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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR NEBIVOLOL BY USING THE DOE APPROACH

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ABSTRACT
Background: Nebivolol (NBV), classified as a third-generation β1-adrenergic receptor antagonist, is commonly prescribed for managing hypertension. Accurate and prompt quantification of NBV in bulk materials and pharmaceutical formulations is crucial for quality assurance. This research focuses on developing and validating a stability-indicating RP-HPLC method for the quantification of NBV, with an emphasis on sensitivity, precision, accuracy, and robustness. **Methodology:** A stability-indicating reversed-phase high-performance liquid chromatography (RP-HPLC) method was established using an Agilent 1260 Infinity II HPLC system equipped with a Diode Array Detector (DAD). The chromatographic analysis was conducted on an Agilent Zorbax Bonus RP column (250 × 4.6 mm, 5 µm). The mobile phase comprised acetonitrile and 0.1% perchloric acid in a 55:45 (v/v) ratio, delivered at a flow rate of 1 mL/min. Detection was performed at a wavelength of 282 nm. The method underwent validation according to the guidelines provided by the International Council for Harmonisation (ICH), including assessments of linearity, precision, accuracy, robustness, and forced degradation studies. **Results and Discussion:** This method demonstrated improved sensitivity, shorter run time (retention time of 4.22 min), and high precision. Forced degradation studies confirmed Nebivolol's instability under alkaline (15.94%) and oxidative (8.57%) conditions, highlighting the method's stability-indicating capability. The method also gives robust linearity across the concentration range of 80–120 µg/mL, with a correlation coefficient (r^2) of 1.00. The limits of detection (LOD) and quantification (LOQ) were determined to be 0.55 µg/mL and 1.61 µg/mL, respectively. **Conclusion:** The proposed RP-HPLC method proved to be reliable, precise, and stability-indicating, making it a valuable tool for the quality control and stability assessment of Nebivolol formulations in pharmaceutical settings.

INTRODUCTION

Nebivolol (NBV) is a third-generation β1-adrenergic receptor antagonist widely prescribed for the treatment of hypertension and various cardiovascular conditions. Approved by the U.S. Food and Drug Administration (FDA), it belongs to the beta-blocker class of medications, which are commonly used to

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manage hypertension, angina, arrhythmias, anxiety, hyperthyroidism, migraine prophylaxis, and essential tremors. Beta-blockers are categorized into β1-selective and non-selective beta-blockers, depending on whether they primarily target β1-receptors in cardiac muscles, β2-receptors in smooth muscles and the lungs, or both types of receptors. Additionally, beta-blockers are further classified as vasodilators or non-vasodilators, depending on their ability to widen or constrict blood vessels [1]. NBV exhibits a dual mechanism of action, which distinguishes it from traditional beta-blockers. At lower doses, it selectively inhibits β1-receptors, reducing side effects such as bronchoconstriction. However, at higher doses or in individuals with poor metabolism, it can also block β2-receptors, impacting pulmonary and vascular function. Additionally, NBV promotes vasodilation by stimulating the release of nitric oxide, thereby enhancing its effectiveness in reducing blood pressure while maintaining cardiovascular protection. This dual action makes NBV highly effective and well-tolerated, particularly in patients with respiratory conditions [2].

Commercially available under brand names such as Nebistar© and Bystolic©, NBV is often prescribed alone or in fixed-dose combinations with valsartan to enhance its antihypertensive effects. It is a racemic mixture of D-Nebivolol and L-Nebivolol, where the D-enantiomer primarily blocks β1-receptors, and the L-enantiomer facilitates nitric oxide release, leading to vasodilation. This high selectivity towards β1-receptors gives NBV an advantage over other beta-blockers in terms of tolerability and therapeutic efficacy. However, when administered at doses exceeding 10 mg, its β1-selectivity diminishes, and it begins to affect both β1 and β2 receptors, making dose optimization essential for achieving optimal therapeutic benefits [3].

NBV, initially patented in 1983 and introduced for clinical application in 1997, is now widely accessible as a generic drug in various countries, including the United Kingdom. As of 2020, it was ranked as the 239th most frequently prescribed medication in the United States, with approximately one million prescriptions dispensed. This extensive utilization highlights the importance of establishing analytical methods that are precise, efficient, and reliable for its quantification within pharmaceutical formulations. Ensuring the high quality of pharmaceutical products necessitates the development of analytical techniques that are not only accurate and precise but also capable of indicating product stability. Given the intricate nature of NBV's molecular structure, along with challenges related to its solubility and degradation mechanisms, optimizing chromatographic conditions remains a complex task. Conventional approaches such as the One-Factor-at-a-Time (OFAT) technique often result in limitations concerning robustness and reproducibility. To address these issues, modern methodologies like Design of Experiments (DoE) facilitate systematic optimization, thereby enhancing the sensitivity, efficiency, and overall reliability of the analytical process.

Previous RP-HPLC methods for NBV have reported extended retention times (>10 min), lower resolution (asymmetry >2.0), or insufficient sensitivity (LOD >1.5 µg/mL) [4]. For instance, Ghereghlou et al. (2024) reported a 12-minute run time with limited peak symmetry. Unlike these, the present method utilizes a DoE approach, which allows a statistically optimized chromatographic setup, reducing variability and enhancing robustness [5]. Furthermore, studies focusing on forced degradation are scarce, despite being critical for evaluating stability under various stress conditions, including acidic, basic, oxidative, and thermal environments. Understanding degradation profiles is essential for ensuring the drug's stability and efficacy throughout its shelf life [6]. Given the complex stereochemistry and variable solubility of NBV, its analytical profiling remains challenging [7]. Studies have shown its susceptibility to degradation under alkaline and oxidative stress, necessitating advanced stability-indicating methods [8]. This study primarily aims to establish and validate a stability-indicating RP-HPLC method for the accurate quantification of NBV in both bulk and formulated dosage forms. The proposed method aims to achieve rapid separation, enhancing efficiency, precision, and accuracy to ensure consistent and reliable results across varying concentrations. Robustness and reproducibility are validated according to ICH guidelines, and the method is sensitive enough to detect minimal levels of degradation products, thereby ensuring drug safety. By adhering to stringent validation procedures and including stress testing, the developed method offers a robust and compliant approach suitable for routine quality control and regulatory purposes in pharmaceutical analysis.

