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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF BENIDIPINE HCL AND NEBIVOLOL HCL IN BULK DRUG AND TABLET FORM

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ABSTRACT

The present study is aimed towards the evaluation of fixed dose combination of two anti-hypertensive agents in combination in formulation and bulk by a RP-HPLC method and the stability evaluation is done according to ICH guidelines. The method proposed utilizes a WATERS C18 column and the mobile phase consisting K_2HPO_4: Methanol (55:45) adjusted to pH 5.5, with PDA detection at 248 nm. Flow rate was maintained at 1 ml/min and the analysis was carried out at ambient temperature. The run time was 6 minutes and flow rate maintained was 10 µl. With the above parameters as optimized, the method validation parameters were established within the acceptable criteria. Recovery levels of drugs was known to be >100% for benidipine and nebivolol, while the precision in terms of standard deviation was known to be 0.53 and 0.28 respectively. % RSD for system suitability was found to be 0.2% and 0.1% for benidipine and nebivolol respectively. Linearity and range of the method was found in the concentrations of 2-6 µg/ml and 2.5-7.5 µg/ml for benidipine and nebivolol respectively. Robustness of the method was analyzed with the slight modification in the parameters. Stability assessment and degradation studies under assorted conditions was carried out, which is an indicative of the shelf life of the drug.Hence, the proposed method is said to be efficient, selective, robust for the analysis of above said drugs in both forms.

KEYWORDS: Benidipine, Nebivolol, RP-HPLC, Validation, Stability indicating.

INTRODUCTION

A variety of anti-hypertensive agents are available in the market, which act by their own unique mechanisms, but the major obstacle to cure lies in the potential side effects corresponding with these drugs. Hence the treatment can only remain success when negative aftermath effects of the drug is minimized by the use of combination therapy.

Benidipine (3-(3R)-1-benzylpiperidin-3-yl 5-methyl (4R)-2,6-dimethyl-4-(3-nitrophenyl)- 1,4-dihydropyridine-3,5-dicarboxylate hydrochloride), a calcium channel blocker, acts by relaxing the blood vessels thereby reducing the blood pressure, while Nebivolol (1-(6-fluoro- 3,4-dihydro-2*H*-chromen-2-yl)-2-[[2-(6-fluoro-3,4-dihydro-2*H*-chromen-2-yl)-2- hydroxyethyl] amino] ethanol) a beta blocker acts on aldosterone and renin by decreasingblood volume and causing vasoconstriction respectively, thereby reducing blood pressure.[1-10]

MATERIALS AND METHODS

A HPLC system, of Waters 2695 module along with WATERS C18 column; 250 mm × 4.6 mm; 5 µm dimension particle size is used while maintaining the temperature at 25˚C. The studies were performed using Empower 2 Waters software. The detector used was Photodiode array detector (Waters 2699 module). Methanol and potassium dihydrogen phosphate is used in 55:45, in the preparation of mobile phase. Benidipine Tablets with labelled amount 4 mg (manufactured by Synokem Pharmaceuticals limited, Raipur) and Nebivolol with labeled amount 5mg (Abott Pharmaceuticals) were procured from neighbourhood store.

Preparation of mobile phase

Accurately weighed amount of K_2HPO_4 is transferred into a 1000 ml volumetric flask and a small amount of HPLC grade water is added to dissolve it. Make up the volume to 1000 ml with same solvent, mix well and adjust the pH to 5.5 to obtain the mobile phase. Mix 550 ml of above solution with 450 ml of methanol and sonicate for 20 minutes to remove any particulate matter or entrapped air. The prepared solution is then considered as a mobilephase.

Preparation of Benidipine & Nebivolol standard solution

Benidipine HCl 4mg and 5mg Nebivolol HCl are correctly weighed and transferred into 100ml flask; to which was added 10ml Methanol, was made to sonicate 10min and made up the final volume with Methanol. One ml of the resulting solution is taken in a 10ml volumetric flask, followed by diluting it to 10 ml with water.

Preparation of Benidipine & Nebivolol stock solution: Accurately weigh and add 126 mg of (4mg Benidipine HCl and 5mg Nebivolol HCl) into a volumetric flask - 100 ml and add 10ml Methanol and shake 10min (or) sonicate it for 20min and fill with Methanol. Transfer 1 ml of the above solution into 10 ml volumetric flask dilute to 10 ml the volume with water. Filter the solution through 0.45µ filter (prior to injecting into system).

Working linearity calibrated solutions of Benidipine & Nebivolol

Working concentrations solutions of BNP (2-6 µg/ml) and NBV (2.5-7.5 µg/ml) are prepared according to linearity concentration range by diluting stock solutions with suitable amount of methanol.

1. **Solution 1:** Pipette 0.5 ml of stock solution into a volumetric flask, 100 ml and dilute with methanol to obtain concentrations of 2 µg/ml of BNP and 2.5 µg/ml of NBV respectively.
2. **Solution 2:** Pipette 0.75 ml of stock solution into a volumetric flask, 100 ml and dilute with methanol to obtain concentrations of 3 µg/ml of BNP and 3.75 µg/ml of NBV respectively.
3. **Solution 3:** Pipette 1.0 ml of stock solution into a volumetric flask, 100 ml and dilute with methanol to obtain concentrations of 4 µg/ml of BNP and 5 µg/ml of NBV respectively.
4. **Solution 4:** Pipette 1.25 ml of stock solution into a volumetric flask, 100 ml and dilute with methanol to obtain concentrations of 5 µg/ml of BNP and 6.25 µg/ml of NBV respectively.
5. **Solution 5:** Pipette 1.5 ml of stock solution into a volumetric flask, 100 ml and dilute with methanol to obtain concentrations of 6 µg/ml of BNP and 7.5 µg/ml of NBV respectively.

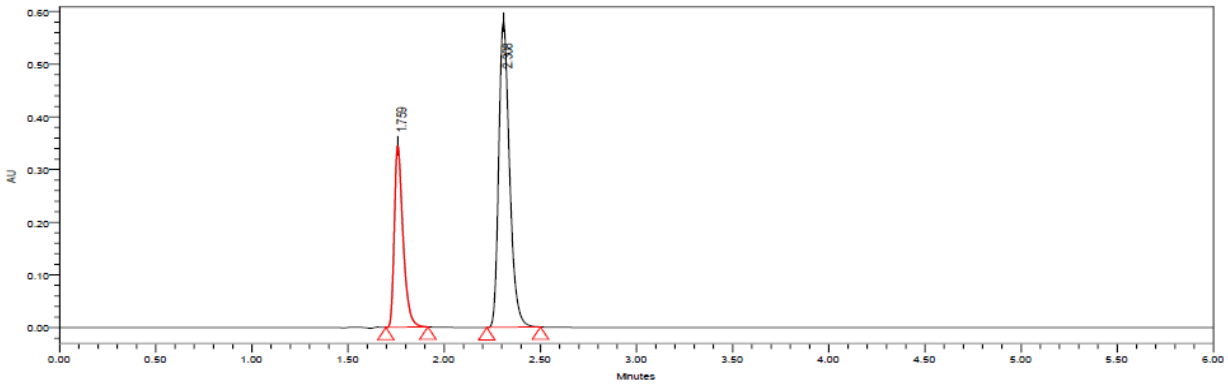
RESULTS AND DISCUSSION

Optimized Method

- Mobile Phase: K_2HPO_4: Methanol (55:45)
- Column: WATERS, C18, 150X4.6mm, 5µm
- Flow Rate: 1.0ml/Min
- Temperature: 25˚C
- Volume: 10µl
- Run time: 6min
- Detector: 248
- PH: 5.5

Procedure

Inject 10µL of standard sample solution into chromatographic system and measure areas for Benidipine HCL and Nebivolol HCL peaks and calculate the % assay.



