https://doi.org/ 10.33472/AFJBS.6.Si2.2024.1576-1590

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT FOR THE SIMULTANEOUS ESTIMATION OF NEBIVOLOL AND CHLORTHALIDONE IN PHARMACEUTICAL DOSAGE FORM

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Article History

Volume 6,Issue Si2, 2024

Received:27 Mar 2024

Accepted : 28 Apr 2024

doi: 10.33472/AFJBS.6.Si2.2024.1576-1590

ABSTRACT

The new stability-indicating high performance liquid chromatography (HPLC) method has been developed and validated with different parameters for Nebivolol (NEB) and Chlorthalidone (CHL) in the combined dosage form. The chromatographic conditions were optimized using a mobile phase of Acetonitrile : 0.01M KH_2PO_4 buffer pH 3(OPA) (60 : 40 %v/v) with a flow rate of 1 mL/min. Cosmosil Column (C18) of 4.6 × 250 mm dimension was used as a stationary phase; the particle size capacity of the column was 5 µm. The detection was carried out at 226 nm. The method was validated according to ICH guidelines for linearity, precision, repeatability, the limit of detection (LOD), and limit of quantitation (LOQ). The response was found to be linear in the concentration range of 12.5 to 75 µg/ml for NEB and 31.25 to 187.5 µg/ml for CHL. The developed method shows the minimum quantity of drugs to be identified (LOD) and minimum drug to be quantified (LOQ). The LOD and LOQ were found to be 0.0059 and 0.018 respectively for NEB and 0.1 and 0.306 respectively for CHL. The method was linear, simple, precise, and accurate, therefore, suitable for routine analysis of drugs in tablet form. The forced degradation studies were also done through the exposure of analyte solution to four different stress conditions.

Keywords: Nebivolol, Chlorthalidone, Development, Forced degradation, High performance liquid chromatography (HPLC), Validation.

INTRODUCTION

For centuries, hypertension has been recognized as a pathological disease. Physicians used to check the arteries to assess the pulse's quality in earlier societies. These days, a "hard" pulse described in this way might be diagnosed as hypertension[1]. Poor treatment of hypertension due to a lack of awareness about the condition has led to renal, cardiovascular, and cerebrovascular diseases. Currently, hypertension is still a major socioeconomic burden, with an estimated 1.13 billion cases and 8.9 million deaths annually in 2022[2-3].

Nebivolol (NEB) is chemically designated as 1-(6-fluorochroman-2-yl)-{[2-(6-fluorochroman-2-yl)-2-hydroxy-ethyl]amino}ethanol [4]. It is a selective β1 receptor blocker used in the treatment of hypertension. Third-generation, long-acting, and highly selective β1 adrenoreceptor antagonist nebivolol also lowers oxidative stress and demonstrates nitric oxide (NO)-mediated vasodilatory effects via β3 receptor agonism[5]. Chlorthalidone (CHL) is Chemically 2-chloro-5-(2,3-dihydro-1-hydroxy-3-oxo-1H-isoindol-1-yl) benzenesulfonamide [6]. As an anti-hypertensive, chlorthalidone (CHL) lowers peripheral vascular resistance and active sodium reabsorption[7]. It is also a diuretic that is frequently referred to as a thiazide diuretic. It is an antihypertensive and diuretic drug used alone or in combination with other drugs to treat hypertension and various renal disorders[8].

Typically, samples for stability-indicating assay techniques are generated via forced deterioration or stress testing. "The stability testing of drug substance and drug product under conditions exceeding those used for accelerated stability testing" is the definition of forced degradation/stress testing[9]. When a single medicine is ineffective for treating hypertension, combinations of NEB and CHL are employed. The therapy of hypertension involves the combination of NEB and CHL. In QC, simultaneous estimate of the both medications is frequently necessary[10]. It also lessens the chance of having a heart attack or stroke in the future. According to the literature review, there aren't many HPLC techniques available for determining each particular NEB and CHL in dose and bulk forms[11]. Analytical techniques for simultaneously estimating NEB and CHL in bulk and their mixed dose forms in the presence of their degradation products have not been described. Therefore, the author tried to create a stability-indicating particular, sensitive, accurate, and precise RP-HPLC technique for these medications' simultaneous measurement with an isocratic elution mode. The developed RP-HPLC technique may be used for quality control as it was verified in accordance with ICH requirements.

