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Stability indicating assay method for simultaneous estimation of nebivolol and valsartan in pharmaceutical dosage form by RP-HPLC

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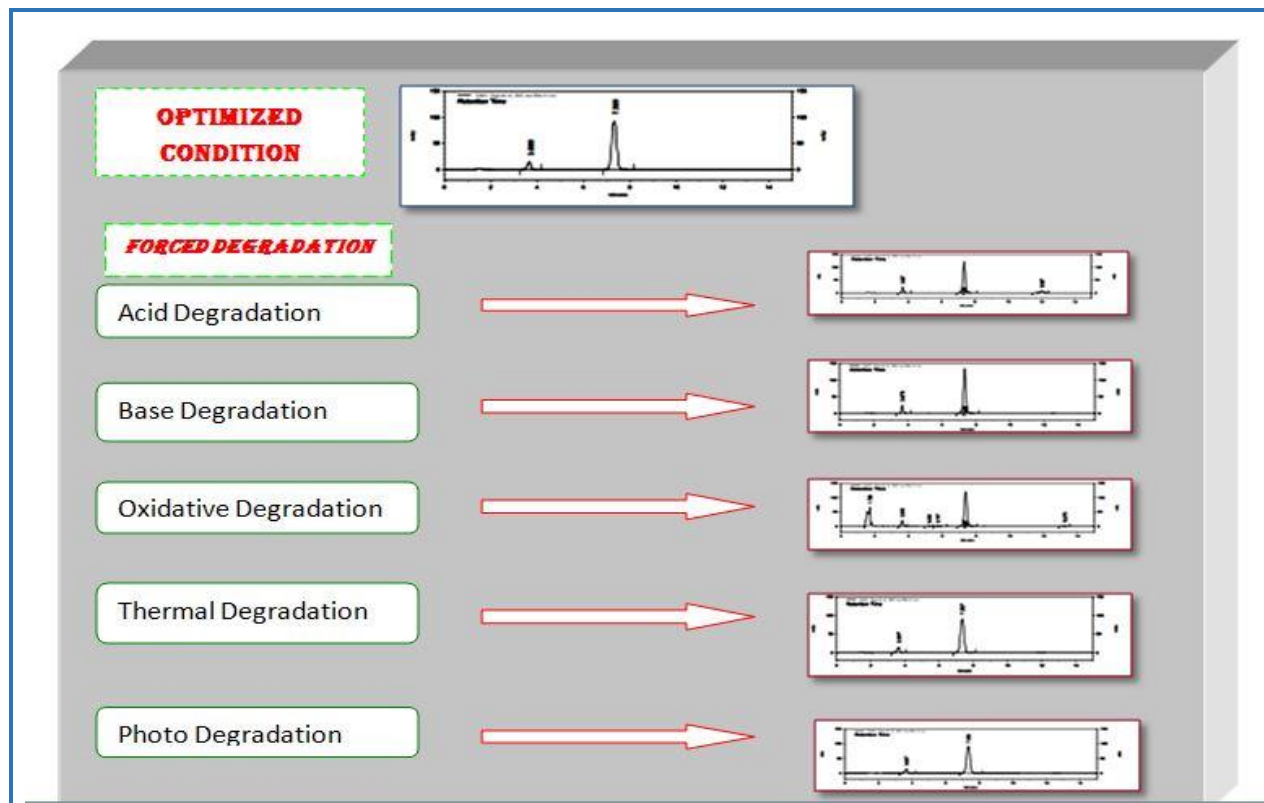
KEYWORDS

Nebivolol
Valsartan
RP-HPLC
Stability indicating
Validation

ABSTRACT

A simple, rapid, precise, and accurate isocratic reversed-phase stability indicating HPLC method was developed and validated for simultaneous determination of the nebivolol and valsartan in the tablet dosage form. The effective chromatographic separation was achieved by a YMC pack pro octadecyl silane (150×4.6 mm, 3 μm) column using a mobile phase composed of methanol: acetonitrile: 0.05 M potassium dihydrogen phosphate buffer (pH=3.0 with 10% ortho phosphoric acid after addition of 0.2% triethylamine) (30:30:40, v/v/v) at a flow rate of 1 mL/min and UV detection at 282 nm. Drugs were subjected to the acid, base, oxidation, heat, and photolysis to apply the stress. Linearity ranges were 5–30 μg/mL ($r^2=0.9989$) for nebivolol and 80–480 μg/mL ($r^2=0.9991$) for valsartan. Limit of detection was 0.38 μg/mL and 1.08 μg/mL for nebivolol and valsartan, respectively. The limit of quantification for the nebivolol and valsartan was 1.15 μg/mL and 3.27 μg/mL, respectively.

Graphical Abstract



Introduction

The nebivolol hydrochloride is a highly selective β_1 -blocker. Nebivolol is rapidly absorbed after the oral doses. It is extensively metabolised in liver by alicyclic and aromatic hydroxylation, *N*-dealkylation, and glucuronidation; hydroxy metabolites are reported to be active. Rate of aromatic hydroxylation by cytochrome P450 iso enzyme CYP_{2D6} is subjected to the genetic polymorphism, and bioavailability and half-life vary widely. The metabolizes elimination half-life of the nebivolol is about 10 h and the hydroxyl metabolites is about 24 h. The peak plasma concentrations of the unchanged drug plus active metabolites are 1.3 to 1.4 times higher in the slow metabolizes and half-lives of the nebivolol and its hydroxy metabolites are prolonged. It has been clinically used for treatment of the hypertension and chronic heart failure. The Valsartan is a AT₁-receptor antagonist.

It has been clinically used for treatment of the hypertension and heart failure. The Valsartan is rapidly absorbed after oral doses, with a high bioavailability of about 23%. The peak plasma concentrations in the Valsartan occur 2 to 4 hours after an oral dose, which is between 94 and 97% bound to plasma proteins [1, 2]. The structures of both drugs are shown in Scheme 1 and 2.

The literature survey of valsartan and nebivolol revealed various analytical methods for determine the valsartan and nebivolol individually and in other combination methods. Moreover,

various analytical methods including spectro photometric methods [3, 4], LC/MS [5-7], HPTLC [8], TLC [9], UPLC [10], and RP-HPLC [11-14] were available. However, no stability indicating RP-HPLC method was reported in literature.

So in the present study, it was decided to develop stability indicating RP-HPLC method for simultaneous estimation of the valsartan and nebivolol in tablet dosage form. The method was validated in compliance with the ICH guideline (Q2 R1).

Experimental

Materials and methods

Nebivolol and valsartan were supplied by the torrent pharmaceutical limited. HPLC grade acetonitrile, methanol, water, triethylamine, ortho phosphoric acid were obtained from Merck. Analytical grade of the hydrochloric acid (Merck), sodium hydroxide (Merck), hydrogen peroxide (Merck) were used. NEBICARD-V tablets purchased from the local market (5 mg NEBIVOLOL and 80 mg VALSARTAN, Torrent pharmaceutical limited).

