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Original Article

Method Development and Validation of Stability Indicating RP-HPLC Method for Simultaneous Estimation of Nebivolol HCL and Valsartan in Bulk and its Pharmaceutical Formulations

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ABSTRACT

A simple, robust, precise HPLC method was developed and the method has been validated for the estimation of valsartan and nebivolol simultaneously in its combined dosage forms as well as individual formulations. Both the compounds are well resolved in a isocratic method by using the mobile phase composition of acetonitrile: methanol: pH4.0 0.02M Potassium hydrogen phosphate buffer in the ratio of 50:20:30 v/v at a flow rate of 1.0mL/min using a C-18 column. The detection was carried out at 210nm.. The retention time of Nebivolol and Valsartan were 2.5 min and 4.3 min respectively. The developed method was validated for specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness as per ICH guidelines. Linearity for Nebivolol and Valsartan were found in the range of 48-112 µg/ml and 3.0-7.0 µg/ml, respectively. The percentage recoveries for Nebivolol and Valsartan ranged from 98.9-100.8 % and 98.9-101.1 %, respectively. The proposed method could be used for routine analysis of Nebivolol and Valsartan in their combined dosage forms.

Keywords: Liquid chromatography, Nebivolol HCl, Valsartan, Combined dosage forms, Simultaneous estimation, Validation.

INTRODUCTION

Nebivolol hydrochloride (NEB) is (±) [2R* R* R* (S *)] œ, œ [imino bis (methylene)] bis- [6- fluoro- 3, 4 - dihydro- 2H- 1- benzopyran- 2-methanol] hydrochloride is an antihypertensive drug, It is a racemate of two enantiomers with four chiral centers. The SRRR-enantiomer (d-nebivolol) is a potent and cardio selective

β1-adrenergicblocker[1,3,4]. The RSSS-enantiomer (nebivolol) has a favorable hemodynamic profile[1,3]. Valsartan (VAS) (N-valeryl-N[[2-(1H-tetrazol-5-yl) biphenyl-4-yl]methyl] valine,[2,12,14] is an orally active, potent and specific competitive angiotensin II antagonist acting at the ATI receptor, which mediates all known effects of angiotensin II on the cardiovascular system. Valsartan is widely used in the treatment of hypertension[1,3]. Combination of NEB and VAS is used as cardiovascular and 1-adrenergic blocker. The chemical structures of NEB, VAS are shown in (Fig. 1)[1,2].

To the best of our knowledge, no study has been reported for the simultaneous determination of NEB and VAS in pharmaceutical formulations by UV-spectrophotometer[5,11,13,14,17,18] and RP-HPLC method[4,6-10,12]. The significance of the developed methods is to determine the content of both drugs simultaneously in commercially available capsule dosage form and can be used in future for bioequivalence study for the same formulations. The capsule solid dosage form in combination containing nebivolol HCl (5 mg) and valsartan (80mg) is available in the market. In this paper, we reported two spectrophotometric methods and one reverse-phase HPLC method for the quantification of NEB and VAS simultaneously. The present RP-HPLC method was validated as per ICH guidelines[20].

However, there is no analytical method reported for simultaneous estimation of both drugs in their combined tablet dosage form by reporting forced degradation studies to demonstrate stability indicating nature of the method. Present work describes rapid, simple, sensitive, accurate and reproducible stability indicating method. The present developed method was used to determine the Nebivolol and Valsartan present in the formulation and method validated according to the ICH guidelines[18,19].

MATERIALS AND METHODS

Materials

HPLC grade Potassium dihydrogen phosphate, acetonitrile, methanol and water were procured from Merck India. All dilutions were performed in standard class-A, volumetric glassware. For the estimation of commercial formulation, Nebicard V having (Nebivolol HCl-80mg and Valsarton-5mg) manufactured by Torrent pharmaceuticals Ltd were procured from the local market.

Instrumentation

Agilent 1120 compact LC chromatographic system, with DAD detector and a fixed injector equipped with 20µL loop was used for the chromatographic separation. The chromatogram was recorded at and peaks quantified by means of Ez Chrome software. Chromatographic separation was carried out on a C18 column [Inertsil ODS 3V, 150mm x4.6mm 5µ]. Sartorius electronic balance was used for weighing the samples. Ultrasonic bath sonicator was used for degassing and mixing of the mobile phase.

Chromatographic conditions

Chromatographic separation of Nebivolol and Valsartan was carried on a C18 column. The mobile phase was composed of acetonitrile, methanol and potassium dihydrogen phosphate buffer (pH 4.0) in the ratio of 50:20:30 v/v. It was filtered through a 0.45 µ membrane filter and degassed for 15 minutes. The flow rate of the mobile phase was maintained at 1 ml/min. Detection was carried out at 210 nm at ambient temperature.

