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Validated Stability Indicating RP-HPLC Method for Simultaneous Quantitative Estimation of Hydrochlorothiazide and Nebivolol Hydrochloride in Bulk and Combined Tablet Dosage Form

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ABSTRACT

A simple and precise stability indicating RP-HPLC method was developed and validated for the simultaneous determination of Hydrochlorothiazide (HCTZ) and Nebivolol Hydrochloride (NBV) inbulk and Pharmaceutical dosage forms. Chromatography was carried out on Thermo Hypersil BDS C 18 (150 x 4.6 mm, 5µparticle size) column using a mobile phase of phosphate buffer (adjusted to pH 6.5 with dilute orthophosphoric acid): acetonitrile (40:60% v/v) at a flow rate of 0.8 ml/min. The analyte was monitored using PDA detector at 282 nm. The retention time was found to be2.4 min and 4.0min for Hydrochlorothiazide and Nebivolol Hydrochloride respectively. The proposed method was found to be having linearity in the concentration range of 6.25-37.5 µg/ml for Hydrochlorothiazide (r^2 0.9999) and 2.5-15 µg/ml for Nebivolol Hydrochloride (r^2 0.9999) respectively. The mean % recoveries obtained were found to be 99.93 % for Hydrochlorothiazide and 100.03% for Nebivolol Hydrochloride respectively. Stress testing which covered acid, alkali, peroxide, photolytic and thermal degradation was performed on under test to prove the specificity of the method and the degradation was achieved. The developed method has been statistically validated according to ICH guide lines and found to be simple, precise and accurate with the prescribed values. Thus the proposed method was successfully applied for the stability indicating simultaneous determination of Hydrochlorothiazide and Nebivolol Hydrochloridein bulk and Pharmaceutical formulations and in routine quality control analysis.

Keywords: Hydrochlorothiazide, Nebivolol Hydrochloride, RP-HPLC, Forced degradation, Method validation.

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INTRODUCTION

Hydrochlorothiazide

Chemically it is (figure 1), (6-Chloro-3, 4-dihydro-7-sulfamoyl-2H-1, 2, 4-benzothiadiazin1, 1 dioxide. It has molecular formula of$C_7H_8ClN_3O_4S_2$ and molecular weight is297.739g/mol. Hydrochlorothiazide, a thiazide diuretic, inhibits water reabsorption in the nephron by inhibiting the sodium-chloride symporter (SLC12A3) in the distal convoluted tubule, which is responsible for 5% of total sodium reabsorption. Normally, the sodium-chloride symporter transports sodium and chloride from the lumen into the epithelial cell lining the distal convoluted tubule. The energy for this is provided by a sodium gradient established by sodium-potassium ATPases on the basolateral membrane. Once sodium has entered the cell, it is transported out into the basolateralinterstitium via the sodium-potassium ATPase, causing an increase in the osmolarity of the interstitium, thereby establishing an osmotic gradient for water reabsorption. By blocking the sodium-chloride symporter, hydrochlorothiazide effectively reduces the osmotic gradient and water reabsorption throughout the nephron.

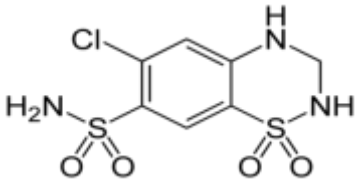

Figure 1: Chemical structure of Hydrochlorothiazide

Nebivolol Hydrochloride

Chemically it is (figure 2), α, α'-(iminodimethylene) bis [6-fluoro-2-chromanmethanol] hydrochloride. It has a molecular formula of$C_{22}H_{25}F_2NO_4$.HCL and molecular weight of 441.9g/mol. Nebivololhydrochloride is a selective β1- adrenoceptor receptor antagonist. Activation of β1-receptors by epinephrine increases the heart rate and the blood pressure, and the heart consumes more oxygen. Nebivolol blocks these receptors which reverses the effects of epinephrine, lowering the heart rate and blood pressure. In addition, beta blockers prevent the release of renin, which is a hormone produced by the kidneys which leads to constriction of blood vessels. At high enough concentrations, this drug may also bind beta 2 receptors.