HPLC instrumentation and conditions

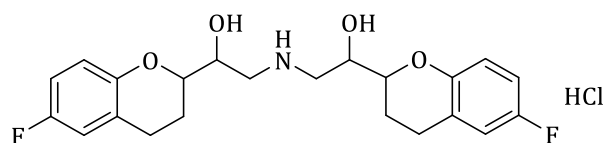
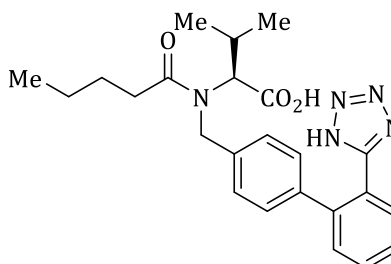
Agilent 1260 infinity series with quaternary pump, vacuum degasser, diode array detector, and a fixed injector equipped with 20 μ L connected to a computer loaded with Ez chrome software were used. Chromatographic separation was achieved on a YMC Pack Pro Octadecylsilane (150 \times 4.6 mm, 3 μ m) column using a mobile phase composed of Methanol/ Acetonitrile: 0.05 M potassium dihydrogen phosphate buffer (pH=3.0 with 10% ortho phosphoric acid after addition of 0.2% triethylamine) (30:30:40, v/v/v) at a flow rate of 1 mL/min. Eluent was monitored using a UV detection at a wavelength of 282 nm. The mobile phase was filtered through 0.45 μ m filter and degassed by ultra sonicator prior to use.

Preparation of stock and standard solutions

Standard stock solution

Valsartan

Stock solution (2000 μ g/mL) of the valsartan was prepared by dissolving accurately weighed 100 mg of valsartan in 50 mL volumetric flask, sonicated and made up to the mark. Further working

Scheme 1. Structure of neбиволол hydrochloride**Scheme 2.** Structure of valsartan

standard (1000 $\mu\text{g/mL}$) was prepared by transferring 25 mL of the stock solution into 50 mL of volumetric flask then diluted up to the mark with methanol and filter through 0.45 μm filter.

Nebivolol

Stock solution (1000 $\mu\text{g/mL}$) of the valsartan was prepared by dissolving accurately weighed 50 mg of valsartan in 50 mL volumetric flask, sonicated and made up to the mark. Further working standard (100 $\mu\text{g/mL}$) was prepared by transferring 5 mL of the stock solution into 50 mL of volumetric flask then diluted up to the mark with methanol and filter through a 0.45 μm filter.

Standard stock solution of API mixture

The standard stock solution (160 $\mu\text{g/mL}$ of valsartan and 10 $\mu\text{g/mL}$ of neбиволол) was prepared by transferring 1.6 mL of the valsartan stock solution and 1 mL of the neбиволол stock solution into 10 mL volumetric flask and diluted up to the mark with methanol and filtered through a 0.45 μm filter.

Preparation of tablets for assay

Weigh 20 tablets and taken average weight were taken. Then, they were crushed and mixed in a mortar and pestle. A portion of powder equivalent to 5 mg of neбиволол and 80 mg of valsartan was accurately weighed and transferred into 50 mL volumetric flasks and 50 mL of HPLC-grade methanol was added to flask. Volumetric flask was sonicated for 20 min to complete the dissolution. Aliquots solution was filtered through a 0.45 μm filter and 1 mL of filtered solution was transferred to a 10 mL volumetric flask and diluted up to mark with methanol to obtain concentration of Neбиволол 10 $\mu\text{g/mL}$ and valsartan 160 $\mu\text{g/mL}$.

Forced degradation studies

Forced degradation studies were carried out on a nebivolol and valsartan API according to the following conditions. The results are reported in [Table 1](#).

Acid degradation studies

1.6 mL of standard stock solution of the valsartan and 1 mL of standard stock solution of nebivolol were pipetted into 10 mL of volumetric flask. Then, 1 mL of 1.5 N hydrochloric acid solution was added and mixed. After 6 h, the solution was neutralized by 1.5 N sodium hydroxide solution and diluted up to the mark with methanol and filter. Total concentration for nebivolol was (10 $\mu\text{g/mL}$) and for valsartan was (160 $\mu\text{g/mL}$). Chromatogram of acid degradation was shown in [Figure 1](#).

Alkali degradation studies

1.6 mL of the standard stock solution of valsartan and 1 mL of the standard stock solution of nebivolol were pipetted into a 10 mL of volumetric flask. Then 1 mL of 1.5 N sodium hydroxide solution was added to the mixture and mixed well. After 6 h neutralizing the solution with 1.5 N hydrochloric acid solution, the mixture was diluted up to the mark with methanol. Total concentration for nebivolol was (10 $\mu\text{g/mL}$) and for valsartan was (160 $\mu\text{g/mL}$). The chromatogram of the base degradation is shown in [Figure 2](#).

Table 1. Summary of degradation studies of nebivolol and valsartan using proposed HPLC method

Degradation Condition	Degradation (%)		Peak purity index	
	Nebivolol	Valsartan	Nebivolol	Valsartan
Acid degradation [HCl (1.5 N), 6 h]	0.87	15.29	1	1
Base degradation [NaOH (1.5 N), 6 h]	0.19	0.38	1	1
H ₂ O ₂ degradation [H ₂ O ₂ (10%), 6 h]	0.31	1.71	1	1
Thermal degradation [80 °C, 24 h]	0	0.03	1	1
Photolytic degradation [24 h]	0	0.13	1	1

Oxidation degradation studies

1.6 mL of standard stock solution of valsartan and 1 mL of standard stock solution of nebivolol were pipetted in to 10 mL of volumetric flask. Then, 1 mL of the 10% hydrogen peroxide was added

to the mixture and slightly stirred for 6 h, and diluted up to the mark with methanol. The concentration of the nebivolol and valsartan was 10 $\mu\text{g}/\text{mL}$ and 160 $\mu\text{g}/\text{mL}$, respectively. The chromatogram of the oxidation degradation is shown in [Figure 3](#).

Thermal degradation studies

1.6 mL of the standard stock solution of valsartan and 1 mL of the standard stock solution of nebivolol were pipetted into a 10 mL of volumetric flask. Then volumetric flask was heated in water bath at 80 °C for 24 h. After an appropriate time period were cooled to ambient temperature and diluted up to mark with methanol. Total concentration for nebivolol (10 $\mu\text{g}/\text{mL}$) and valsartan (160 $\mu\text{g}/\text{mL}$). The chromatogram of thermal degradation is demonstrated in [Figure 4](#).

Photolytic degradation studies

The samples were exposed to the UV light in UV chamber for 24 h, then, diluted with the individual API with methanol in such way that final concentration for the nebivolol and valsartan was 10 $\mu\text{g}/\text{mL}$ and 160 $\mu\text{g}/\text{mL}$, respectively. The chromatogram of the photolytic degradation is revealed in [Figure 5](#).

