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A MODIFIED RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF NEBIVOLOL AND INDAPAMIDE IN BULK AND PHARMACEUTICALDOSAGE FORMS

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ABSTRACT

A simple, precise, accurate, reproducible and economic stability-indicating reverse phase liquid chromatography method was developed and validated for the quantitative simultaneous estimation of Nebivolol and Indapamide in bulk and marketed formulations. Estimation of drugs in this combination was done with a C18 column [Kromasil column. 250mm × 4.6 mm] using mobile phase of composition Acetonitrile and phosphate buffer (60:40 v/v, pH 3). The flow rate was 0.8 ml/min and the effluents were monitored at 226nm. The retention time of Nebivolol and Indapamide were 2.92 min and 4.21 min respectively. The method was found to be linear over a range of 10-50 µg/ml for Nebivolol and 2-10 µg/ml for Indapamide. The established method proved as reproducible one with a %RSD value of less than 2 and having the robustness and accuracy within the specified limits. Assay of marketed formulation was determined and find with 99.08% and 99.87% for Nebivolol and Indapamide respectively. The stressed samples were analyzed and this proposed method was found to be specific and stability indicating as no interfering peaks of degradation compounds and excipients were noticed.

KEYWORDS: Nebivolol, Indapamide, RP-HPLC, Stability and Method validation.

INTRODUCTION

Nebivolol is chemically designated as 1-(6-flo2zuro chroman-2-yl) - [2-(6-fluorochroman-2-yl)-2-hydroxy-ethyl] amino} ethanol. It is a third generation vasodilating cardio selective

blocking agent used in the treatment of hypertension.[1] Its molecular formula is $C_{22}H_{25}F_2NO_4$and it has a molecular weight of 444.90gm/mole. Nebivolol is a white odorless powder used for the treatment of hypertension. It's a mode of action is lowering blood pressure by reducing the peripheral vascular resistance and significantly increases the stroke volume with preservation of cardiac output. The net hemodynamic effect of Nebivolol is the result of a balance between the depressant effects of a beta-blockade and an action that maintains the cardiac output.

Indapamide is chemically designated as 4-chloro-N-(2-methyl-2,3-dihydroindol-1-yl)-3-sulfamoyl-benzamide. It is a non-thiazide sulphonamide diuretic drug generally used in the treatment of hypertension, as well as decompensate cardiac failure. Its molecule contains a both a polar sulfonyl chloro benzamide moiety and a lipid soluble methyl-indoline moiety. It differs chemically from thiazide is that it does not possess the thiazide ring system contains only one sulfonamide group. The molecular formula is C_{16} $H_{16}ClN_3O_3S$ and molecular weight is 365.8gm/mol. It is awhite to off-white crystalline powder. That is soluble in methanol, ethanol, acetic acid and ethyl acetate, very slightly soluble in ether, chloroform,and benzene, particularly insoluble in water. It is official drug in British Pharmacopoeia 2000 and United States Pharmacopoeia 2007.

Extensive literature survey proved that very few methods were reported for the determination of Nebivolol and Indapamide by RP-HPLC.[2-12] So we attempted to develop an accurate, rapid, precise, stable, sensitive and economically viable liquid chromatographic method for the simultaneous determination of selected drugs in the present research.

MATERIALS AND METHODS

Equipment

The chromatographic separation was performed on Agilent 1120 compact liquid chromatographic system integrated with a variable wavelength programmable UV detector and a Rheodyne injector equipped with 20µl fixed loop. A reverse phase C18[Agilent ODS UG 5 column, 250mm × 4.5 mm]was used. Lab India 3000$^+$double beam UV visible spectrophotometer and Axis AGN204-PO electronic balance were used for spectrophotometric determinations and weighing purposes respectively.

Chromatographic conditions

Kromasil 100-5C_{18} column [250mm x 4.6mm] was used for the chromatographic separation at a detection wave length of 226 nm. Mobile phase of composition Acetonitrile and Phosphate buffer pH 3in a ratio of 60:40 v/v was selected for elution and same mixture was used in the preparation of standard and sample solutions. Flow rate was adjusted to 0.8ml/min and the injection volume was 20µl.

