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Research Article

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Method development and validation for the simultaneous estimation of Nebivolol hydrochloride and S(-) Amlodipine besilate in Tablet dosage form by RP–HPLC

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ABSTRACT

High Performance Liquid Chromatography method was developed and validated in a simple, specific and precise manner for Nebivolol HCl and Amlodipine besilate in a combined solid dosage form. Nebivolol hydrochloride, a long acting, cardioselective β – blocker and Amlodipine besilate, a calcium channel blocker was considered in combination for the treatment of hypertension. The HPLC (WATERS ALLIANCE 2695) – Separation module with photo diode array detector (WATERS 2996) with CN (Zorbax) column (250 x 4.6 mm), 5 µ (Agilent) was used. The retention times of Nebivolol HCl and Amlodipine besilate was found to be 13.568 min and 9.554 min respectively. The developed method was validated as per ICH Guidelines for specificity, linearity, accuracy, precision, range, detection limit, quantitation limit, ruggedness and robustness. The applicability of the developed method for the quantification of Nebivolol HCl and Amlodipine besilate present from marketed tablet formulation was evaluated. The results confirmed the suitability of developed method for routine analysis of Nebivolol HCl and Amlodipine besilate in combined dosage forms.

Key words: HPLC, hypertension, retention time, validation

INTRODUCTION

Nebivolol hydrochloride is an antihypertensive, which is a competitive and selective β_1 receptor antagonist. It is chemically known as α, α^1 – [imino bis (methylene)] bis [6 – fluoro-3, 4-dihydro-2H-1-benzopyran-2-methanol]. Nebivolol HCl is commonly used for the management of mild to moderate hypertension and the pharmacological activity is attributable to the d – enantiomer[1].

S (-) Amlodipine besilate is 2 – [(2- aminoethoxy) methyl] – 4 – (2 – chlorophenyl) – 1, 4 – dihydro – 6 – methyl – 3, 5 – pyridine carboxylic acid 3 – ethyl 5 – methyl ester, is a dihydropyridine calcium antagonist used in the treatment of hypertension. It is a long acting calcium channel blocker, which inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle.

Literature survey revealed that HPLC method[2, 3, 4, 5] is the commonly used analytical method[6] for the estimation of Nebivolol hydrochloride and Amlodipine besilate, either as individual drugs or in combination with other drugs. However, no method has been reported for the simultaneous estimation of Nebivolol HCl and Amlodipine besilate in pharmaceutical dosage forms. Hence, the purpose of the present work was intended to develop and validate[7, 8] a simple, sensitive analytical method for simultaneous quantification of Nebivolol HCl and Amlodipine besilate in tablet dosage form.

MATERIALS AND METHODS

Materials and Equipments:

Nebivolol hydrochloride and S (-) Amlodipine besilate were procured from Cadila Healthcare Pvt. Ltd., Ahmedabad and Emcure Pharmaceuticals, Pune respectively. Each tablet contains Nebivolol hydrochloride 2.5 mg and S (-) Amlodipine besilate equivalent to Amlodipine 5 mg, manufactured by The Madras Pharmaceuticals, Research and Development Department. Each mg of Amlodipine besilate is equivalent to Amlodipine 0.721mg. Other materials required were HPLC water, Acetonitrile HPLC grade (Merck Speciality, Mumbai), Ammonium acetate HPLC grade (Hipure fine chemicals, Chennai) and Glacial acetic acid Analytical grade (Ranbaxy Chemicals, New Delhi). HPLC separation module (WATERS ALLIANCE 2695) with Photodiode array detector (WATERS, 2996) was employed for method development and *Waters Empower* was used as the chromatographic data software.

Methods

The method was developed with mobile phase system containing a mixture of 0.01M ammonium acetate buffer (pH 4.5) and acetonitrile in the ratio of (1:1) v/v with a flow rate of 1 ml/min on Zorbax CN column (250 x 4.6 mm), 5 µm particle size along with the use of Photo diode array detection at 274 nm.

Preparation of Mobile Phase:

Buffer and Acetonitrile was mixed in 1:1 ratio, filtered through 0.45 µ nylon membrane filter, degassed and used as mobile phase.

Preparation of buffer: Ammonium acetate (0.77 g) was accurately weighed and dissolved in 1000 ml HPLC grade water and pH was adjusted to 4.5 with glacial acetic acid. The resultant solution was filtered through 0.45 µ nylon membrane filter and degassed.

Nebivolol hydrochloride standard solution:

Stock solution: Nebivolol hydrochloride (100 mg) was accurately weighed and dissolved with mobile phase in a 50 ml standard flask and the volume was made up with the same.