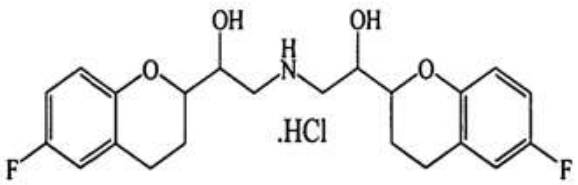

Figure 2: Chemicalstructure of Nebivolol Hydrochloride

Literature survey reveals that few analytical methods were reported like spectrophotometric methods[1-4], RP-HPLC methods[5-7] and HPTLC methods[8-9]in alone or in combination in pharmaceutical dosage forms but no simple stability indicating RP-HPLC method for the simultaneous estimation of Hydrochlorothiazide and Nebivolol Hydrochloridein pharmaceutical dosage forms have been reported so far. Hence author has planned to develop a simple, accurate, precise and sensitive stability indicating RP-HPLC method for the simultaneous estimation of Hydrochlorothiazide and Nebivolol Hydrochloridein bulk and pharmaceutical dosage forms suitable for routine quality control analysis.

MATERIALS AND METHOD

Chemicals

Hydrochlorothiazide (HCTZ) and Nebivolol Hydrochloride (NBV) were obtained as gift samples from Micro Labs Limited, Bangalore. HPLC grade methanol and acetonitrile were purchased from E.Merck. Chem.ltd. Mumbai and HPLC grade water (E.Merck) was used throughout the study. All the chemicals used were of analytical reagent grade. Fixed dose combination tablet formulation (Nebicard-H) containing 12.5 mg of Hydrochlorothiazide and 5 mg of Nebivolol Hydrochloride was procured from local market.

Instrumentation

Quantitative HPLC was performed on Waters technologies2695 series, PDA detector module equipped with auto injector usingempower software. A reverse phaseThermo Hypersil BDSC18 (150x 4.6mm, particle size 5µm) analytical column was used. Weighing was done on shimadzu balance (AX 200) and pH adjustments doneusing pH meter (Unichem AD102U) was used.

Chromatographic conditions

Preliminary studies were conducted and trails were made for the method development. Separation and analysis was carried out on Thermo Hypersil BDS C 18 (150 x 4.6mm, 5µm particle size) column. The optimized mobile phase consisting ofphosphate buffer (pH adjusted to 6.5 with diluteorthophosphoric acid) and acetonitrilein the ratio of 40:60 %v/v. Flow rate was maintained at 0.8ml/min. Prior to sample injection, column was saturated with mobile phase for 30 min and injection volume was 20µl injected into the chromatographic system using auto sampler. The detection response was measured at 282 nm and maintained column at ambient temperature.

Preparation of mobile phase

Mix buffer (0.02M Potassium dihydrogen orthophosphate adjusted to pH 6.5 with dilute ortho phosphoric acid) and acetonitrile in the ratio of 40: 60 %v/v , sonicated for 5min, followed by

degassing using vacuum filtration containing 0.45 µm membrane filter.

Preparation of standard stock solution

Accurately weighed and transferred 12.5mgof HCTZ and 5 mg of NBV working standards into a 50 ml clean and dry volumetric flask, 25ml of diluent (mobile phase) was added, sonicated to dissolve for 5 minutes and then made up to the final volume with diluent. From the above stock solution, 1.0 ml was pipette out in to a 10ml volumetric flask and then made up to the final volume with diluent.

Preparation of sample solution

20 tablets were weighed accurately and average weight of each tablet was taken. Then crushed the tablets into fine powder in a mortar with pestle and then transferred sample powder equivalent to 12.5 mg of Hydrochlorothiazide and 5 mg of Nebivolol hydrochloride into a 50ml volumetric flask, 25ml of diluent was added and sonicated for 5 min, further the volume made up with diluent and filtered. From the filtered solution, 1.0 ml was pipette out into a 10 ml volumetric flask and made up to volume with diluent.20µL of the standard and sample solutions were injected into chromatographic system, chromatograms were recorded and peak areas were measured.