Preparation of Mobile phase

Phosphate buffer pH 3 was prepared by dissolve 0.136gm of Potassium dihydrogen phosphate and 2 ml of Triethyl amine in 80ml of HPLC grade water and adjusts the pH to 3.0 with orthophosphoric acid and sufficient water was added to produce100 ml filtered through 0.45µ membrane filter and sonicated for 20 minutes.

Preparation of Standard solutions

25mg each of Nebivolol and Indapamide were accurately weighed and transferred into two 25ml volumetric flasks, dissolved using mobile phase and the volume was made up with the same solvent to obtain primary stock solutions A (Nebivolol) and B (Indapamide) of concentration 1000µg/ml of each drug. From the primary stock solutions , 5ml and 1ml were pipette out from A and B respectively, transferred to a 10ml volumetric flask and the volume was made up with the mobile phase to obtain final concentrations of 50 µg/ml and 10µg/ml of Nebivolol and Indapamide respectively and this solution is (working stock solution A).

Preparation of Sample Solution

Twenty tablets of Nebivolol and Indapamide were weighed and crushed. Tablet powder equivalent to 5mg of Nebivolol and 1.5mg of Indapamide was weighed accurately and transferred to a 25ml volumetric flask. The content was dissolved with 10ml of mobile phase and then sonicated for 15min. The volume was made up with the mobile phase and filtered with 0.45µmembrane filter and sonicated for 20min.0.5ml of this solution was pipette out and transferred to a 10mlvolumetric flask and the volume was made up with the mobile phase to obtain a concentration of 60µg/ml of Nebivolol and 22.5µg/ml of Indapamide (working stock solution B).

Optimization of RP-HPLC method

The HPLC method was optimized with an aim to develop a simultaneous estimation procedure for the assay of Nebivolol and Indapamide. For the method optimization, different

mobile phases were tried, but acceptable retention times, theoretical plates and good resolution were observed with Acetonitrile ,Phosphate buffer pH3 (60:40 v/v) using Kromasil 100-5C_{18} column [250mm x 4.6mm].

Validation of the RP-HPLC method

Validation of the optimized method was performed according to the ICH Q2 (B) guidelines.

System suitability

System suitability was carried out with five injections of solution of 100% concentration having 50 µg/ml of Nebivolol and 10 µg/ml of Indapamide in to the chromatographic system. Number of theoretical plates (N) obtained and calculated tailing factors (T) were reported in table 1.

Linearity

For the determination of linearity, appropriate aliquots were pipette out from working stock solution A to a series of 10ml volumetric flasks and volume was made up with the solvent to obtain concentration ranging from 10-50µg/ml of Nebivolol and 2-10µg/ml of Indapamide. Each solution was injected in triplicate. Calibration curves were plotted with observed peak areas against concentration followed by the determination of regression equations and calculation of the correlation coefficients. The calibration curves for Nebivolol and Indapamide were shown in figure 3 and figure 4 their corresponding linearity parameters were given in table 2.

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

The LOD and LOQ were calculated from the slope(s) of the calibration plot and the standard deviation (SD) of the peak areas using the formulae LOD = 3.3 σ/s and LOQ = 10 σ/s. The results were given in table 2.

Precision

The repeatability of the method was verified by calculating the %RSD of six replicate injections of 100% concentration (50 µg/ml of Nebivololand 10µg/ml of Indapamide) on the same day and for intermediate precision % RSD was calculated from repeated studies on different days. The results were given in table 3.

Accuracy

To ensure the reliability and accuracy of the method recovery studies were carried out by standard addition method. A known quantity of pure drug was added to pre-analyzed sample and contents were reanalyzed by the proposed method and the percent recovery was reported. The results were given in table 4.

Specificity

Specificity of a method was determined by testing standard substances against potential interferences. The method was found to be specific when the test solution was injected and no interferences were found because of the presence of excipients. The optimized chromatogram of Nebivolol and Indapamide without any interference was shown in figure 2.

Robustness

Robustness of the method was verified by altering the chromatographic conditions like mobile phase composition, wave length detection, flow rate, etc. and the % RSD should be reported. Small changes in the operational conditions were allowed and the extent to which the method was robust was determined. A deviation of ±2nm in the detection wave length and ±0.2ml/min in the flow rate, were tried individually. A solution of 100% test concentration with the specified changes in the operational conditions was injected to the instrument in triplicate. %RSD was reported in the table 5.