Working standard solution: 5 ml of above standard stock solution was diluted to 50 ml with mobile phase to get a concentration of 200 µg/ml of Nebivolol hydrochloride. 20 µl of this solution was injected and the chromatogram was recorded.

Amlodipine besilate standard solution:

Stock solution: Amlodipine besilate equivalent to 100mg of Amlodipine was weighed precisely and dissolved with sufficient volume of mobile phase in a 50 ml standard flask and the volume was made up. *Working standard*

solution: 10 ml of above standard stock solution was diluted to 50 ml with mobile phase to get the concentration of 400 µg/ml of Amlodipine. 20 µl of this solution was injected and the chromatogram was recorded.

Preparation of mixed standard stock solution:

Nebivolol hydrochloride (100 mg) and Amlodipine besilate (equivalent to 200 mg of Amlodipine) were weighed precisely into a 50 ml volumetric flask and dissolved with sufficient mobile phase and the volume was made up with the same. *Working standard solution:* 5 ml above standard stock solution was taken in a 50 ml standard flask and diluted to 50 ml to get a concentration 200 µg/ml of Nebivolol HCl and 400 µg/ml of Amlodipine besilate. 20 µl of this solution was injected and the chromatogram was recorded (Fig.1).

Preparation of working sample solution:

Nebivolol hydrochloride and Amlodipine besilate tablets (20 tablets) were weighed accurately and crushed to fine powder. Each tablet contains 2.5 mg Nebivolol hydrochloride and 5 mg Amlodipine as Amlodipine besilate. The quantity of powder equivalent to 10 mg Amlodipine was weighed and dissolved by using sufficient amount of mobile phase in a 25 ml volumetric flask and the volume was made up to give a concentration 400 µg/ml of Amlodipine and 200 µg/ml of Nebivolol hydrochloride. The solution was filtered through 0.45 µ nylon membrane filter paper and 20 µl of this solution was injected and the chromatogram was recorded (Fig.2). The amount of Nebivolol hydrochloride and Amlodipine besilate present in each tablet formulation was calculated by comparing the peak area of the standard. The amount of drug in each tablet was calculated separately using the given formula:

Method validation:

The developed method was validated for the following parameters as per ICH Guidelines.

Specificity:

Specificity was confirmed by injecting the placebo and placebo with the spiked standard. Placebo (260 mg, prepared

$$\frac{\text{Sample area} \times \text{Standard dilution factor} \times \text{Potency} \times \text{Average weight of tablet} \times \%\ \text{purity} \times \text{Conversion factor}}{\text{Standard area} \times \text{Sample dilution factor} \times 100 \times \text{Label Claim}}$$

by mixing all excipients without active ingredient) was weighed accurately and dissolved with mobile phase in 25 ml volumetric flask and the volume was made up. The resultant solution was filtered through 0.45 µ nylon membrane filter paper and 20 µl of this solution was injected and chromatogram was recorded.

Mixed standard stock solution A (5 ml) was taken in a 50 ml volumetric flask and 130 mg of placebo was added and diluted to 50 ml with mobile phase to get a concentration of 200 µg of Nebivolol HCl and 400 µg of Amlodipine. The solution was filtered through 0.45 µ nylon membrane filter paper and 20 µl of this solution was injected and chromatogram was recorded (Fig.3).

Linearity and range:

Mixed standard stock solution A was suitably diluted with the mobile phase to obtain the concentration of 120, 160, 200, 240, 280, 320 µg/ml of Nebivolol HCl and 240, 320, 400, 480, 560, 640 µg/ml of Amlodipine. The solution was filtered through 0.45 µ nylon membrane filter paper and 20 µl of each was injected and the chromatogram was recorded. Peak area was plotted against concentration and the correlation coefficient (R) (graph says R^2) was calculated from the graph (Fig.4 and Fig. 5).

Limit of Detection (LOD):

Standard stock solution of Nebivolol hydrochloride: Nebivolol hydrochloride (25 mg) was dissolved with 25 ml of mobile phase in a 25 ml volumetric flask and 1 ml was diluted to 100 ml with the mobile phase in a 100 ml volumetric flask.

Standard stock solution of Amlodipine besilate: Amlodipine (25 mg) was transferred into a 25 ml volumetric flask and the volume was made up with 25 ml mobile phase and 1 ml was diluted to 10 ml with the mobile phase in a 10 ml volumetric flask.