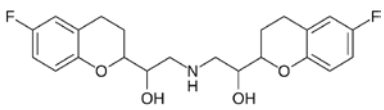

Figure 1: Chemical Structure of Nebivolol (NBV)

MATERIALS AND METHODS

Chemicals and Reagents

NBV was obtained as a complimentary sample from Hetero Drug Ltd. (Unit-I), Sangareddy, TS, India. HPLC grade Acetonitrile was received from Thermofisher Scientific India Pvt. Ltd., Molychem, India, and provided with perchloric acid. HPLC-grade water was obtained from an internal Milli-Q system. Calibrated NABL scales were used for all weight measurements. Analytical balance and type 'A' glassware were used for sample preparation.

Rationale for HPLC Conditions

Optimizing high-performance liquid chromatography (HPLC) conditions is crucial for achieving reliable, precise, and consistent analysis of NBV in both bulk materials and pharmaceutical formulations. The method was refined to facilitate effective separation, minimize peak tailing, and enhance resolution, all while maintaining a brief run time to improve analytical efficiency. A reverse-phase HPLC (RP-HPLC) method was established utilizing an Agilent Zorbax Bonus-RP column (250 × 4.6 mm, 5 µm), recognized for its durability, peak symmetry, and high theoretical plate count. The mobile phase comprises acetonitrile and 0.1% perchloric acid. The 55:45 ratio was selected based on preliminary trials showing superior peak shape and theoretical plates. The DoE design tested flow rates (0.45–0.55 mL/min) and acetonitrile concentrations (60–70%) to identify optimal conditions that minimize tailing and maximize resolution. These values were based on preliminary scouting experiments and literature precedents [9]. The inclusion of 0.1% perchloric acid served to stabilize pH, decrease peak tailing, and enhance resolution, thereby contributing to a robust and consistent method.

A flow rate of 1 mL/min was employed, as increasing the flow rate resulted in decreased retention time but compromised peak separation, whereas lowering it extended the analysis duration. The detection wavelength was set at 282 nm, corresponding to the UV absorption maxima of NBV, to ensure high sensitivity and selectivity. An injection volume of 10 µL was maintained to provide consistent peak responses while avoiding column overloading. The developed method featured a run time of 10 minutes, promoting efficient analysis and preventing carryover. These optimized conditions rendered the RP-HPLC method robust, reproducible, and suitable for routine quality control assessment of NBV in pharmaceutical formulations [5, 6].

REAGENTS AND SOLUTIONS PREPARATION

Stock Solution Preparation

A NBV Standard Stock Solution-I (NSSS-I) was prepared by dissolving 10 mg of NBV in 100 mL of diluent (0.1% perchloric acid: acetonitrile, 50:50 v/v) in a volumetric flask. The solution was sonicated for 5 minutes to ensure complete dissolution and then diluted to obtain a final concentration of 100 µg/mL.

Drug product sample preparation for assay

A total of ten Nebistar® 5 tablets (each containing 5 mg of NBV) were weighed, and the average weight was recorded. The tablets were then finely ground using a mortar and pestle to ensure uniformity. A sample containing an equivalent of 10 mg NBV was transferred into a 100 mL volumetric flask, followed by the addition of 50 mL of diluent. The solution was sonicated for 5 minutes to ensure complete extraction, and the final volume was adjusted to 100 mL with diluent, yielding a 100 µg/mL concentration [7].

Chromatographic Conditions

- **Column:** Agilent Zorbax BonusRP(250×4.6mm,5 µm)
- **HPLC System:** Agilent 1260 Infinity II HPLC
- **Detector:** Diode Array Detector (DAD) - G7115A
- **Mobile Phase:** 0.1% Perchloric Acid: Acetonitrile (55:45 v/v)
- **Flow Rate:** 1.0 mL/min
- **Injection Volume:** 10 µL
- **Oven Temperature:** 30°C
- **Detection Wavelength:** 282 nm
- **Run Time:** 10 minutes
- **Diluent:** 0.1% Perchloric Acid: Acetonitrile(50:50 v/v)

Data Analysis

The collected data was processed using Agilent OpenLAB CDS software (version 2.8), which ensured precise peak integration, calibration curve generation, and accurate quantification. Calibration curves were constructed by plotting peak area versus NBV concentration, confirming method linearity over the 80–120 µg/mL range.

Preparation of Calibration Standards and Quality Control Samples

Calibration standards were prepared by serial dilution of NSSS-I to obtain concentrations ranging from 80% to 120% (80–120 µg/mL). The samples were prepared as shown in Table 1.

Table 1: Preparation of Calibration Standards and Quality Control Samples for NBV

%Level	NBV Conc.(µg/mL)	NSSS-I (mL)	Final Vol. (mL)
80%	80	0.8	10
90%	90	0.9	10
100%	100	1.0	10
110%	110	1.1	10
120%	120	1.2	10

Quality control (QC) samples at low (80 µg/mL), medium (100 µg/mL), and high (120 µg/mL) levels were used to assess precision and reproducibility [8].

Optimization Study: This study aimed to enhance chromatographic performance by identifying the optimal combination of Critical Method Variables (CMVs). Initial screening revealed that the percentage of acetonitrile in the mobile phase and the flow rate had a significant impact on peak asymmetry and the number of theoretical plates. A three-level, two-factor full factorial design was employed to assess these variables systematically. The acetonitrile concentrations tested were 70% v/v, 65% v/v, and 60% v/v, corresponding to high, intermediate, and low levels, respectively. Similarly, flow rates of 0.55 mL/min, 0.50 mL/min, and 0.45 mL/min were evaluated. A design matrix comprising 13 experimental runs was generated using Design-Expert software to examine possible nonlinear relationships (Table 2) [9, 10].

