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VALIDATION OF A NOVEL RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF AMLODIPINE BESYLATE AND NEBIVOLOL HYDROCHLORIDE IN BULK AND PHARMACEUTICAL DOSAGE FORMS

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Keywords:

Amlodipine Besylate, Nebivolol Hydrochloride, RP-HPLC, Method validation

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ABSTRACT: This experimental work present the development and validation of a simple, rapid, accurate and precise RP-HPLC method for the simultaneous estimation of amlodipine besylate (AMB) and nebivolol hydrochloride (NBH) in bulk drug and pharmaceutical dosage forms. The chromatographic separation was carried out with AGILENT 1120 liquid chromatograph in an isocratic mode using Kromasil ODS column (250 x 4.6mm x 5µ particle size) with a mobile phase of mixed acetate Buffer pH 5: acetonitrile (60:40v/v) and the eluents were monitored at 268nm. The retention times of AMB and NBH were 5.26min and 6.84min respectively. The method was found to be linear over the concentration range of 5-25µg/ml for amlodipine besylate and 10-50µg/ml for nebivolol hydrochloride with a correlation coefficient of 0.999. The percentage recoveries of AMB and NBH were found to be 99.01-101.5 % and 99.2-101.0% respectively. The proposed RP-HPLC method was validated according to ICH guidelines and was employed for routine quality control analysis in bulk and combined dosage forms.

INTRODUCTION: Nebivolol hydrochloride (NBH) is chemically, α,α -[imino bis (methylene)] bis[6-fluoro-3,4-dihydro-2 *H* -1-benzopyran-2- methanol] hydrochloride (**Fig. 1**) which is a highly selective β1 receptor antagonist without partial agonist activity. It is official in Martindale, the extra pharmacopeia. Amlodipine besylate (AMB) is chemically 3-Ethyl 5-methyl 2-(2-aminoethoxymethyl)4-(2-chlorophenyl)-1,4-dihydro-6-methylpyridine-3,5dicarboxylate mono benzene sulphonate (**Fig. 2**) used in the treatment of hypertension and congestive heart failure [1-4]. It is official in British pharmacopoeia.

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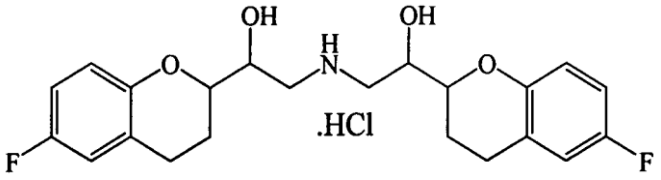

FIG. 1: CHEMICAL STRUCTURE OF NEBIVOLOL HYDROCHLORIDE

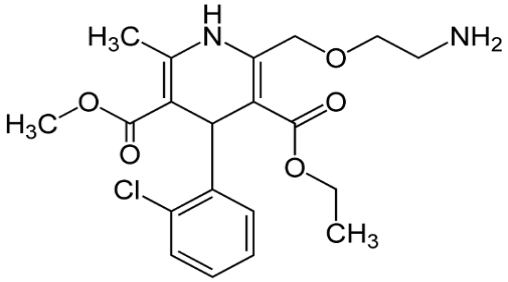

FIG. 2: CHEMICAL STRUCTURE OF AMLODIPINE BESYLATE

A literature search revealed that very few methods [5-12] are published for the determination of combination of Amlodipine and Nebivolol. The objective of the present work was to design a validation procedure which can efficiently determine both drugs in tablet dosage form within a short run time and with good resolution. The present RP-HPLC method was validated by following ICH guidelines.

Instrumentation: An Agilent model 1120 compact LC system equipped with a VWD detector was used. The HPLC method uses a kromasil ODS 250 x 4.6mm x 5µ particle size column with a mobile phase consisting 60:40 (v/v) Acetate buffer pH 5 and Acetonitrile respectively. Detection was carried out at 268 nm and the flow rate 1.2 ml/min. Data was recorded by using EZchrome software.