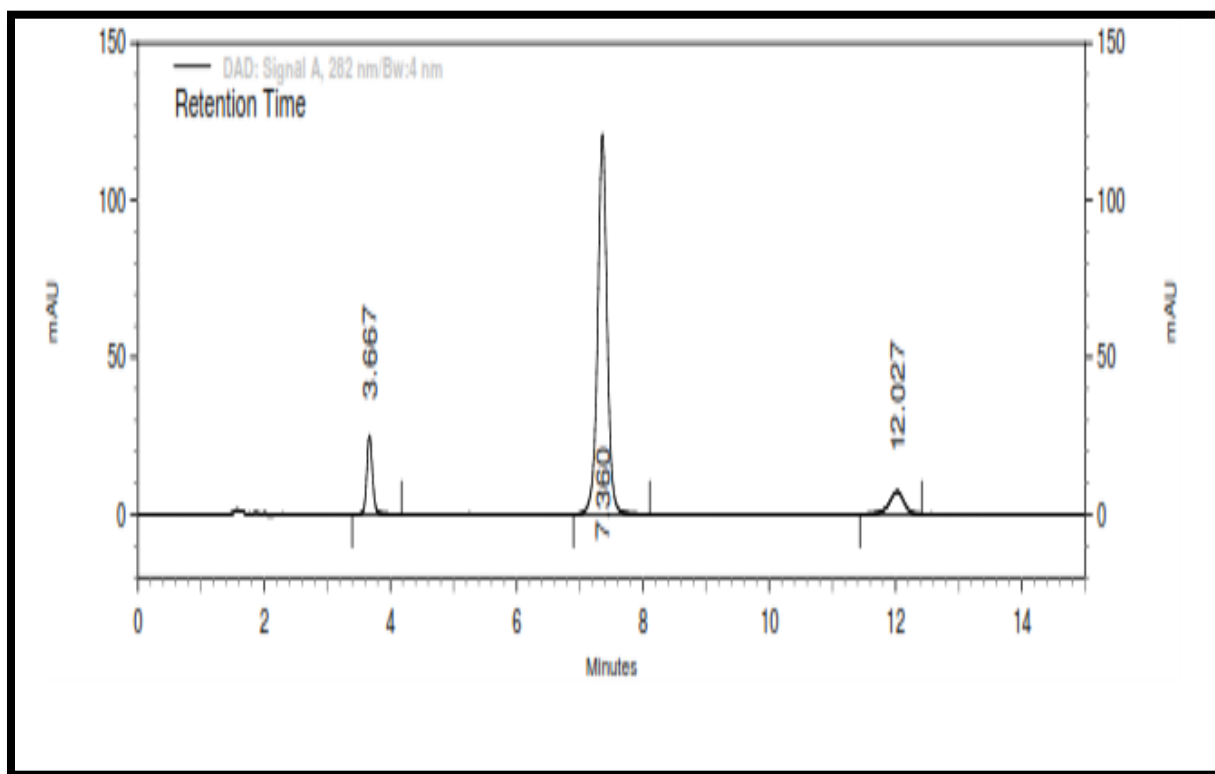


Figure 1. Chromatogram of acid degradation

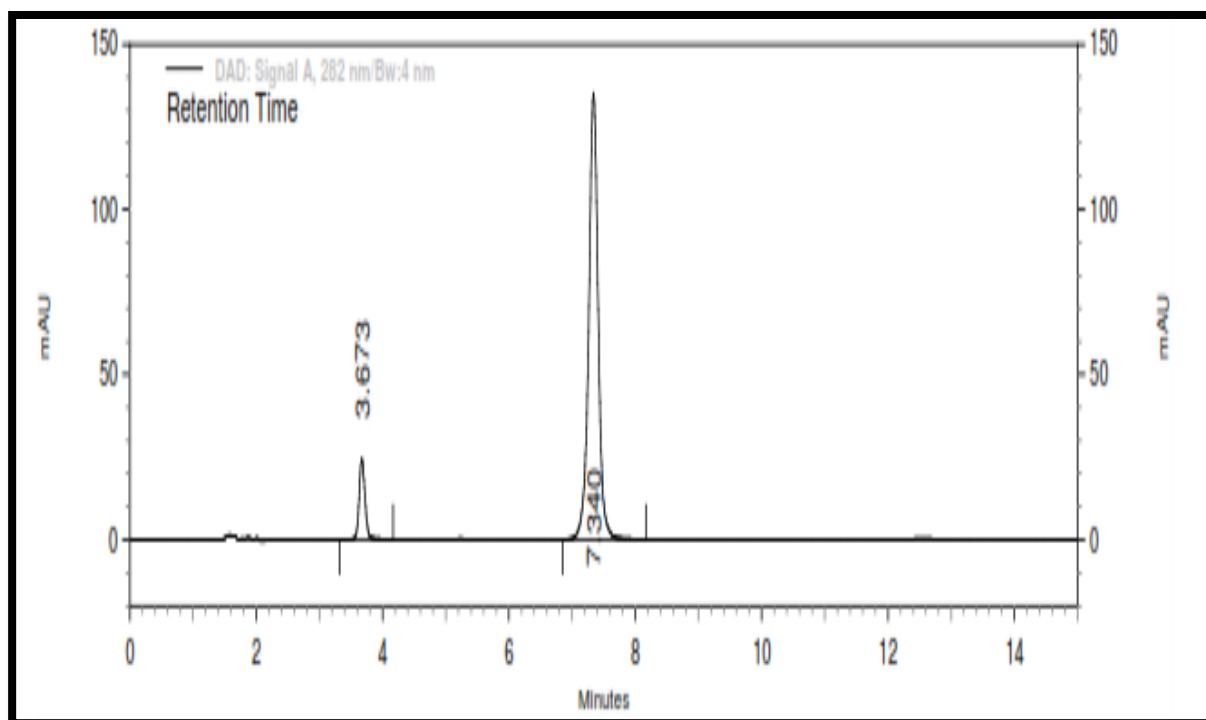


Figure 2. Chromatogram of base degradation

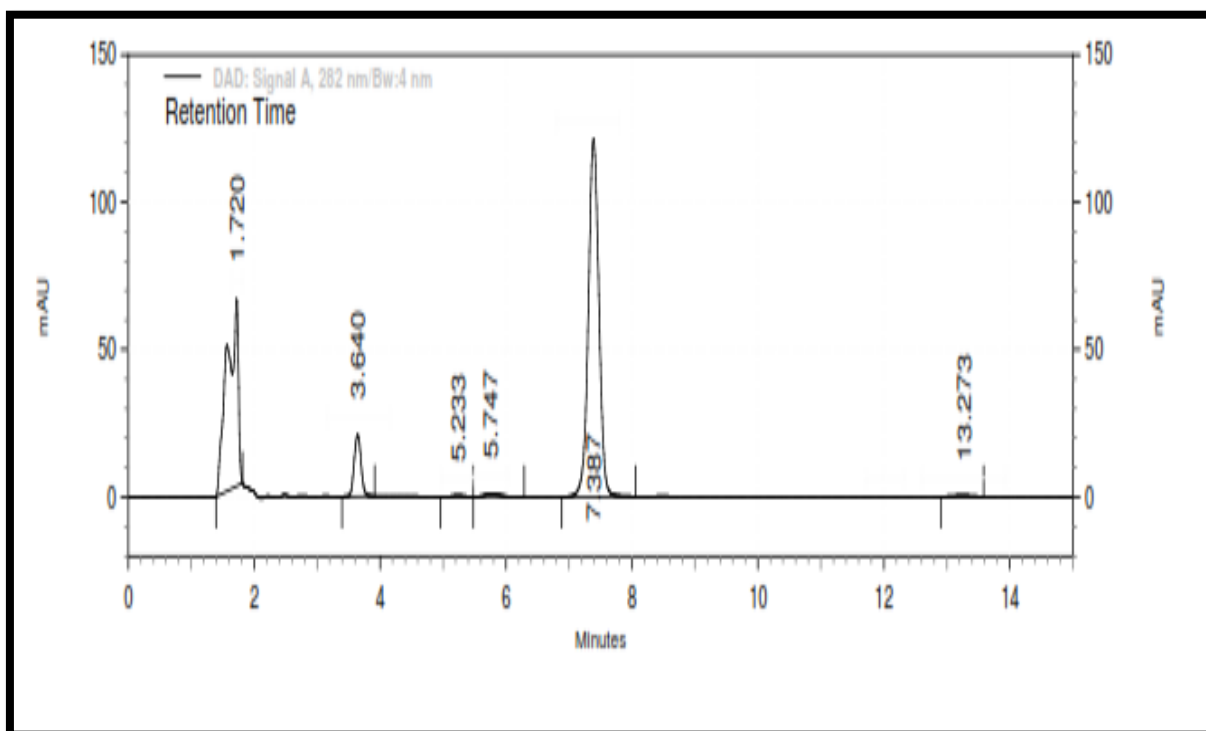


Figure 3. Chromatogram of oxidation degradation

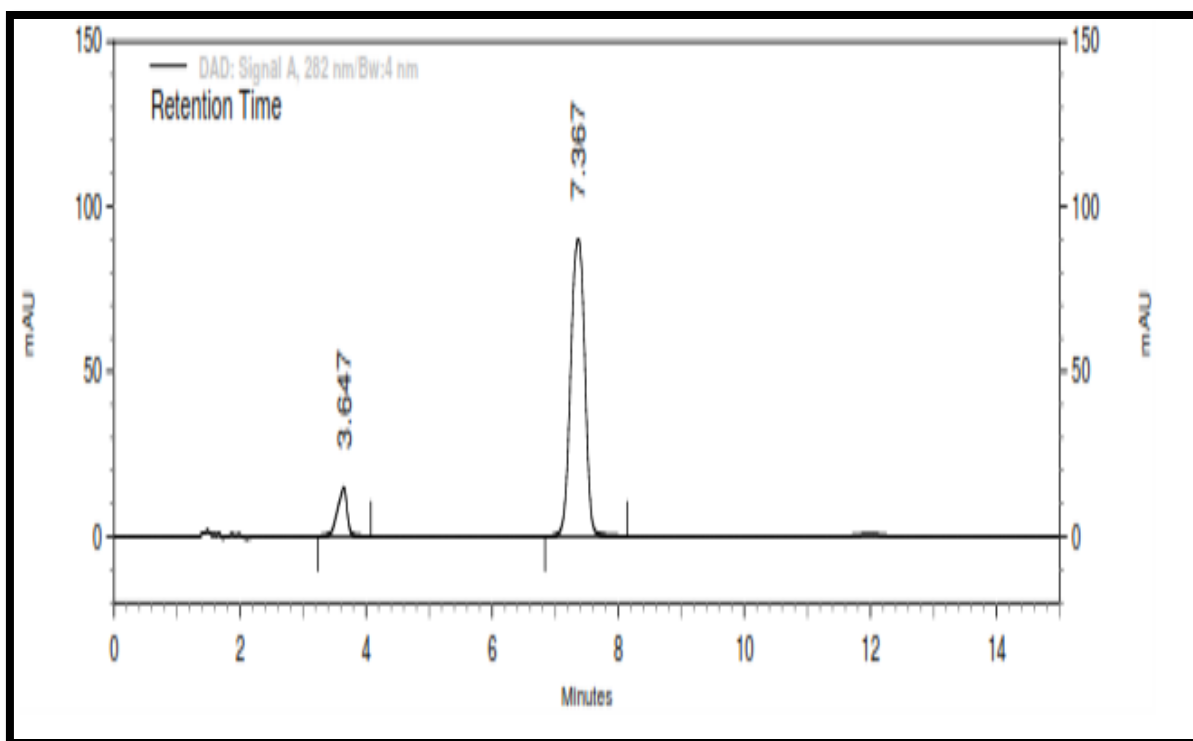


Figure 4. Chromatogram of thermal degradation

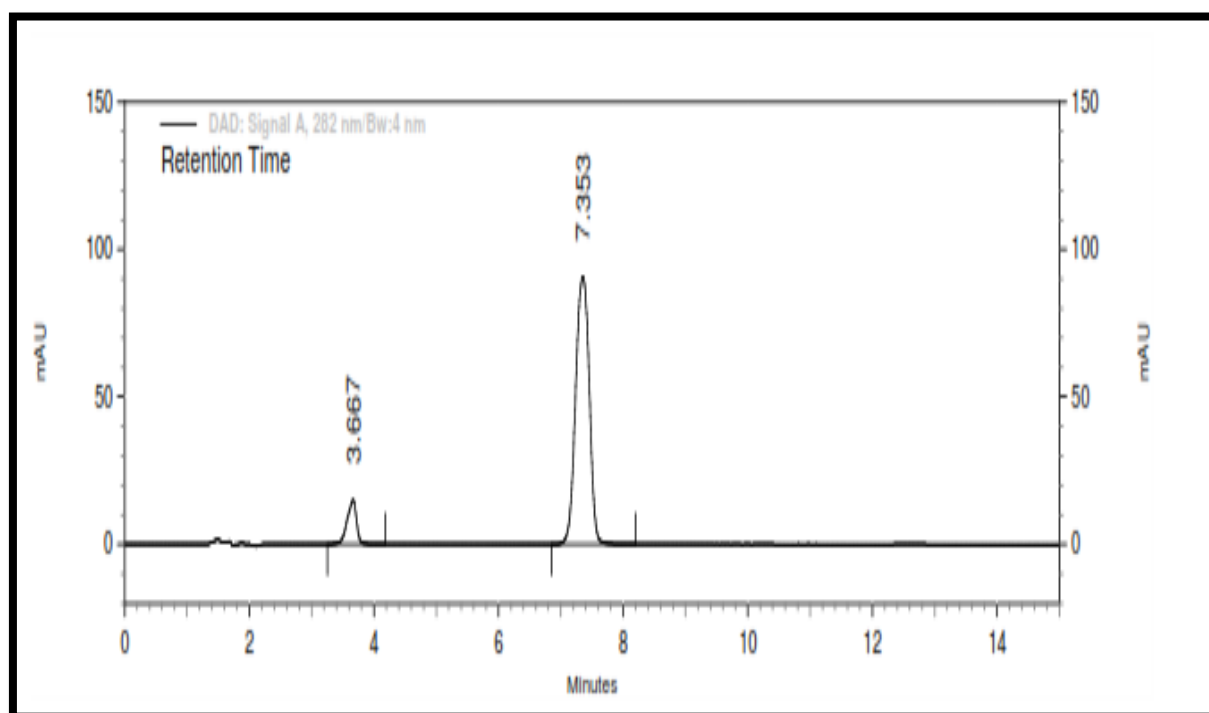


Figure 5. Chromatogram of photolytic degradation

Results and discussion

Optimization of chromatographic conditions

YMC pack pro octadecylsilane (150×4.6 mm, 3 μm) column maintained at the ambient temperature was used for separation and method validated for determination of the nebivolol and valsartan in tablets. A mobile phase composed of Methanol/ Acetonitrile: 0.05 M potassium dihydrogen phosphate buffer (Ph=3.0 with 10% ortho phosphoric acid after addition of 0.2% triethylamine) (30:30:40, v/v/v) which was set at a flow rate of 1 mL/min was selected for use in further studies after several preliminary investigatory chromatographic runs. Under described experimental conditions, all peaks were well defined and free from tailing. The effects of the small deliberate changes in the mobile phase composition, pH, and the wavelength and flow rate were evaluated as a part of testing for method robustness. In addition, this work describes development and validation of new stability indicating RP-HPLC assay method for simultaneous estimation of nebivolol and valsartan in tablet dosage form. The method was validated for its specificity, linearity, accuracy, precision, limit of detection (LOD), Limit of quantification (LOQ), robustness, system suitability and stability indicating properties by resolution of cited drug from their forced degradation product. System suitability was determined by 5 replicate injection of the standard preparation. The results are reported in [Table 2](#).

In the given reported stability, indicating RP-HPLC method, none of the chromatogram mentioned degradants peak. Degradation data, information of actual retention time of valsartan, calibration curve for Nebivolol, and thermal degradation temperature and time were found adequate [[11](#)].