Statistical Analysis of Data

A statistical evaluation was performed using a three-level, two-factor full factorial design, focusing on two response variables: theoretical plates (R1) and asymmetry (R2). The quadratic model was used to correlate experimental conditions with response variables. Analysis of Variance (ANOVA) confirmed model suitability, with significant F-values supporting its predictive strength. The model equations derived were:

$$\textit{Theoretical Plates (R1)} = +11678.59 + 196.33A + 533.33B - 142.50AB - 377.55A^2 - 2545.55B^2$$

$$\textit{Asymmetry (R2)} = +1.39 - 0.0050A + 0.0700B + 0.0050AB + 0.0012A^2 - 0.0338B^2$$

Residual analysis, normal probability plots, and 3D response surface plots further validated the statistical accuracy of the method [11-13].

Table 2. Optimization study of Nebivolol

SN	Mobile Phase	Mobile Phase Ratio	Observation
1.	HPLC Water - Acetonitrile	50-50	No peak was observed within 20 minutes of run time.
2.	HPLC Water - Acetonitrile	40-60	The peak of nebivolol was observed at RT 3.87 min., but the peak was not sharp due to high asymmetry, and the theoretical count was below the limit.
3.	HPLC Water - Acetonitrile	20-80	The peak of nebivolol was observed at RT 3.96 min, but the peak was not sharp due to high asymmetry, and the theoretical count was below the limit. To achieve good results, the mobile phase was changed to 0.1% Perchloric
4.	0.1% Perchloric acid-Acetonitrile	50-50	A sharp peak of nebivolol was observed at RT 3.13 min.
5.	0.1% Perchloric acid-Acetonitrile	55-45	A sharp peak of nebivolol was observed at RT 4.26 min., with good theoretical plate count, improved asymmetry, and peak purity.
6.	0.1% Perchloric acid-Acetonitrile	60-40	A sharp peak of nebivolol was observed at RT 6.71 min, with a good theoretical plate count and peak purity.

Method Validation [14-16]

The stability-indicating RP-HPLC method developed for the quantification of NBV was validated following the ICH guidelines, ensuring that the process meets the necessary criteria for accuracy, precision, linearity, robustness, specificity, and sensitivity. The validation process encompassed the assessment of various parameters, including system suitability, specificity, assay, precision, accuracy, linearity, robustness, and forced degradation studies, thereby confirming the method's reliability for analyzing Nebivolol in pharmaceutical formulations.

Specificity and Assay

The specificity of the proposed method was established by analyzing blank, standard, and drug product solutions. Separate

chromatographic runs were performed to verify the absence of interference at the retention time corresponding to NBV. Injection of a blank sample confirmed that no extraneous peaks coincided with the analyte's retention time, thereby establishing the method's capability to differentiate NBV from excipients and potential degradation products effectively. The assay of the marketed formulation was calculated.

$$\%\textbf{Assay} = \frac{\textbf{Sample Area}}{\textbf{Standard Area}} \times \textbf{100}$$

System Suitability and Repeatability

System suitability tests were performed to ensure consistent method performance before sample analysis. A single working standard solution was injected six times, and critical system suitability parameters were evaluated, including:

- Retention Time (RT)
- Theoretical Plates (≥ 2000)
- Peak Tailing Factor (≤ 2.0)
- Resolution (≥ 2.0 between adjacent peaks)
- Peak Purity (1.0, confirming no co-elution of impurities)

These parameters were within the acceptable ICH limits, ensuring the method's suitability for routine analysis.

Linearity and Range

The linearity of the method was evaluated by analyzing five calibration levels within the concentration range of 80–120 µg/mL. A calibration curve was generated by plotting peak area vs. NBV concentration, and the correlation coefficient (r^2) was determined using the least-squares regression method. The method exhibited excellent linearity with an r^2 value close to 1, confirming a direct relationship between concentration and detector response.

Accuracy

Accuracy was determined at three concentration levels (80%, 100%, and 120%) by spiking known amounts of NBV into the diluent. Each level was analyzed in triplicate, and the percentage recovery and relative standard deviation (RSD) were calculated. The recovery percentages were found to be within the acceptable range (98%–102%), indicating the method's ability to provide accurate results across the tested concentration range.

Inter-day and Intraday Precision

Precision was assessed by evaluating repeatability (intraday precision) and intermediate precision (inter-day precision) using multiple injections of the same concentration on different days. Intraday precision was evaluated by analyzing the working standard solution at two different time intervals (morning and evening) on the same day, and the percentage relative standard deviation (RSD) was calculated. Interday precision was assessed by reanalyzing the same standard solution on the following day and comparing the results. The method demonstrated reproducibility, as indicated by %RSD values for both intraday and inter-day precision being below 2.0%.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The sensitivity of the method was assessed by determining LOD and LOQ, calculated using the standard error of the regression line:

$$\boldsymbol{LOD} = \frac{\boldsymbol{3.3 \times Std.\ Error\ of\ Intercept}}{\boldsymbol{Slope\ of\ calibration\ curve}}$$

$$\boldsymbol{LOQ} = \frac{\boldsymbol{10 \times Std.\ Error\ of\ Intercept}}{\boldsymbol{Slope\ of\ calibration\ curve}}$$

The low values of LOD and LOQ indicate the method's ability to detect and quantify NBV at very low concentrations, making it suitable for trace-level analysis in pharmaceutical formulations.

Robustness

The robustness of the method was evaluated by deliberately varying critical parameters, such as column oven temperature (±2°C) and detection wavelength (±2 nm), to determine the method's stability under minor changes in conditions (Table 3).

Table 3: Robustness Study – Effect of Column Temperature and Wavelength on Method Performance

Condition	Increased	Normal	Decreased
Column Temp. (°C)	32°C	30°C	28°C
Wavelength (nm)	230 nm	228 nm	226 nm

Variations in column temperature and wavelength did not significantly impact the retention time, peak area, or assay results, confirming the method's robustness and reliability for routine use.