Reagents and Chemicals: Acetonitrile HPLC grade, HPLC grade water was procured from Merck specialities (India) Ltd., Mumbai. Working standard of NBH was provided by Yarrow chem. Laboratories Ltd., Mumbai and AMB was provided by Mylan Laboratories Ltd., Hyderabad, Sodium acetate A.R. grade from Merck chemicals Mumbai, India.

Optimized Chromatographic Condition: Kromasil ODS column (250 × 4.6mm, 5µ particle size) Agilent column was used as the stationary phase. The mobile phase comprised of acetonitrile and acetate buffer pH 5 and in proportion of 40:60 (v/v) with pH adjusted to 5.0 by using glacial acetic acid. Injection volume was 20µl and run time was 20min and flow rate 1.2ml/min. The column was maintained at ambient temperature and the eluent was detected at 268nm.

Preparation of Mobile Phase: Mobile phase was prepared by mixing Acetonitrile and Acetate buffer pH 5 in the ratio of 40:60 v/v was sonicated for 15 min and filtered through a 0.45µm membrane filter paper.

Preparation of Standard Solutions: Standard stock solution (1000µg/ml) of Nebivolol hydrochloride and Amlodipine besylate were prepared separately in mobile phase comprised of acetonitrile and acetate buffer pH 5 and in proportion of 40:60 (v/v) with pH adjusted to 5.0 by using glacial acetic acid. The working standard solutions were prepared and further diluted in mobile phase to Nebivolol hydrochloride and Amlodipine besylate contain a mixture of in over the linearity ranges from 10-50µg/ml and 5-25µg/ml respectively (working stock solution A).

Preparation of Sample Solution: Twenty tablets were weighed and finely powdered. A quantity of powder equivalent to 5mg of NBH and 2.5mg of AMB was weighed and transferred to a 25 ml volumetric standard flask and added 10 ml of mobile phase. The sample was kept in an ultrasonic bath for 20 min and further diluted to 25 ml by using mobile phase to get 50µg/ml of NBH and 25µg/ml of AMB. Then it is filtered through 0.45µ membrane filter paper (Working stock solution B).

Method Validation:

1. **System Suitability:** The system was deemed suitable if the following acceptance criteria were satisfied. The relative standard deviation (RSD) of the peak area responses for Amlodipine and Nebivolol from six standard solution injections was not more than 2.0%. The relative standard deviation (RSD) of the retention time of Amlodipine and Nebivolol was not more than 1.0%. The tailing factor for the Amlodipine and Nebivolol in the peak was not more than 2.0. The resolution between Amlodipine and Nebivolol peaks was not less than 1.5. Theoretical plate counts in standard solution were not less than 1000. Results were reported in **Table 1**.

2. **Specificity:** Specificity demonstrated that, the solvents peaks, diluents and placebo peaks are not interfering with the analyte peak and suitability of analytical method for stability of Amlodipine and Nebivolol. To evaluate the interference injected individual standard solutions, diluent & placebo to ensure that the method used for determination of Amlodipine and Nebivolol is specific.

3. **Linearity:** The response for the detector was determined to be linear over the range of 5-25µg/ml of AMB and 10-50µg/ml for NBH. Calibration curves were plotted between concentration and the area response.

The proposed method was evaluated by its correlation coefficient and intercept value calculated in the statistical study. The calibration curves for AMB and NBH were shown in figure 5&6 respectively and their corresponding linearity parameters were given in **table 2**.

4. **Precision:** The %RSD of interday and intraday precision obtained was less than2%for both the drugs. The intraday and interday precision of AMB was 0.182 and 0.331 and NBH was 0.231 and 0.452 respectively. From the data obtained, the developed HPLC method was found to be precise and the values are given in **table 3**.

5. **Accuracy:** Recovery studies were carried out by applying the standard addition method. A known amount of standard NBH and AMB corresponding to 50%, 100%, and 150% of the label claim was added to pre analyze sample of tablet dosage form separately. The recovery studies were carried out six times at each level of recovery. From the data obtained from **table 4**, recoveries of standard drugs were found to be accurate.