Working mixed standard solution: Standard solution of Nebivolol hydrochloride (7 ml) and Amlodipine besilate standard solution (3 ml) were transferred to a 100 ml volumetric flask and diluted with the mobile phase to give a concentration of 0.7 µg/ml of Nebivolol hydrochloride and 3 µg/ml of Amlodipine. The solution was filtered through a 0.45 µ nylon membrane filter paper and 20 µl of each was injected and the chromatogram was recorded.

Limit of Quantitation (LOQ):

Standard stock solution of Nebivolol hydrochloride: Nebivolol hydrochloride (25 mg) was dissolved with 25 ml of mobile phase in a 25 ml volumetric flask and 1 ml was diluted to 100 ml with the mobile phase in a 100 ml volumetric flask.

Standard stock solution of Amlodipine besilate: Amlodipine (25 mg) was transferred into a 25 ml volumetric flask and the volume was made up with 25 ml mobile phase and 1 ml was diluted to 10 ml with the mobile phase in a 10 ml volumetric flask.

Working mixed standard solution: In a 50 ml volumetric flask, 10 ml standard solution of Nebivolol HCl and 5 ml standard solution of Amlodipine besilate were taken and the rest of the volume was made up using mobile phase to give a concentration of 2µg/ml of Nebivolol hydrochloride and 10 µg/ml of Amlodipine. 20 µl of solution was injected and the chromatogram is presented in Fig.6.

Accuracy:

Four, five, six ml of mixed standard solution (A) was taken in 3 different 50 ml volumetric flasks and 130 mg of placebo was added to each and mixed with mobile phase to obtain concentration of 160, 200, 240 µg/ml of Nebivolol HCl and 320, 400, 480 µg/ml of Amlodipine that gives 80%, 100%, 120% of working concentration. 20 µl of this solution was injected and the chromatogram was recorded.

Precision:

System Precision: Standard stock solution A (5 ml) was diluted to 50 ml to get concentration 200 µg of Nebivolol hydrochloride and 400 µg of S (-) Amlodipine. System precision was evaluated by measuring the peak response of Nebivolol hydrochloride and Amlodipine besilate for 6 replicate injections and chromatogram was recorded.

Method Precision: It was determined by quantifying the amount of Nebivolol hydrochloride and Amlodipine besilate present in tablet formulation repeatedly for 6 times and chromatogram was recorded.

Ruggedness:

Ruggedness was done by carrying out the analysis on two different days by two different analysts to check the reproducibility of the present analytical method and chromatograms were recorded.

Robustness:

Quantification of Nebivolol hydrochloride and Amlodipine besilate was done under different chromatographic conditions. The mobile phase ratio and the flow rate were altered slightly and the chromatograms were recorded.

System suitability parameters:

A solution of 200 µg/ml of Nebivolol hydrochloride and 400 µg/ml of Amlodipine was prepared using mobile phase and the chromatogram was recorded (Fig.7).

RESULTS AND DISCUSSIONS

The combination of Nebivolol HCl and Amlodipine besilate has been increasingly finding its use in the treatment of hypertension. However there is no official method for estimating Nebivolol hydrochloride and Amlodipine besilate in combined dosage form. Consequently it was necessary to develop a sensitive method for simultaneous estimation of Nebivolol hydrochloride and Amlodipine besilate.

The chromatogram recorded showed that the retention times of Amlodipine besilate and Nebivolol HCl was 9.554 min and 13.568 min respectively. The present quantitative estimation gave a satisfactory result for both Nebivolol HCl (99.4 % w/w) and Amlodipine besilate (100.52 % w/w) for explained in Table No.1.

The method was validated based on US pharmacopoeia and ICH parameters such as accuracy, precision, linearity, specificity, limit of detection, limit of quantitation, ruggedness and robustness.

The specificity results showed the absence of interference due to placebo materials and are tabulated in Table No.2.

The linearity parameters such as linear dynamic range, correlation coefficient and % curve fitting slope for Nebivolol HCl and Amlodipine besilate was calculated and the linearity curve is illustrated in Fig.4 and Fig.5. The method was found to be linear in the concentration range of 120 – 320 µg/ml for Nebivolol HCl and 240 – 640 µg/ml for Amlodipine. The LOD for Nebivolol hydrochloride and Amlodipine was found to be 0.7 µg/ml and 3 µg/ml respectively and the LOQ for Nebivolol HCl was found to be 2 µg/ml and Amlodipine 10 µg/ml.