Forced Degradation Studies

Forced degradation studies were conducted to assess the stability of Nebivolol under various stress conditions by ICH Q1A(R2) guidelines. The stress conditions applied included:

- Acidic degradation (1N HCl, 10 min at room temperature)
- Basic degradation (0.1M NaOH, 60°C for 3 hours)
- Oxidative degradation (30% H_2O_2, 60°C for 30 min)

- Thermal degradation (100°C for 6 hours)
- UV degradation (254 nm for 6 hours)

The percentage of degradation under each condition was calculated based on the difference in peak areas before and after stress exposure (Table 4).

Table 4: Forced Degradation Study of Nebivolol Under Different Stress Conditions

Condition	Degradation (%)
Acidic (1N HCl, 10 min, RT)	No degradation
Basic (0.1M NaOH, 60°C, 3h)	15.94%
Oxidative (30% H_2O_2, 60°C, 30 min)	8.57%
Thermal (100°C, 6h)	No degradation
UV (254 nm, 6h)	No degradation

RESULTS AND DISCUSSION

Method Development

A reversed-phase HPLC column (Zorbax Bonus RP) was used to achieve a well-resolved nebivolol peak. Six trials were conducted (Table 1), where adjustments to the mobile phase ratio affected peak shape and retention time. Acetonitrile was used due to its strong elution force and compatibility with buffers, thereby reducing the retention time of perchloric acid. Trials with varying mobile phase ratios identified a 0.1% perchloric acid and acetonitrile mixture (55:45) as the optimal condition, achieving a nebivolol retention time of 4.26 minutes with good peak shape (Figure 2), high theoretical plate count, optimal asymmetry, and superior system suitability compared to other trials (Table 5).

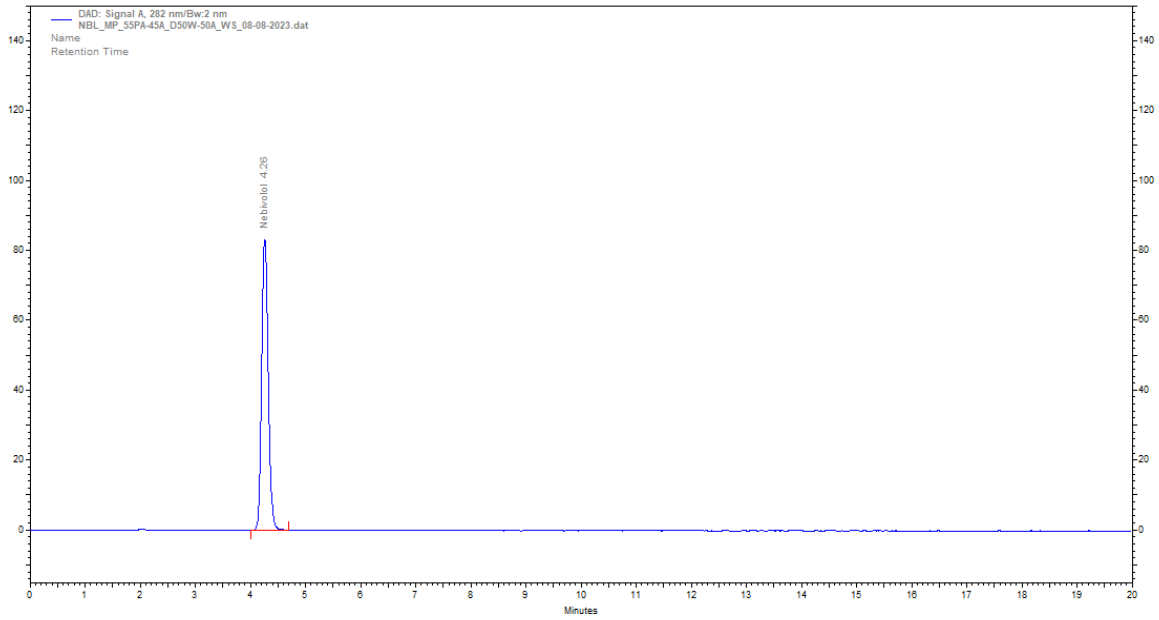

Figure 2: Chromatogram of Standard Drug Nebivolol

Table 5: Method development results of nebivolol

Method Development Trial. No.	Nebivolol			
	RT mins	Theoretical Plates	Asymmetry	Peak Purity
1	No peak observed			
2	3.87	76	2.44	0.98
3	3.96	604	1.58	0.99
4	3.13	1834	1.18	1.00
5	4.26	6113	1.15	1.00
6	6.71	6228	1.14	1.00

Design of Analytical Method through Design Expert.

A three-level, two-factor full factorial design was employed to optimize the chromatographic method, resulting in a 13-run experimental plan generated using Design-Expert software. The data from these experiments were analyzed using Design of Experiments (DoE) software. Among multiple statistical models, the optimal model was selected based on a high F-value, low P-value, and high R-squared value (Table 6).

Table 6: ANOVA results obtained from a three-level, two-factor full factorial design for Theoretical Plates and Retention Time (Quadratic model)

	R1: Theoretical Plates		R2: Retention Time	
Source	**F-value**	**p-value**	**F-value**	**p-value**
Model	2163.95	<0.0001	**F-value**	**p-value**
A-Mobile phase A (0.1% PA)	0.0852	0.7789	546.68	<0.0001
B-Flow rate	7146.98	<0.0001	0.1107	0.7490
AB	0.2358	0.6421	2482.19	<0.0001
A^2	0.6382	0.4506	4.90	0.0626
B^2	3173.15	<0.0001	21.17	0.0025
Residual				
Lack of Fit	11.74	0.0188	244.05	<0.0001

Design of Analytical Method through Design Expert.