MATERIALS AND METHODS

Reagents and Chemicals

NEB and CHL reference standards were supplied Reliable Lab Jalgaon. Pharmaceutical dosage form (Nodon CH tablet), containing NEB and CHL, was obtained commercially. This tablet contained NEB 5 mg and CHL 12.5 mg. Acetonitrile and o-phosphoric as HPLC grade were used as solvents.

Instrumentation

The study was conducted using a HPLC Binary Gradient System 3000 Series HPLC chromatographic system with a reverse-phase Cosmosil C18 (250mm x 4.6ID, Particle size: 5

micron). The system included a photodiode array detector, a Quaternary pump, and a 20 µL injection loop. The LC Solution software was used for the analysis. Weighing was done using a Shimadzu electronic balance (ELB-300) and a UV double spectrophotometer (UV-1650PC). Samples were prepared and degassed using an ultra sonicator. Class 'A' borosilicate glassware was employed for volumetric and general purpose in the study.

Optimised Chromatographic Condition

Nebivolol (NEB) and Chlorthalidone (CHL) was analyzed with Cosmosil C_{18} (250mm x 4.6ID, Particle size: 5 micron) for the chromatographic separation, and the column was maintained at ambient temperature. The mobile phase composition comprising of Acetonitrile : 0.01 M KH_2PO_4 buffer pH 3 (OPA) (60 : 40 %v/v) delivered at a flow rate of 1.0 mL/min and detection was monitored at 226 nm with a PDA detector. The mobile phase was used as a diluent. The injection volume was 20 µL. The run time was set at 10 min. The retention time of NEB and CHL was found to be 9.97 minutes respectively.

Standard Stock Solution of NEB and CHL (Mixed)

5 mg of NEB and 12.5 mg of CHL were dissolved in the mobile phase and then added to 10 mL standard flasks. To ensure that the medication was completely dissolved, it was sonicated for ten minutes. The stock solution has been produced at a concentration of 1250 µg/mL of CHL and 500 µg/mL of NEB (Solution I).

Calibration of standards

The concentration range of NEB and CHL calibration curves (12.5 to 75 µg/mL and 31.25 to 187.5 µg/mL) were created by pipetting out various quantities from each stock solution and diluting with mobile phase until the markings were reached[12].

Method validation

The process of demonstrating that an analytical technique is appropriate for its intended use is known as method validation. The International Conference on Harmonization (ICH) and the United States Pharmacopeia (USP) provide rules for pharmaceutical processes. Studies on linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ), robustness are often required for techniques for regulatory submission[13-14].

Linearity

When sample solutions are in a concentration range where the analyte response is exactly proportional to the concentration, the linearity of a method of analysis is confirmed. To assess the linearity of the analytical procedure, a linear concentration range of a standard mixed solution was prepared. NEB (Nebivolol) and CHL (Chlorthalidone) calibration curves were chromatographed between 12.5 and 75 µg/mL and 31.25 and 187.5 µg/mL, respectively. To obtain a linear curve, the area was plotted as a function of drug concentration [15].

Accuracy

The accuracy of the procedure was determined by using the analytical technique on examined samples spiked with known concentrations of analyte. Accuracy was calculated

samples spiked with known concentrations of analyte. Accuracy was calculated based on test results, specifically the proportion of analyte recovered by the assay. To verify the accuracy of the proposed method, recovery tests using the standard addition procedure were conducted. This approach involved adding a known amount of a pure drug to pre-analyzed sample solutions at three distinct concentrations (i.e., 80%, 100%, and 120%), and then calculating the recovery of NEB (Nebivolol) and CHL (Chlorthalidone) for each concentration [16].

Precision

By calculating the relative standard deviation and assaying an adequate number of samples, the analytical method's accuracy was confirmed. The method's intra-day (repeatability, measured by evaluating the drug solutions on the same day) and inter-day (measured by injecting the samples on three successive days) variations were used to measure precision. Six duplicates of 100% concentration (50 µg/ml of NEB and 125 µg/ml of CHL) were injected during the study. Within two percent should be the relative standard deviation.