	Name	Retention Time	Area	% Area	Height	Int Type	USP Resolution	USP Tailing	USP Plate Count
1		1.759	1090605	32.50	344786	BB		1.47	7370
2		2.308	2265174	67.50	579410	BB	5.81	1.31	8245

Fig 10: Chromatogram - optimized method.

RESULT: Two peaks eluted and all the system suitability parameters were established within limits.

Method Validation System suitability tests

Six injections of working concentrations of BNP-NBV are repeatedly passed into the HPLC system equipped with a WATERS C18, 150 × 4.6mm, 5µm and mobile phase K_2HPO_4:Methanol (55:45). Various parameters confined to HPLC, such as peak tailing and resolution, number of theoretical plates, retention times of the sample were evaluated and are as follows.

Table 1: System suitability results for Benidipine.

S.no	Peak Name	Rt	Area	USP Plate Count	USP Tailing
1	Benidipine HCl	1.756	1087512	7503	1.49
2	Benidipine HCl	1.757	1090098	7435	1.48
3	Benidipine HCl	1.757	1089964	7464	1.48
4	Benidipine HCl	1.758	1085610	7495	1.48
5	Benidipine HCl	1.760	1084496	7497	1.48
Mean			1087535.9		
% RSD			0.2		

Table 2: System suitability results for Nebivolol.

S.no	Peak Name	Rt	Area	USP Resolution	USP Plate Count	USP Tailing
1	Nebivolol HCl	2.302	2256840	5.87	8558	1.32
2	Nebivolol HCl	2.306	2257242	5.86	8459	1.32
3	Nebivolol HCl	2.306	2260953	5.83	8303	1.32
4	Nebivolol HCl	2.307	2259273	5.86	8495	1.32
5	Nebivolol HCl	2.307	2261642	5.85	8358	1.32
Mean			2259190			
% RSD			0.1			

Selectivity

Blank and working concentration of working concentration solutions are passed into the HPLC system equipped with a WATERS C18, 150 × 4.6mm, 5µm and solvent system K_2HPO_4:Methanol (55:45). The parameters are adjusted as per the specifications of optimization conditions. The selectivity of the method is evaluated by comparing the chromatograms of the blank with the sample/placebo.

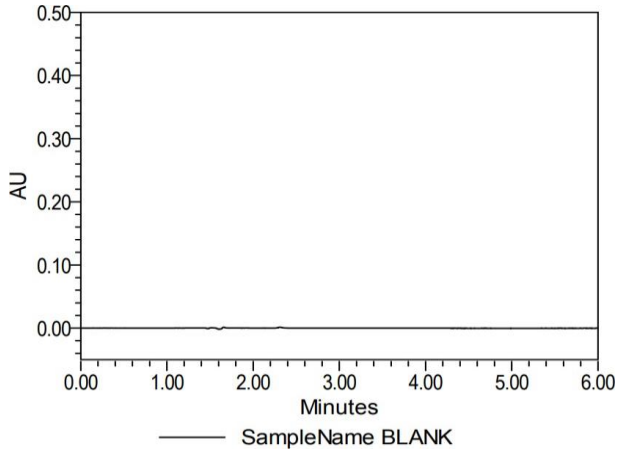


Fig 3: Chromatogram – Blank.

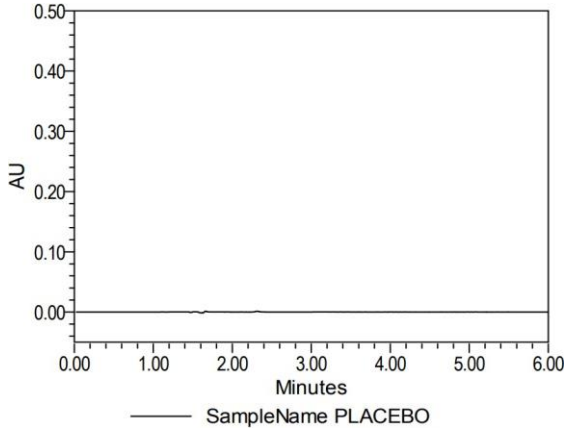


Fig 4: Chromatogram – Placebo.

Linearity

Five different concentrations of BNP & NBV are prepared in the level of upper interval and lower interval of 2-6 µg/ml and 2.5-7.5 µg/ml respectively are fed into the HPLC system equipped with WATERS C18, 150 × 4.6mm, 5µm. The peak areas for the respective concentrations were recorded, plot of concentration against peak areas is plotted and the R^2 values were found below 1, which defines that the method is inferred to be linear in the above said range.

Table 3: Results of Linearity and range of BNP & NBV.

BNP			NBV		
CONC%	Concentration (µg/ml)	Peak area	CONC%	Concentration(µg/ml)	Peak area
50	2	541083	50	2.5	1105140
75	3	813669	75	3.75	1675423
100	4	1061247	100	5	2233693
125	5	1330576	125	6.25	2802591
150	6	1611742	150	7.5	3368096