Method development

Preparation of standard stock solutions

Standard stock solutions were prepared by dissolving 40 mg of Valsartan

and 25 mg Nebivolol working standard in two separate each 25 mL and 100 mL volumetric flasks using 30mL of mobile phase and made up to the mark with mobile phase to obtain a final concentration of 1600µg/mL and 250 µg/mL of each Nebivolol and Valsartan . From the above stock solutions, each 5ml and 2mL of aliquots of Nebivolol and Valsartan were pipette in to a 100mLvolumetric flask and dissolved in 25mL of the mobile phase and made up to the mark with the solvent to obtain a final concentration of 80 µg/mL and 5 µg/mL for Nebivolol and Valsartan respectively.

Preparation of sample solutions

Weighed and finely powdered 20 Tablets. Accurately weighed and transferred equivalent to 400mg Nebivolol and 25mg of valsartan into a 250 mL volumetric flask, added 150 mL of diluent, and sonicated for 30minutes with intermittent shaking at controlled temperature and diluted to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transferred 5.0 mL of the above solution into a 100 mL volumetric flask and diluted to volume with diluent to obtain a concentration of 80 and 5 µg/mL of Nebivolol and Valsartan respectively.

Method validation

The developed HPLC method for the simultaneous determination of Nebivolol and Valsartan was validated as per the ICHguidelines[13,14].

As part of method validation as per ICH guidelines, the following parameters are studied.

1. System Suitability and System Precision
2. Specificity Studies
   A. Blank Interference
   B. Placebo Interference
   C. Forced degradation studies in different stress conditions to establishing stability indication of the developed method.
3. Method Precision
4. Accuracy studies
5. Linearity Studies including LOD/LOQ determination
6. Ruggedness
7. Robustness
8. Analysis of Marketed samples by applying the developed method.

Each parameter was explained separately in different sections under results and discussions.

RESULTS AND DISCUSSION

System suitability and system precision[20]

System suitability for chromatographic separation was checked in each day of validation to evaluate the components of the analytical system in order to show that the performance of the system meet the standards required by the method. System suitability parameters established for the developed method include number of theoretical plates (efficiency), Resolution, Tailing factor. The HPLC system was equilibrated using the initial mobile phase composition, followed by 5 injections of the standard solution of 100% concentration containing 80 µg/mL Nebivolol and 5 µg/ml Valsartan. These 5 consecutive injections were used to evaluate the system suitability on each day of method validation. The result was given in the Table 1.

Specificity[20]

Blank interference

A study to establish the interference of the blank was conducted. Diluent was injected into the chromatograph in the defined above chromatographic conditions and the blank chromatograms were recorded. Chromatogram of Blank solution (Fig. no. 2) showed no peaks at the retention time of Nebivolol and Valsartan peak. This indicates

that the diluent solution used in sample preparation does not interfere in the estimation of Nebivolol and Valsartan in Nebivolol and Valsartan tablets. Similarly, typical representative chromatogram of standard is also shown (Fig. No. 3).

Forced degradation[20]

Control sample

Weighed and finely powdered 20 Tablets. Accurately weighed and transferred equivalent to 400mg Nebivolol and 25mg of Valsartan into a 250 mL volumetric flask, added 150 mL of diluent, and sonicated for 30minutes with intermittent shaking at controlled temperature and diluted to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transferred 5.0 mL of the above solution into a 100 mL volumetric flask and diluted to volume with diluent. Refer (Fig. no. 4A)

Acid degradation sample

Weighed and finely powdered 20 Tablets. Accurately weighed and transferred equivalent to 400mg Nebivolol and 25mg of Valsartan into a 250 mL volumetric flask, added 150 mL of diluent, and sonicated for 30minutes with intermittent shaking at controlled temperature. Then add 5mL of 1N acid, refluxed for 30min at 60°C, then cooled to room temperature, neutralize with 1N NaOH and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 5.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent. Refer (Fig. no. 4B)

Base degradation sample

Weighed and finely powdered 20 Tablets. Accurately weighed and transferred equivalent to 400mg Nebivolol and 25mg of Valsartan into a 250 mL volumetric flask, added 150 mL of diluent, and sonicated for 30minutes with intermittent shaking at controlled temperature. Then add 5mL of 1N NaOH, refluxed for 30min at 60°C, then cooled to room temperature, neutralize with 1N NaOH and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 5.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent. Refer (Fig. no. 4C)

Peroxide degradation sample

Weighed and finely powdered 20 Tablets. Accurately weighed and transferred equivalent to 400mg Nebivolol and 25mg of Valsartan into a 250 mL volumetric flask, added 150 mL of diluent, and sonicated for 30minutes with intermittent shaking at controlled temperature. Then add 5mL of Hydrogen Peroxide, refluxed for 30min at 60°C, then cooled to room temperature, and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 5.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent. Refer (Fig. no. 4D)

Thermal degradation sample

Weighed and finely powdered 20 Tablets. Accurately weighed and transferred equivalent to 400mg Nebivolol and 25mg of Valsartan into a 250 mL volumetric flask, added 150 mL of diluent, and sonicated for 30minutes with intermittent shaking at controlled temperature and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter. Transfer 5.0 mL of the above solution into a 100 mL volumetric flask and dilute to volume with diluent. Refer (Fig. no. 4E)

Similarly Humidity, UV-Light exposure, Sunlight exposure and Water hydrolysis stress samples are prepared and checked for their purity by the proposed method.