A three-level, two-factor full factorial design was implemented to optimize the chromatographic method. Design-Expert software was utilized to create a 13-run experimental plan. Following the completion of all experiments, the data were analyzed using Design of Experiments (DoE) software. Multiple statistical models were evaluated, and the optimal model was identified based on selection criteria, including a high F-value, low P-value, and high R-squared value. Among the tested ratios, 55:45 acetonitrile: perchloric acid achieved optimal peak shape and a high plate count (6113), with minimal asymmetry (1.15). The positive coefficients in the ANOVA model for acetonitrile and flow rate indicate a direct relationship with plate count. Negative quadratic coefficients suggest diminishing returns beyond a threshold.

For retention time, flow rate had a significant inverse effect, as evident from its large negative coefficient. The validity of the selected model was further confirmed through a two-way analysis of variance (ANOVA).

Statistical Analysis of Data

Table 3 summarizes the statistical analysis and model performance for the two response variables: the number of theoretical plates (R1) and retention time (R2). A quadratic model demonstrated statistical significance for both responses, with F-values of 2163.95 for R1 and 546.68 for R2. The adjusted R-squared (R^2adj) values confirmed a strong fit between the predicted and actual data, validating the model's reliability. The equations derived using coded factors are:

$$\textit{Theoretical Plates (R1) : +6103.10+ +8.83A++2558.67B+ 18.00 AB+35.64A}^2\textit{-2512.86B}^2\textit{.}$$

$$\textit{Retention Time(R2): +4.29-0.0117A+1.75B-0.2378A}^2\textit{+0.8072 B}^2$$

3D response surface plots (Figure 3C and 3D) illustrate the relationships between independent variables and responses, highlighting the influence of critical method variables (CMVs) on R1 and R2. A counterplot (Figure 3A and 3B) provides a graphical representation of the response surface, showing how changes in perchloric acid concentration (x-axis) and flow rate (y-axis) affect the responses. Figure 4A, B shows the normal probability plot of residuals for retention time, indicating a normal distribution and strong agreement between predicted and actual values. This confirms the model's statistical validity, accuracy, and reliability.

Specificity and Assay

Specificity was established by comparing retention times and peak purity of the standard, blank, and drug sample chromatograms. The absence of extraneous peaks at the analyte's retention time (4.22 min) and a peak purity index of 1.000 confirmed no interference from excipients or degradation products. Figure 3 displays the specificity of chromatograms for the blank, working standard, and drug product. The main peak for Nebivolol in both the API and drug product was identified with a retention time of 4.22 minutes and an asymmetry of 1.15. No peak was observed at 4.22 minutes in the blank chromatogram, confirming the absence of interference. The assay for the marketed drug product was determined to be 99.52%.

Assessment of Instrumental Precision, Methodological Precision, Intermediate Precision, and System Suitability

The suitability of the HPLC instrument for validation was assessed, and the %RSD values demonstrated the accuracy and reliability of the procedure. The equipment was deemed suitable for further validation based on system suitability limits.

The %RSD values for instrument precision, method precision, and intermediate precision were 0.50%, 0.53%, and 0.50%, respectively. Additionally, the cumulative %RSD for method precision and intermediate precision was 0.03%. These results highlight the procedure's reliability across multiple analysts and samples at the same concentration (Table 7).

R1: Theoretical plate **R2: Retention Time**

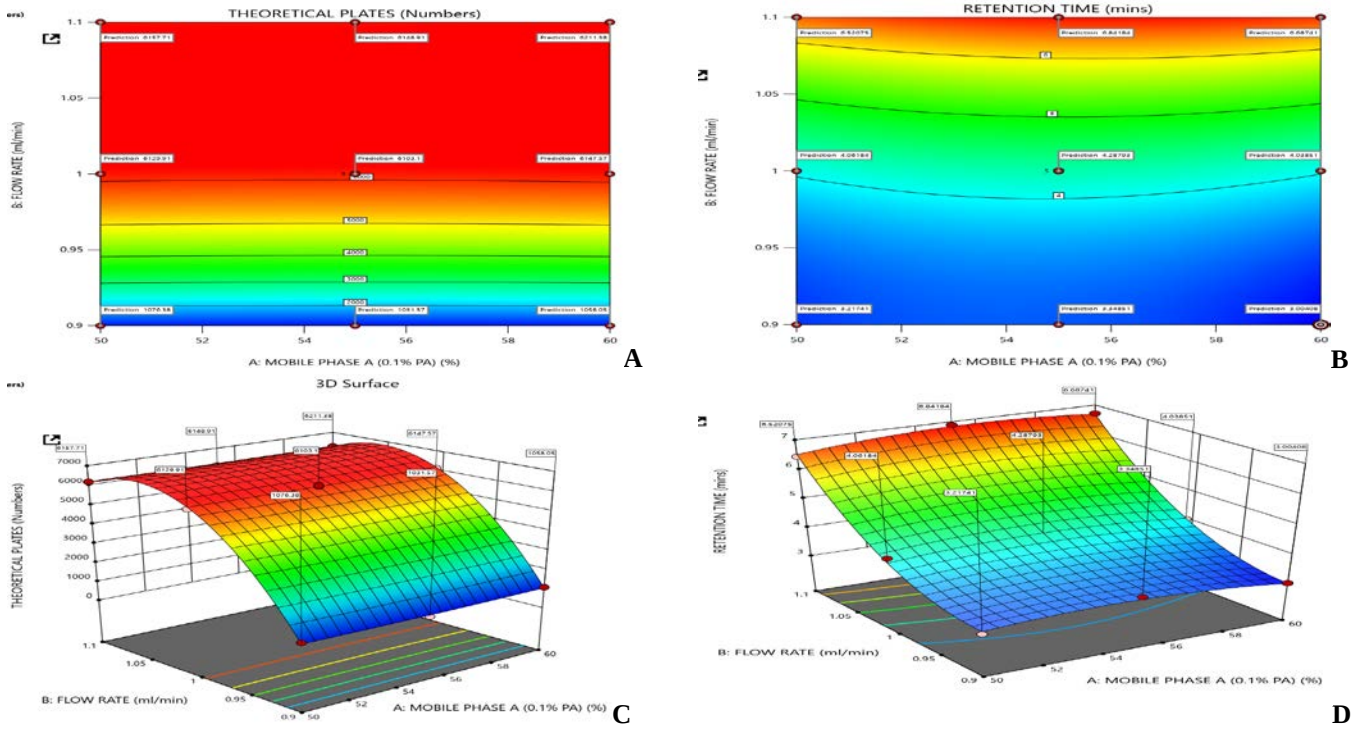

Figure 3: Relation Plot: (A, B) Counter plot, (C and D)