LOD and LOQ

The ability of the analytical method to determine the analyte's lowest concentration is known as its limit of detection, or LOD. The limit of quantification, or LOQ, is the lowest quantity of analyte that can be quantitatively evaluated with a sufficient degree of accuracy and precision. It was calculated using the blank response and the slope of the calibration curve in compliance with ICH regulations. The basis for measuring LOQ is the S/N ratio (signal/noise), which is frequently used for HPLC processes. Generally speaking, a signal-to-noise ratio (S/N) of three was acceptable for calculating LOD and ten for determining LOQ. LOD and LOQ were calculated based on the slope and response standard deviation[17-18].

$$\textbf{LOD} = 3.3 \times \text{avg SD/slope} \quad \text{.....eq.1}$$

$$\textbf{LOQ} = 10 \times \text{avg SD/slope} \quad \text{.....eq.2}$$

Robustness

Analyzing aliquots from homogeneous batches using varying physical parameters—such as flow rate, mobile phase, and lambda max—that may vary but remain within the assay's defined parameters allowed for the determination of an analytical method's robustness. Here, we have used 500µg/ml and 1250µg/ml of NEB and CHL, respectively, to make a little change in the flow rate (± 0.1 ml/min), Change in mobile phase 60:40 (±1) and wavelength (226 ±1 nm).

System Suitability

A fundamental feature of the chromatographic system is system appropriateness. It is described as tests for evaluating a procedure's ability to provide results with a sufficient level of precision and accuracy. Following the completion of method development and validation, the system appropriateness was assessed. Parameters like Plate number (N), Resolution (R), tailing factor, capacity factor, HETP, and sample peak symmetry were assessed in order to achieve this.

Specificity

The capacity of an analytical technique to detect analytes properly in the presence of chemicals that would be expected to be present in the sample matrix is known as its specificity. By injecting the diluents or excipients solution under the same experimental circumstances as the test, the specificity of the analytical technique was confirmed. Excipients that are often used in tablet manufacture were spiked with a pre-weighed quantity of medicines, and the area was measured and the drug quantity was calculated[19-20].

Assay of marketed formulation

The peak response was ascertained following the injection of 20 µl of sample solution into the chromatographic equipment. The solution was injected into the column three times. It was possible to calculate the quantity in each tablet by comparing the test and standard regions. By comparing the test and standard areas, the amount in each tablet was computed, and the results for NEB and CHL were determined to be 99.95% and 99.97%, respectively[21-22].

Forced Degradation Sample Preparation

The purpose of this study was to evaluate how well NEB, CHL, and their degradation product could be separated. A forced degradation study was conducted to assess the method's stability-indicating qualities. Samples were treated with acid, base, hydrogen peroxide (oxidative degradation), photolytic, and thermal degradation agents in order to conduct a forced degradation research[23]. These are spoken about in the section below:

Acidic Degradation

From the stock solution-I, a sample (0.2 mL) was obtained and put into a 10 mL volumetric flask. This was followed by the addition of 5 mL of 1 N hydrochloric acid (HCl), mobile phase to bring the solution up to the appropriate level, and 30 minutes of heating at 60 °C. The flask was taken out and allowed to cool to room temperature after 30 minutes. This solution's chromatogram was recorded.

Base Degradation

From stock solution-I, a sample (0.2 mL) was obtained and put into a 10 mL volumetric flask. After adding 5 milliliters of 1 N sodium hydroxide (NaOH) and adding enough mobile phase to bring the solution up to temperature, the mixture was heated for 30 minutes at 60 degrees Celsius. The flask was taken out and allowed to cool to room temperature after 30 minutes. This solution's chromatogram was recorded.

Oxidative Degradation

From stock solution I, a 0.2 mL sample was obtained and put into a 10 mL volumetric flask. After adding 5 milliliters of 3% hydrogen peroxide (H2O2) and adding enough mobile phase to bring the solution up to temperature, the mixture was heated for 30 minutes at 60 degrees Celsius. The flask was taken out and allowed to cool to room temperature after 30 minutes. This solution's chromatogram was recorded.