Linearity and range[20]

The standard curve was obtained in the concentration range of 48-112 µg/ml for Nebivolol and 3-7 µg/mL for Valsartan. The linearity of this method was evaluated by linear regression analysis. Slope, intercept and correlation coefficient [r2] of standard curve were calculated and given in Figure-5A (For Nebivolol) and Figure-5B (For Valsartan) to demonstrate the linearity of the proposed method. The result of regression analysis was given in the Table 2.

From the data obtained which given in Table-2 (For Nebivolol and Valsartan) the method was found to be linear within the proposed range.

Accuracy[20]

The accuracy of an analytical method is the closeness of the results obtained by that method to the true value of the sample. It is expressed as recovery (%), which is determined by the standard addition method. In the current study recovery at three spike levels 80%, 100% and 120% were carried out. The % recovery at each spike level was calculated and was given in Table 3.

Method precision[20]

The precision of an analytical method is the closeness of replicating results obtained from analysis of the same homogeneous sample. Precision was considered at different levels, i.e. method, system.

Inter day and Intraday. Precision of the developed method was assessed by measuring the response on the same day (Intraday precision) and next two consecutive days (inter day precision). The precision of the method was assessed by six replicate injections of 100% test concentration. Intra and inter-day precision of the method was assessed by determination of standard deviation and % RSD for the analyst response. The result was given in Table 4.

LOD and LOQ[20]

LOD and LOQ values were determined by the formulae LOD = 3.3 σ/S and LOQ = 10 σ/S(Where, σ is the standard deviation of the responses and S is the slope of the calibration curves). In the present method σ is the mean of standard deviation of y intercepts of the three calibration curves and S is the mean of the slopes of the calibration curves. The result was given in Table5.

Robustness[20]

The robustness of the method were determined by assessing the ability of the developed method to remain unaffected by the small changes in the parameters such as percent organic content, pH of the mobile phase, buffer concentration, temperature, injection volume and flow rate. A deviation of ± 2nm in the detection wavelength, ± 0.1 mL/min in the flow rate, ± 5%change in the organic phase were tried individually. The result was given in the Table 5.

OVER ALL SUMMARY OF THE METHOD

Column chemistry, solvent selectivity, solvent strength (volume fraction of organic solvent (s) in the mobile phase), detection wavelength and flow rate were varied to determine the chromatographic conditions for giving the best separation[20]. Several mobile phase compositions were trying to resolve the peaks of Nebivolol and Valsarton. The optimum results were attained with acetonitrile, methanol and potassium dihydrogen phosphate buffer (pH 4.0) in the ratio of 50:20:30 (v/v) because it could resolve the peaks of Nebivolol with retention time of 2.5 min and Valsortan retention time at 5.3 min. The two peaks were symmetric and sufficiently resolved. System suitability was carried out by injecting 5 replicate injections of 100% concentration of Nebivolol and Valsarton. The resolution was

found to be greater than 2 and the other parameters are presented in Table 1.

Specificity[20] of the chromatographic method was tested by injecting mobile phase as blank and sample concentration prepared from marketed formulation. The response was compared with that obtained from the standard drug. The chromatogram confirms the presence of Nebivolol and Valsarton at 2.5 min and 5.3 min respectively without any interference. Thus the developed method was specifically for analyzing the commercial formulations for Nebivolol and Valsarton. An optimized chromatogram with the retention times of Nebivolol and Valsarton was shown in the Figure 2.

The peak areas corresponding to the concentration range of Nebivolol 48-112 µg/mL and Valsarton 3-7 µg/ml prepared in triplicate were plotted against the respective concentrations. The calibration curves were linear in the range studied for Nebivolol and Valsarton, respectively, with mean correlation coefficients (n=3) of 0.999 and higher, the representative calibration curve is shown in Figure3. The regression analysis was given in Table 2.

Accuracy[20] of the proposed method was assessed by the standard addition method at 80%, 100% and 120% levels of recovery to the pre analyzed sample in triplicate. The recovery of the added standard to the sample was calculated and it was found to be 98.9-100.8 %w/w for Nebivolol and 98.9-101.1 %w/w for Valsarton respectively and the % RSD was less than 2 for both the drugs which indicates good accuracy of the method. The result of recovery was given in table 3.