Forced degradation

The results of the stress testing indicated that the developed method was highly specific in nature. The valsartan drug was unstable in acidic and oxidation condition. Acidic stress leads to 15.29% degradation with one unknown degradation peaks at 12.02 min. The oxidative stress leads to 1.71% degradation with three unknown degradation peaks at 5.23, 5.74, and 13.27 min. The results revealed that the nebivolol drug was stable in all conditions. Here only in oxidative and acidic conditions degradants peaks were generated while in others peak area was decreased showing the degradation of drugs. In this study, pathway of the degradation and structure elucidation were not part of the work. Results of the degradation study of each stress condition are shown in [Table 1](#). Chromatograms of each degradation study are shown in [Figure 1](#), [Figure 2](#), [Figure 3](#), [Figure 4](#), and [Figure 5](#), respectively.

Validation of method

The analytical method was validated with respect to the parameters such as linearity, limit of quantification (LOQ), limit of detection (LOD), precision, accuracy, specificity, and robustness.

Linearity

The linearity was established by the least squares linear regression analysis of the calibration curve. The constructed calibration curves were linear over the concentration range of 5-30 $\mu\text{g/mL}$ and 80-480 $\mu\text{g/mL}$ for nebivolol (n=6) and valsartan (n=6), respectively. The peak areas of the nebivolol or valsartan were plotted versus their respective concentrations and linear regression analysis performed on resultant curves. The correlation coefficients (n=6) were found to be 0.9989 for the nebivolol and 0.9991 for the valsartan with % RSD values ranging from 0.08 to 1.19% across the studied concentration ranges. Typically, regression equations were: $y=32753x + 9583.2$ ($r^2=0.9989$) for nebivolol and $y=20263x-122781$ ($r^2=0.9991$) for valsartan, respectively. The results are reported in [Table 3](#). (Calibration curve of nebivolol was shown in [Figure 6](#), Calibration curve of valsartan was shown in [Figure 7](#), Overlay chromatogram was shown in [Figure 8](#)).

Limit of quantification (LOQ) and limit of detection (LOD)

The LOD and LOQ values were determined by using the formula $\text{LOD}=3.3 \sigma/S$ and $\text{LOQ}=10 \sigma/S$ (where, σ was standard deviation of responses and S was slope of calibration curves). In this method, σ is mean of standard deviation of y intercepts of six calibration curves and S is mean of slopes of calibration curves. Limits of detection was 0.38 $\mu\text{g/mL}$ and 1.08 $\mu\text{g/mL}$ for the nebivolol and valsartan, respectively. Limits of quantification was 1.15 $\mu\text{g/mL}$ and 3.27 $\mu\text{g/mL}$ for the nebivolol and valsartan, respectively. The results are represented in [Table 4](#).

Precision

Within-day (Intra-day) precision for the proposed method was studied at three concentration (5 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, 15 $\mu\text{g/mL}$ for nebivolol and 80 $\mu\text{g/mL}$, 160 $\mu\text{g/mL}$, 240 $\mu\text{g/mL}$ for valsartan) levels for each compound using three replicate determinations for each concentration within one day (0 h, 3 h, 6 h). Similarly, between-day (Inter-day) precision was tested by analysing the same three concentrations for each compound using three replicate determinations repeated on three days. Repeatability for proposed method was studied by 6 determinations at 100% of test concentration. Developed method was found to be precise as % RSD value for repeatability and precision studies were <2 as recommended by ICH guidelines. The results are reported in [Table 5](#) and [6](#).

Table 2. System suitability parameter

System suitability parameter	Nebivolol	Valsartan
Retention times (Rt)	3.67 ± 0.01	7.35 ± 0.02
Theoretical plates (N)	2812.40 ± 28.16	5057.20 ± 77.26
		10.66 ± 0.02
Asymmetry	0.94 ± 0.01	0.96 ± 0.01

Table 3. Linearity data of nebivolol and valsartan

Parameter	Nebivolol	Valsartan
Linearity range	5-30 µg/mL	80-480 µg/mL
Regression equation	$y = 32753x + 9583.2$	$y = 20263x - 122781$
Slope	32753	20263
Intercept	9583.2	122781
r^2	0.9989	0.9991

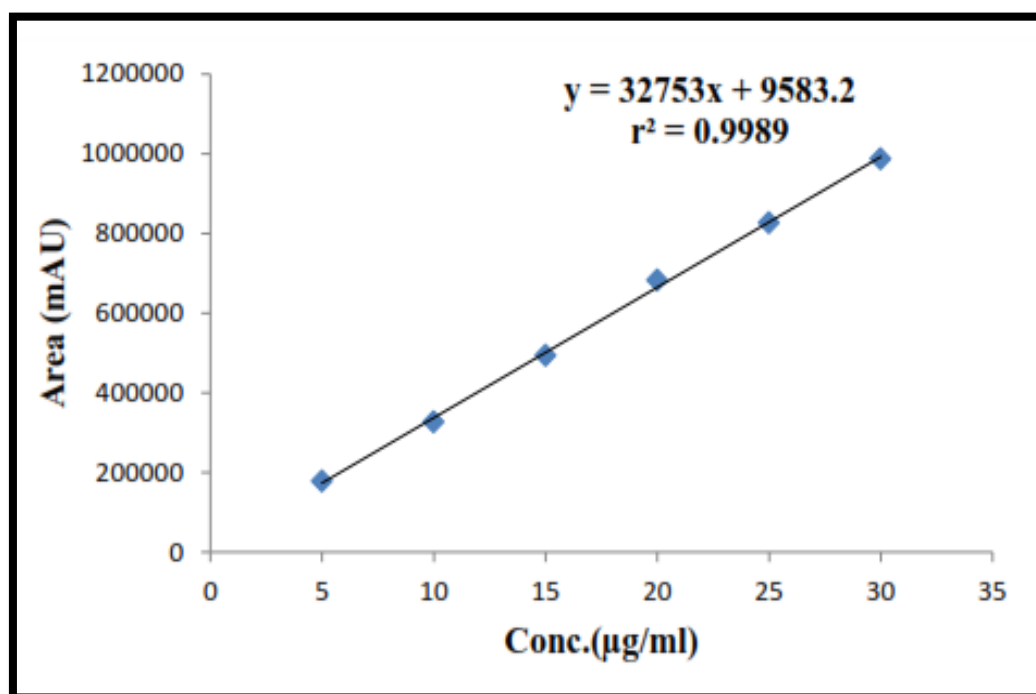
**Figure 6.** Calibration curve of nebivolol