Photolytic

From stock solution I, a 0.2 mL sample was obtained and put into a 10 mL volumetric flask. It spent ten hours in the UV chamber. Following the time period, the volume was changed using diluent to get 12.5µg/ml of chlorthalidone and 5µg/ml of nebivolol.

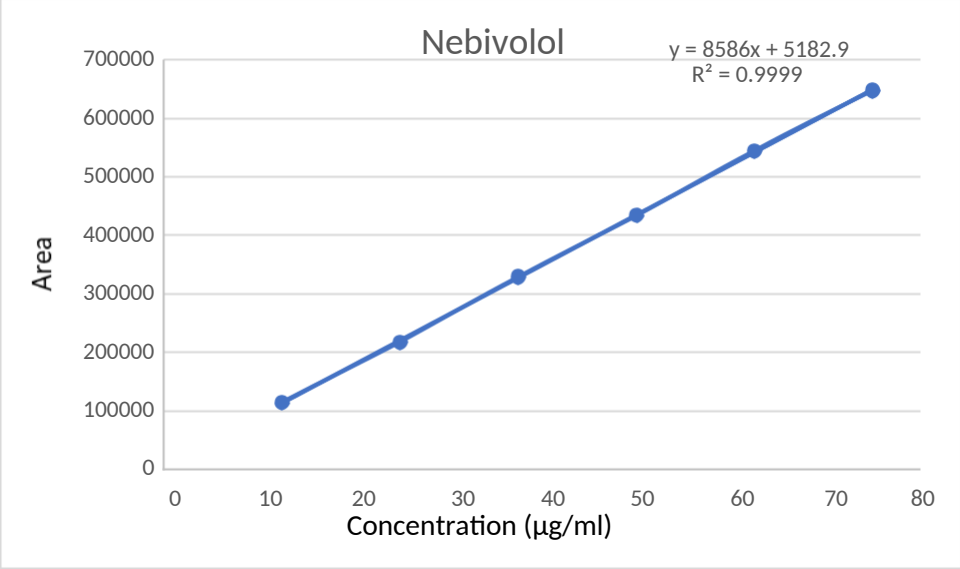
Thermal

From stock solution I, a 0.2 mL sample was obtained and put into a 10 mL volumetric flask. After adding 5 mL of water and adding enough mobile phase to make the solution level, the mixture was heated to 60 °C for 30 minutes. The flask was taken out and allowed to cool to room temperature after 30 minutes. This solution's chromatogram was recorded[24-26].