METHOD VALIDATION

System suitability

System suitability was carried out by injecting standard solutions five times into the chromatographic system. The system suitability parameters were then evaluated using tailing factor, retention time and theoretical plates of standard chromatograms.

Accuracy

The accuracy of the test method was demonstrated by % recovery across its range by making three different concentrations at 80%, 100% and 120 levels using standard addition method where sample preparations were spiked with known amount of standard and then each concentration was injected three times into the chromatographic system.

System Precision

The system precision was carried out by injecting standard solution preparations six times into the chromatographic system and calculated %RSD of retention time and peak areas for both HCTZ and NBV.

Method precision

In method precision, a homogenous sample of a single batch was analyzed by injecting sample solution preparations six times into the chromatographic system and calculated %RSD of retention

time and peak area for both HCTZ and NBV.

Specificity

Specificity is the ability to assess unequivocally the analytes in the presence of compounds that may be expected to present, such as impurities, degradation products and matrix components. The specificity of the method was assessed by comparing the chromatograms obtained from standard and sample solutions. The retention times of the analytes in standard and the sample solutions were found to be same, so the method was specific and free from interference from excipients present in the tablets.

Linearity

The linearity of an analytical method was carried out to check its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Different levels of standard solutions (25-150%) were prepared by diluting aliquots (0.25- 1.5 ml) of standard stock solution (250µg/ml) in to 10 ml volumetric flasks with diluent and injected each level into the chromatographic system and the chromatograms were recorded.

Robustness

The robustness of the proposed method was determined by analyzing aliquots from homogenous lots by differing physical parameters like mobile phase composition, flow rate and column temperature. The standard and sample solutions were injected into the chromatograph at varied conditions of flow rate ± 0.2 ml/min, mobile phase buffer pH ± 0.2 units and temp. by $\pm$ 5 ^{o}c.

Ruggedness (Intermediate precision)

It is carried out by injecting standard solution preparations six times into the chromatographic system on two different days. %RSD was determined for retention time and peak areas of standard and sample solutions of HCTZ and NBV.

Forced degradation

Stress testing of the drug substance can help in identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule.

Acid degradation studies

Transferred sample quantitatively equivalent to 25 mg of HCTZ and 10 mg of NBV in to a 200 ml round bottom (RB) flask, added 100 ml of freshly prepared 0.1 N HCL. After keeping the solution for 10 hrs, filtered and then neutralize the solution up to the volume with 0.1 N NaOH. Again diluted 1.0 ml of the above solution to 10 ml in a 10 ml volumetric flask with diluents and

injected20 µl of the solutioninto the chromatographic system and the chromatogram was recorded as shown in figure 7.

Alkali degradation studies

Transferred sample quantitatively equivalent to 25 mg of HCTZ and 10 mg of NBV in to a 200 ml RB flask, added 100 ml of freshly prepared 0.1 N NaOH. After keeping the solution for 10 hrs, filtered and then neutralize the solution up to the volume with 0.1 N HCL. Again diluted 1.0 ml of the above solution to 10 ml in a 10 ml volumetric flask with diluent and then injected 20 µl of the solution into the chromatographic system and the chromatogram was recorded as shown in figure 8.

Oxidation studies

Transferred sample quantitatively equivalent to 25 mg of HCTZ and 10 mg of NBV in to a 200 ml RB flask, added 100 ml of freshly prepared 1% Hydrogen peroxide solution. After keeping the solution for 10 hrs on a bench top, filtered and then again diluted 1.0 ml of the filtrate to 10 ml in a 10 ml volumetric flask with diluent and then injected20 µl of the solution into the chromatographic system and the chromatogram was recorded as shown in figure 9.