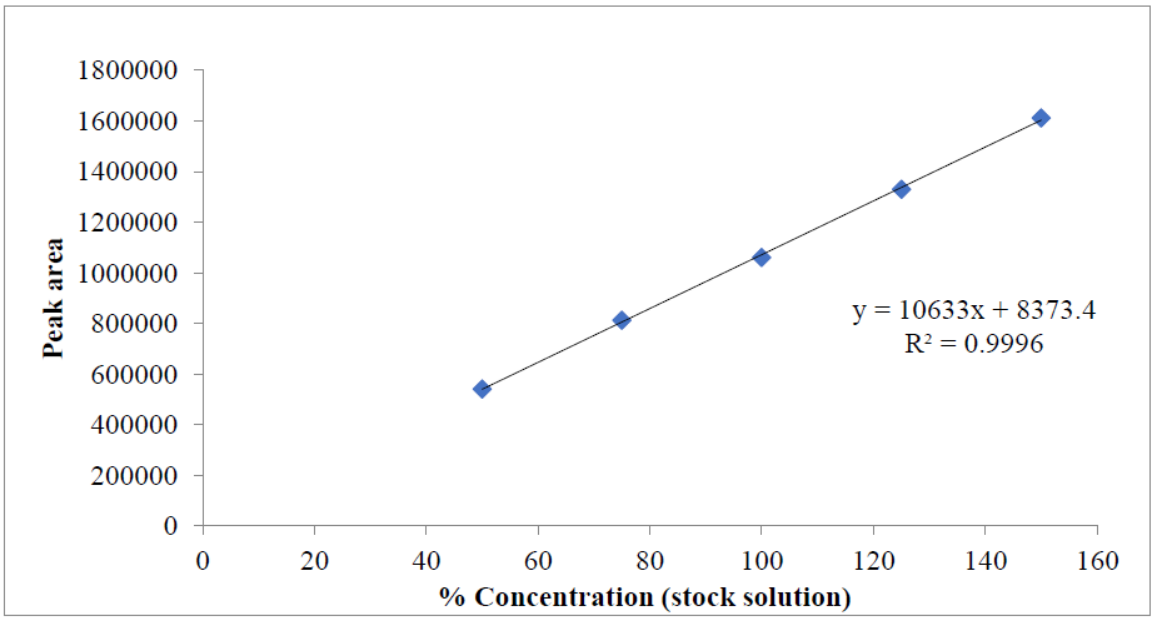


Fig 5: Calibration curve for BNP.

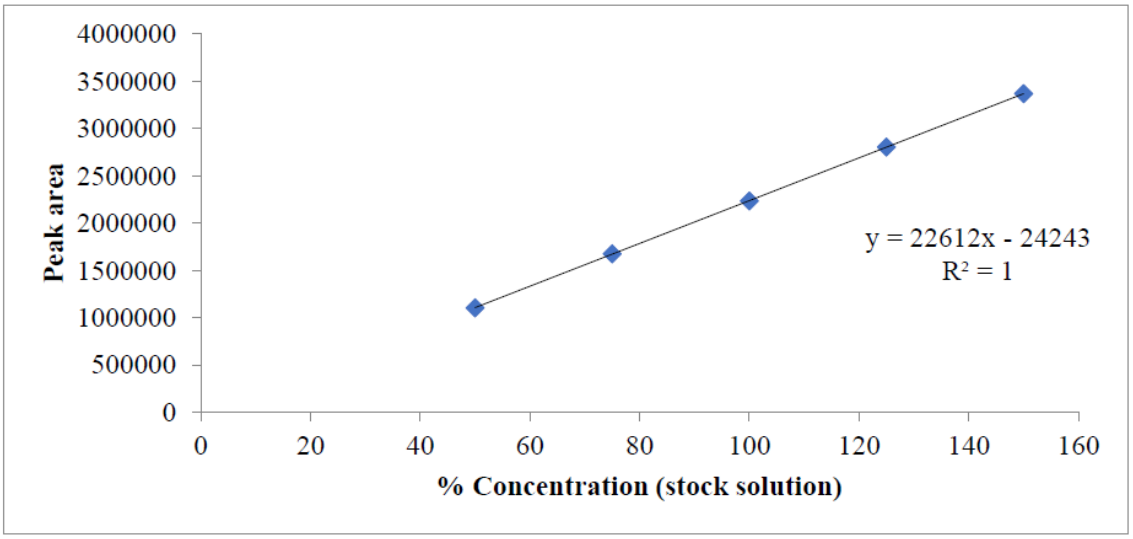
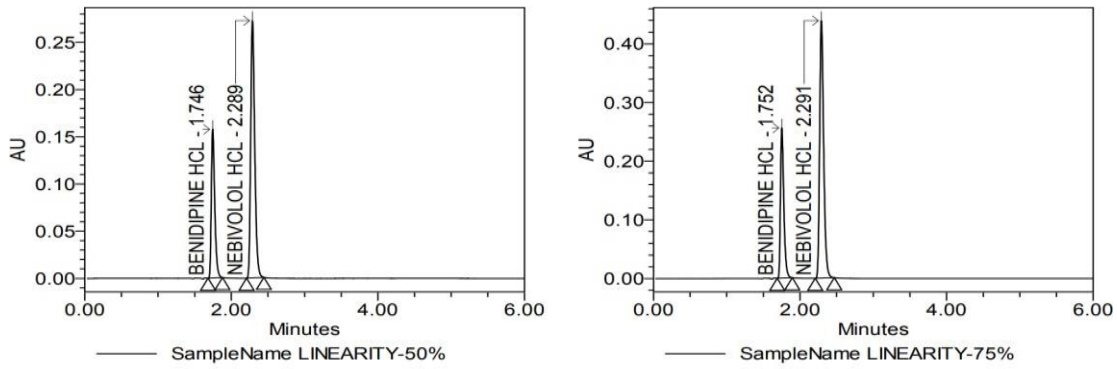


Fig 6: Calibration curve for NB.



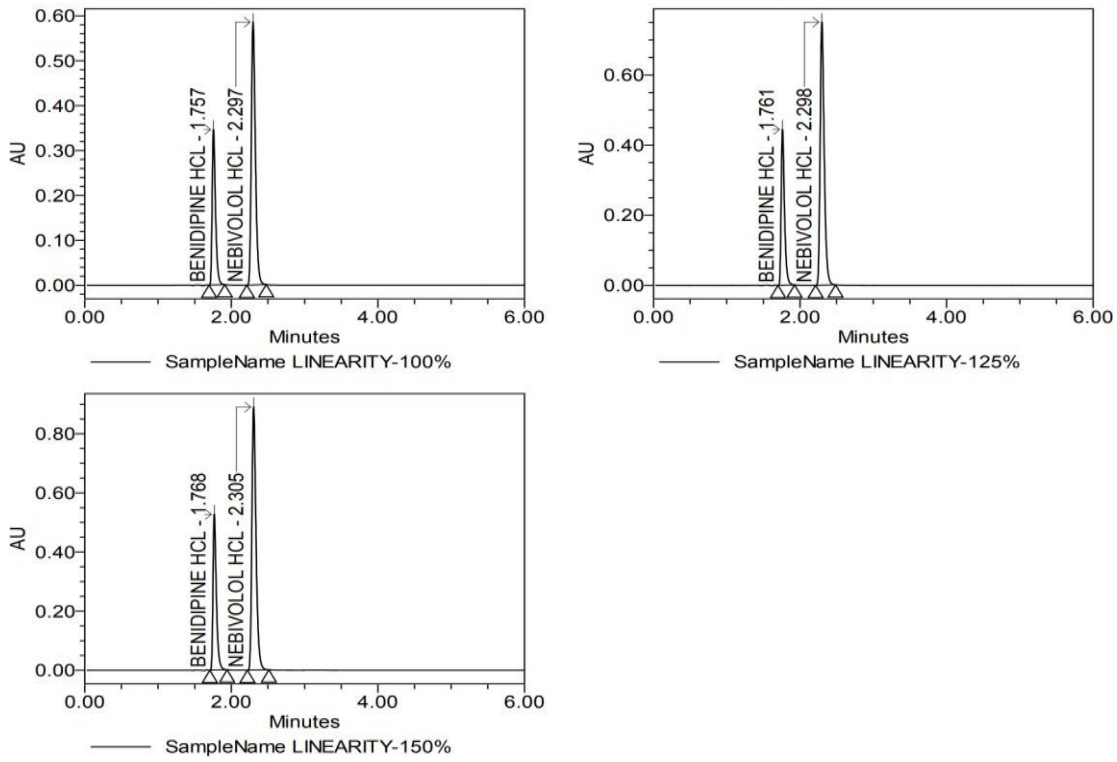


Fig 7: Chromatograms for linearity and range of BNP & NBV.