Figure 7. Calibration curve of valsartan

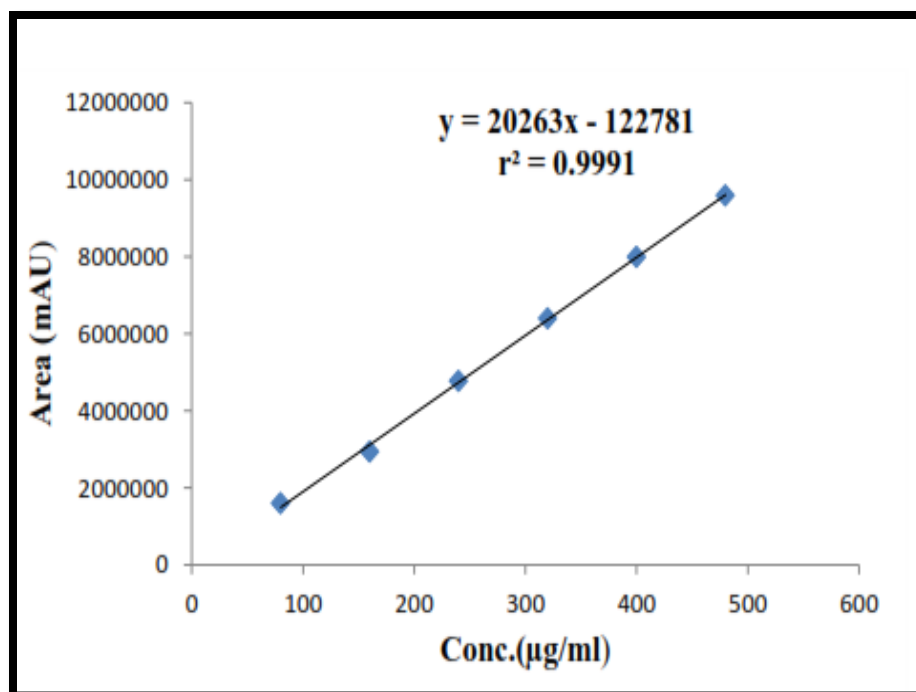


Figure 8. Overlay Chromatogram for linearity of nebivolol and valsartan

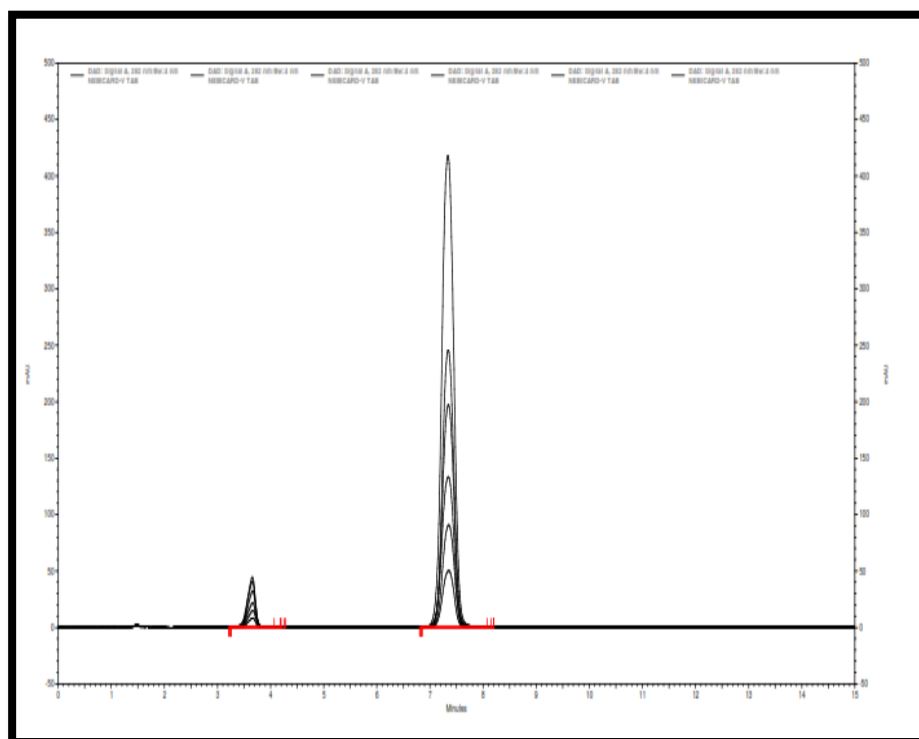


Table 4. LOD and LOQ

Parameter	Nebivolol	Valsartan
LOD ($\mu\text{g/mL}$)	0.38	1.08
LOQ ($\mu\text{g/mL}$)	1.15	3.27

Table 5. Repeatability data of nebivolol and valsartan

Drug	Concentration ($\mu\text{g/mL}$)	Mean Concentration found ($\mu\text{g/mL}$) \pm SD (n=6)	% RSD
Nebivolol	10	10.01 \pm 0.08	0.84
Valsartan	160	159.87 \pm 1.23	0.77

Table 6. Data of intraday and interday precision of nebivolol and valsartan

Drug	Concentration ($\mu\text{g/mL}$)	Intraday precision		Interday precision	
		Mean Concentration found ($\mu\text{g/mL}$) \pm SD (n=3)	% RSD	Mean Concentration found ($\mu\text{g/mL}$) \pm SD (n=3)	% RSD
Nebivolol	5	4.98 \pm 0.02	0.43	4.98 \pm 0.04	0.97
	10	9.97 \pm 0.06	0.62	9.94 \pm 0.04	0.42
	15	15.03 \pm 0.05	0.38	15.09 \pm 0.13	0.87
Valsartan	80	80.50 \pm 0.75	0.93	79.68 \pm 0.93	1.26
	160	160.38 \pm 1.63	1.02	160.90 \pm 1.06	0.68
	240	239.59 \pm 1.41	0.59	239.32 \pm 2.56	1.07

Accuracy

Accuracy of the method was examined by performing recovery studies and by standard addition method at three levels 50%, 100%, 150%. Accuracy was evaluated using a minimum of 9 determinations over a minimum of 3 concentration levels covering specified range. The results of the recovery studies were in range of 98% to 102%. The results are reported in [Table 7](#).

Specificity

Specificity of method was ascertained by diluents, analysis of drug individual standard (10 $\mu\text{g/mL}$ for nebivolol, 160 $\mu\text{g/mL}$ for valsartan), standard mixture (10 $\mu\text{g/mL}$ for nebivolol and 160 $\mu\text{g/mL}$ for valsartan) and sample. Chromatograms were shown in [Figure 9-13](#) respectively. Mobile

phase resolved both drugs very efficiently. Specificity was determined in various stress conditions like acidic, basic, oxidation, thermal, and photolytic.

Robustness

Robustness was examined by evaluating influence of small variations in different conditions such as concentration of working wavelength (± 2 nm), flow rate (± 0.1 mL/min), pH (± 0.1 units), mobile phase variation ($\pm 1\%$ for methanol/ acetonitrile mixture). These variations did not have any significant effect on measured responses or chromatographic resolution. % RSD for measured peak areas using these variations did not exceed 2%. The results are reported in [Table 8](#).

Assay

Validated method was applied to determine the nebivolol and valsartan in the commercially available tablets. The observed % assay of the nebivolol and valsartan were found to be 99.68 ± 0.55 and 99.85 ± 0.49 (mean \pm SD), respectively. The % RSD value of the nebivolol and valsartan were 0.55% and 0.49%, respectively. The results of the assay indicate that the method was selective for analysis of both nebivolol and valsartan without interference from excipients used to formulate and produce these tablets. The results are demonstrated in [Table 9](#).

Table 7. Accuracy data for nebivolol and valsartan

Nebivolol			
Recovery level (%)	Amount taken ($\mu\text{g/mL}$)	Amount added ($\mu\text{g/mL}$)	% Mean recovery \pm SD (n=3)
50	10	5	99.45 ± 0.83
100	10	10	100.22 ± 0.93
150	10	15	99.50 ± 1.07
Valsartan			
Recovery level (%)	Amount taken ($\mu\text{g/mL}$)	Amount added ($\mu\text{g/mL}$)	% Mean recovery \pm SD (n=3)
50	160	80	100.28 ± 0.61
100	160	160	99.77 ± 0.75
150	160	240	100.14 ± 0.97