Assay of Marketed Formulations

Weigh accurately Nebula-D tablets (Nebivolol or Indapamide) were initially powdered and an amount equivalent to 5 mg of Nebivolol and 1.5 mg of Indapamide was accurately weighed into a 25ml volumetric flask, mixed with 10ml of mobile phase. The solution was made up to the volume with mobile phase and sonicated for 5 minutes. The solution was then filtered through 0.45µm Millipore membrane filter. Final stock containing 30µg/ml and 30µg/ml of Nebivolol and Indapamide respectively was prepared by subsequent dilution with the same mobile phase. 20µl of sample solution was injected into chromatographic system and the peak responses were measured. The solution was injected three times into the column. The amount present in each tablet was calculated by comparing the areas of test with that of the standard. A typical chromatogram for assay of marketed formulation was shown in figure 5and the obtained values were reported in the table 6.

STABILITY STUDIES

Acid degradation studies

Prepared each 1mg/ml stock solution of Nebivolol and Indapamide by using mobile phase as solvent, and then filtered through 0.45µm membrane filter paper. Stock solutions of 5 ml and 1ml of Nebivolol and Indapamide stock solution was transferred into 10ml volumetric flask and added 1 ml of 0.1N HCL and diluted to volume with mobile phase. The resultant solution was injected into the system; there was no acid degradation products were found the obtained chromatogram was shown in figure 6.

Alkaline degradation studies

Prepared each 1mg/ml of stock solution with Nebivolol and Indapamide then filtered through 0.45µm membrane filter paper. Stock solutions of 5 ml and 1ml of Nebivolol and Indapamide stock solution was transferred into 10ml volumetric flask and added 1 ml of 0.1NNaOH and diluted to volume with mobile phase. The obtained non interfered chromatogram was represented in figure 7.

Oxide degradation studies

Prepared each 1mg/ml of stock solution of Nebivololand Indapamide then filtered through 0.45µm membrane filter paper. Stock solutions of 5 ml and 1ml of Nebivolol and Indapamide stock solution was transferred into 10ml volumetric flask and added 1 ml of H_2O_2 and diluted to volume with mobile phase. In this investigation no identifiable oxidative degradants were found and the chromatogram was shown in figure 8.

Thermal degradation studies

Prepared each 1mg/ml of stock solution with Nebivolol and Indapamide and then filtered through 0.45µm membrane filter paper. Stock solutions of 5 ml and 1ml of Nebivolol and Indapamide10ml volumetric flask and diluted to volume with mobile phase and kept for 60min at 60^0c in hot air oven. From the obtained chromatogram it was proved that the selected samples were stable against thermal conditions. The chromatogram was shown in figure 8.

RESULTS AND DISCUSSION

After a number of trials with mobile phases of different composition, Acetonitrile, Phosphate buffer pH 3.0 in the ratio 60:40 v/v was selected as mobile phase because of better resolution and symmetric peaks. Nebivolol and Indapamide were found to show appreciable absorbance

at 226nm when determined spectrophotometrically and hence it was selected as the detection wavelength. An optimized chromatogram showing the separation of Nebivolol and Indapamide at different R_Ts was shown in figure 2.

System suitability was carried out by injecting 5 replicate injections of 100% test concentration, number of theoretical plates, HETP and resolution were satisfactory. The chromatograms confirm the presence of Nebivolol and Indapamide at 2.92min and 4.21min respectively without any interference. The parameters were given in table 1.

Concentration range of 10-50µg/ml for Nebivolol and 2-10µg/ml of Indapamide values where be tabulated in table-2, found to be linear with correlation coefficients 0.999 and 0.999 for Nebivolol and Indapamide respectively. The results were given in table 3.

The limits of detection for Nebivolol and Indapamide were found to be 0.16µg/ml and 10.33µg/ml respectively and the limit of Quantitation were 0.49µg/ml and 1.01µg/ml respectively. Values were represented in table 2.

The proposed method was found to be precise and reproducible with %RSD of 0.87 and 1.64 for Nebivolol and Indapamide respectively. %RSD was reported in table 4.

Accuracy of the method was verified by performing recovery studies by standard addition method. The percent recovery of the standard added to the pre-analysed sample was calculated and it was found to be 98.8% to 99.4% for Nebivolol and 98.6 to 100.8% for Indapamide. This indicates that the method was accurate. Values obtained were given in table 5.