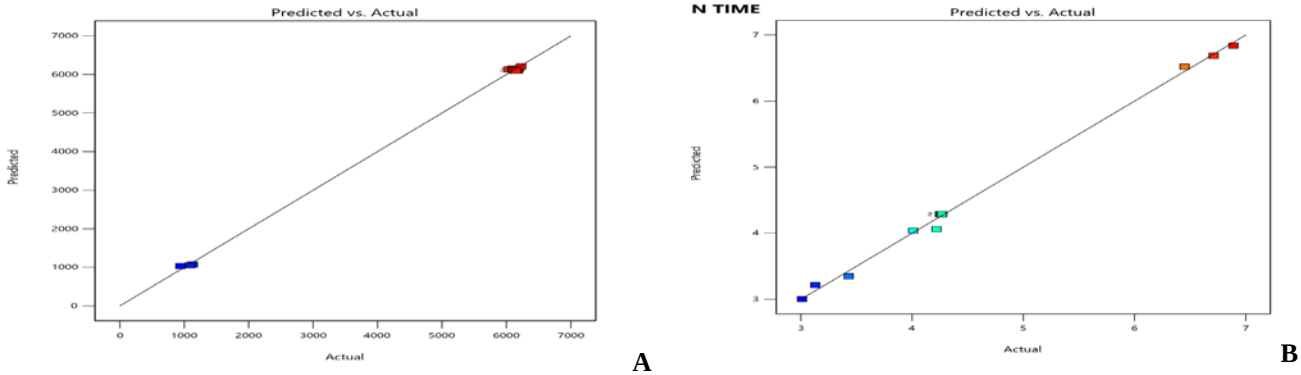

Figure 4: Relation Plot: (A) Predicated vs Actual values of showing Theoretical Plates; (B) Predicated vs Actual values of showing Retention time.

Table 7: Instrumental Precision, Methodological Precision, Intermediate Precision, and System Suitability for Nebivolol

Nebivolol: System Suitability					Peak Area		
Sample ID	**RT**	**TP**	**Asymmetry**	**Peak Purity**	**Instrument Precision**	**Method Precision**	**Intermediate Precision**
Rep 1	4.22	6012	1.12	1.00	1443567	1425471	1445217
Rep 2	4.22	6026	1.10	1.00	1435521	1442547	1443567
Rep 3	4.22	6057	1.12	1.00	1452647	1425417	1425417
Rep 4	4.22	6004	1.13	1.00	1445412	1443567	1436524
Rep 5	4.22	6047	1.09	1.00	1452211	1435754	1436687
Rep 6	4.22	6021	1.11	1.00	1436897	1442412	1442325
Average	4.22				1444376	1437939	1436904
STDEV	0				7292.063	7656.541	7175.051
%RSD	0.00				0.50	0.53	0.50

Linearity of nebivolol

Linearity was evaluated across various concentration levels. A graph depicting the relationship between peak area and concentration demonstrated linearity, with the correlation coefficient presented in Figure 4. Linearity was assessed for nebivolol with concentrations ranging from 80% to 120% (80 µg/mL to 120 µg/mL), yielding a correlation coefficient of 1, as shown in Figure 5.

LOD & LOQ of nebivolol

Linearity assessment was conducted for nebivolol across a concentration range of 80% to 120% (80 µg/mL to 120 µg/mL). A calibration curve was constructed by plotting peak area against concentration, demonstrating a linear relationship with a correlation coefficient of 1, as illustrated in Figure 5.

Accuracy

The accuracy assessment for nebivolol was performed in triplicate at concentration levels of 80%, 100%, and 120% of the specified test concentration. The method yielded an average percentage recovery ranging from 99% to 100%, as presented in Table 8. The relative standard deviation (%RSD) values corresponding to the 80%, 100%, and 120% levels were calculated to be 0.17%, 0.59%, and 0.41%, respectively.

Robustness

Robustness testing was conducted to assess the method's resilience to variations in critical parameters, including column temperature and wavelength. Changes in column temperature (±2℃) and wavelength (±2 nm) did not result in significant changes in retention time or peak area for the API and marketed product, as shown in Table 9. Changes in retention time (<2%), peak area (<2.5%), and % assay (<2%) were used as criteria. All observed values were within these limits, indicating method robustness.

Intra & Inter-day precision

Intra- and inter-day precision studies were conducted to evaluate the consistency of the assay and changes in peak area for the API and marketed product at various time points. The %RSD for both intra- and inter-day precision was 0.21%, which meets the acceptance criteria of %RSD <2%, as shown in Table 10. Variability may arise from operator technique, instrument fluctuations, or minor temperature fluctuations. The low %RSD values (<0.6%) across intraday and interday evaluations demonstrate the method's robustness against such variations.

Forced degradation

Forced degradation studies of nebivolol were performed under various stress conditions, with the results summarized in Table 11. Significant degradation was observed under basic conditions, with up to 81% degradation in the initial test. To meet ICH guidelines (degradation between 5% and 25%), the experiment was repeated using 0.1M sodium hydroxide, resulting in 15.94% degradation. Oxidative conditions showed 8.57% degradation. No significant degradation was observed under acidic, heat, or photolysis conditions. Stress conditions were chosen based on ICH Q1A(R2) guidelines and literature practices. Base degradation was initially performed with 5 M NaOH, but exceeded 25%, so it was optimized to 0.1 M NaOH to meet regulatory guidelines (5–20% degradation range).