The percentage recovery was found to be 99.2 – 102 % w/w for Nebivolol hydrochloride and 99 – 100.8 % w/w of Amlodipine besilate showed the accuracy of the proposed method (Table No.3). Percent relative standard deviation for replicated injections was found to be 0.121% for Nebivolol hydrochloride and 0.245% for Amlodipine, which are within the acceptance criteria of 2 %. Thus the proposed method showed a high degree of precision and reproducibility (Table No.4).

The result of ruggedness was found to be 98.6 – 99.5 % w/w for Nebivolol hydrochloride and 99.1 – 101% w/w for Amlodipine besilate (Table No.5) and the results confirmed the reproducibility of the developed method. The results of robustness signify that the present analytical method was robust in-spite of slight variations in mobile phase ratio and flow rate (Table No.6).

System suitability parameters were calculated to ascertain the suitability of the proposed methods on the given system on CN column and mobile phase of ammonium acetate buffer (pH 4.5) and acetonitrile (1:1) ratio. The value of resolution was found to be 4.93, which indicate a complete separation of Nebivolol HCl and Amlodipine besilate from each other with a well defined base line.

CONCLUSION

A HPLC method was developed for simultaneous estimation of Nebivolol hydrochloride and S (-) Amlodipine besilate in a combined dosage form. The HPLC (WATERS ALLIANCE 2695) – Separation module with photo diode array detector (WATERS 2996) with CN (Zorbax) column (4.6 x 250 mm), 5 µ (Agilent) was used. 20 µl was injected and eluted with mobile phase and detected by UV detector at 274 nm.

The peaks of Amlodipine besilate and Nebivolol hydrochloride were found well separated at 9.554 and 13.568 min respectively. The developed method was validated for various parameters as per ICH guidelines. The results obtained were within the acceptance criteria.

The proposed method was applied for the determination of Nebivolol hydrochloride and S (-) Amlodipine besilate in marketed formulation. The assay results confirm with the label claim of formulation. Hence, the proposed method was found to be satisfactory and could be used for the routine analysis of Nebivolol HCl and S (-) Amlodipine besilate in a tablet dosage form.

Legends

Table Legends

Table No.1 Quantitative estimation
Table No.2 Specificity for Nebivolol hydrochloride and S (-) Amlodipine
Table No.3 Recovery study data for Nebivolol hydrochloride and S (-) Amlodipine besilate
Table No.4 Precision report for Nebivolol hydrochloride and S (-) Amlodipine besilate
Table No.5 Ruggedness report for Nebivolol hydrochloride and S (-) Amlodipine besilate
Table No.6 Robustness report for Nebivolol hydrochloride and S (-) Amlodipine besilate

Figure Legends

Fig.1 Chromatogram illustrating the mixed standard solution used in the assay of amlodipine besilate and Nebivolol hydrochloride.
Fig.2 Chromatogram representing the sample assay of Amlodipine and Nebivolol hydrochloride.
Fig.3 Illustration explains the specificity of placebo and standard of Nebivolol HCl and Amlodipine.
Fig.4 Linearity curve of Nebivolol
Fig.5 Linearity curve of Amlodipine
Fig.6 Chromatogram shows validating the limit of quantitation for Nebivolol hydrochloride and Amlodipine.
Fig.7 Illustration describes the chromatogram recorded for the system suitability parameters of Nebivolol hydrochloride and Amlodipine

Table No.1 Quantitative estimation

S. No.	Tablet Sample	Label Claim (mg/tab)	Peak area*	Amount present (mg/tab)	% Label Claim (w/w)
1	Amlodipine besylate	5 mg	1455597	5	100.52 % w/w
2	Nebivolol hydrochloride	2.5 mg	2285150	2.5	99.4 % w/w

**** Each value is the mean of 6 readings***
**** Acceptance criteria: 90 – 110 % w/w***

Table No.2 Specificity for Nebivolol HCl and Amlodipine

S. No.	Sample	Area Obtained		% Content of drug (w/w)	
		Nebivolol hydrochloride	Amlodipine	Nebivolol HCl	Amlodipine
1	Standard	2279848	1458224	100.10 w/w	100.20 w/w
2	Standard + Placebo	2248879	1426780	99.90 w/w	99.94 w/w
3	Placebo	0	0	0	0

Table No.3 Recovery study data for Nebivolol HCl and Amlodipine besilate

S.No.	Recovery (%)	Average area		Amount recovered (mg)		% Recovery (w/w)	
		Nebivolol HCl	Amlodipine	Nebivolol HCl	Amlodipine	Nebivolol HCl	Amlodipine
1	80	1865287.6	1156397.6	1.98	3.96	99.01 %	99 %
2	100	2329412.6	1474332.3	2.55	5.06	102 %	101.2 %
3	120	2740983.0	1726149.3	2.97	6.05	99 %	100.85 %