LOD and LOQ were calculated from the average slope and standard deviation of y intercepts of the calibration curve. Limit of detection for Nebivolol and Valsarton were 2.20 µg/mL and 1.65 µg/mL, respectively where as the limit of quantitation of Nebivolol and Valsarton were 6.67 µg/mL and 5.00 µg/mL respectively indicating the high sensitivity of the method. LOD and LOQ value was given in table 2. The method is precise with a %RSD of less than 2 for both Nebivolol and Valsarton respectively. The results of Intraday and inter day precision was given in table 4. Robustness were carried out by change in the flow rate (±1mL/min), mobile phase variation (±5%) and variation in wavelength (± 2 nm). Solution of 100% concentration is prepared and injected in triplicate for each varied operational condition and % R.S.D was found to be less than 2. The result was given in table 5. The proposed method was applied for the assay of commercial formulation containing Nebivolol and Valsarton. Each sample was analyzed in triplicate. The mean recovery values were 100.2 and 101.0 for Nebivolol and Valsarton. The result of estimation was given in table 6.

CONCLUSION

The proposed RP-HPLC method for simultaneous assay Nebivolol and Valsartan in combined dosage forms was validated, and found to be applicable for routine quantitative analysis of Nebivolol and Valsartan. The results of linearity, precision, accuracy and specificity, were proved to be within the limits. The method provides selective quantification of Nebivolol and Valsartan with no interference from other formulation excipients. Therefore, this method can be employed for the routine analysis for simultaneous estimation Nebivolol and Valsartan in quality control of formulations and also in the dissolution studies.

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Table 1. System suitability parameters for nebivolol and valsartan by proposed method

Name of the compound	Retention time	Tailing factor	Theoretical plate	USP resolution
Nebivolol	2.510	1.375	2761	-
Valsartan	4.397	1.156	4883	10.248

Table 2. Linearity studies for nebivolol and valsartan by proposed method

Linearity of response				
% Level (approx.)	For nebivolol		For valsartan	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
60	48	1402.1622	3	158.2464
80	64	1899.5496	4	210.9952
100	80	2336.937	5	263.744
120	96	2804.3244	6	316.4928
140	112	3271.7118	7	379.2416
	Slope	29	Slope	55
	Intercept	21	Intercept	8
	% Y-Intercept	0.7	% Y-Intercept	14.5
	STYEX	14	STYEX	4
	CC	0.9999	CC	0.9993
	RSQ	0.9997	RSQ	0.9987
	Residual sum of squares	14	Residual sum of squares	4
	LLD	2.20	LLD	1.65
	LLQ	6.67	LLQ	5.00

Table 3A. Recovery studies for nebivolol by proposed method

% Level	Recovery range	% RSD at each level	Over all %RSD
80	99.5-100.8	0.72	0.57
100	99.1-99.8	0.36	
120	98.9-99.2	0.15	

Table 3B. Recovery studies for valsartan by proposed method

% Level	Recovery range	% RSD at each level	Over all %RSD
80	98.9-99.8	0.48	0.88
100	99.7-101.5	0.98	
120	100.8-101.1	0.62	

Table 4. Method precision (inter and intraday) studies for nebivolol and valsartan by proposed method

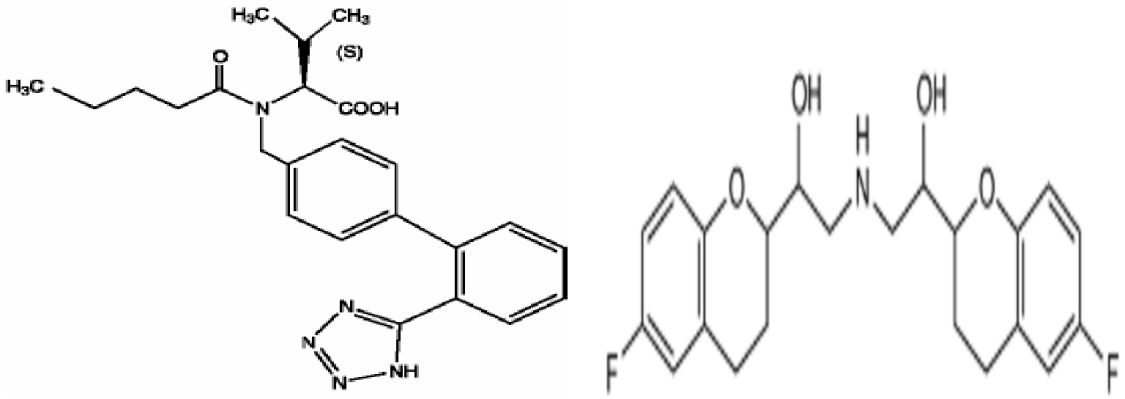
Summary showing method precision by proposed method			
For nebivolol		For valsartan	
Method precision (Inter &Intra day)		Method precision (Inter &Intra day)	
100.8	98.9	98.9	98.1
100.1	99.5	98.7	99.8
100.6	99.1	98.6	99.9
99.9	100.7	99.8	100.1
98.9	100.1	99.9	100.8
100.4	98.7	98.9	99.1
Overall avg.	99.81		99.38
Overage std dev.	0.76		0.78
Over all %RSD	0.76		0.78

Table 5. Robustness studies for nebivolol and valsartan by proposed method

Parameter		% RSD	
		Nebivolol	Valsartan
Wavelength ±2	208 nm	0.46	0.87
	212 nm	0.59	0.24
Flow Rate mL /min	0.8 mL/min	0.75	0.68
	1.2mL.min	0.12	0.43