The method was found to be robust after changing the conditions like detection wavelength (± 2nm) and flow rate (± 0.2 ml). %RSD was calculated for each variation and reported. Values obtained were given in table 6.

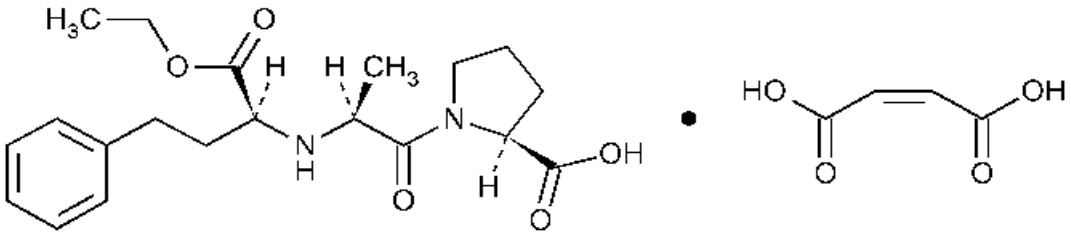
The method was found to be specific for the combination of interest after verifying the chromatograms showing no interference of the excipients present. Hence, the method was well suitable for the estimation of the commercial formulations of the selected combination with a percentage purity of 99.08% for Nebivolol and 99.87% for Indapamide. The typical chromatogram for assay of marketed formulations was shown in figure.5 and Values obtained were given in table 7.

FORCED DEGRADATION STUDY

Degradation studies indicated the specificity of developed method in presence of degradation products. Degradation was carried out in combination of two drugs and purity of drug peaks was confirmed by purity angles. Their combination drug products were exposed to acid, base, oxidative and thermal stress conditions .Then found to be no degradable substances presence and proved that the proposed method was stable towards acid, alkali, peroxide and thermal conditions. The obtained values were reported in table 8.

FIGURES AND TABLES

a) Nebivolol



b) Indapamide

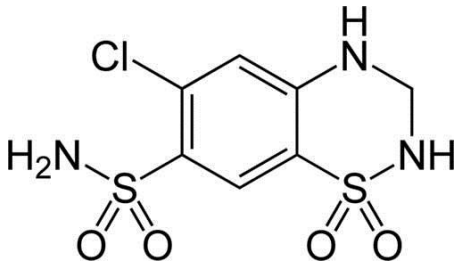


Fig 1: Chemical Structures of a) Nebivolol and b) Indapamide

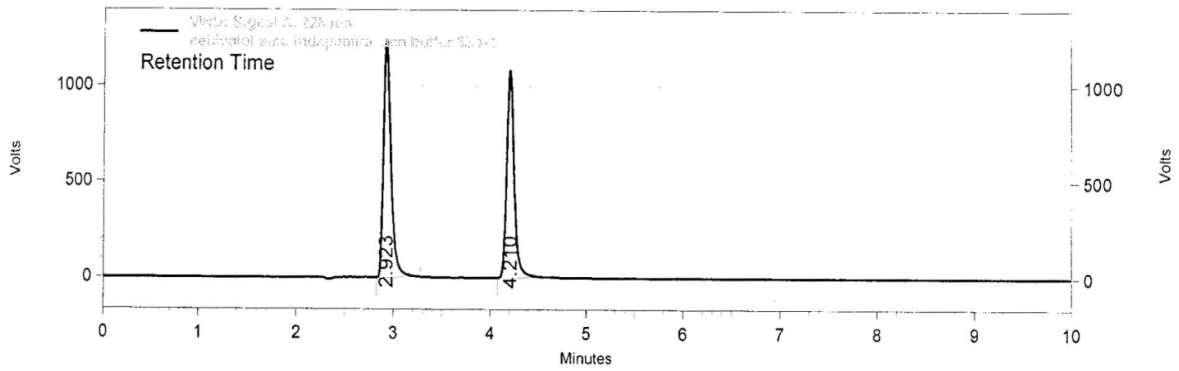


Fig 2: Optimized chromatogram of Nebivolol and Indapamide.