Limit of detection

Working concentrations of BNP & NBV were passed into the system on a WATERS C18, 150 × 4.6mm, 5µm, adjusting the settings according to the optimized parameters selected for the method. Evaluation of LOD was done based on signal to noise ratio. The formula for calculation of LOD is 3.3* and was deduced as 0.004 µg/ml and 0.003 µg/ml respectively. Thus, the procedure employed was proved to be sensitive for analyzing the above-mentioned drugs.

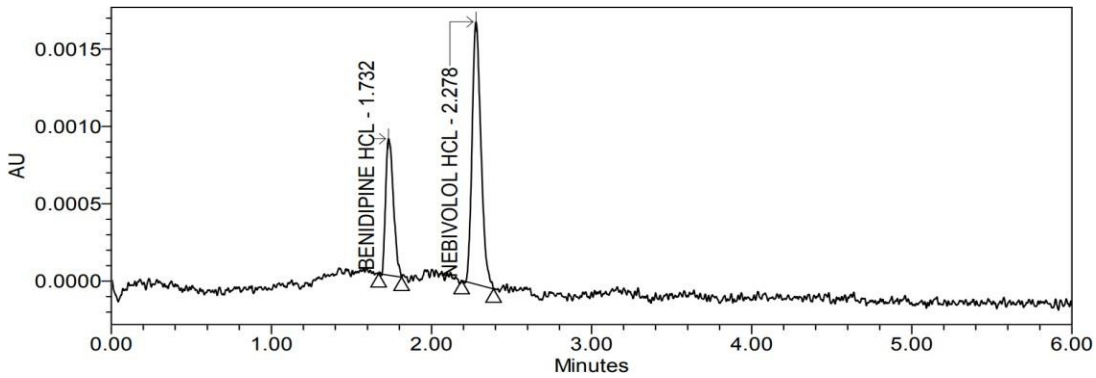


Fig 8: Chromatogram showing LOD of BNP and NBV.

Limit of Quantification

Working concentrations of BNP & NBV were passed into the system on a WATERS C18, 150 × 4.6mm, 5µm, adjusting the settings according to the optimized parameters selected for the method. Evaluation of LOQ was done based on signal to noise ratio and was deduced as 0.013 µg/ml and 0.010 µg/ml respectively. Thus, the procedure was proved to be sensitive for analyzing the above-mentioned drugs.

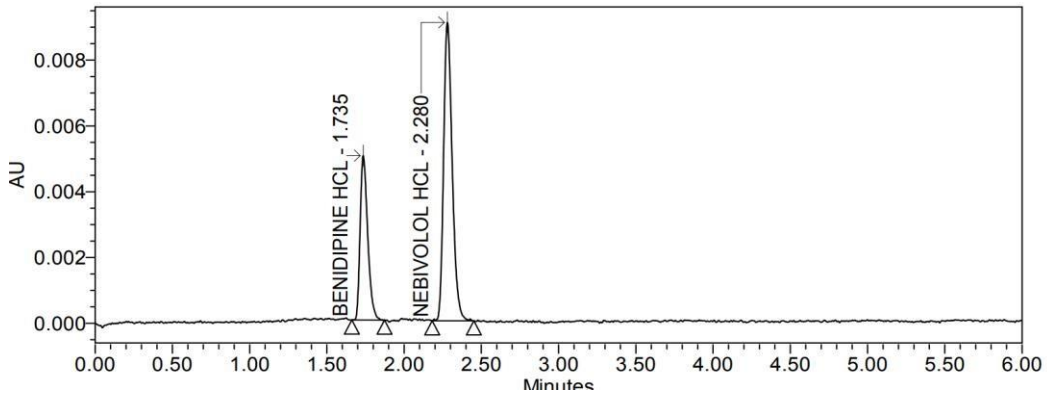


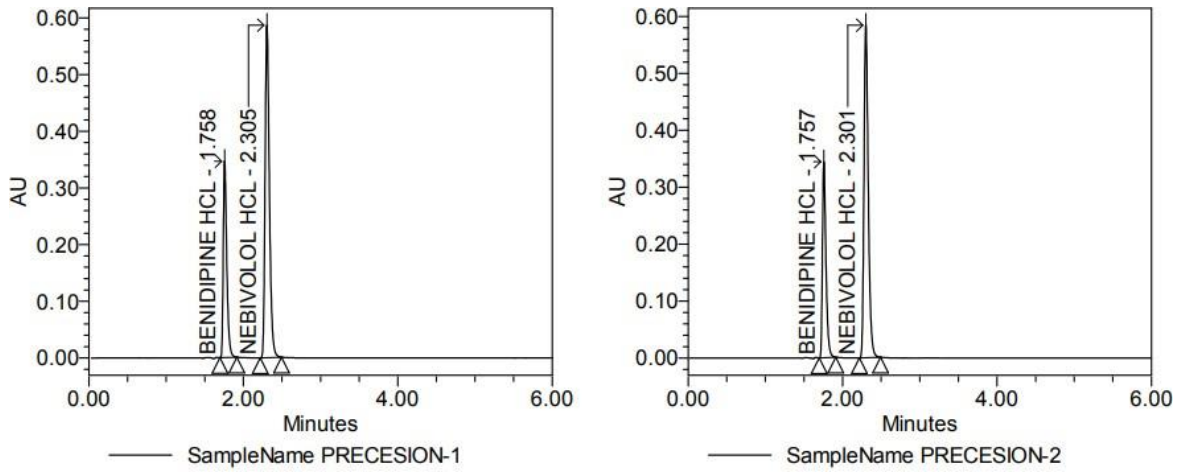
Fig 9: Chromatogram showing LOQ of BNP and NBV.

Precision

The precision of the suggested method is evaluated based on the repeatability results. Six injections of working concentrations of BNP & NBV were passed into the HPLC system on a WATERS C18, 150 × 4.6mm, 5µm column and the settings were adjusted according to the optimized parameters. Repeatability of the method was assessed based on the % RSD obtained from the peak areas found as 0.54 and 0.28 respectively, indicating that this method is apt for the analysis of BNP and NBV.

Table 4: Precision results for BNP & NBV.