Photolytic studies

Transferred sample quantitatively equivalent to 25 mg of HCTZ and 10 mg of NBV on to a clean and dry petri plate.Kept the petri plate in UV Chamber for 7days or 200 Watt hours/m2.Then transferred contents in to a 100 ml volumetric flask, added 50 ml of diluent and sonicate it for 10 minutes and made up to volume with diluent. Filtered and again diluted1.0 ml of the filtrate to 10 ml with a mobile phase and then injected 20 µl of the solution into the chromatographic system and the chromatogram was recorded as shown in figure 10.

Thermal studies

Transferred sample quantitatively equivalent to 25 mg of HCTZ and 10 mg of NBV on to clean and dry petri plate. Kept the petri plate in an oven at 100°C for 10 hrs. Then transferred the contents in to a 100 ml volumetric flask, added 50 ml of diluent and sonicate it for 10 minutes and made up to volume with diluent. Filtered and again diluted 1.0 ml of the filtrate to 10 ml with a mobile phase (diluent) and then injected 20 µl of the solution into the chromatographic system and the chromatogram was recorded as shown in figure 11.

RESULTS AND DISCUSSION

From this study, it was found that a simple, precise, accurate, sensitive and efficient stability indicating RP-HPLC method has been developed and validated for simultaneous estimation of

HCTZ and NBV in bulk and pharmaceutical dosage form. Separation was done by using mobile phase composed of phosphate buffer (adjusted to pH 6.5 with dilute orthophosphoric acid) and acetonitrile in the ratio of 40:60 % v/von Thermo Hypersil BDSC 18 column (150x 4.6mm, 5µ particle size) at a flow rate 0.8 ml/min using PDA detection at 282 nm. The retention times of HCTZ and NBV were found to be 2.4 and 4.0 min respectively. Linearity was evaluated in the concentration range of 6.25-37.50 µg/ml forHCTZand 2.5-15 µg/ml for NBV. The calibration curves of HCTZ and NBV were described by the equation y = 83009x + 1753.5and y=12355x+4051.1 with correlation coefficient of 0.9999 as shown in figure 3 and figure 4 respectively. System suitability results are shown in table 1. The %RSD in precision, accuracy and robustness studies were found to be less than 2.0%, indicating that the method is precise, accurate and robust. Accuracy data as shown in table 2. The validation summary parameters and assay results obtained from the marketed formulations are shown in table 3 and robustness study as shown in table 4. The stress testing results for both HCTZ and NBV are shown from figure 7 to figure 11 and results are presented in table 5 and table 6.

Table 1: System Suitability Results

S. No.	System Suitability Parameters	Results	
		HCTZ	NBV
1	USP Tailing	1.16	1.15
2	USP Resolution (Rs)	6.80	
3	Retention time(Min.) min.	2.48	4.0
4	USP Plate Count	3330	4488

Table 2: Accuracy Study

Sample	Level	Peak	Amount added	Amount	Mean % Recovery *±
HCTZ	80%	1651166	10.0	9.42	99.42 ±0.22
	100%	2074911	12.50	12.49	99.94±0.36
	120%	2502124	15.0	15.07	100.42± 0.42
NBV	80%	988473	4.0	3.98	99.58 ±0.35
	100%	1239202	5.0	4.99	99.87 ±0.57
	120%	1303235	6.0	6.04	100.66 ±0.23

***Mean of three determinations**

Linearity

R^2 values was found to be 0.9999 and regression equation y = 83009x + 1753.5for HCTZand y=12355x+4051.1for NBV.

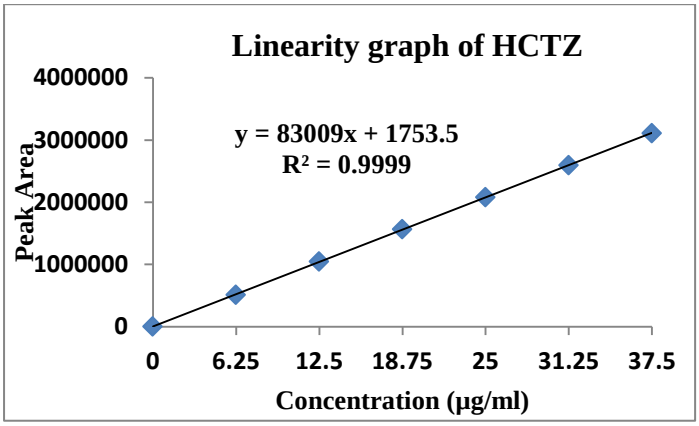

Figure 3: Linearity Graph of HCTZ (6.25-37.50 µg/ml)

