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DEVELOPMENT AND VALIDATION OF A ROBUST RP-HPLC METHOD FOR ESTIMATION OF NEBIVOLOL IN MARKETED FORMULATION

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ABSTRACT

An accurate and sensitive RP-HPLC method has been developed for the measurement of Nebivolol (NEB) in its commercial formulation. The wavelength maximum for NEB was estimated to be 280 nm. RP-HPLC separations were conducted using a Phenomenex Gemini 5 µm C18 110 Å column (150 mm x 4.6 mm, 5 µm), with a mobile phase consisting of Methanol and KH2PO4 buffer at pH 4.5 (adjusted with a 5% Sodium Hydroxide solution) in a 55:45 ratio. The measurement was conducted at 280 nm with a UV detector, maintaining a flow rate of 1.3 ml/min and an injection volume of 20µl. The existing technique exhibits strong linearity within the range of 12.5-37.5 µg/ml for NEB. No influence from excipients was seen throughout the estimate. The suggested procedures were validated and found to be particular, accurate, and precise. The assay of NEB in the commercial formulation was determined to be 99.82%, indicating the correctness of the procedures used. The methods were efficiently used for the measurement of drug in pharmaceutical formulations, making them appropriate for routine quality control assessments.

KEYWORDS: Nebivolol (NEB), RP-HPLC method, ICH Validation, Liquid Chromatography, Marketed Formulation.

1. INTRODUCTION

Nebivolol is a third-generation beta-blocker largely used in the treatment of hypertension and heart failure owing to its cardio selective characteristics and supplementary vasodilatory effects. Nebivolol, distinct in its structure and pharmacological properties from traditional beta-blockers, is acknowledged for its advantageous side-effect profile and improved effectiveness in certain cardiovascular disorders.[1-4]

Chemically Nebivolol is 1-(6-fluoro-chroman-2-yl)-2-[(2RS)-6-(4-hydroxyphenyl)-2, 5, 7, 8-tetramethylchroman-2-yl] ethanol. The chemical structure has a chromanol backbone (**Figure 1**) and a fluorochroman moiety, endowing it with both hydrophobic and hydrophilic characteristics, hence enhancing its affinity for adrenergic receptors and contact with endothelial cells. It is a combination of two racemic mixture d-nebivolol and I-nebivolol. D-nebivolol is one of the two enantiomers that make up nebivolol, which helps stop beta-1 adrenergic receptors. L-nebivolol, which opens up blood vessels by releasing nitric oxide.[1-10] Nebivolol exhibits a dual mechanism of action, Beta-1 Adrenergic Receptor Blockade and Nitric Oxide (NO) Release. Nebivolol selectively antagonizes beta-1 adrenergic receptors in the myocardium. By inhibiting these receptors, it diminishes the effects of catecholamines (such as adrenaline), resulting in a decreased heart rate and myocardial contractility, eventually leading to reduced cardiac output and blood pressure.[7-10] Nebivolol, in contrast to several other beta-blockers, promotes the endothelial synthesis of nitric oxide, a powerful vasodilator. The NO-mediated vasodilation facilitates the relaxation of smooth muscles in blood arteries, leading to enhanced blood flow and a subsequent decrease in blood pressure. The vasodilatory action also contributes to its beneficial influence on endothelial function and arterial stiffness. Nebivolol, when taken orally, is well absorbed, reaching peak plasma concentrations within 1.5 to 4 hours. It undergoes significant first-pass metabolism, mostly via the CYP2D6 enzyme, resulting in variable plasma concentrations based on individual metabolic rates. The half-life is around 10–12 hours in substantial metabolizers and may extend to 48 hours in poor metabolizers. It is mostly excreted via urine and feces. Nebivolol is largely used in the management of cardiovascular disorders, including those associated with hypertension and cardiac function abnormalities. Its selective blockage of beta-1 receptors and nitric oxide-

mediated vasodilatory characteristics render it useful in controlling these disorders with reduced adverse effects relative to earlier beta-blockers. It is often used in hypertension, heart failure, the prevention of cardiovascular events, and endothelial dysfunction.