Drug name	Peak area	Average	Standard deviation	% RSD
BNP	1067453	1068904.167	5752.187	0.53
	1077998			
	1063102			
	1064756			
	1073739			
	1066377			
NBV	2230741	2238885.167	6273.206721	0.28
	2247971			
	2243441			
	2233705			
	2238376			
	2239077			



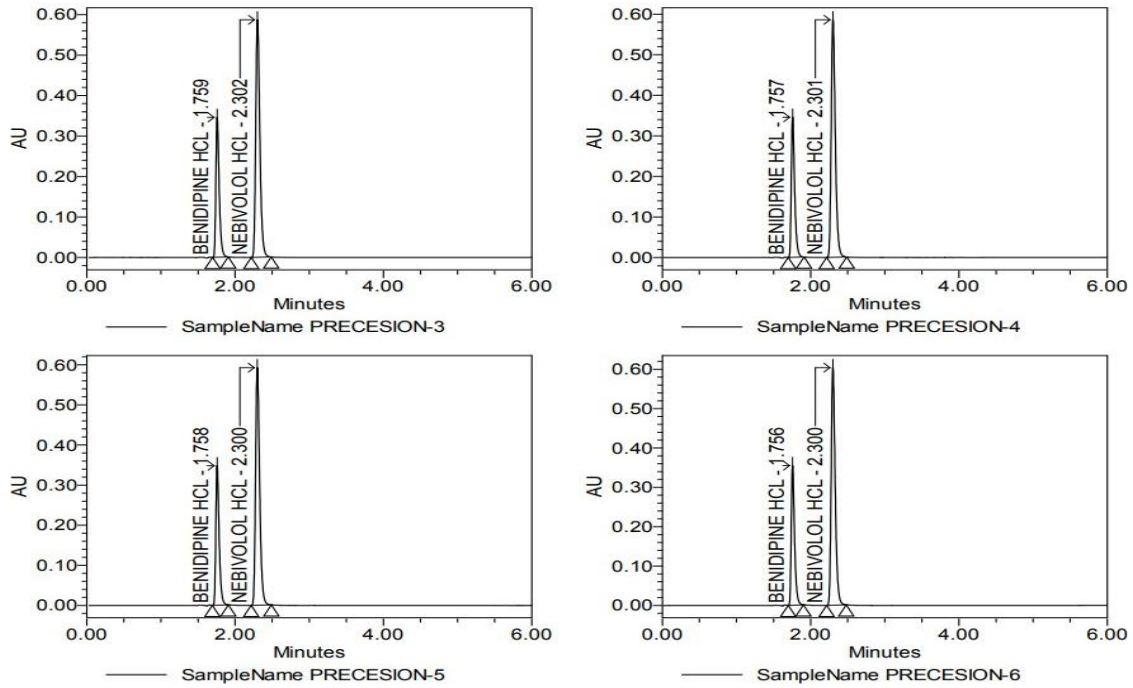


Fig 10: Chromatograms showing precision results of BNP & NBV.

Accuracy and recovery

Sample solutions of BNP & NBV were inculcated into the HPLC system on a WATERS C18, 150 × 4.6mm, 5µm column, the settings were adjusted according to the optimized conditions. The % RSD and assay of the sample are calculated from the peak areas obtained, indicating that the method is accurate for estimating BNP & NBV simultaneously.

Recovery of the method is valuated by spiking the sample solutions of BNP & NBV at 50%, 100% and 150% and the % recovery after spiking was made in contrast with that of the drug content amount in sample before spiking. Results obtained indicating the method is selective for the concomitant quantification of BNP & NBV in the formulations.

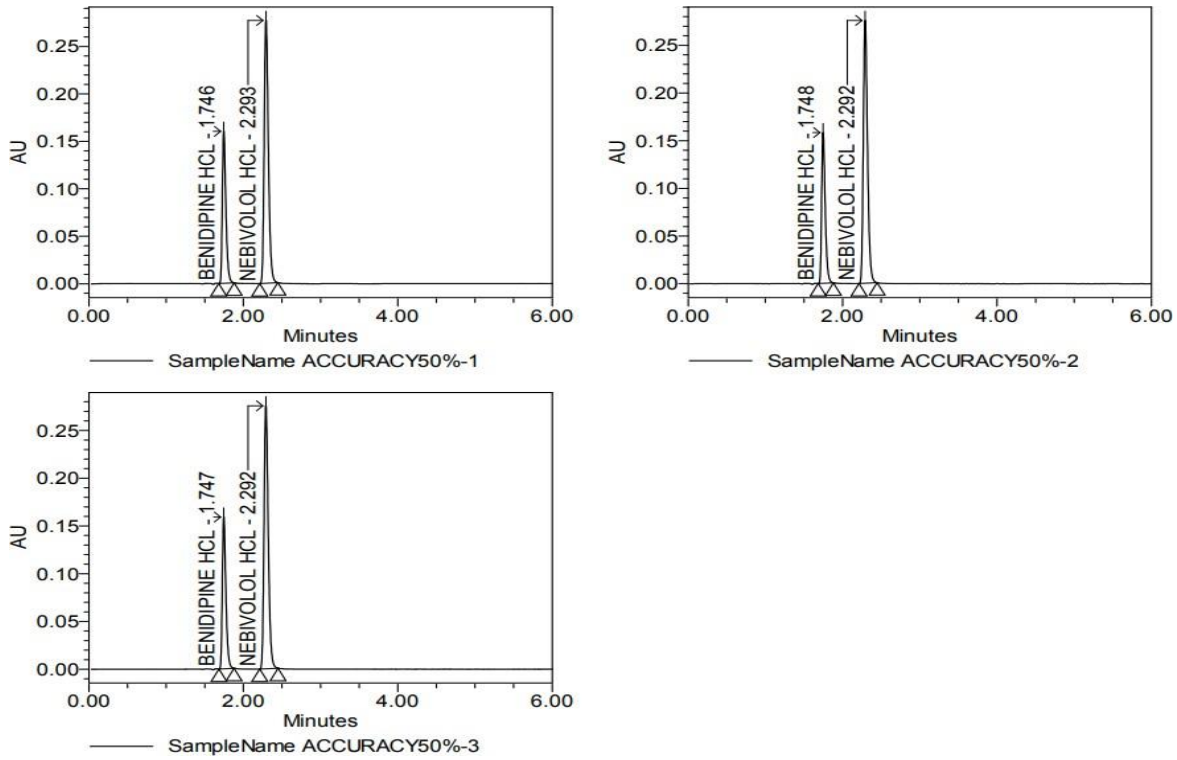


Fig 11: Chromatograms showing recovery of BNP & NBV at 50% spiking.