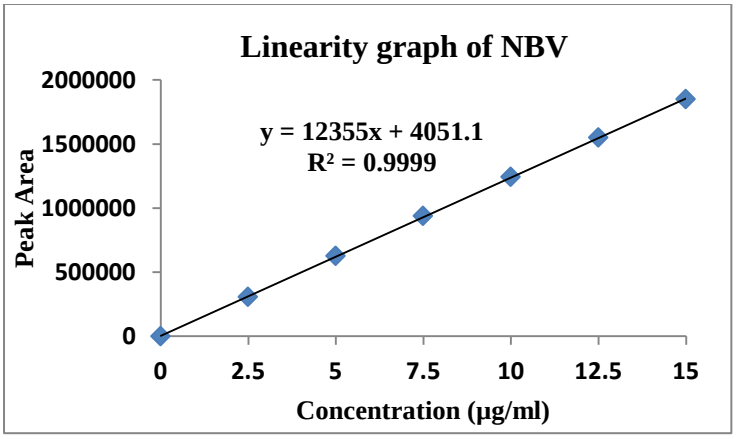

Figure 4: Linearity Graph of NBV (2.5-15 µg/ml)

Specificity

The chromatograms of standard and sample were identical to each other as shown in figure 5 and figure 6. The blank and placebo injections were also identical without any interference from the excipients.