Table 6. Assay of marketed samples for nebivolol and valsartan by proposed method

Drug	Amount claimed in mg per tablet	Estimated amount in mg/tablet	% Assay
Nebivolol	80	80.12	100.2
Valsartan	5	5.05	101.0



(a) (b)

Figure 1. (A) Valsartan, (B) Nebivolol HCl

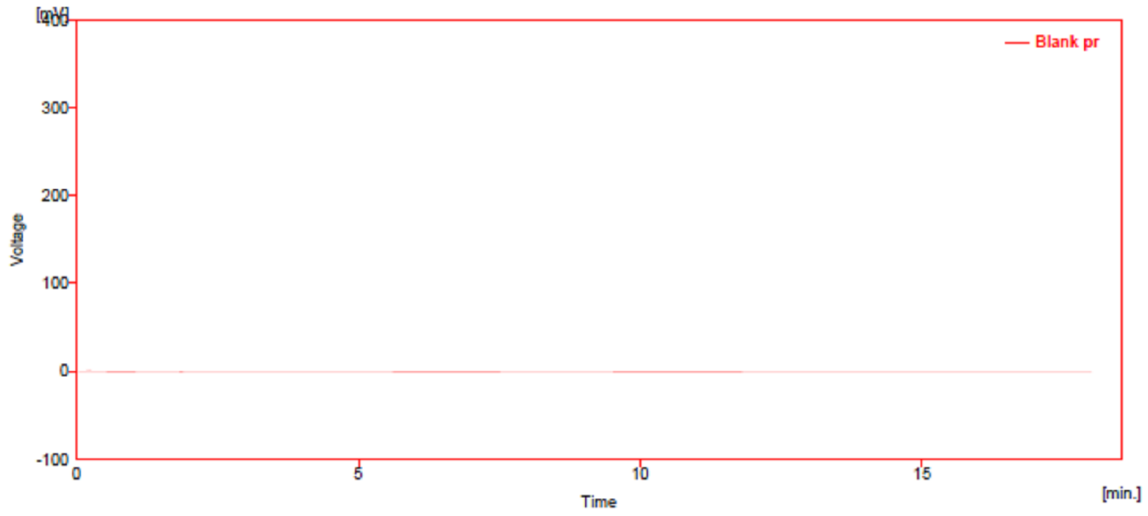


Figure 2. A typical HPLC Chromatogram showing the no interference of diluent for Nebivolol and Valsartan

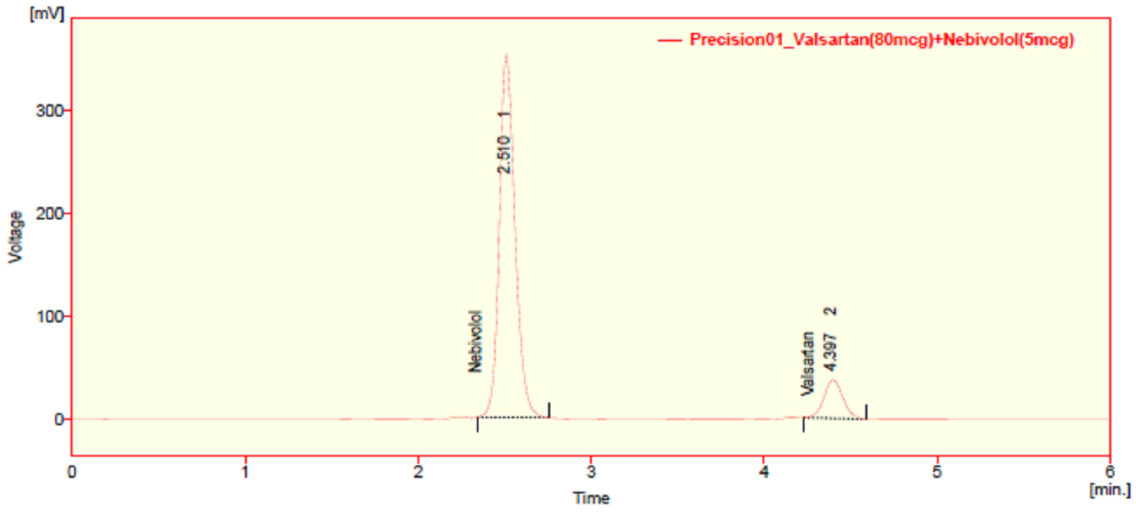


Figure 3. A typical HPLC chromatogram showing the peak of nebivolol and Valsartan