Table No.4 Precision report for Nebivolol HCl and Amlodipine besilate

S. No.	Precision Parameters	Nebivolol HCl	Amlodipine
System Precision			
1	Mean (area)	2282359.23	1449983.32
2	Standard deviation	2276.309	3554.7
3	% Relative Standard deviation (Not More Than 2 %)	0.121 %	0.245 %
Method Precision			
1	Mean (% Label Claim)	100.525	99.83
2	Standard deviation	0.594768863	0.311833289
3	% Relative standard deviation (Not More Than 5 %)	0.312 %	0.591 %

Table No.5 Ruggedness report for Nebivolol HCl and Amlodipine besilate

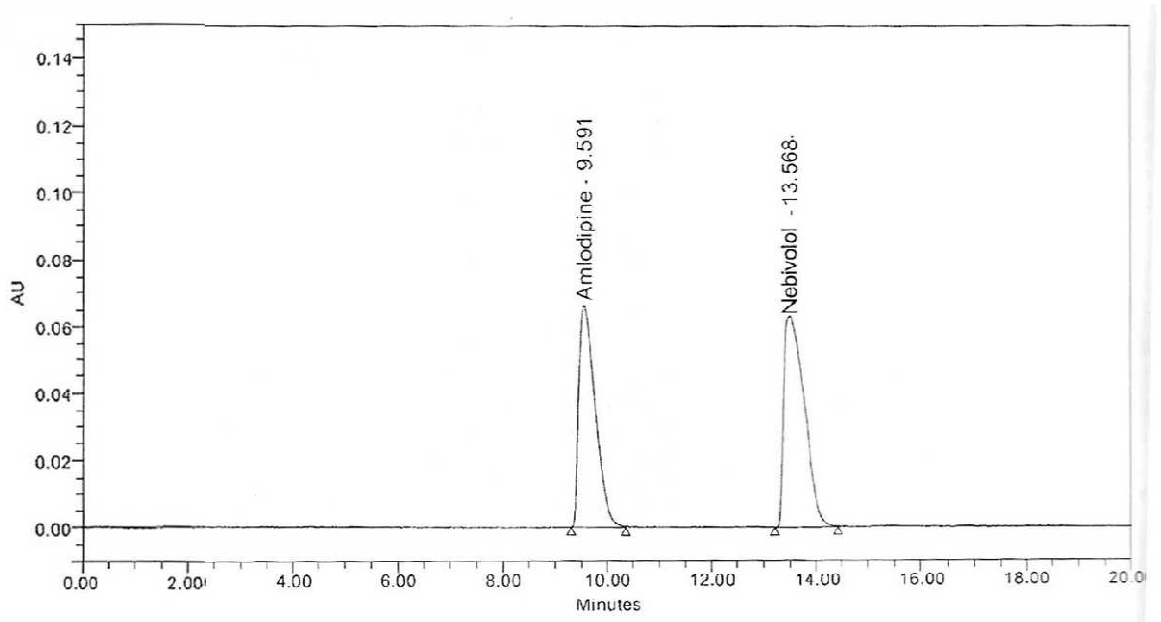
S. No.	Parameters	Nebivolol HCl	Amlodipine
1	Percentage contents	99.03 % w/w	99.73 % w/w
2	Acceptance criteria	90 – 110 % w/w	90 – 110 % w/w

Table No.6 Robustness report for Nebivolol HCl and Amlodipine besilate

S. No.	Parameters	% Relative standard deviation	
		Nebivolol HCl	Amlodipine
1	Change in flow rate (0.9 ml/min)	1.06 %	0.58 %
2	Change in flow rate (1.1 ml/min)	1.05 %	1.48 %
3	Change in mobile phase ratio (49: 51)	1.25 %	0.76 %