A comprehensive literature review was conducted using databases such as Scholar, Sci Finder, PubMed, Scopus, and Web of Science.[10-18] The literature review indicated that numerous analytical techniques have been documented for NEB, including High-Performance Liquid Chromatography (HPLC), High-Performance Thin-Layer Chromatography (HPTLC), Liquid Chromatography coupled with Mass Spectrometry (LC-MS/MS) and Ultraviolet/Visible Spectrophotometry. Yunoos Mohammad and colleagues developed a stability-indicating method for the simultaneous determination of hydrochlorothiazide (HCT) and nebivolol (NEB) in both pure and tablet dosage forms. S. N. Meyyanathan et al developed RP-HPLC method for Nebivolol and HCTZ estimation[15], while Bhatia M. S. and team developed RP-HPLC method for quantification of Nebivolol and Valsartan.[16] Kancherla P and team developed and validated process of Related Impurities of Nebivolol and Structural Characterization by LC-MS/MS.[17] Vaishali Gohel et al developed stability indicating method for Nebivolol and Valsartan[18], Kaveri T. V and colleagues developed method for Nebivolol Hydrochloride in Human Plasma using RP-HPLC.[19] In this research, we have developed and validated method for estimation of Nebivolol form its bulk drug and marketed formulation.

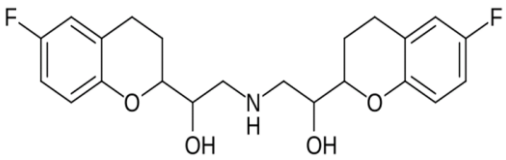

Figure 1: Structure of Nebivolol.

2. MATERIALS AND METHOD

2.1 Reagents and chemicals

The NEB sample was received as gift sample by Step up Pharmaceuticals for this testing. HPLC-grade methanol and acetonitrile were procured from Merck. The sodium hydroxide pellets and hydrochloric acid were of analytical grade procured from Loba Chemicals Ltd. The experiment used just Milli-Q water. The Nebimac 2.5mg Tablet brand was acquired from the local market.

2.2 Preparation of Solution

Preparation of Standard solution of NEB

Accurately weighed 25 mg NEB was transferred into 100 ml volumetric flask. Sufficient mobile phase was added to dissolve the drug and the volume was made up to mark with mobile phase to obtained stock solution (250µg/ml) and labeled as Standard Solution 1 (SS1). Further pipetted 5.0 ml of this Standard Solution 1 (SS1) to 50 ml volumetric flask and diluted with mobile phase to make final concentration 25 µg/ml.

Preparation of Sample Solution

Weighed and transferred 20 intact NEB tablets into 100 ml volumetric flask and added about 65 mL of mobile phase to it. Sonicate the resultant mixture for 30 minutes with intermediate shaking to prevent creation of lumps. The solution was cooled at room temperature and make volume up to mark with mobile phase. (The solution contains 250µg/ml of NEB. An aliquot of 5.0 ml from this solution was transferred in 50 ml volumetric flask and diluted up to mark with mobile phase, mix well. (The final solution contains 25 µg/ml of NEB). The solution was filtered through 0.45 µm PVDF syringe filter and first 3 ml of filtrate were discarded.

2.3 Optimization of chromatographic conditions

A trial-and-error methodology was used to refine the chromatographic parameters. A variety of experiments were conducted using various buffer ranges in conjunction with appropriate solvents to achieve effective elution of NEB within a brief runtime. In conclusion, the mobile phase comprising Methanol and KH2PO4 buffer at pH 4.5 (adjusted with 5% Sodium Hydroxide solution) in a 55:45 ratio, with a flow rate of 1.3 mL/minute, using a Phenomenex Gemini 5 µm C18 110 Å column (150mm x 4.6mm, 5 µm), was selected for optimal elution pattern. Isocratic mode of elution was developed for rapid elution of NEB. The injection volume was 20 µL, and a wavelength of 280 nm was used to ensure the symmetry of the analyte peak. The optimized chromatography was implemented for estimation of NEB in finished drug product and same has been validated as per ICH Q2 (R1) guidelines.

2.4 Validation of the analytical method

With the use of ICH recommendations Q2 (R1), the analytical method was validated in terms of its specificity, linearity, accuracy, precision, limit of detection, limit of quantitation, and robustness.

Specificity

The specificity of the analytical method was employed to eliminate any potential interference from blank samples at the retention times of the NEB analyte peak.

Linearity

A series of dilutions were prepared from the Stock Standard Solution (SSS1) in order to evaluate the linearity of Nebivolol (NEB). These dilutions were prepared with NEB concentrations ranging from 12.5 to 37.5µg/ml, particularly 12.5, 17.5, 25, 31.5 and 37.5 µg/ml. Plotting the peak area against the concentration that corresponded to it resulted in the generation of a calibration curve. Additionally, the correlation coefficient and the regression line equation for NEB were found.