6. **LOD and LOQ:** Limit of detection (LOD) and limit of quantification (LOQ) were calculated as 3.3 σ/*S* and 10 σ/*S*, respectively as per ICH guidelines, where σ is the standard deviation of the response (*Y*-intercept) and *S* is the slope of the calibration plot. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3).The LOD for AMB and NBH was found to be 0.36µg/ml and 1.43µg/ml, respectively. The LOQ is the smallest concentration of the analyte which gives response that can be accurately quantified (signal to noise ratio of 10).The LOQ was 1.10µg/ml and 4.41µg/ml for AMB and NBH respectively (Table 2).

7. **Ruggedness:** The Ruggedness of the method was evaluated using different analyst and different instrument in the same laboratory. The average % variation of six measurement of test sample of analyst-1 and analyst-2 was 0.11 % and 0.10% for Amlodipine, 0.19 % and 0.45% for Nebivolol (NMT 2.0%).

8. **Robustness:** The method was found to be robust with respect to flow rate and column temperature without any changes in system suitability parameters such as resolution, tailing factor and theoretical plate. The values are given in **Table 5**.

RESULTS AND DISCUSSION: From the UV spectrophotometric identifications for simultaneous estimation of Nebivolol hydrochloride and Amlodipine besylate in overlay mode showed that both the drugs absorb appreciably at 268nm, hence 268nm was selected as the detection wavelength. Several mobile phases were tried initially for the simultaneous quantitation of both the drugs, but optimum results were observed with a mobile phase composition of acetate buffer with pH 5 and acetonitrile in the ration of 60:40v/v. The two peaks were symmetric and sufficiently resolved.

System suitability was assessed by injecting 5 replicate injections of 100% test concentration. Number of theoretical plates was more than 2000 for both the drugs and tailing factor was less than 1.5 for both Nebivolol hydrochloride and Amlodipine besylate was reported. A resolution of greater than 2 was observed. The results were given in the **Table 1**.

TABLE 1: SYSTEM SUITABILITY

Parameters	Amlodipine Besylate	Nebivolol Hydrochloride
Retention Time (min)	5.26	6.76
Resolution (R_s)	1.5	
Tailing Factor (T)	1.12	1.03
Theoretical Plates (N)	11230	10463

Specificity of the chromatographic method was tested by injecting sample concentration prepared from marketed formulation. The response was compared with that obtained from the standard drug. The chromatogram confirms the presence of Nebivolol hydrochloride and Amlodipine besylate at 5.26 min and 6.76 min respectively without any interference. Thus, the developed method was specific to Nebivolol hydrochloride and Amlodipine besylate. An optimised chromatogram with the retention times of Nebivolol hydrochloride and Amlodipine besylate was given in the **Figure 2**.

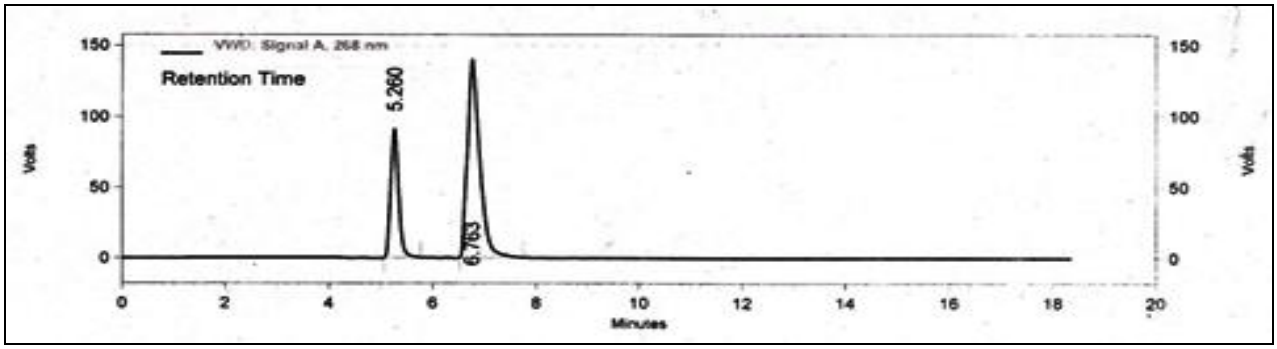

FIG. 2: TYPICAL CHROMATOGRAM OF STANDARD SOLUTION OF 25µg/ml OF AMLODIPINE BESYLATE AND 50µg/ml OF NEBIVOLOL HYDROCHLORIDE