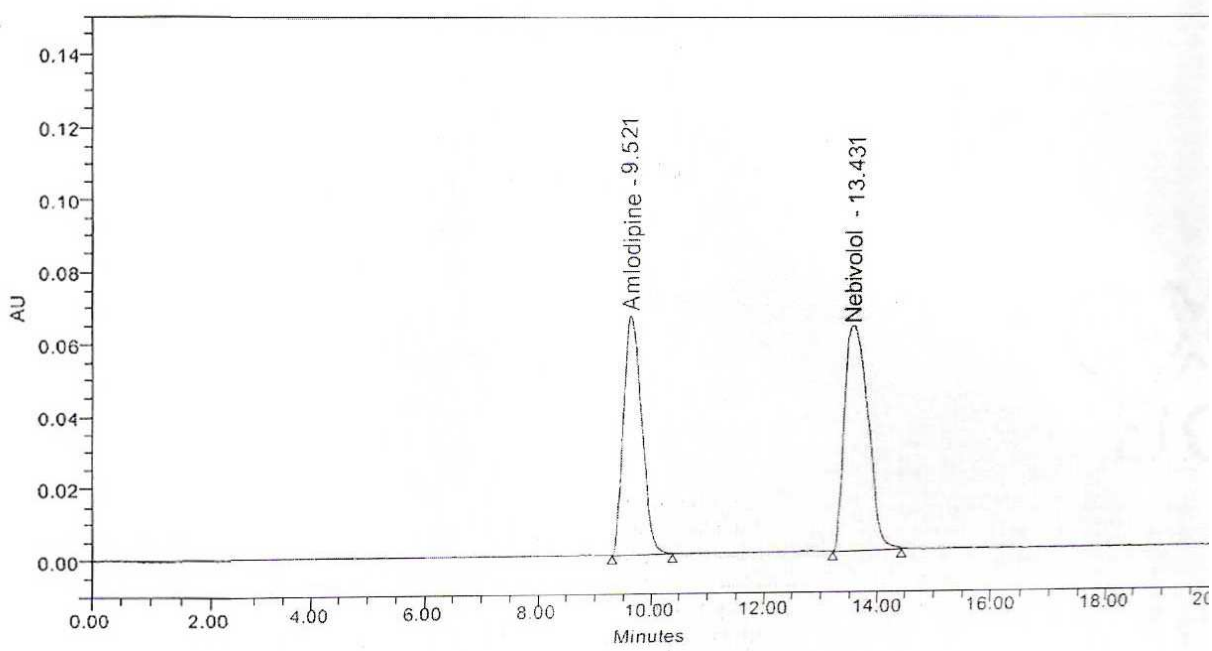
**** Acceptance criteria: Not More Than 2 %***

Fig.1 Chromatogram illustrating the mixed standard solution used in the assay of amlodipine besilate and Nebivolol HCl.



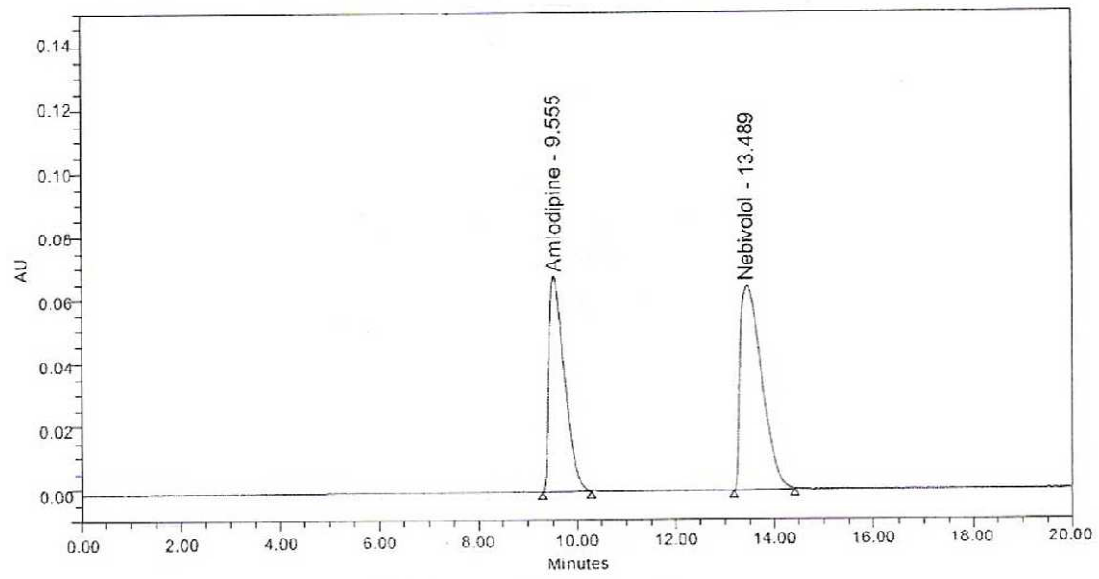
	Peak Name	RT	Area	% Area	Height
1	Amlodipine	9.591	1458224	38.96	66909
2	Nebivolol	13.568	2279848	61.04	63169

Fig.2 Chromatogram representing the sample assay of Amlodipine and Nebivolol.