DISCUSSION

A stability-indicating RP-HPLC method for the analysis of Nebivolol was successfully developed and optimized using a Zorbax Bonus RP column. The mobile phase consisted of 0.1% perchloric acid and acetonitrile in a 55:45 v/v ratio, which resulted in improved peak resolution, better symmetry, and a retention time of 4.26 minutes. The method demonstrated high efficiency, as evidenced by a theoretical plate count of 6113 and an asymmetry factor of 1.15, both of which meet the ICH guidelines for validation.

To fine-tune the method parameters, a full factorial design with three levels and two factors was employed. ANOVA-based statistical analysis confirmed the significant effect of flow rate and mobile phase composition on theoretical plates and retention time. The quadratic model demonstrated considerable statistical significance, with F-values of 2163.95 for theoretical plates and 546.68 for retention time, along with adjusted R^2 values indicating a strong relationship between experimental and predicted data. Further validation through residual analysis and response surface plots supported the model's robustness and precision. Method validation was conducted by the ICH guidelines, confirming the method's specificity, precision, accuracy, linearity, robustness, and sensitivity. Specificity studies revealed no interference from excipients, with Nebivolol showing a retention time of 4.22 minutes in both standard and drug product solutions. The linearity test exhibited a correlation coefficient of 1.00 across a concentration range of 80–120 µg/mL, establishing its effectiveness for quantitative analysis.

Recovery experiments at 80%, 100%, and 120% concentrations yielded average recoveries of 99–100%, with relative standard deviation (RSD) values of less than 1.0%, indicating excellent reproducibility. Sensitivity was demonstrated through low LOD and LOQ values, confirming the method's ability to detect and quantify trace amounts of the analyte. Robustness tests demonstrated that minor variations in column temperature (±2°C) and detection wavelength (±2 nm) had no significant impact on peak area, retention time, or assay results, thereby validating the method's consistency and reliability.

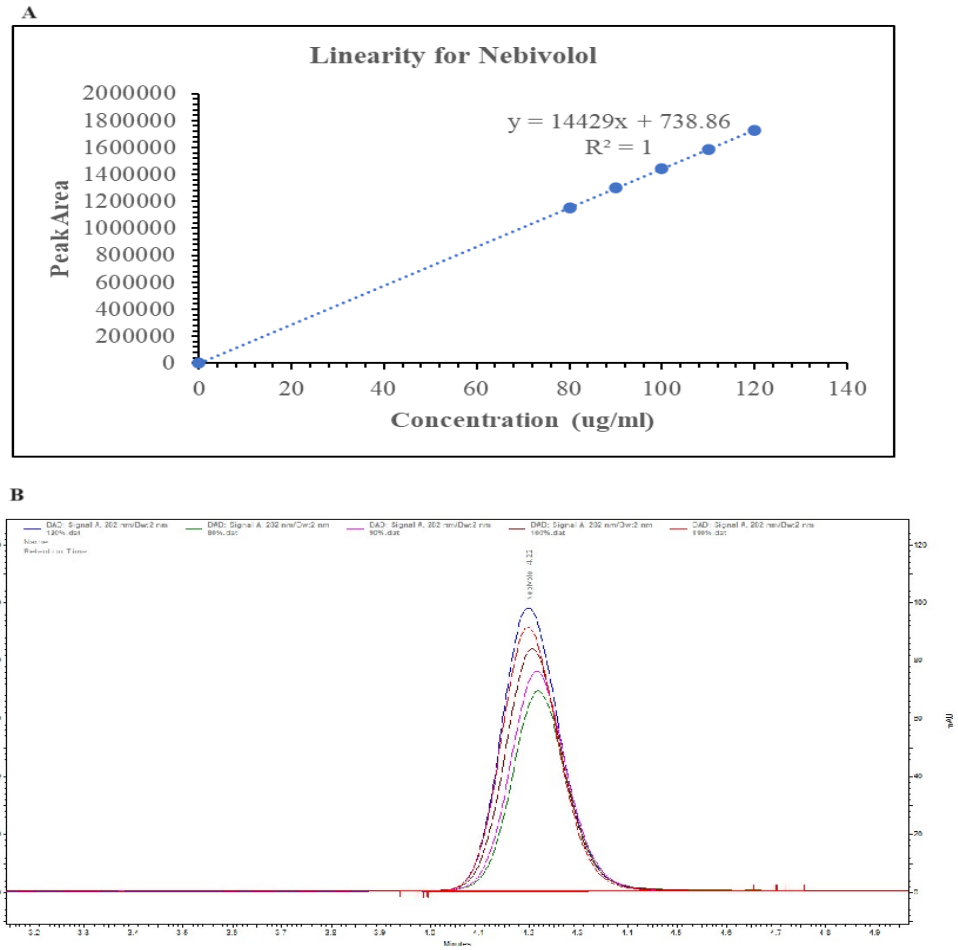

Figure 5: Linearity (A) Linearity scale (B) Linearity overlay of nebivolol standard solution

Table 8: Accuracy for Nebivolol [Spiked Conc(ug/mL) and Amt Recovered(ug/mL)]

Sample ID	Reps	Spiked Conc.	Peak Area	Amt Recovered	%Recovery	%Average	STDEV	%RSD
80%	Rep 1	79.92	1156027	79.96	100.05	99.94	0.1651	0.17
	Rep 2	79.92	1152647	79.72	99.75			
	Rep 3	79.92	1155874	79.95	100.03			
100%	Rep 1	99.9	1443567	99.84	99.94	99.97	0.5932	0.59
	Rep 2	99.9	1435521	99.29	99.39			
	Rep 3	99.9	1452647	100.47	100.57			
120%	Rep 1	119.88	1730494	119.69	99.84	99.90	0.4090	0.41
	Rep 2	119.88	1726547	119.42	99.61			
	Rep 3	119.88	1736574	120.11	100.19			