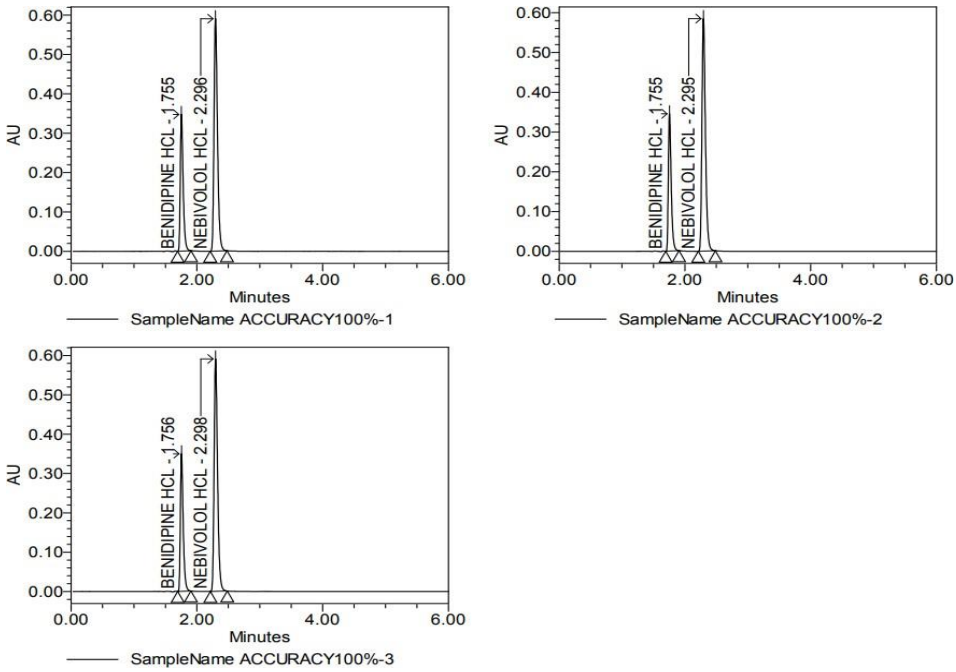


Fig 12: Chromatograms showing recovery of BNP & NBV at 100% spiking.

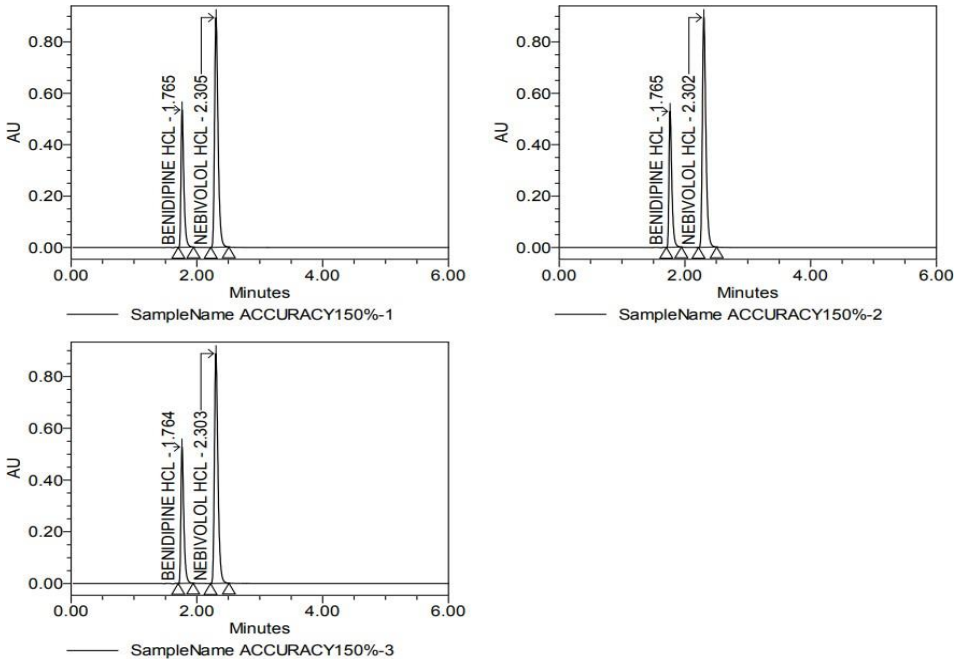


Fig 13: Chromatograms showing recovery of BNP & NBV at 150% spiking.

Table 5: Recovery results for Benidipine.

Spiking value (%)	Peak Area	Amount of drug added	Total amount of drug found	% Recovery of drug	Mean recovery value
50	541502	1.960	1.98	101	101
50	541902	1.960	1.99	101	
50	542402	1.960	1.99	101	
100	1068493	3.920	3.91	100	100
100	1078499	3.920	9.95	101	
100	1066692	3.920	3.91	100	
150	1627858	5.880	5.96	101	101
150	1618090	5.880	5.93	101	
150	1618546	5.880	5.93	101	

Table 6: Recovery results for Nebivolol.

Spiking value (%)	Peak Area	Amount of drug added	Total amount of drug found	% Recovery of drug	Mean recovery value
50	1101040	2.475	2.43	98	99
50	1113692	2.475	2.46	99	
50	1101758	2.475	2.43	98	
100	2231218	4.950	4.92	99	100
100	2242075	4.950	4.95	100	
100	2236627	4.950	4.94	100	
150	3379951	7.425	7.46	100	100
150	3360779	7.425	7.42	100	
150	3364371	7.425	7.42	100	

Robustness

Working concentration solutions of BNP & NBV were passed into the HPLC system equipped with a WATERS C18, 150 × 4.6mm, 5µm column, and modification of the selected parameters such as wavelength, rate of flow, pH, mobile phase ratio and temperature was done to evaluate the robustness of the designated method. Number of theoretical plates, peak tailing, peak resolution, retention times and peak areas of BNP & NBV after modification were calculated and the results show that the method is robust for the concomitant quantification of BNP & NBV in the dosage forms.

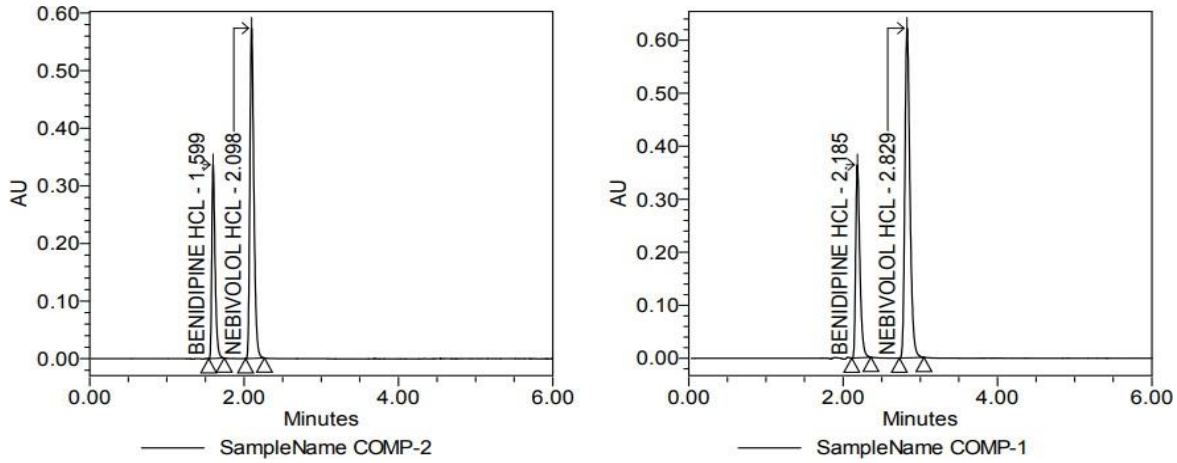


Fig 14: Chromatograms showing robustness of BNP & NBV with modification of mobile phase composition.

