromatograms of serial dilutions of NEB and VAL in optimized chromatographic conditions

Table 3: Response of Valsartan and Nebivolol at various linearity levels

Valsartan		Nebivolol	
Concentration (µg/ml)	Peak Area mv	Concentration (µg/ml)	Peak Area mv
0	0	0	0
32	547	2	47
64	1052	4	89
96	1598	6	137
128	2095	8	184
160	2303	10	203

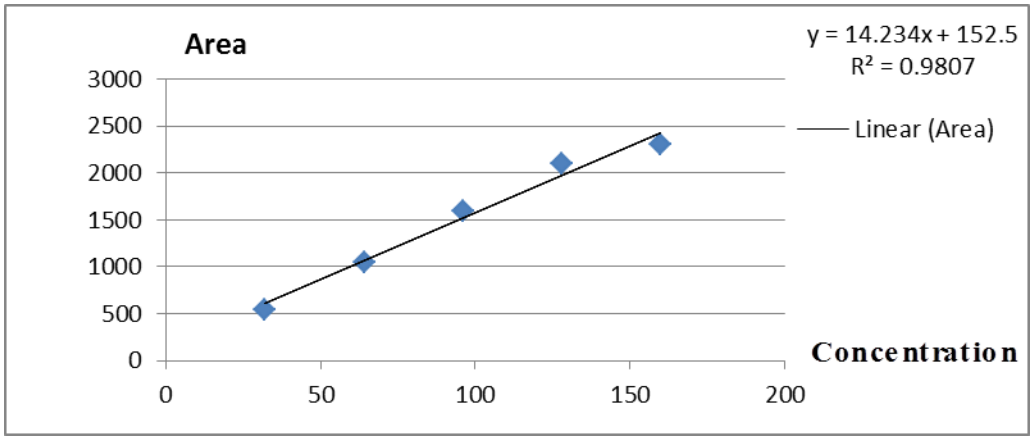

Fig.8. Calibration curve of Valsartan of RP-HPLC method

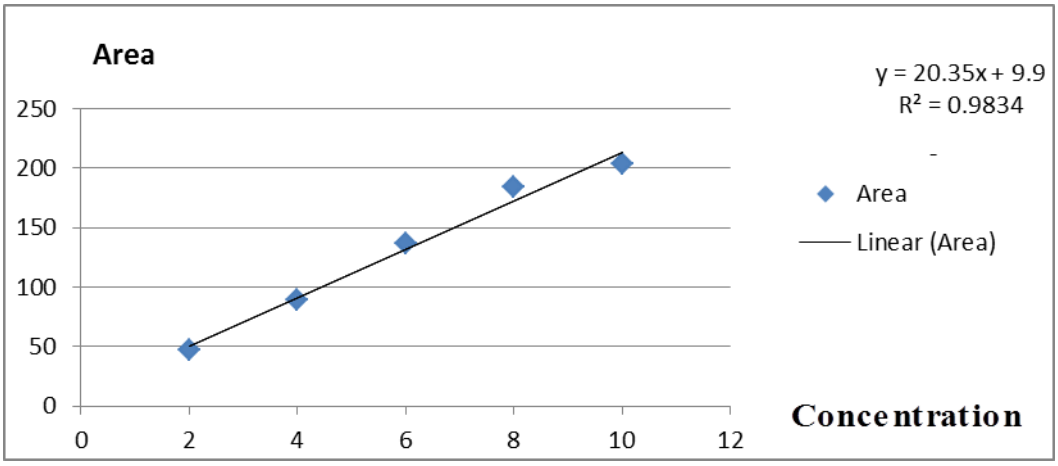

Fig.9. Calibration curve of Nebivolol of RP-HPLC method

Table 4: Shows the range is from 32-160µg/ml for VAL and 2-10µg/ml for NEB

Parameters	VAL	NEB
Linearity Range (µg/ml)	32-160	2-10

The data for precision is represented in Table – 5 and 6. The %RSD was found to be at 8am- 1.41 for Valsartan and 0.433 for Nebivolol and at 6pm- 0.93 and 1.69 for Valsartan and Nebivolol for intraday. The % RSD was found to be 0.93 and 1.69 of Valsartan and Nebivolol for inter day precision studies. Thus the developed method was found to be accurate and precise as the % RSD value was less than 2.