Precision

The accuracy of the suggested approach was assessed using intra-day and inter-day evaluations by monitoring the responses of three distinct concentrations of nebivolol (12.5, 25, and 37.5 µg/ml) three times within a

single day. The outcomes were articulated as relative standard deviation (RSD). To evaluate the method's repeatability, the answers for the 100% test concentration of nebivolol (25µg/ml) were recorded six times, and the associated RSD values were documented.

Accuracy

Accuracy was evaluated via the standard addition technique, whereby specified amounts of nebivolol (NEB) standards at 50%, 100%, and 150% concentration levels were included into the diluent. The quantity of NEB recovered at each level was quantified, and the % recovery was documented to assess the method's precision.

Limit of Detection and Limit of Quantification

The sensitivity of analytical methods is commonly evaluated using two key parameters: Limit of Detection (LOD) and Limit of Quantification (LOQ). These crucial metrics were determined by utilizing the calibration curve's slope (S) and the standard deviation of response (SD).

LOD=3.3*SD/S

LOQ=10*SD/S

Robustness

The robustness analysis was conducted by modifying the method's flow rate, wavelength, and mobile phase ratio parameters. To evaluate robustness, adjustments were made to the mobile phase ratio (± 2% absolute), wavelength (±2 nm), and flow rate (± 0.1ml/min). The effects of these modifications were examined and compared to standard conditions.

Estimation of NEB in market formulation by the proposed method

The NEB concentration was assessed using the previously described sample preparation method and calculated assay. The chromatogram's apex regions of NEB were extracted after injecting the Blank solution, followed by the standard and sample preparations. These extracted regions were then utilized for quantifying NEB in the commercial formulation.

3. RESULT AND DISCUSSION

3.1 Selection of Chromatographic Conditions

Various experiments were performed using different mobile phase ratios and flow rates across multiple USP column types, including C8 and C18, to achieve optimal symmetry of the analyte peaks, NEB. Multiple experiments were performed to select the mobile phase by assessing the pKa value and solubility of the compound. The modification of pH in the buffer, along with the solvent composition in the mobile phase, is essential for optimizing the separation of the analyte peak from the degradation peak. A methanol and KH2PO4 buffer at pH 4.5, adjusted with a 5% sodium hydroxide solution in a 55: 45 ratios, was utilized with a flow rate of 1.3 mL/min. The Phenomenex Gemini 5 µm C18 110 Å column (150mm x 4.6mm, 5 µm) was chosen to achieve optimal elution patterns. The injection volume was 20 µL, and a wavelength of 280 nm was employed to ensure the symmetry of the analyte peak. The analyte peak elutes within a 20-minute time frame.

3.2 Results of validated analytical method

Specificity

There was no interference from the diluent during the retention of NEB, demonstrating specificity of the analytical method. The chromatograms of the blank and standard solutions, as well as an overlay graph of the two sets of data, are shown in Figures 2, 3, and 4.

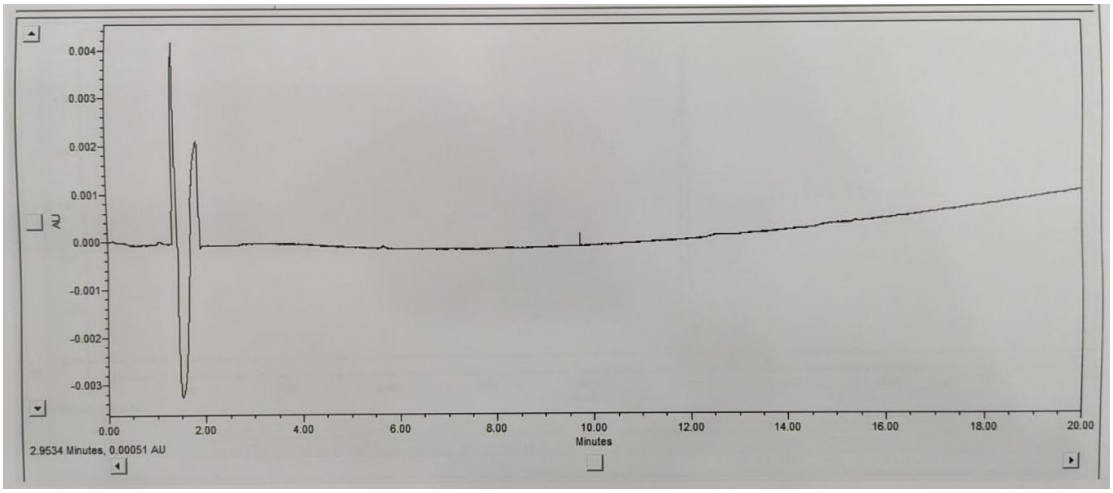

Figure 2: Chromatogram of NEB Blank Solution.