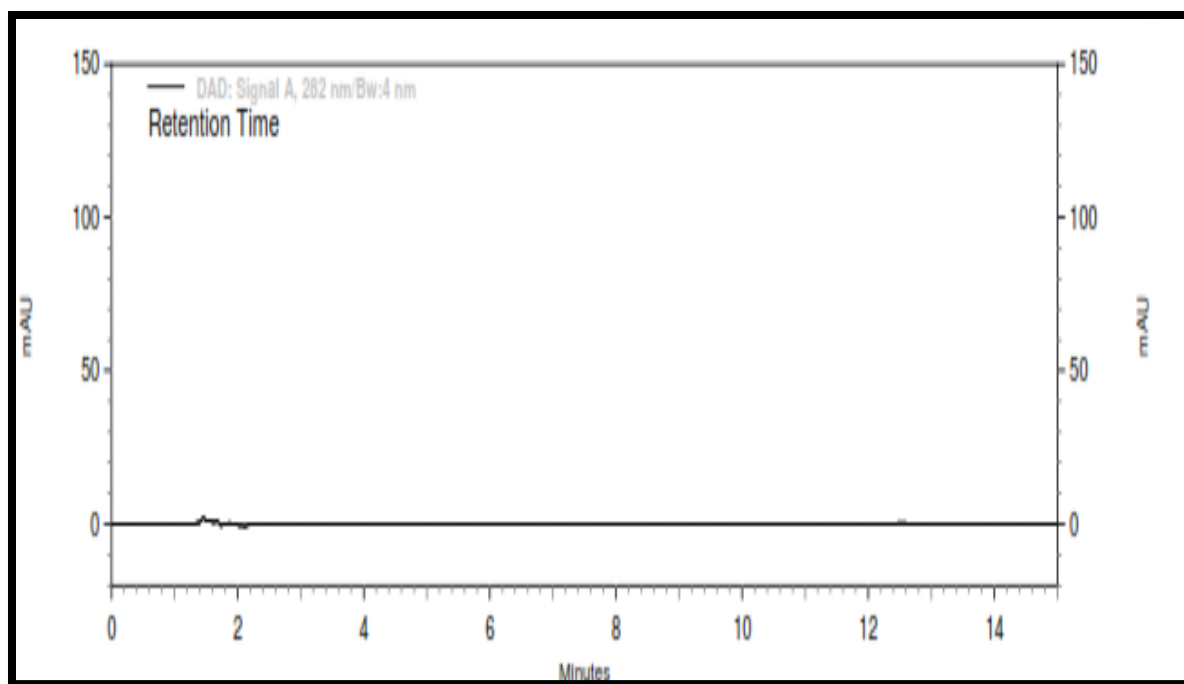


Figure 9. Chromatogram of diluents

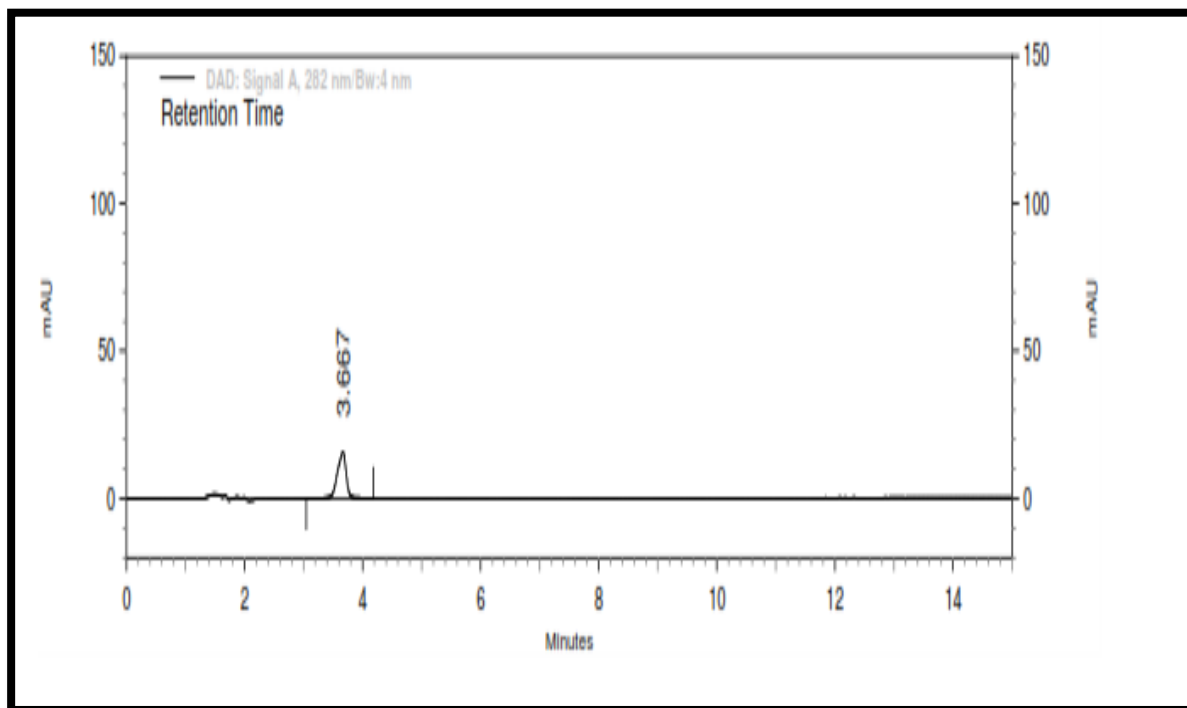


Figure 10. Chromatogram of standard solution of Nebivolol (10 µg/mL)

Figure 11. Chromatogram of standard solution of valsartan ($160 \mu\text{g/mL}$)

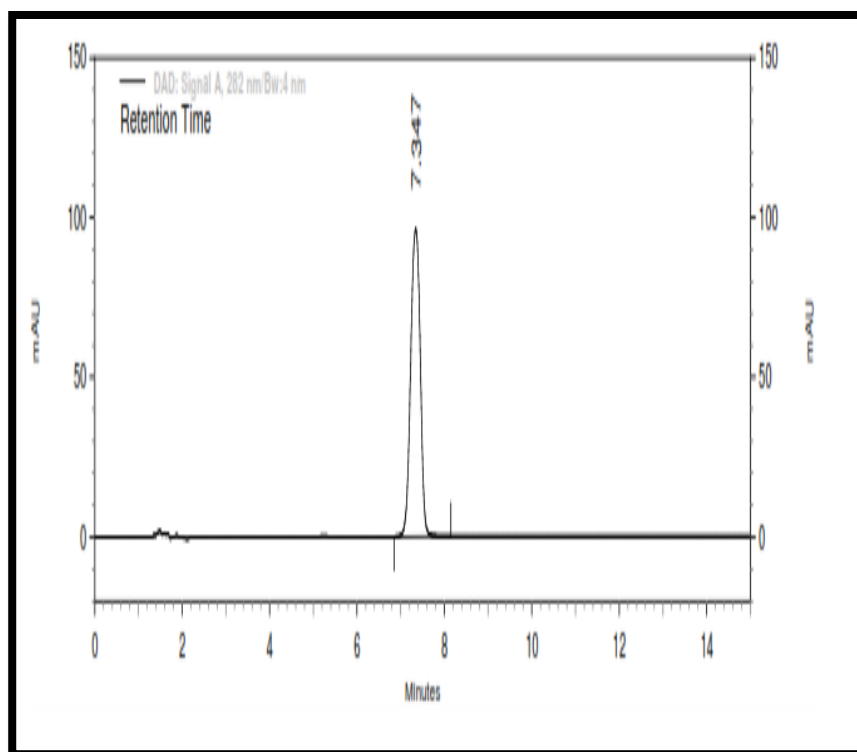
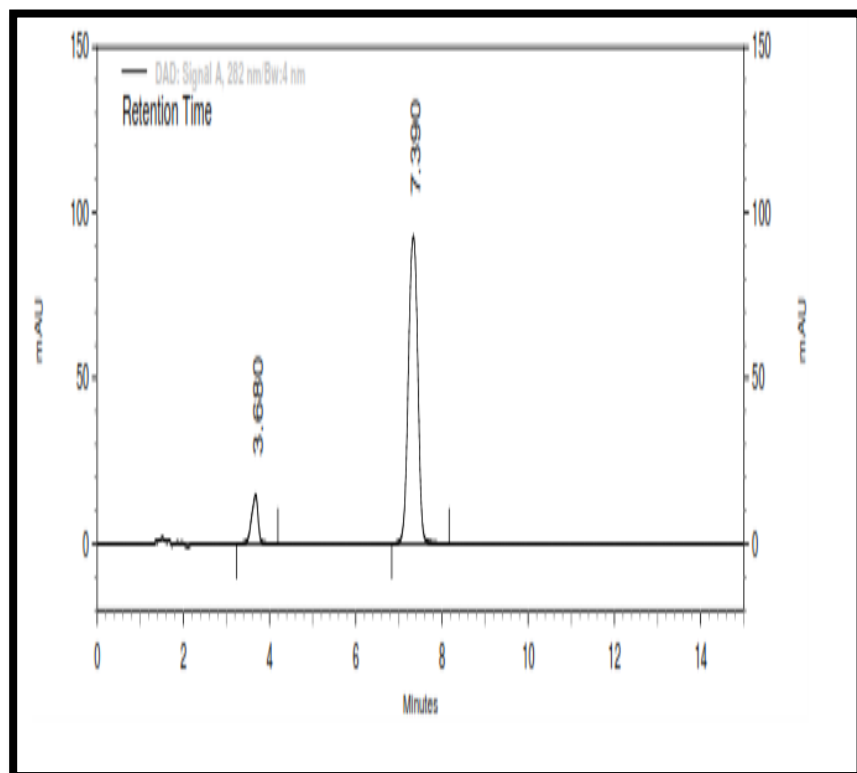