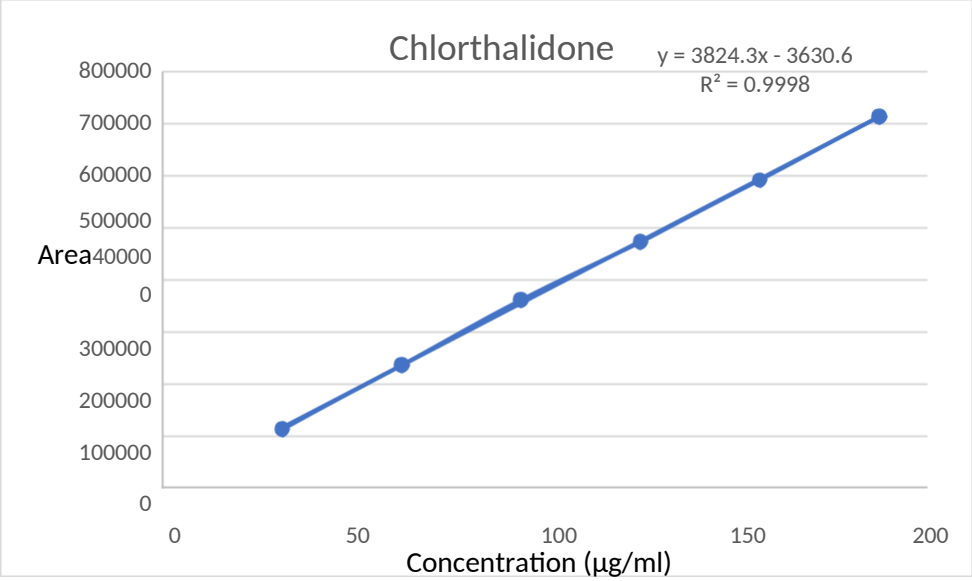
RESULTS AND DISCUSSION

Linearity

The calibration curve of samples at various concentrations of NEB (Nebivolol) and CHL (Chlorthalidone) was used to assess the linearity of the procedure. The ranges of the NEB and CHL calibration curves were 12.5 to 75 µg/mL and 31.25 to 187.5 µg/mL, respectively, on chromatography. The area was plotted graphically as a function of drug concentration in order to create a linear curve. NEB and CHL were found to have correlation values of 0.9999 and 0.9998, respectively (Figure 1A and 1B), indicating satisfactory linearity in the designated concentration range (Table 1 and 2).



(A)



(B)

Figure 1. Calibration curve of (A) Nebivolol (B) Chlorthalidone

Table 1. Linearity data for Nebivolol and Chlorthalidone

Sr. No.	Nebivolol (µg/ml)	Peak Area* ± S.D.(n=6)	Chlorthalidone (µg/ml)	Peak Area* ± S.D.(n=6)
1	12.5	113853	31.25	113202
2	25	216902	62.5	235513
3	37.5	328613	93.75	360807
4	50	433721	125	473121
5	62.5	543979	156.25	591687
6	75	647863	187.5	713609

Table 2. Regression analysis data for Nebivolol and Chlorthalidone

Regression Analysis	Nebivolol	Chlorthalidone
Regression equation	y=8586x + 5182.9	y=3824.3x - 3630.6
Correlation co-efficient (R^2)	0.9999	0.9998
Slope(S)	8586	3824.3
Intercept (б)	5182.9	3630.6

Accuracy

To confirm the procedure's accuracy, recovery trials were carried out by mixing a standard drug with pre-analysed samples. The accuracy of the proposed method was tested in triplicate at 80%, 100%, and 120% recovery levels to the pre-analyzed sample using the standard addition procedure. The recovery percentages for NEB and CHL were found to be 99.76-100.01 % and 99.97-100.04 %, respectively, when the supplementary standard was calculated. The method's accuracy matched favorably to the label claim for both drugs, for which the indicated RSD was less than 2 (Table 3).

Table 3. Accuracy Data for NEB and CHL

NEB	CHL	NEB	CHL	NEB	CHL	NEB	CHL
50	125	50	125	436755.4 ±689.42	446811.9 ±41.01	99.76	99.98
50	125	62.5	156.25	639197.8± 380.42	528330.4 ±72.83	99.90	99.97
50	125	75	187.5	839221.8± 56.56	619137.4 ±170.41	100.01	100.04
Amt. of sample (µg/ml)		**Amt. of drug added (µg/ml)**		**Amt. Recovered Mean ± S.D (µg/ml)**		**%Recovery**	

Precision

To ascertain intraday and interday variances, a thorough research was conducted. In the intraday and interday precision investigation of NEB and CHL, the correspondence response was calculated three times on the same day and three separate days for three different concentrations. All results are within the acceptable limits (RSD < 2), although the data are expressed as a percentage relative standard deviation (% RSD). The precision of the current procedure was demonstrated by the minimal deviation in the percentage RSD values obtained (Tables 4 and 5).

Table 4. Intraday Precision for NEB and CHL

NEB	CHL	NEB	CHL	NEB	CHL	NEB	CHL
50	125	389485.37± 1401.49	427940.52± 2194.62	100.52±1.02	100.30±0.93	0.36	0.51
50	125	389744.01± 2442.16	426070.44± 1997.22	100.34±1.46	100.78±1.28	0.63	0.47
50	125	391555.98± 1003.74	427696.82± 1122.78	101.78±1.97	99.59±1.64	0.26	0.26
Conc. (µg/ml)		**Mean Peak Area*± S.D.**		**Mean %Assay* ± S.D.**		**%RSD**	