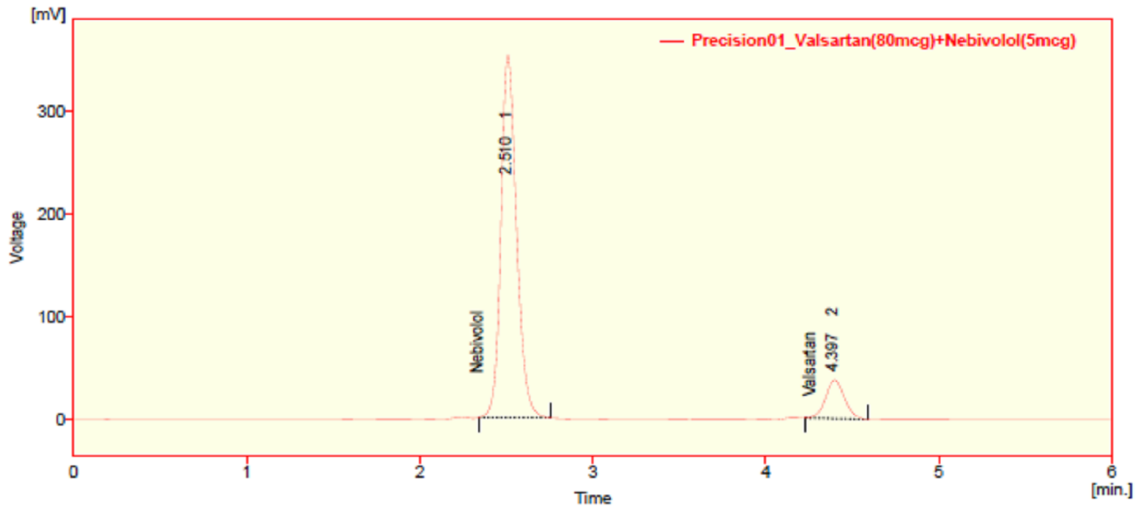


Figure 4A. A typical HPLC chromatogram showing the control sample profile of nebivolol and valsartan by proposed method

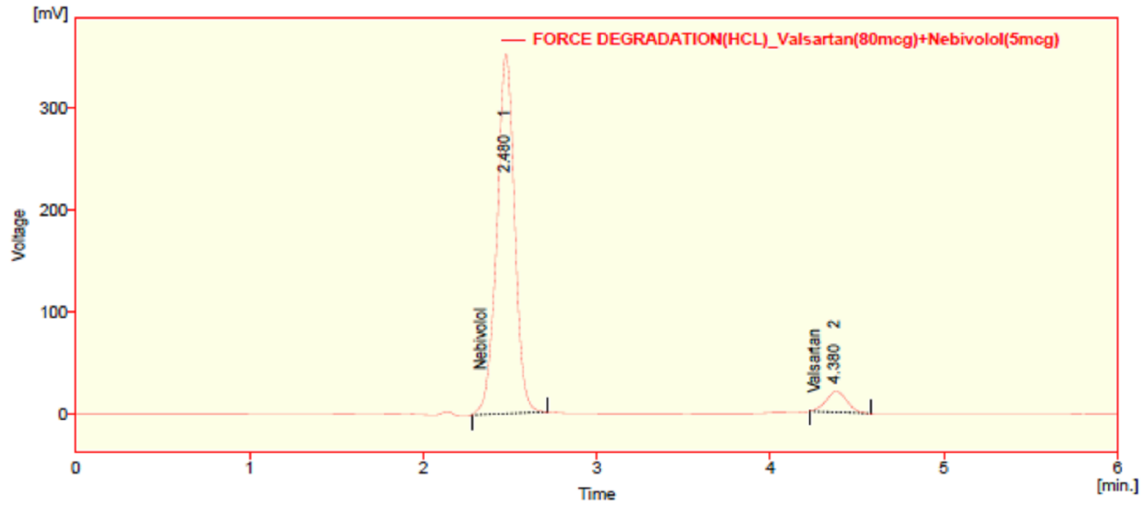


Figure 4B. A typical HPLC chromatogram showing the profile of nebivolol and valsartan in acidic hydrolysis by proposed method