Table 7: Robustness results- mobile phase composition modification.

Mobile phase (Methanol)	Sample name	Rt	Peak area	USP resolution	USP plate count	USP tailing
35%	BNP	2.185	1363098		8166	1.45
55%	BNP	1.599	993706		6983	1.47
35%	NBV	2.829	2833813	5.82	9103	1.29
55%	NBV	2.098	2067183	5.68	7931	1.32

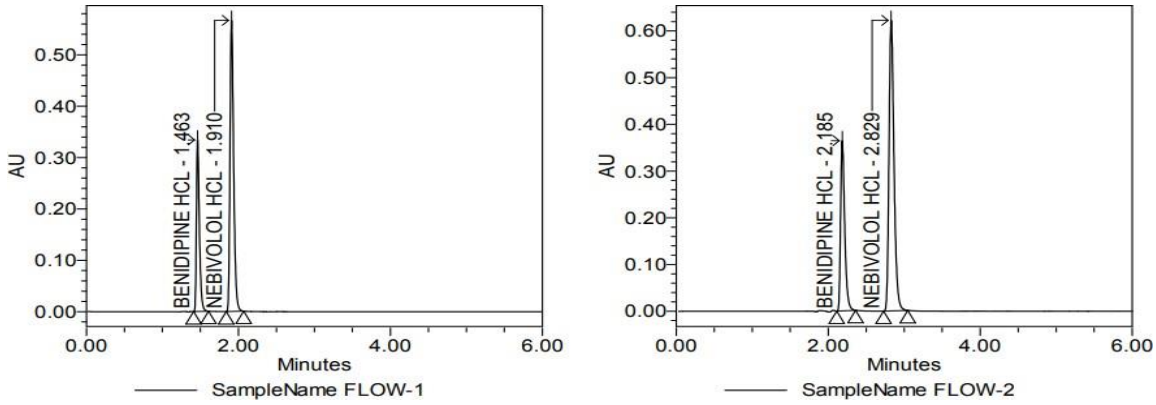


Fig 15: Chromatograms showing robustness of BNP & NBV with modification of flow rate.

Table 8: Robustness results- Flow rate modification.

Flow rate (ml/min)	Sample name	Rt	Peak area	USP resolution	USP plate count	USP tailing
0.9	BNP	1.463	912052		6865	1.49
1.1	BNP	2.185	1363098		8166	1.45
0.9	NBV	1.910	1885873	5.53	7769	1.34
1.1	NBV	2.829	2833813	5.82	9103	1.29

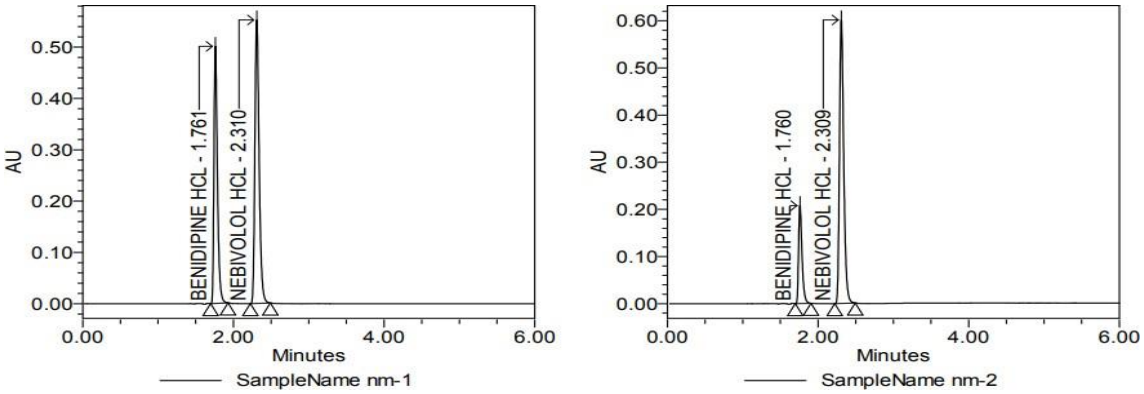


Fig 16: Chromatograms showing robustness of BNP & NBV with modification of wavelength.

Table 9: Robustness results- wavelength modification.

Wavelength (nm)	Sample name	Rt	Peak area	USP resolution	USP plate count	USP tailing
246	BNP	1.761	1584445		7413	1.47
250	BNP	1.760	655507		7446	1.48
246	NBV	2.310	2157770	5.82	8264	1.31
250	NBV	2.309	2343027	5.83	8326	1.32

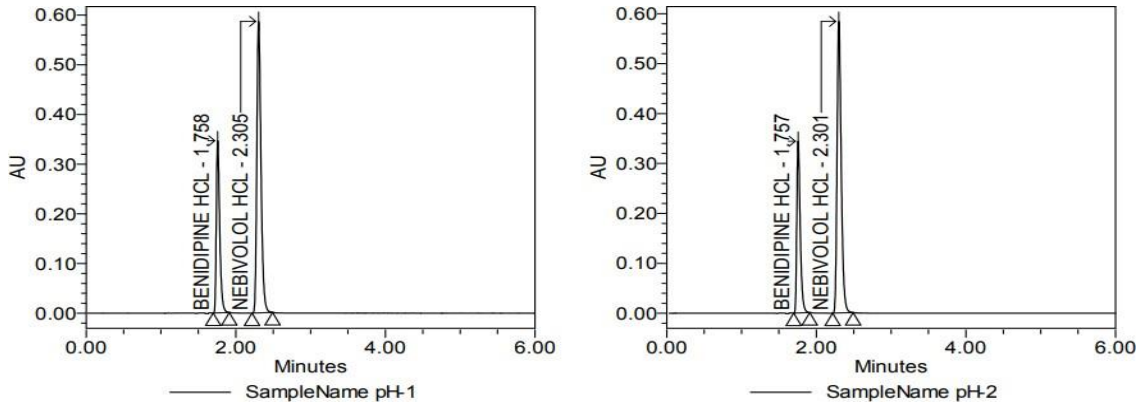


Fig 17: Chromatograms showing robustness of BNP & NBV with modification of pH.