Table 5. Interday Precision for NEB and CHL

NEB	CHL	NEB	CHL	NEB	CHL	NEB	CHL
50	125	389388.75± 1264.67	429437.32± 78.23	99.76±0.68	99.97±1.14	0.32	0.02
50	125	384743.54± 2822.11	429915.89± 3440.94	100.26±1.03	99.79±1.48	0.73	0.80
50	125	387936.09± 773.70	429386.72± 1267.54	101.14±1.78	100.54±1.95	0.20	0.30
Conc. (µg/ml)		**Mean Peak Area*± S.D.**		**Mean %Assay* ± S.D.**		**%RSD**	

LOD and LOQ

Table 6 shows the minimum detection limit and the minimum quantitation limit of NEB and CHL. LOD of NEB and CHL were found to be 0.0059 and 0.018, respectively. These LOD values indicate that the method is suitable to determine a lower concentration of NEB and CHL, and it confirms that the developed method is sensitive for determination. The minimum quantitation limit of NEB and CHL. LOQ of NEB and CHL was found to be 0.1 and 0.306, respectively. These LOQ values indicate that the method is suitable to determine a lower concentration of NEB and CHL and confirms that the developed method is sensitive for determination.

Table 6. Limit of Detection and Limit of Quantitation of NEB and CHL

Sr No.	Drug	LOD	LOQ
1	Nebivolol	0.0059	0.018
2	Chlorthalidone	0.1	0.306

Robustness

The following parameters were adjusted in order to look into robustness. The effects of each parameter adjustment on the assay and suitability of the system were then examined. In order to test for resilience, the flow rate (1 ml/min), mobile phase 60:40 (±1), and wavelength (226 ±1 nm) were varied. A 100% concentration solution was prepared for each condition and injected three times; it was discovered that the percentage RSD for each condition was less than two (Table 7).

Table 7. Robustness study for NEB and CHL

Condition	Variation	NEB			CHL		
		Area Mean	SD	% RSD	Area Mean	SD	% RSD
Change in Flow Rate (1ml/min)	0.9	429065.5	951.06	0.22	188283.00	1442.50	0.77
	1.1	429335.50	1332.90	0.31	186551.50	1006.21	0.54
Change in Mobile Phase (60+40)	61:39	428233.50	2127.68	0.50	189130.5	243.95	0.13
	59:41	427881.50	1629.88	0.38	189243.00	403.05	0.21
Change in Wavelength (226±1nm)	227	429043.50	919.95	0.21	188282.50	863.38	0.46
	225	429086.00	859.84	0.20	188172.50	707.81	0.38

System Suitability

As a method of validation parameter, the number of area ratio, retention duration, and peak areas were also ascertained. Table 8 lists the values that were obtained. The devised technique and the optimized chromatographic conditions were determined to be suitable, as evidenced by the method's computed % RSD of less than 2% (Figure 2).

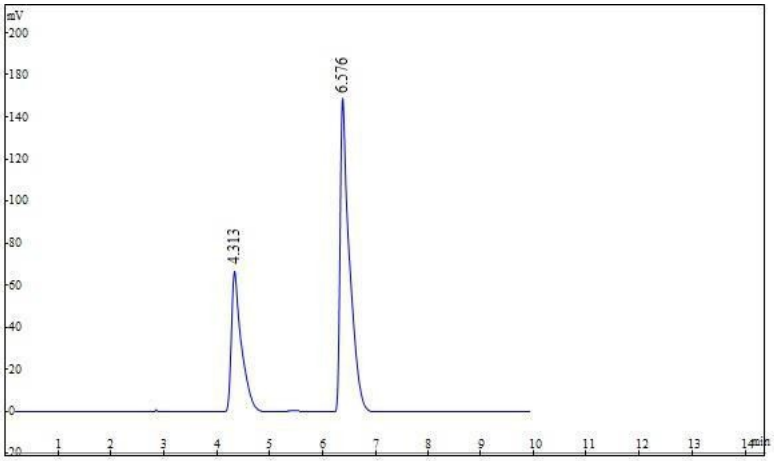


Figure 2. Optimized mobile phase trial for Nebivolol and Chlorthalidone

Table 8. System Suitability for NEB and CHL

Drug	Resolution	Retention Time	Area (mAU x min) (USP)	Asymmetry (USP)	Theoretical Plate
Nebivolol	0.00	4.313	279497	1.25	8372
Chlorthalidone	6.435	6.576	528473	1.33	8905

Specificity

The specificity of the method was assessed by looking at the chromatograms extracted from the sample solution. The method was verified as specific as none of the excipients reacted with the target analyte. As a result, it was decided that the method was suitable for looking at the formulation of the commercial drug (Table 9).