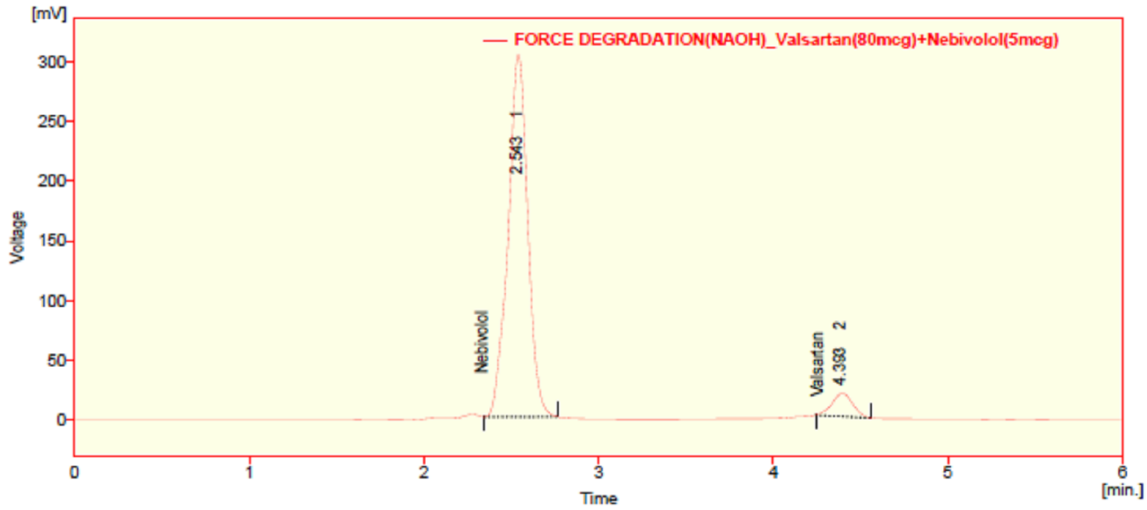


Figure 4C. A typical HPLC chromatogram showing the profile of nebivolol and valsartan in base hydrolysis by proposed method

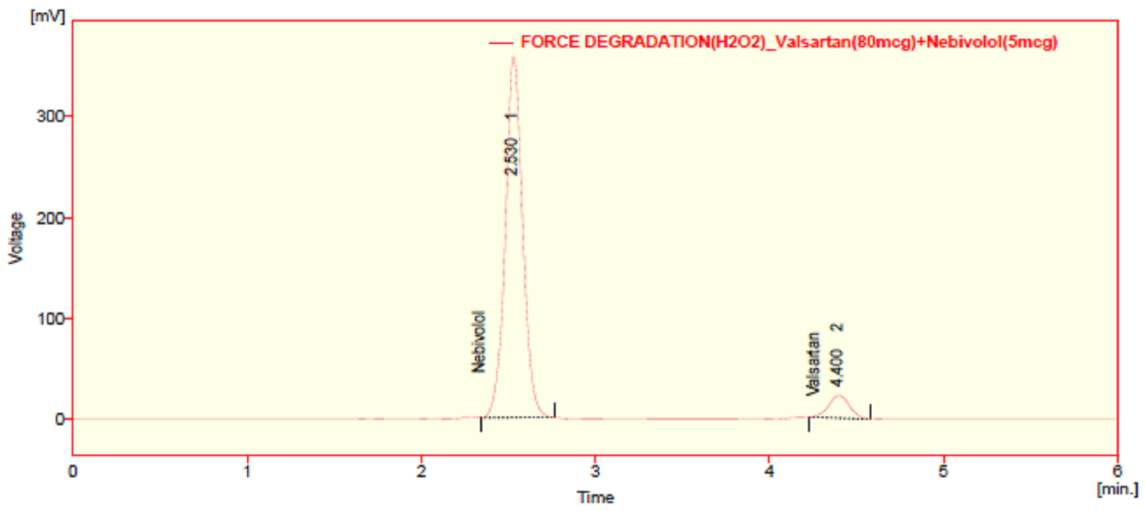


Figure 4D. A typical HPLC chromatogram showing the profile of nebivolol and valsartan in peroxide hydrolysis by proposed method

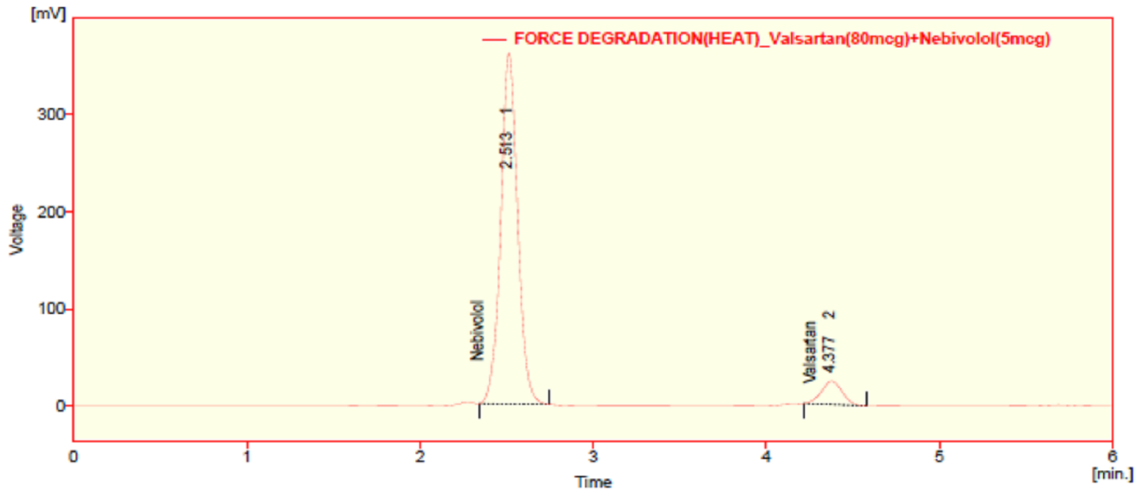


Figure 4E. A typical HPLC chromatogram showing the profile of nebivolol and valsartan in thermal hydrolysis by proposed method