	Peak Name	RT	Area	% Area	Height
1	Amlodipine	9.521	1484680	38.86	68625
2	Nebivolol	13.431	2297848	61.14	61392

Fig.3 Illustration explains the specificity of placebo and standard of Nebivolol and Amlodipine



	Peak Name	RT	Area	% Area	Height
1	Amlodipine	9.555	1426780	38.15	66582
2	Nebivolol	13.489	2248879	61.85	61232

Fig.4 Linearity curve of Nebivolol

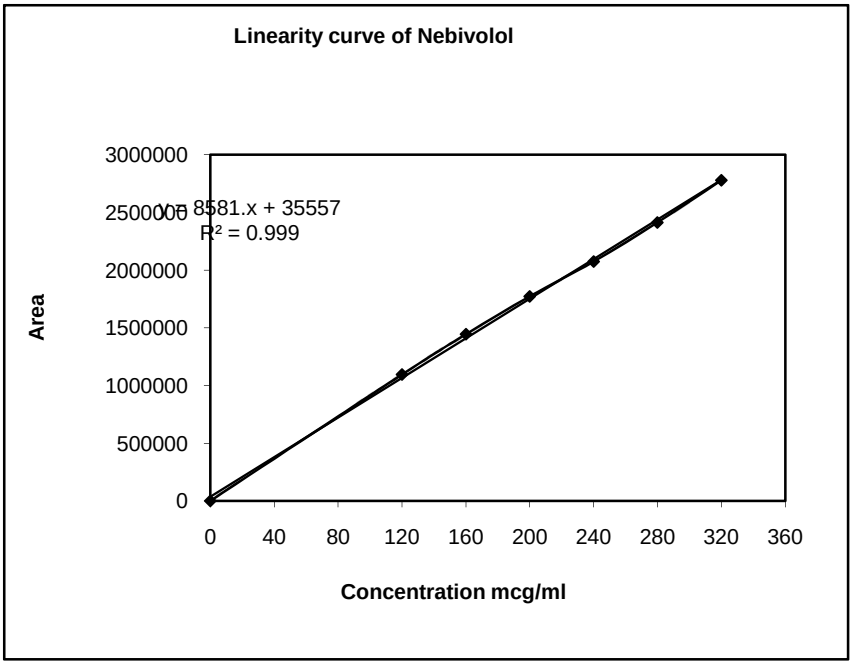


Fig.5 Linearity curve of Amlodipine

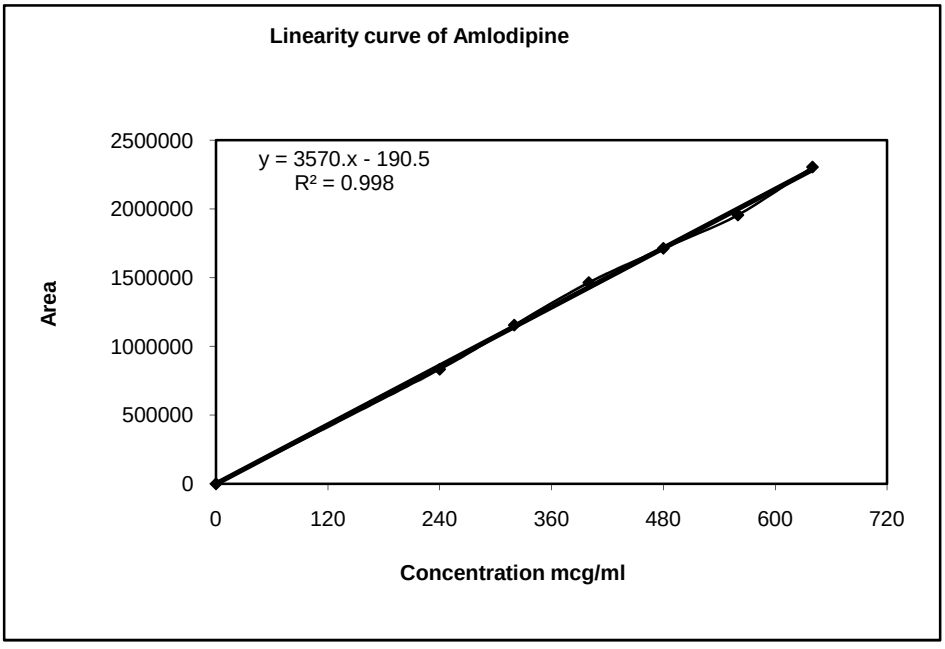
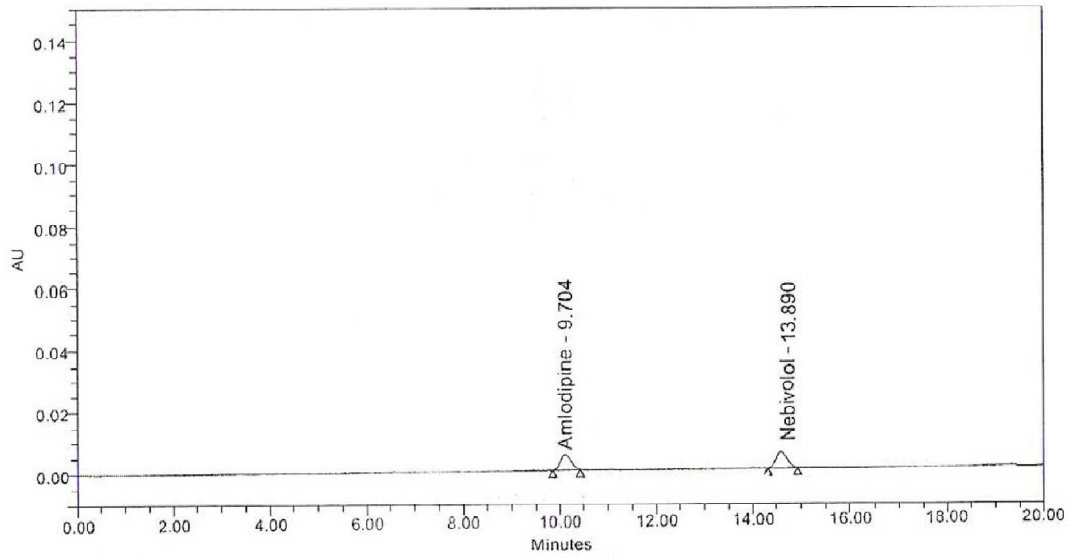
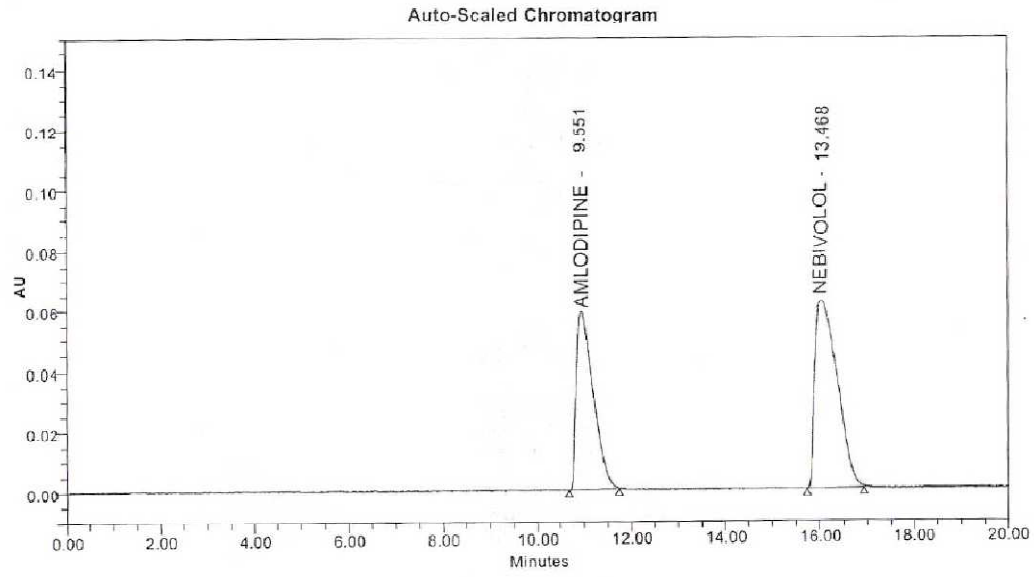


Fig.6 Chromatogram shows validating the limit of quantitation for Nebivolol and Amlodipine.



	Peak Name	RT	Area	% Area	Height
1	Amlodipine	9.704	80854	41.90	5229
2	Nebivolol	13.890	112126	58.10	3769

Fig.7 Illustration describes the chromatogram recorded for the system suitability parameters of Nebivolol and Amlodipin



Peak Results

	Name	RT	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Amlodipine	9.551	1457220	66909	4046.35	1.32	
2	Nebivolol	13.468	2282948	63169	2876.63	1.17	4.93

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