Table 9. Repeatability data for estimation of NEB and CHL

Sr. No.	Drug	Conc.(µg/ml)	Mean Peak Area	Mean % Assay	%RSD
1	Nebivolol	50	434632.16	99.16	0.276%
2	Cholrthalidone	125	496,090.16	99.1	0.393%

Assay of marketed formulation

After injecting a total of 20 µl of sample solution into the chromatographic apparatus, the peak response was determined. Three injections of the solution were made into the column. By comparing the test and standard areas, the quantity in each tablet was determined, and the results for NEB and CHL were 99.95 %, and 99.97%, respectively. The findings are displayed in a Table 10.

Table 10. Analysis of market formulation

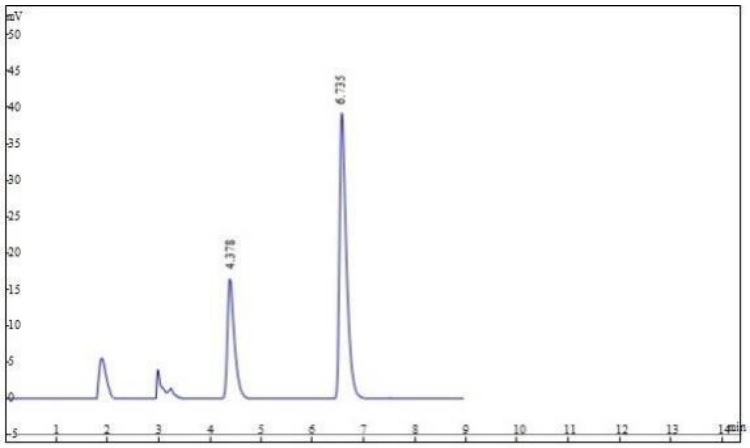
Tablet	Label Claim (in mg/ml)	Conc.	Area of Standard	Area of Sample	% Assay
Nodon CH	Nebivolol 5 mg	50ppm	437253	437037	99.95
	Chlorthalidone 12.5 mg	125ppm	446851	446728	99.97

Forced Degradation Sample Preparation

All forced degradation studies were analyzed for the samples at a concentration of 1.6 ml and 1 ml of NEB and CHL with HPLC conditions mentioned earlier using PDA detector to ensure the homogeneity and purity of drug peak. NEB and CHL standard sample were undergone acidic, alkaline, oxidative, and hydrolytic degradation. The sample shows 19.16, 14.41, 6.92,10.66 and 5.73% for NEB and 12.32, 09.67, 5.66, 5.25 and 4.77% for CHL degradation in acidic, alkaline, oxidative, photolytic and dry heat, respectively. The degradation was under acceptance criteria. It shows stability-indicating the properties of the method. The mass balance (% assay + % sum of all compounds + % sum of all degradants) results were calculated for all stressed samples and found to be more than 99%. The purity and assay of NEB and CHL was unaffected by the presence of its degradation products and thus confirms the stability indicating power of the developed method. Table 11 shows the results of forced degradation studies. Figures 3A, B, C, D, and E shows the chromatograms of forced degradation studies.

Table 11. Stability data

Sr. No.	Degradation	NEB		CHL	
		Degraded upto %	Actual % Degradation	Degraded upto %	Actual % Degradation
1	Acid Degradation	80.83	19.16	87.67	12.32
2	Base Degradation	85.58	14.41	90.32	9.67
3	H2O2 Degradation	93.07	6.92	94.33	5.66
4	Photolytic Degradation	89.33	10.66	94.74	5.25
5	Thermal Degradation	94.26	5.73	95.22	4.77