The peak areas corresponding to the concentration range of amlodipine besylate 5-25 µg/ml and nebivolol hydrochloride 10-50 µg/ml prepared in triplicate were plotted against the respective concentrations. The calibration curves were linear in the range studied for amlodipine besylate and nebivolol hydrochloride, respectively, with mean correlation coefficients (n = 3) of 0.999 and higher, the representative calibration curve was shown in **Figure 3 and 4** respectively. The regression analysis was given in the Table 2.

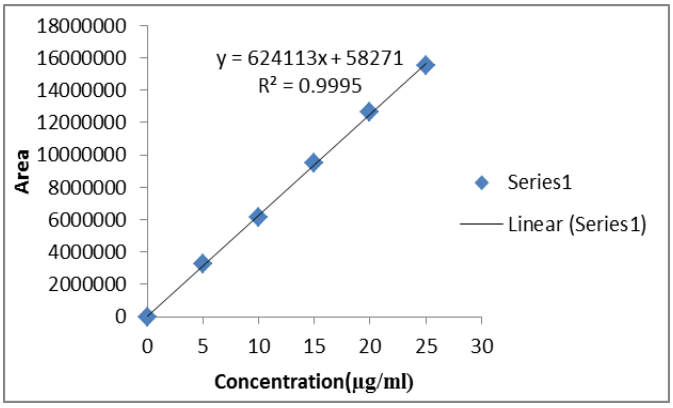

FIG. 3: CALIBRATION CURVE OF AMLODIPINE BESYLATE

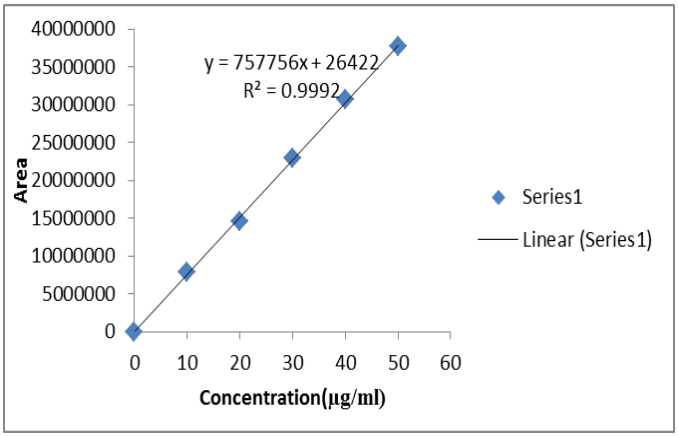

FIG. 4: CALIBRATION CURVE OF NEBIVOLOL HYDROCHLORIDE

TABLE 2: RESULTS FOR LINEARITY (n=6)

Parameters	Amlodipine besylate	Nebivolol Hydrochloride
Slope	62411	75775
Y- intercept	58271	26422
Correlation coefficient	0.999	0.999
Regression Equation	Y=62411x + 58271	Y=75775x + 26422
Linearity range	5-25 µg/ml	10-50 µg/ml
LOD	0.36	1.43
LOQ	1.10	4.41

∗n= No. of determinants

Accuracy of the method was examined by performing recovery studies by standard addition method for drug product. The recovery of the added standard to the drug product sample was calculated and it was found to be 97.9-103.44 %w/w and 98.9-103.1%w/w for Cinitapride and Pantoprazole respectively and the % RSD was less than 2 for both the drugs which indicates a good accuracy of the method. The results of recovery were given in the Table 3. The method was precise with a %RSD of less than 2 for both Cinitapride and Pantoprazole respectively. The results of intraday and inter day precision were given in the Table 4.