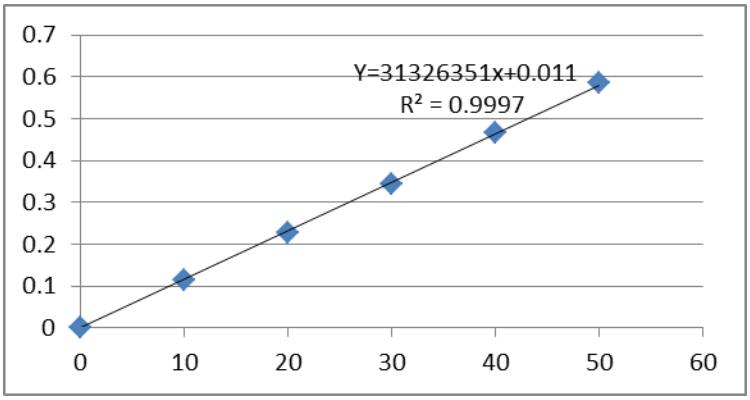


Fig 3: Calibration plot of Nebivolol.

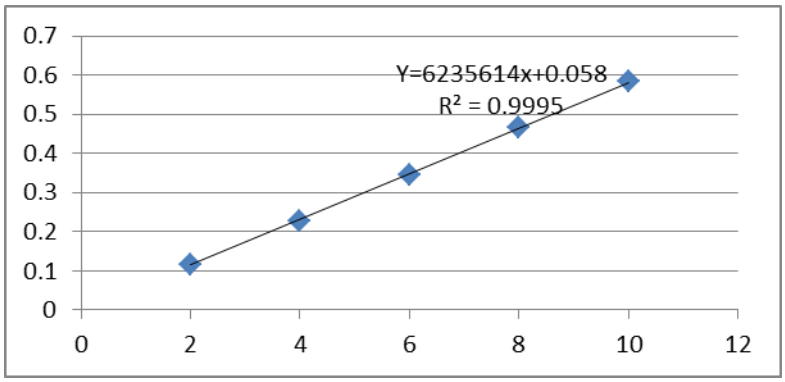


Fig 4: Calibration plot of Indapamide.

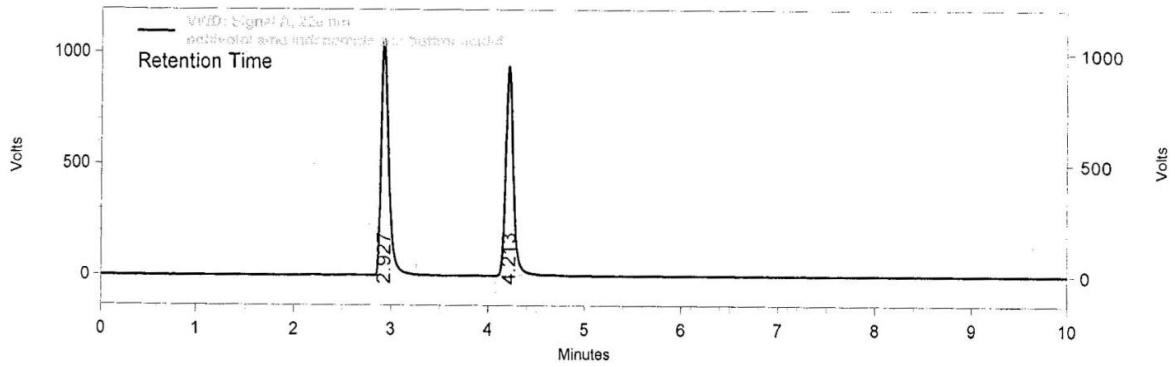


Figure 5: A typical chromatogram for assay of marketed formulation containing 50µg/ml of Nebivololand 10 µg/ml Indapamide.