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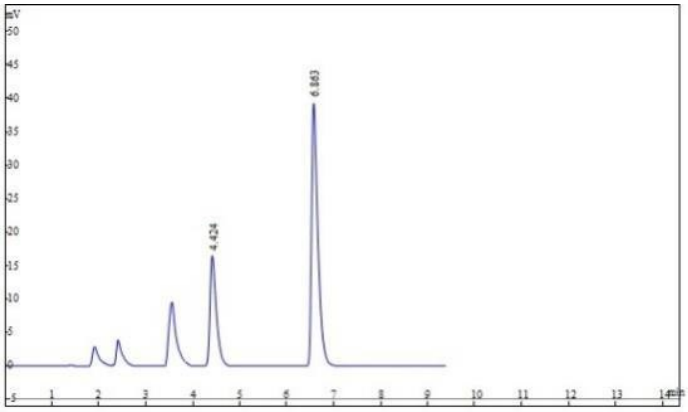

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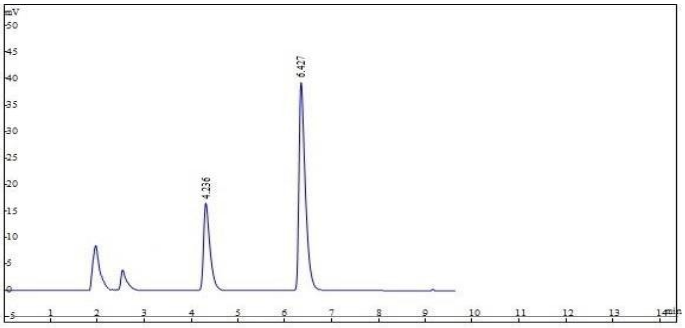

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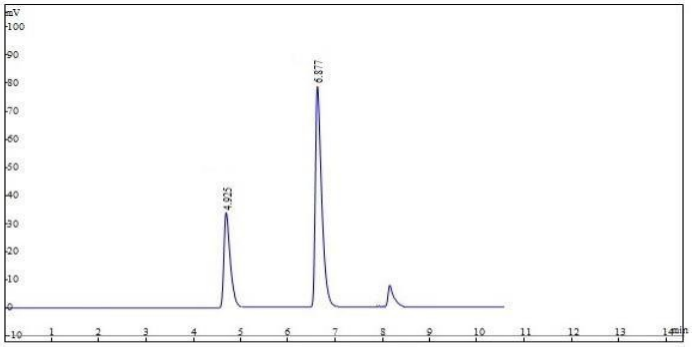

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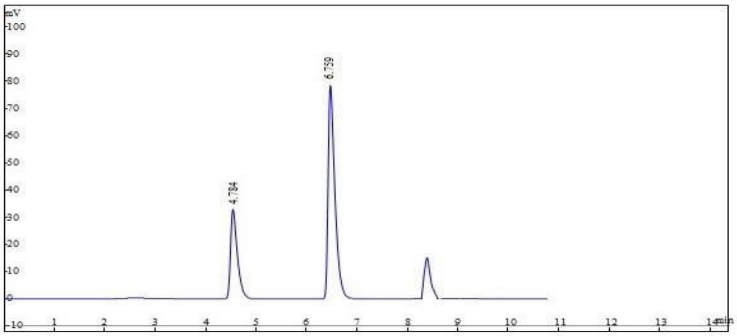

(E)

Figure 3. Chromatogram of degradation study in (A) 0.1 N HCl (B) 0.1 N NaOH (C) 3% H_2O_2 (D) Dry Heat and (E) Photolytic

CONCLUSION

The current chromatographic study was aimed to provide a stability-indicating high-performance liquid chromatography (HPLC) approach for NEB and CHL dosage form estimation. For LOD and LOQ, the stability-indicating RP-HPLC technique has been created and verified as a result. Research on forced degradation study was also conducted under five distinct stress scenarios. Based on the chromatographic analysis, we have determined that the established technique for routine analysis of NEB and CHL tablet dosage form is more accurate, precise, repeatable, and linear. Therefore, forced degradation studies and validation may be carried out.

ACKNOWLEDGEMENT

This study was done in collaboration with Sankalp Foundation, Dhule. The necessary infrastructure was provided by Gangamai College of Pharmacy, Nagaon, Dhule-424005.

The authors have no conflict of interest.

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