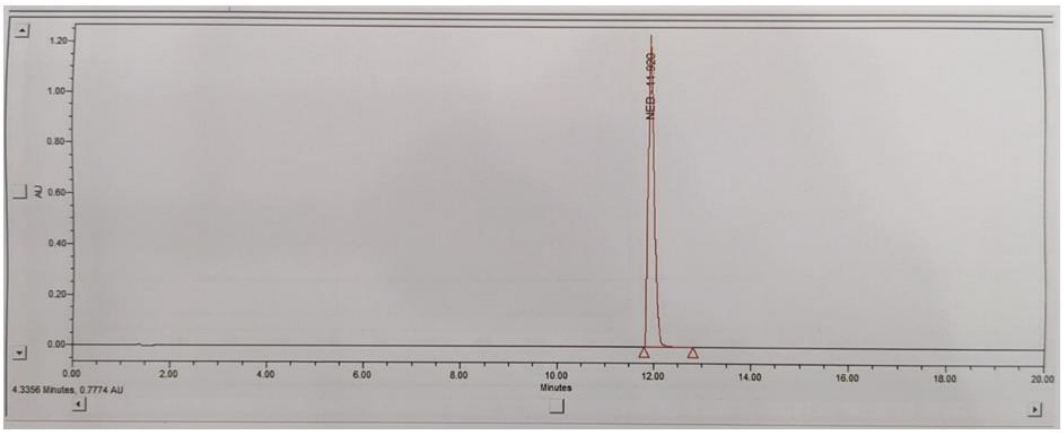

Figure 3: Chromatogram of NEB Standard Solution.

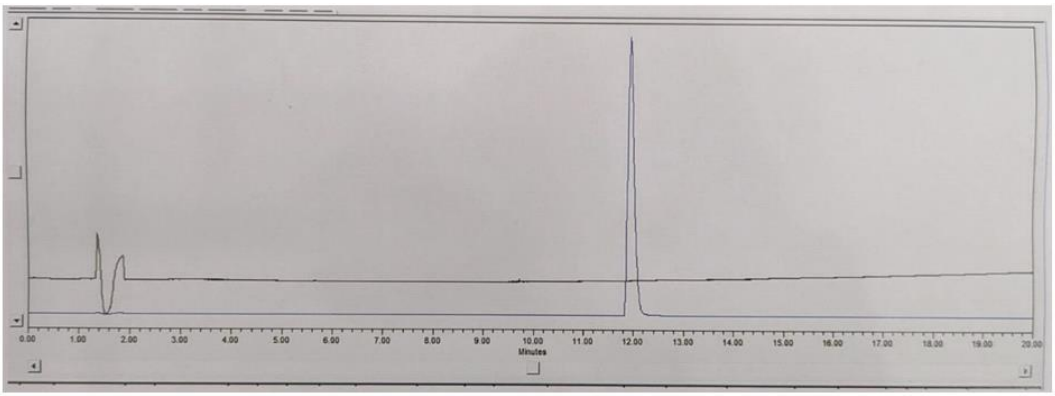

Figure 4: Overlaid Chromatogram of NEB for Specificity.

Linearity

The peak area versus concentration was used to establish linearity, with a concentration range of 12.5-37.5 µg/ml for NEB. The correlation coefficient was determined to be 0.999, consistent with the guidelines shown in the linearity plots presented in Figures 5 with corresponding results displayed in Table 1.

Table 1: Result of NEB Linearity.