Table 10: Robustness results- pH modification.

pH	Sample name	Rt	Peak area	USP resolution	USP plate count	USP tailing
5.3	BNP	1.758	1087453		7532	1.48
5.7	BNP	1.757	1087998		7370	1.48
5.3	NBV	2.305	2260741	5.87	8508	1.32
5.7	NBV	2.301	2257971	5.78	8343	1.32

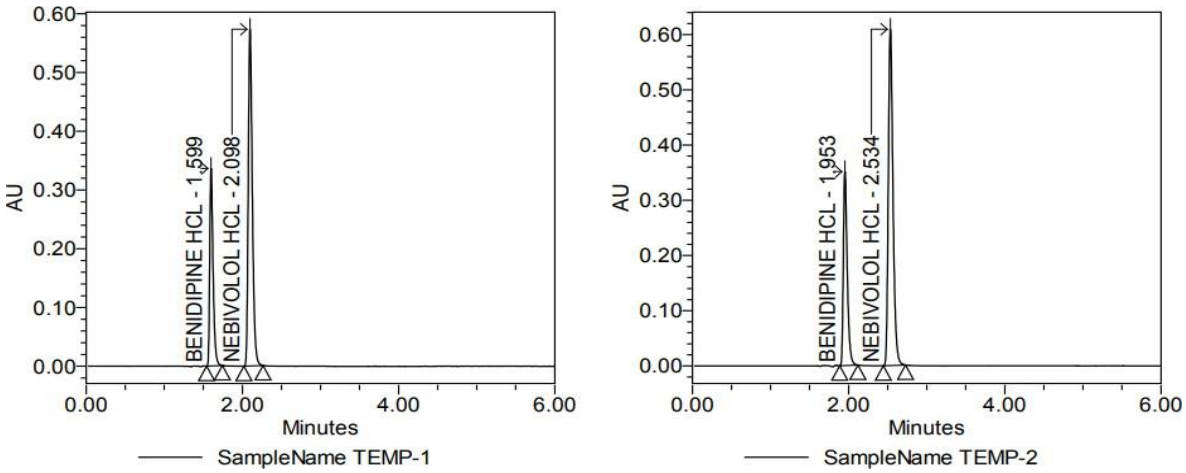


Fig 18: Chromatograms showing robustness of BNP & NBV with modification of temperature.

Table 11: Robustness results- Temperature modification.

Temperature (˚C)	Sample name	Rt	Peak area	USP resolution	USP plate count	USP tailing
23	BNP	1.599	993706		6983	1.47
27	BNP	1.953	1211916		7669	1.45
23	NBV	2.098	2067183	5.68	7931	1.32
27	NBV	2.534	2523716	5.74	8812	1.30

Table 12: Results for degradation studies of BNP & NBV - Order of Stability.

Stress Applied	BNP			NBV		
	Response	% Remained	% Degraded	Response	% Remained	% Degraded
No stress	1090605	100	0	2265174	100	0
Acid	979496	89.71	10.29	2070270	91.36	8.64
Base	1012755	92.75	7.25	2129482	93.98	6.02
Peroxide	1030133	94.34	5.66	2147898	94.79	5.21
Thermal	969181	88.76	11.24	2027203	89.46	10.54
Sunlight	1020164	93.43	6.57	2054872	90.68	9.32
Humidity	1084092	99.28	0.72	2226696	98.27	1.73

BNP: Humidity > Peroxide > Sunlight > Base > Acid > Thermal
NBV: Humidity > Peroxide > Base > Acid > Sunlight > Thermal

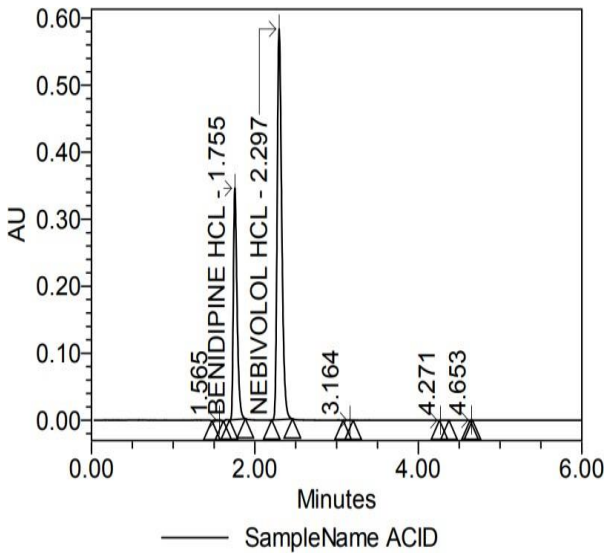


Fig 19: Chromatogram showing acid degradation of BNP & NBV.

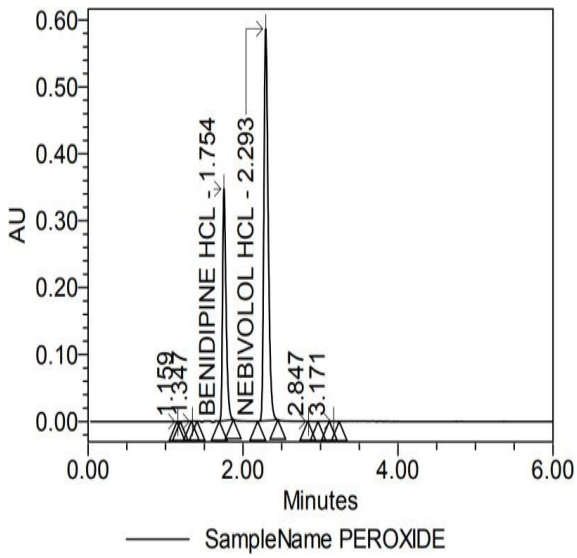


Fig 20: Chromatogram showing base degradation of BNP & NBV.

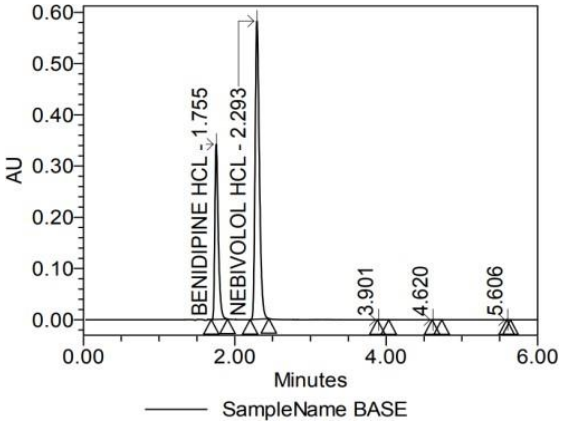


Fig 21: Chromatogram showing peroxide degradation of BNP & NBV.

CONCLUSION

The method proposed for the concomitant quantification of Benidipine and Nebivolol, is validated according to the ICH guidelines and safety assessment was done under various conditions. The procedure employed was proved to be cost-effective as it utilizes simple mobile phase and the short run time, making the less use of mobile phase. It is suitable for the routine estimation of the proposed drugs in bulk and formulations as all the parameters met the ICH acceptance criteria using the specified parameters. Results of degradation studies under the influence of various conditions are reported, which indicate the stability of the drug which is of prime importance to estimate the shelf-life of the drug and expiry of the formulation on marketing.

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