Table 5: Intra-day Precision of Valsartan and Nebivolol

Inj.	Peak Area(mV) at 8am		Peak Area(mV) at 4pm	
	VAL	NEB	VAL	NEB
1	2510	206	2431	205
2	2452	205	2425	200
3	2445	207	2464	209
4	2435	207	2455	208
5	2419	207	2400	208
6	2445	207	2430	208
SD	31.09	0.83	22.81	3.50
RSD	1.41	0.433	0.93	1.69

Table 6: Inter day Study for Valsartan and Nebivolol

Inj.	Peak Area(mV)	
	VAL	NEB
1	1598	137
2	1636	143
3	1623	142
4	1593	140
5	1588	143
6	1631	145
SD	20.92	2.80
RSD	1.29	1.97

The limit of detection and limit of quantification for Valsartan and Nebivolol are given in Table 8. The results of robustness study are given in Table – 7 & 8. It was found that there was no drastic change in the resolution of Valsartan and Nebivolol, thus confirming robustness of the developed method.

Table 7: Limit of Detection and Limit of Quantitation data of Valsartan and Nebivolol

Parameter	VAL	NEB
LOD (µg/ml)	24.56	1.42
LOQ (µg/ml)	81.89	4.73

Table 8: Result of Robustness Study: Variation in Flow Rate (ml/min)

Flow Rate (ml/min)	Analyte	Retention Time (min)	Tailing Factor (T)	Theoretical Plates (N)	Resolution (R)
0.9	VAL	3.9	1.24	5043	8.38
	NEB	2.3	1.61	2889	
1.0	VAL	4.7	1.217	4616	5.53
	NEB	2.5	0.798	1832	
1.1	VAL	3.5	1.16	5454	8.01
	NEB	2.0	1.24	2108	

Table 9: Result of Robustness Study: Variation in Wavelength (nm)

Wavelength (nm)	Analyte	Retention Time (min)	Tailing Factor (T)	Theoretical Plates (N)	Resolution (R)
282	VAL	3.9	1.25	4908	8.3
	NEB	2.2	1.3	2841	
286	VAL	3.9	1.25	5780	8.8
	NEB	2.2	1.28	2997	
284	VAL	3.9	1.24	5043	8.38
	NEB	2.3	1.61	2889	

System suitability testing was studied at parameter of resolution, retention time, tailing factor, and capacity factor shows system is suitable for this method.

Table 10: Results of System Suitability Parameters

Analyte	Retention Time (min)	Tailing Factor (T)	Theoretical Plates (N)	Resolution (R)
VAL	4.7	1.217	4909	4.48
NEB	2.6	1.325	2374	2.46
Required limits	--	T < 2	N > 2000	R >2

The report for the assay of combined tablet of Valsartan and Nebivolol is presented in Table no. 11.

Table 11: Assay Results of Tablet Formulation by RP-HPLC Method

Sr. No.	Amount (Therotical) (µg/ml)		Amount Found (µg/ml)		% Label Claim	
	VAL	NEB	VAL	NEB	VAL	NEB
1	120	7.5	62.08	7.05	51.73	94.41

4. CONCLUSION

In conclusion, the HPLC method is simple, accurate, reproducible method for estimation of VAL and NEB in bulk and pharmaceutical formulation. The short chromatographic time makes this method suitable for processing of multiple samples in short time. The method shows no interference by the excipients. The statistical parameters and recovery data reveals the good accuracy and precision. This method could be useful and suitable for the estimation of the VAL&NEB in bulk and pharmaceutical formulations

5. ACKNOWLEDGEMENT

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