Limit of detection of Cinitapride and Pantoprazole were 0.34 µg/mL and 3.38µg/mL respectively. Limit of detection of Cinitapride and Pantoprazole were 1.04 µg/mL and10.15 µg/mL respectively. LOD and LOQ values were given in the Table 5. Robustness was carried out by change in the flow rate (±1mL/min), percent organic phase (±5%) and variation in wavelength (± 2 nm). Solution of 100% concentration was prepared and injected in triplicate for each varied operational condition and % R.S.D was found to be less than 2. The Results were given in the **Table 5**.

TABLE 3: RESULTS FOR ACCURACY (n=3)

Recovery level	Amount of Standard drug added (µg/ml)		Amount of test added(µg/ml)		Total Amount Recovered (µg/ml)		% Recovery w/w	
	AMB	NBH	AMB	NBH	AMB	NBH	AMB	NBH
50%	2.5	5	2.5	5	4.95	9.92	99.0	99.2
100%	7.5	15	2.5	5	10.12	20.02	101.2	100.1
150%	12.5	25	2.5	5	15.23	30.3	101.5	101.0

TABLE 4: RESULTS OF PRECISION (n=6)

Drug	Intraday Precision (%RSD)
Amlodipine besylate	0.182
Nebivolol hydrochloride	0.231

∗n= No. of determinants

TABLE 5: RESULTS FOR ROBUSTNESS

Parameters (n=3)	%RSD	
	Amlodipine Besylate	Nebivolol Hydrochloride
Detection wavelength at 266nm	1.14	1.05
Detection wavelength at 270m	0.14	0.80
Flow rate 1.0ml/min	0.41	1.42
Flow rate 1.4ml/min	0.23	0.81

∗n= No. of determinants

Assay of marketed formulation: A 20 µL injection volume of test concentration containing 25 µg/mL AMB and 50 µg/mL NBH solution was injected in triplicate to the chromatographic system and the peak response was measured. The content of each component in the formulation was estimated by comparing the peak area of the test sample with that of the peak area of the standard. The results of estimation were given in **Table 6**.

TABLE 6: TABLE FOR ASSAY

S. No.	Amount Present in (mg/tab)		Amount Obtained in (mg/tab)		Label Claim %w/w	
	AMB	NBH	AMB	NBH	AMB	NBH
1	2.5	5	2.49	4.98	99.8	99.2

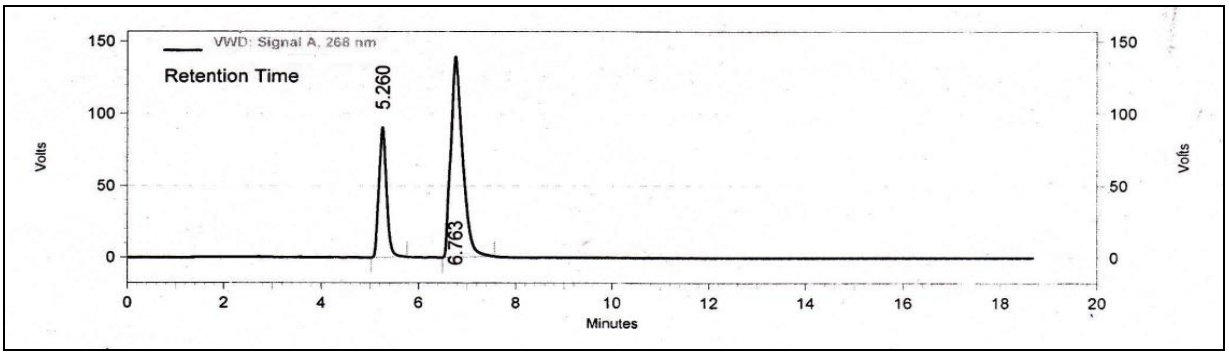

FIG 5: TYPICAL CHROMATOGRAM OF TEST SOLUTION OF 25µg/ml OF AMLODIPINE BESYLATE AND 50µg/ml OF NEBIVOLOL HYDROCHLORIDE

CONCLUSION: The proposed RP-HPLC method for the simultaneous estimation of nebivolol hydrochloride and amlodipine besylate in combined dosage forms is accurate, precise, linear, rugged, robust, simple and rapid. Hence the present RP-HPLC method is suitable as a quality control of the analysis of raw materials, formulations and in dissolution studies.

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