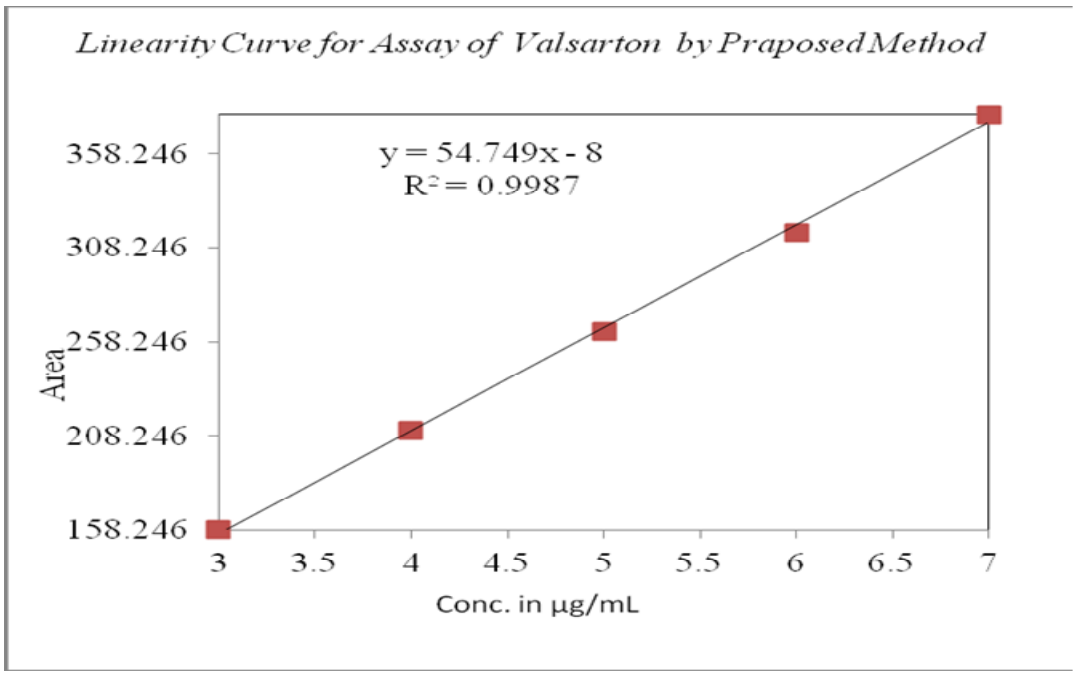


Figure 5A. Calibration curve for nebivolol HCl

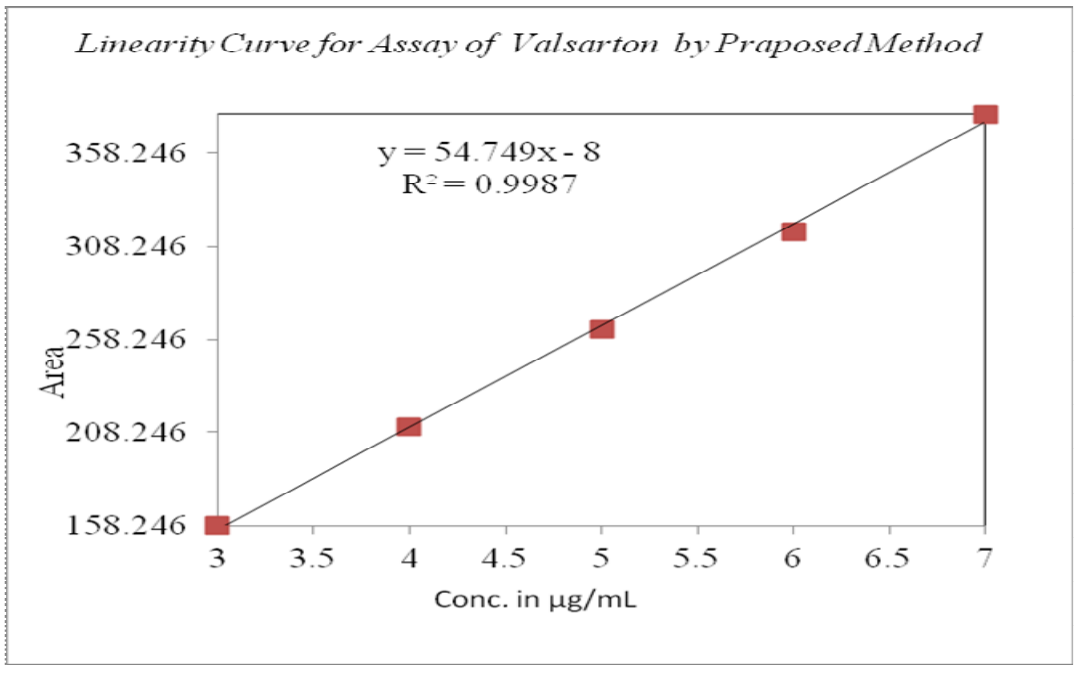


Figure 5B. Calibration curve for valsartan