Table 9: Robustness: Change in Column Oven Temperature and wavelength

Column Oven Temp Change				
Condition	**Sample**	**Nebivolol**		
		Retention Time	**Peak Area**	**%Assay**
28℃	Working Standard	4.22	1421454	-
	Drug Product	4.22	1414574	99.52
30℃	Working Standard	4.22	1443567	-
	Drug Product	4.21	1436594	99.52
32℃	Working Standard	4.22	1412547	-
	Drug Product	4.22	1400324	99.13
Change in Wavelength (nm)				
Condition	Sample	Nebivolol		
		Retention Time	Peak Area	%Assay
280 nm	Working Standard	4.22	1365744	-
	Drug Product	4.22	1325475	97.05
282 nm	Working Standard	4.22	1443567	-
	Drug Product	4.22	1436594	99.52
284 nm	Working Standard	4.22	1412451	-
	Drug Product	4.22	1402574	99.30

Table 10: Intra & inter-day Precision of nebivolol

Intra-day precision			
Day 1	Sample ID	Nebivolol	
		Peak Area	% Assay
Morning	Working Standard	1443567	-
	Drug Product	1436594	99.52
Evening	Working Standard	1422547	-
	Drug Product	1410358	99.14
Inter-day precision			
Day 0	Sample ID	Nebivolol	
		Peak Area	% Assay
Day 2	Working Standard	1412023	-
	Drug Product	1400741	99.20
Average			99.29
STDEV			0.2043
%RSD			0.21

Forced degradation testing was performed under various stress conditions, including acidic, basic, oxidative, thermal, and UV treatments, to evaluate the stability of Nebivolol. The findings indicated that Nebivolol remained stable under acidic, thermal, and UV conditions, but significant degradation occurred under basic (15.94%) and oxidative (8.57%) conditions. The most pronounced degradation occurred under strongly basic conditions (81% degradation at 5.0 M NaOH), which was optimized to 15.94% using 0.1 M NaOH, in line with ICH forced degradation study guidelines. These results underscore the importance of controlling storage conditions to minimize degradation, especially in alkaline and oxidative environments.

The RP-HPLC method developed in this study is efficient, accurate, precise, and robust for the quantification of Nebivolol. The validated method complies with ICH standards, making it suitable for routine pharmaceutical quality control and regulatory purposes. Additionally, the forced degradation studies have outlined the stability profile of Nebivolol, emphasizing the need for appropriate storage conditions to preserve its integrity. This analytical approach offers a reliable and scientifically sound solution for analyzing Nebivolol in both bulk and pharmaceutical formulations. Compared to previous studies reporting retention times of more than 10 minutes and limited specificity, the present method offers faster analysis (4.22 minutes) with higher sensitivity (LOD: 0.55 µg/mL). The observed degradation in alkaline (15.94%) and oxidative (8.57%) conditions aligns with known instability profiles,

informing formulation strategies and shelf-life assessments. Although degradation products were not characterized in this study, future work could involve LC-MS to identify these by-products for better formulation insights.

Table 11: Forced Degradation in Nebivolol

Sample ID	Condition	% Assay	% Degradation
Working Standard		100.00	-
Acid Condition 1	Condition I: 5.0 N HCl (1mL) at Room Temperature for 10m	100.67	No degradation
Acid Condition 2	Condition II: 5.0 N HCl (1mL) at 60˚C (Water bath) for 3 hours.	103.03	No degradation
Base Condition 1	Condition II: 5.0 M NaOH (1mL) at 60˚C (Water bath) for 3 hours.	19.00	81.00
Base Condition 2	Condition II: 0.1 M NaOH (1mL) at 60˚C (Water bath) for 3 hours.	84.06	15.94
Oxidative Peroxide Condition 1	Condition I: 30% Hydrogen peroxide 1.0 mL at Room Temperature for 10 min.	101.27	No degradation
Oxidative Peroxide Condition 2	Condition II: 30% Hydrogen peroxide 1.0 mL at 60˚C (Water bath) for 3 hours.	91.43	8.57
Heat Condition	Thermal (100℃ for 6 hours in an oven).	100.59	No degradation
UV Condition	Photolysis at 254 nm for 6 hours	101.37	No degradation

CONCLUSION

The RP-HPLC method developed in this research provides a precise, reliable, and efficient technique for quantifying nebivolol in pharmaceutical preparations. The optimized procedure, employing a Zorbax Bonus RP column and a mobile phase composed of 0.1% perchloric acid and acetonitrile (55:45 v/v), resulted in a short retention time of 4.26 minutes. This method also ensured excellent peak resolution, minimal asymmetry, and a high theoretical plate count. A three-level, two-factor full factorial design was utilized to fine-tune the method parameters, ensuring consistent reliability and reproducibility. Validation studies conducted by ICH guidelines confirmed the method's specificity, linearity, precision, accuracy, and robustness. The method exhibited high sensitivity with low LOD and LOQ values, making it suitable for detecting trace amounts. Forced degradation studies indicated that Nebivolol remains stable under acidic, thermal, and UV conditions. However, it showed considerable degradation under basic (15.94%) and oxidative (8.57%) conditions. This RP-HPLC method is highly effective for routine quality control and regulatory compliance, providing accurate assessments of the stability and potency of Nebivolol. These findings underscore the significance of controlled storage conditions, particularly protecting the drug from alkaline and oxidative environments, to preserve its therapeutic efficacy. The developed RP-HPLC method offers high sensitivity, short run time, and robust performance. Its ability to detect degradation under stress conditions makes it valuable for stability and quality control of Nebivolol. Compared to existing methods, it provides superior resolution, reproducibility, and regulatory compliance.

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Nil

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding this research study.

AUTHOR CONTRIBUTION

Anant Ghongade collected data, performed experiments, and wrote the first draft of the manuscript. Shruti Borat conducted the analysis. All authors contributed to the study’s conception, design, revision of the previous drafts, and gave final approval.

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