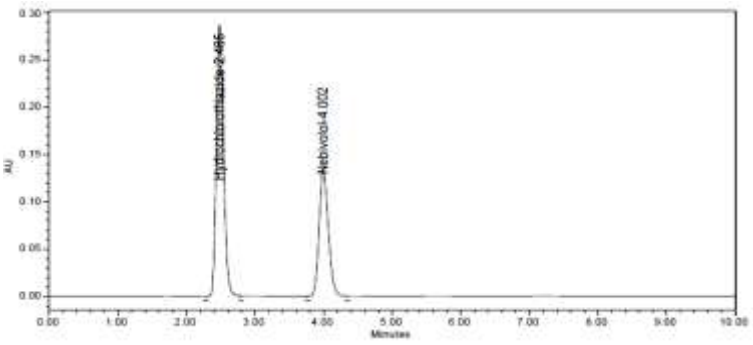

Figure 5:Typical chromatogram of standard

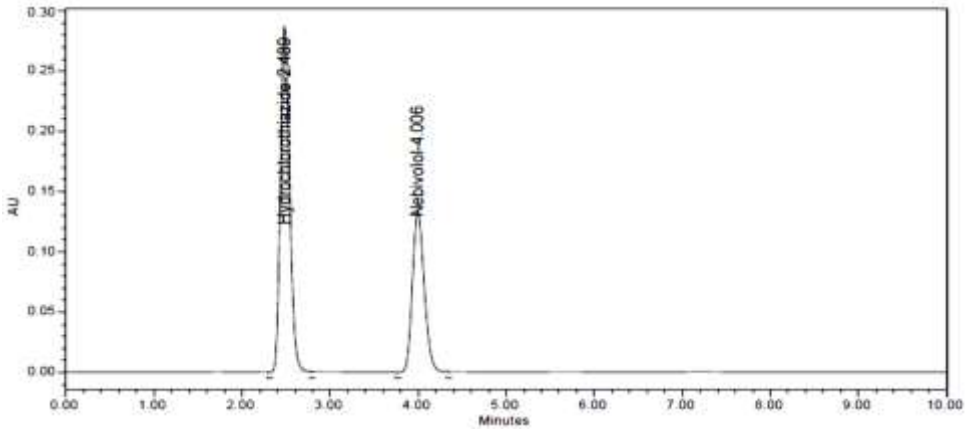

Figure 6: Typical chromatogram of sample

Table 3: Summary of Validation Parameters of the Proposed RP-HPLC Method

Parameter	HCTZ	NBV
Linearity range (µg/ml)	6.25-37.50	2.5-15
Regression equation	y=83009x+1753.5	y = 12355x + 4051.1
Correlation coefficient (r)	0.9999	0.9999
LOD (µg/ml)	0.145	0.078
LOQ (µg/ml)	0.502	0.237
Inter-day precision (% RSD)	0.135	0.102
Intra-day precision(% RSD)	0.172	0.097
% Assay	99.70-100.3%	99.64-100.2%

Table 4: Results of Robustness Study

S. No.	Parameter	Change Level	HCTZ Rt	HCTZ Peak	HCTZ Tailing	NBV Rt	NBV Peak	NBV Tailing
1.	Flow rate (±0.2ml/min)	0.6	2.831	2367484	1.17	4.541	1420226	1.16
		1.0	2.218	1846870	1.16	3.572	1104259	1.15
2.	Mobile organic phase composition	50:50	2.772	2245823	1.16	4.325	1325841	1.12
		30:70	2.134	1965237	1.14	3.776	1203222	1.13
3.	Temperature (±5°C)	25°C	2.513	2066195	1.16	3.846	1238190	1.16
		35°C	2.467	2074970	1.17	4.125	1245049	1.15