Figure 12. Chromatogram of standard mixture Solution of nebivolol ($10 \mu\text{g/mL}$) and Valsartan ($160 \mu\text{g/mL}$)



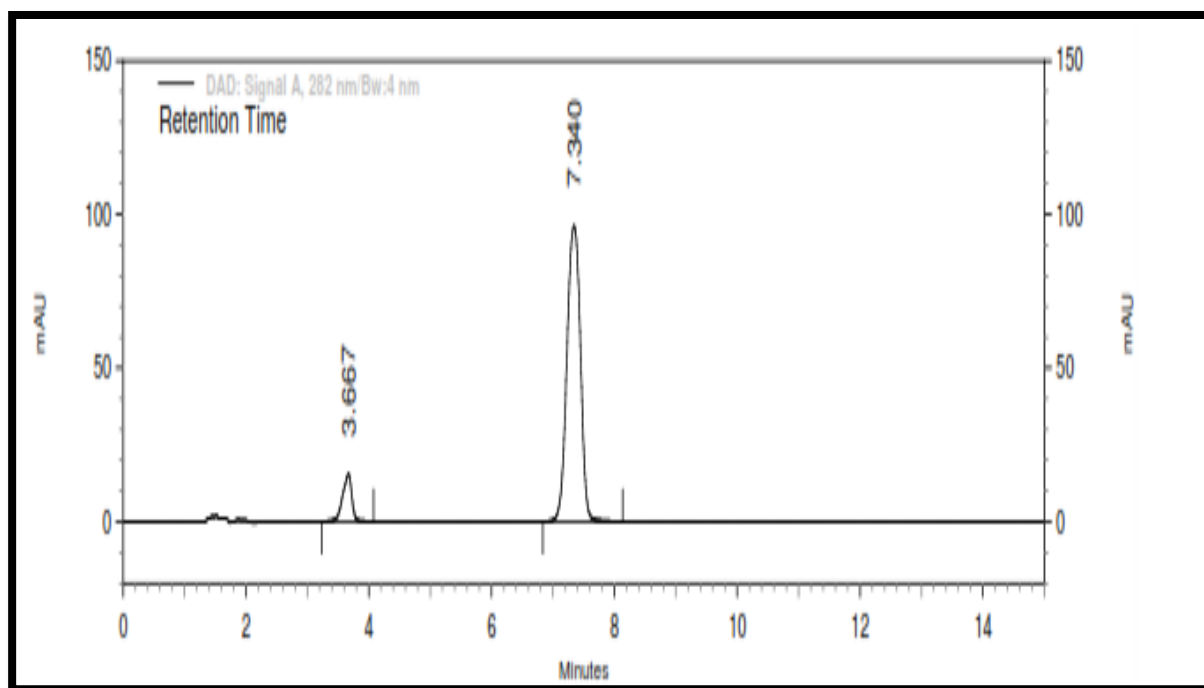


Figure 13. Chromatogram of sample solution of nebivolol ($10 \mu\text{g/mL}$) and Valsartan ($160 \mu\text{g/mL}$)

Table 8. Robustness data for nebivolol and valsartan

Entry	Parameter	Nebivolol			Valsartan		
		Normal condition	Variable 1	Variable 2	Normal condition	Variable 1	Variable 2
1	pH	3.0	2.9	3.1	3.0	2.9	3.1
	Area	339682.33	333959.66	338166	3123297	3118498	3116564
	± SD	± 1959.74	± 1758.19	± 3106.86	± 21563	± 27782	± 30641
	% RSD	0.57	0.52	0.91	0.69	0.89	0.98
2	wavelength	282 nm	280 nm	284 nm	282 nm	280 nm	284 nm
	Area	339682.33	336789.66	335793.66	3123297	3141561	3116229
	± SD	± 1959.74	± 1232.50	± 2017.00	± 21563	± 9753	± 4284
	% RSD	0.57	0.36	0.60	0.69	0.31	0.13
3	Flow rate	1.0 mL/min	0.9 mL/min	1.1 mL/min	1.0 mL/min	0.9 mL/min	1.1 mL/min
	Area	339682.33	339410.33	335968.66	3123297	3129518	3107961
	± SD	± 1959.74	± 899.34	± 1535.42	± 21563	± 9139	± 12320
	% RSD	0.57	0.26	0.45	0.69	0.29	0.39

	% RSD	0.57	0.26	0.45	0.69	0.29	0.39
4	Mobile phase (Methanol -CAN/ Buffer)	60:40	59:41	61:39	60:40	59:41	61:39
	Area	339682.33	335097	336195.66	3123297	3117315	3117904
	± SD	± 1959.74	± 2112.34	± 1031.34	± 21563	± 17467	± 4484
	% RSD	0.57	0.63	0.31	0.69	0.56	0.14

Table 9. Assay result of NEB and VAL

Drug	Label claim (mg)	Amount found (mg)	% Assay ± SD (n=6)	% RSD
Nebivolol	5	4.98	99.68 ± 0.55	0.55
Valsartan	80	79.88	99.85 ± 0.49	0.49

Conclusion

In this study, a simple, specific, and reliable isocratic elution HPLC-DAD procedure was developed for assay of the nebivolol and valsartan in their tablet dosage form. Two analyses were subjected to the forced degradation using several stress (Acidic, basic, oxidative, photolytic and thermal) conditions, and the proposed method was successfully employed for resolution of the analyses peaks from those of forced degradation products. The most important feature in the proposed method is specificity and stability-indicating. The developed method made use of DAD as a tool for peak identity and purity confirmation; however, it could be adapted to conventional HPLC with UV which is most popular in quality control laboratories. Finally, the method was thoroughly validated, therefore, it can be recommended for routine analysis and for checking quality during the stability studies of the cited drugs.

Disclosure statement

No potential conflict of interest was reported by the authors.

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