Parameter	Result for AZI
Linearity Range	12.5-37.5 µg/ml
Linearity	Y = 59566x + 33352
Correlation coefficient	0.9994
LOQ µg/mL	2.5 (µg/mL)
LOD µg/mL	0.75 (µg/mL)

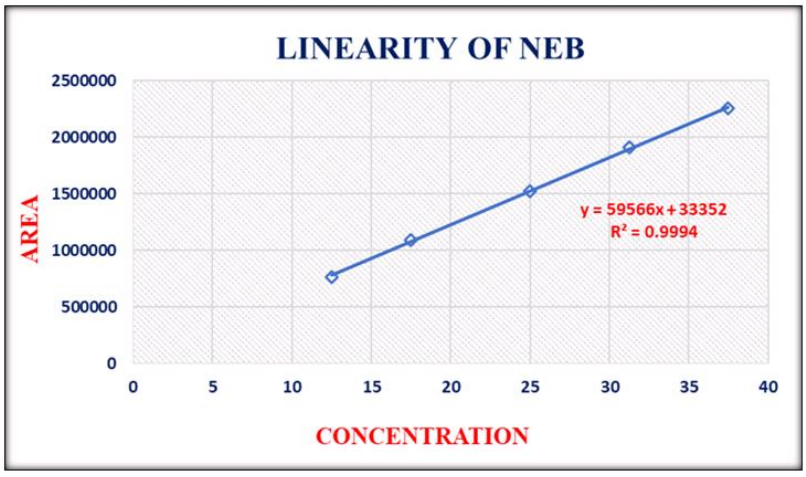

Figure 5: Linearity plot of NEB.

Precision

The % RSD of six replicates of the standard solution was determined to be 0.98% for NEB. The inter-day precision for NEB was determined to have a % RSD of 1.21%, whereas the intra-day precision exhibited % RSD of 0.86%. The results indicate that the procedure is inherently precise in nature.

Accuracy

Recovery was performed at drug concentrations of 50%, 100%, and 150% for NEB. The recovery values were found to be within the expected range of 95% to 105%. The results of the recovery are presented in Table 2. The results demonstrate that the procedure is fundamentally accurate.

Table 2: Result of NEB Recovery.

Name of Compound	Recovery Level	% Recovery			%Recovery Avg.	%RSD
		Set- 1	Set- 2	Set- 3		
NEB	50	98.7	99.1	100.5	99.4	0.95
	100	99.7	101.9	100.8	100.8	1.09
	150	101.1	100.7	99.4	100.4	0.89

Robustness

The approach was considered reliable as minor alterations in the mobile phase composition, flow rate, and detector wavelength do not significantly impact the results. Results are presented in Table 3.

Table 3: Result of NEB Robustness.

Drug Name	Condition	As Such (Normal)	Flow Plus 1.4 mL/min	Flow Minus 1.2 mL/min	Plus Wavelength (282nm)	Minus Wavelength (278 nm)	Plus Organic Me OH: Buffer (57:43 %v/v)	Minus Organic Me OH: Buffer (53:47 %v/v)
NEB	%RSD	0.95	1.20	1.01	1.43	1.09	0.89	1.13
	USP Plates	12530	11253	13321	11989	12563	9289	14523
	USP Tailing	1.1	1.0	0.9	1.1	1.1	0.9	1.0

3.3 Estimation of NEB in Marketed Formulation

The RP-HPLC method recommended for measuring NEB was found to be effective for the commercialized formulation. The analyzed percentage of NEB was found to be acceptable and consistent with the label claim. The result of all five analyses was found with 98.0%-102% and table 4 presents the results of the conducted analysis.

Table 4: Market formulation analysis (n=5).

Dosage Formulation	NEB		
Tablet	Label Value (2.5 mg)	% Drug found	Mean ± SD
Spl-1	2.469	98.76	99.82 ± 1.18
Spl-2	2.508	100.32	
Spl-3	2.459	98.36	
Spl-4	2.523	100.92	
Spl-5	2.519	100.76	

4. CONCLUSION

In order to properly estimate the amount of NEB present in the formulation that is already on the market, an RP-HPLC approach that is accurate, selective, and specific was successfully designed and validated. Specificity, precision, linearity, and accuracy are the findings that demonstrate the dependability of the technique that was specifically devised. It was determined that there was no significant interference found at the retention periods of NEB in either the standard solution or the sample solution. Additionally, it was observed that the NEB had a linear response throughout the calibration range of 12.5-37.5 µg/ml, followed by a correlation value of around 0.9994. Within the acceptable range of 95%-105%, the recovery for NEB at 50%, 100%, and 150% levels was considered to be satisfactory. The assay of marketed formulation was found 99.8%, which clearly represents methods accuracy. Furthermore, the findings on robustness indicate that even a little change in a chromatographic parameter does not have an impact on the effectiveness of the approach. The method described above is reliable and has the potential to be used for the purpose of estimating NEB based on laboratory conditions for future applications.

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Not applicable.

CONFLICTS OF INTEREST

The authors affirm that they have no known financial or interpersonal conflicts that would have appeared to have an impact on the research presented in this study.

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