Forced degradation studies

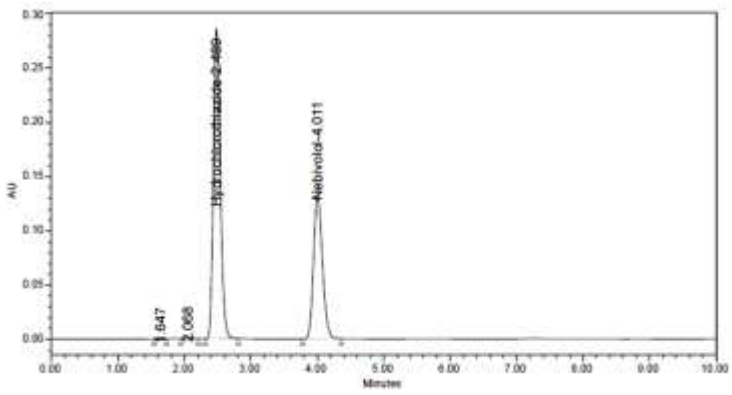

Figure 7: Chromatogram of Acid hydrolysis

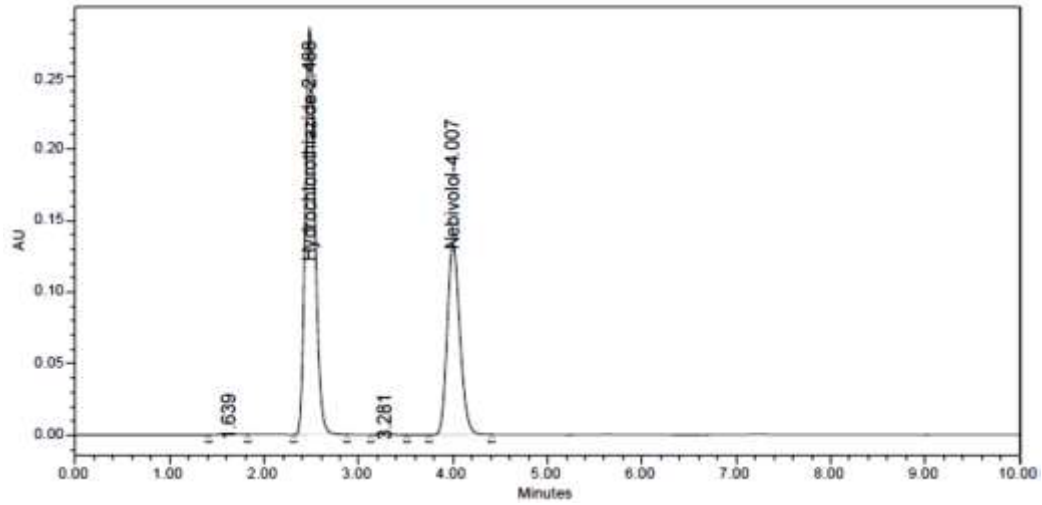

Figure 8: Chromatogram of Base Hydrolysis

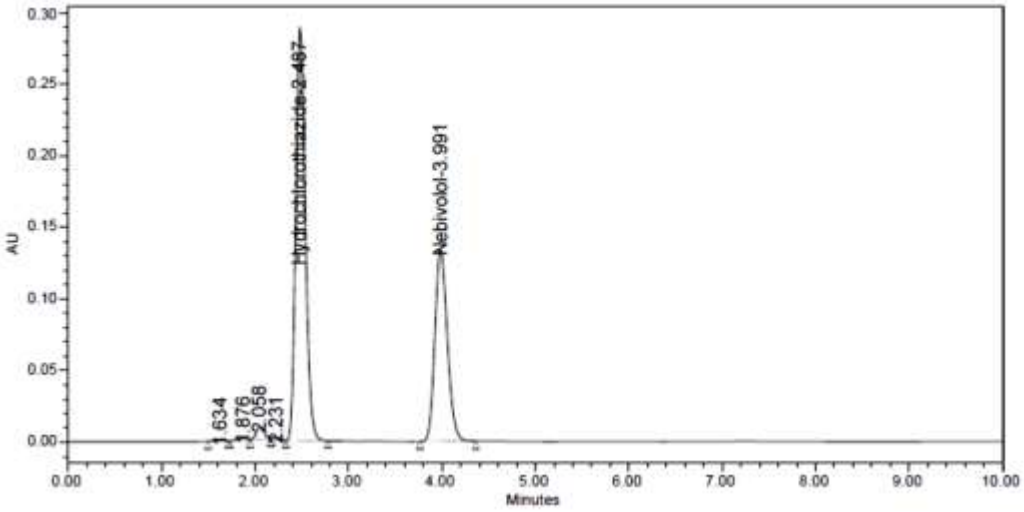

Figure 9: Chromatogram of Oxidation (peroxide)