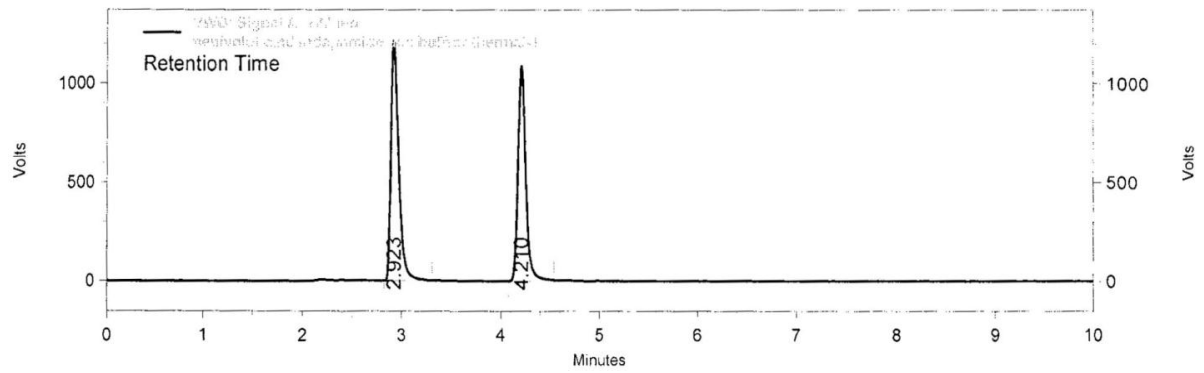


Figure 6: Chromatogram of acid degradation.

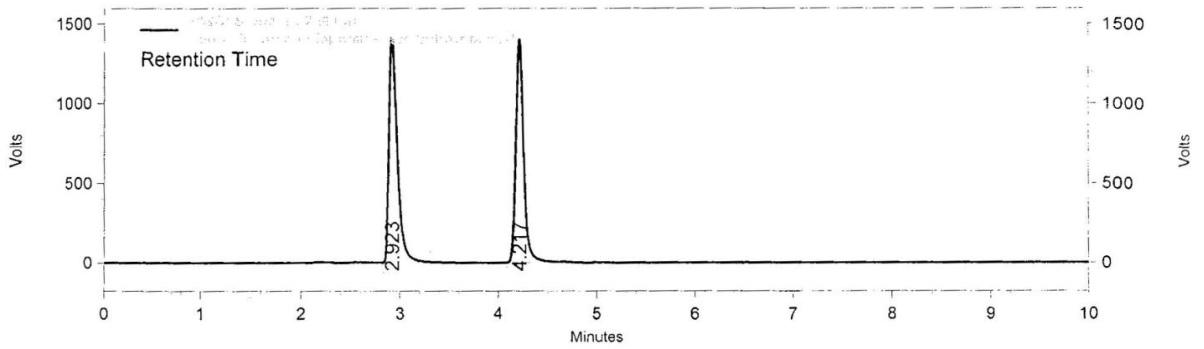


Figure 7: Chromatogram of alkaline degradation.

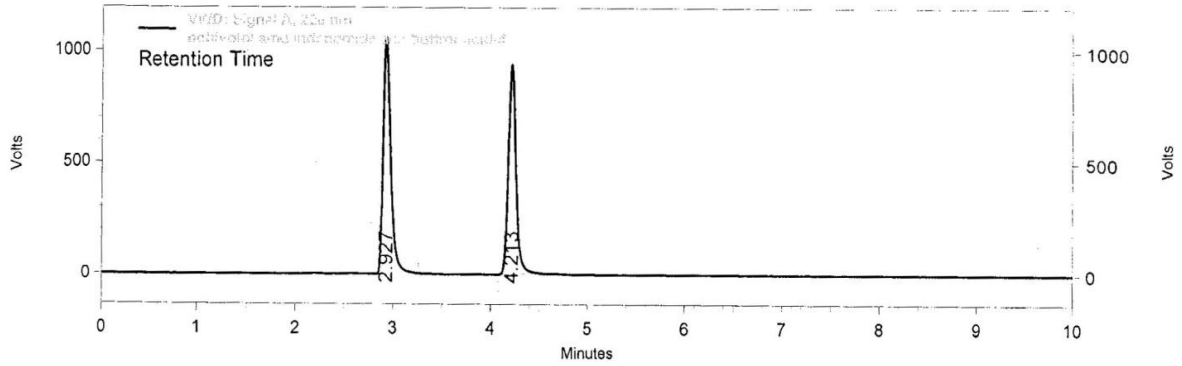


Figure 8: Chromatogram of peroxide degradation.

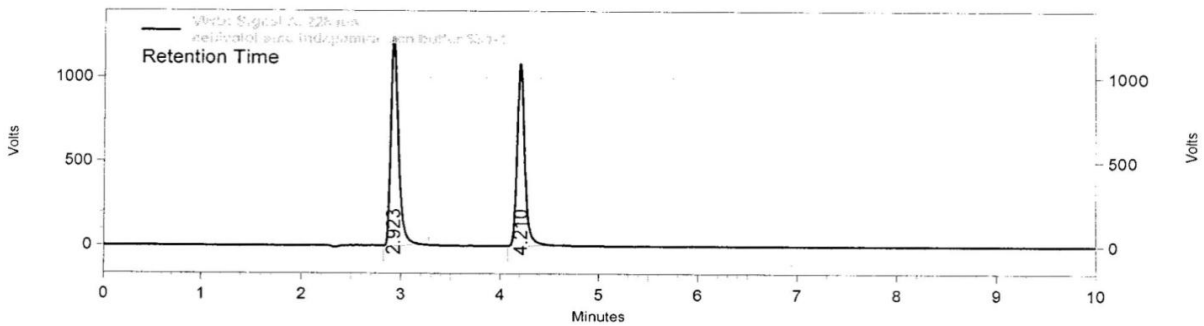


Figure 9: Chromatogram of thermal degradation.

Table 1: System Suitability Parameters.

Parameters	Nebivolol	Indapamide
Retention time (min)	2.92	4.21
Theoretical plates (N)	11456	10366
Tailing factor (T)	1.2	1.4
Resolution (R_s)	2.89	

Table 2: Linearity Data of Nebivolol and Indapamide at 226nm by RP−HPLC Method.

S.No	Nebivolol			Indapamide		
	Conc (µg/ml)	R_t(min)	Peak area	Conc (µg/ml)	R_t(min)	Peak area
0	0	0	0	0	0	0
1	10	2.9	26256208	2	4.2	20086186
2	20	2.9	50513368	4	4.2	39707157
3	30	2.9	69010259	6	4.2	57930305
4	40	2.9	81579573	8	4.2	67431332
5	50	2.9	11317585	10	4.2	98311810

Table 3: Results for Linearity.

Parameters	Nebivolol	Indapamide
Slope	0.0122	0.0119
y intercept	0.011+0.0122=0.0232	0.058+0.0119=0.0699
Correlation coefficient r^2	0.999	0.999
Regression Equation	Y=31326351x+0.011	Y=6235614x+0.058
Linearity range	10-50µg/ml	2-10µg/ml
LOD	0.16µg/ml	0.33µg/ml
LOQ	0.49µg/ml	1.01µg/ml

Table 4: Results of Precision.

Drug	Intraday Precision (%RSD)	Interday Precision (%RSD)
Nebivolol	0.64	0.87
Indapamide	0.78	1.64

Table 5: Results for Accuracy.

Recovery level	Nebivolol				Indapamide			
	Amount Added (µg/ml)		Amount Found (µg/ml)	% Recovery	Amount Added (µg/ml)		Amount Found (µg/ml)	% Recovery
	std	test			std	Test		
80%	40	60	99.98	99.98	8	22.5	30.1	98.6
100%	50	60	109.0	99.09	10	22.5	32.0	98.4
120%	60	60	120.1	100.0	12	22.5	34.3	99.4
Mean recovery	98.8-99.4%				98.6-100.8%			

Table 6: Results for Robustness.

Parameters	%RSD	
	Nebivolol	Indapamide
Detection wavelength at 228nm	0.93	0.56
Detection wavelength at 224nm	0.72	0.98
Flow rate 0.6ml/min	0.86	0.56
Flow rate 1.0ml/min	0.51	0.48

Table 7: Results for Assay of Marketed formulation.

Drug	Label claim (mg/tab)	Amount recovered	% Amount found in drug
Nebivolol	5	4.67	99.08%
Indapamide	1.5	1.42	99.87%

Table 8: Results for Stability studies of Nebivolol and Indapamide combined form.

Parameters	% of degradation Nebivolol Indapamide	
Acid degradation	0.125	0.196
Alkali degradation	0.112	0.156
Peroxide degradation	0.268	0.341
Thermal Degradation	0.262	0.192

CONCLUSION

The RP-HPLC method developed and validated allows a simple and fast quantitative determination of Nebivolol and Indapamide from their formulations. All the validation parameters were found to be within the limits according to ICH guidelines. The proposed method was found to be specific for the drugs of interest irrespective of the excipients present and the method was found to be simple, accurate, precise, rugged, robust and stable under forced degradation stress conditions. So the established method can be employed in the routine analysis of the marketed formulations.

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