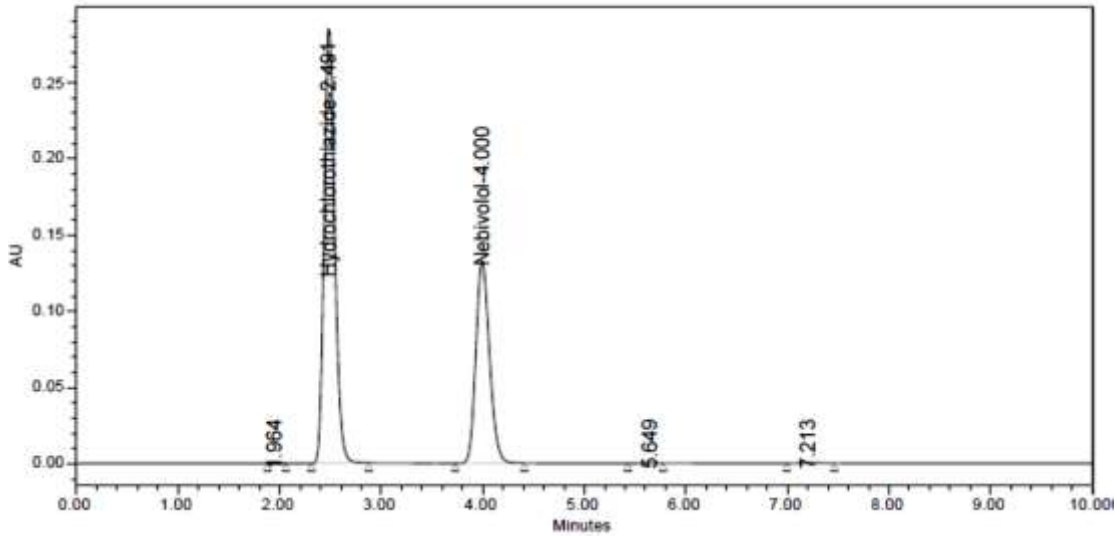

Figure 10: Chromatogram of UV Exposure

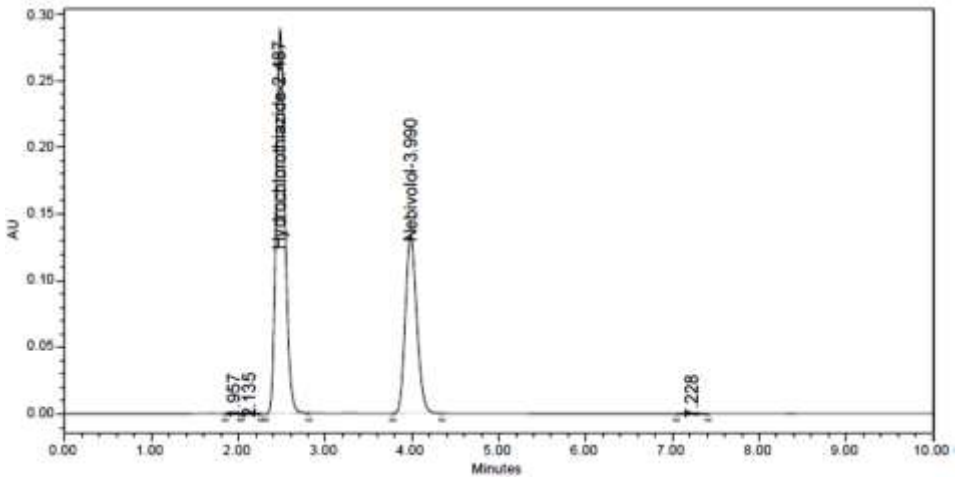

Figure 11: Chromatogram of Heat Exposure

The study was intended to ensure the effective separation of HCTZ and NBV and its degradation peaks of formulation ingredients at the retention time of HCTZ and NBV. Forced degradation studies were performed to evaluate the stability indicating properties and specificity of the method. During the acidic degradation, 2.05% of HCTZ and 2.38% of NBV were decomposed. During the alkaline degradation, 2.92% of HCTZ and 4.39% of NBV were decomposed without any major degradants. HCTZ and NBV have undergone thermal, oxidation and UV degradation slightly i.e less than 7.0% (as shown in Table 5 and Table 6). Typical chromatograms obtained following the assay of stressed samples are shown from Figure 7 to Figure 11

Table 5: Degradation Study of Hydrochlorothiazide

S.No.	Name	Peak Area	Degradation % Assay	% Net Degradation
1	Acid Hydrolysis	2029684	97.65	2.05
2	Base Hydrolysis	3740187	96.78	2.92
3	Heat Exposure	2021567	97.26	2.94
4	Oxidation (peroxide)	2011165	97.26	2.44
5	UV Exposure	2021829	97.27	2.43

Table 6: Degradation Study of Nebivolol Hydrochloride

S.No	Name	Peak Area	Degradation % Assay	% Net Degradation
1	Acid Hydrolysis	1208959	97.25	2.38
2	Base Hydrolysis	1183974	95.24	4.39
3	Heat Exposure	1202752	93.60	6.03
4	Oxidation (peroxide)	1163551	96.75	2.88
5	UV Exposure	1212980	97.57	2.06

CONCLUSION

From this study it is concluded that the proposed Stability Indicating RP-HPLC method was found to be simple, accurate, precise, rapid and useful for routine analysis of Hydrochlorothiazide and

Nebivolol Hydrochloride in bulk &pharmaceutical dosage form. The statistical parameters and recovery studies were carried out and reported. The obtained results were satisfactory as per ICH guidelines.

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