Research journal of Multidisciplinary Bulletin
(An Open Access International Peer Reviewed Journal)
ISSN: 2945-4166

Research Article

Development and Validation of a Reverse Phase HPLC method for Simultaneous Estimation of Nebivolol and Amlodipine in Bulk and Tablet Dosage Form

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Abstract: A simple, accurate, precise, and reproducible reverse phase high performance liquid chromatographic (RP-HPLC) method was developed and validated for the simultaneous estimation of Nebivolol and Amlodipine in bulk drug and pharmaceutical tablet dosage form. Chromatographic separation was achieved using an Inertsil C18 column (4.6 × 250 mm, 5 µm particle size) with a mobile phase consisting of Acetonitrile and Phosphate Buffer (60:40 v/v, pH 4.0) at a flow rate of 1.0 mL/min. The detection wavelength was selected at 265 nm using a UV detector. The retention time of Amlodipine and Nebivolol was found to be 2.987 min and 5.177 min respectively. The developed method was validated according to ICH guidelines for parameters such as linearity, accuracy, precision, specificity, robustness, ruggedness, limit of detection (LOD), and limit of quantitation (LOQ). The method exhibited excellent linearity within the concentration range of 2–20 µg/mL for both drugs with correlation coefficients greater than 0.999. Recovery studies indicated good accuracy with percentage recovery close to 100%. The proposed method was found to be simple, rapid, reliable, and suitable for routine quantitative analysis of Nebivolol and Amlodipine in combined pharmaceutical dosage forms.

Keywords: RP-HPLC, Nebivolol, Amlodipine, Tablet

How to cite this article: Prayag R. Dandge, Satish S. Padgilwar, Lokesh R. Gandhi, Nitin S. Bhajipale. Development and Validation of a Reverse Phase HPLC method for Simultaneous Estimation of Nebivolol and Amlodipine in Bulk and Tablet Dosage Form. **Research journal of Multidisciplinary Bulletin. 2026 May, 5(2):1-11**

Source of support: Nil.

Conflict of interest: None

DOI: doi.org/10.58924/rjmb.v5.iss2.p1

Received: 08-05-2026
Revised: 11-05-2026
Accepted: 13-05-2026
Published: 25-05-2026

1. Introduction

Mikhail Tswett, a Russian botanist separated plant pigments through calcium carbonate column in the year 1903 which formed the basis for Chromatography. The term chromatography has been derived from the Greek words chroma means color and graphy it means to write i.e. color writing or chromatography [1].

Chromatography is considered as one of the most vibrant and versatile analytical methods. The separation of two or more components in a mixture can be well established by employing chromatography with the aid of two phases namely stationary and mobile phases. High Performance or High-pressure Liquid Chromatography (HPLC) is extensively available analytical technique and also ranks high as the most sensitive and unique analytical procedures which easily deal with combination mixtures. C18Chemicallybondedoctadecyl silane (ODS)is a basic compound containing eighteen carbon atoms. HPLC plays an escalator role in quality assurance, stability testing, quantitative analysis of drugs in bulk and formulations. This study focuses on the development of RP-HPLC method for analysis of combined drugs.

1. Materials And Methods:

1. Materials

In HPLC method development, careful consideration of the quality reagents, instrumentation, and analytical conditions is essential to ensure reproducible, accurate,

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and precise results and for reliability of analytical method. The drug used for present investigation was obtained from MG lab Hyderabad as gift sample.

- **Details of Pure Drug:** Drugs selected were Nebivolol (Purity-99.6) and Amlodipine (Purity-99.3) both were taken in quantity of 10g each and were supplied by MG Lab India
- **Marketed Preparation:** Marketed preparation selected was of Brand name 'Nodon AM' containing the contents of drug Nebivolol and Amlodipine 5mg each, and manufactured by Cadila Pharmaceuticals Ltd.
- **Reagents and Chemicals:** Chemicals were used such as Acetonitrile HPLC grade, Methanol HPLC grade, Ortho-phosphoric acid, water HPLC grade, manufactured by Merck Ltd., India.
- **Instruments:** Instruments used were UV spectrophotometer (Mfg. by Shimadzu, Model-Doublebeam-UV1900I), HPLC (Mfg. by Agilent, Model - 1220 series infinity LC gradient system VL- G4288C UV Detector), pH Meter (Mfg. by Equip-tronich, Model-EQ-610), Analytical column (Mfg. by Intersil ODS, MODEL- C18, (4.6 x 150 mm)), Balance (Mfg. by Phoenix Gold, Model- 300 P), Ultrasonicator (Mfg. by Labman, Model – LMUC2).

2. Methods & Procedure

- **Identification and characterization of drug:** Previous to commenced the experimental work it is necessary to determine the different physical and chemical property of the drug which provide information regarding the purity and the nature of the drug. This will help in selection of solvents and procedures for the robust and stable analytical method development of Nebivolol & Amlodipine.
- **Selection and procurement of drug:** Nebivolol (NEB) & Amlodipine (AML) were selected as model drug candidate for the method development and validation. The procured drug was analyzed for different physical properties viz. color, odor, melting point, etc.
- **Solubility Studies:** Solubility of both drugs was tested in different solvents to obtain a common solvent which can be used for simultaneous estimation of both drugs in a mixture.
- **Melting point range determination:** Melting point of Nebivolol (NEB) & Amlodipine (AML) were determined by placing small amount of sample in capillary tube closed at one end and holds the capillary on melting point apparatus.
- **UV Spectroscopy Analysis:** The ultraviolet absorption spectrums of NEB & AML were obtained using Shimadzu 1800-UV visible spectrophotometer and 1 cm quartz cells, over a wavelength range of 400 to 200 nm.

1. Selection of mobile phase:

a. Preparation of standard solutions:

NEB standard solution: Accurately weighed quantity 5mg of NEB was dissolved in methanol and volume was made up to 25 ml mark (200µg/ml).

AML standard solution: Accurately weighed quantity 5mg of AML was dissolved in methanol and volume was made up to 25 ml mark (200µg/ml).

b. **Procedure:** The standard solution containing mixture of NEB and AML was run and different individual solvents have been tried to get a good separation and stable peak. Each phase was filtered through Whatman filter paper No. 42. From various mobile phase tried, mobile phase containing Acetonitrile: Phosphate buffer (60:40) pH was selected, since it gives sharp reproducible retention time for NEB & AML.

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- **Chromatographic conditions:**

Column: Intersil 4.6(id) x 250mm
Particle size packing: 5µm
Stationary phases: C18 Intersil
Mobile phase: Acetonitrile: Buffer (60:40) pH4
Detection wavelength : 265 nm
Flow rate: 1 ml/min.
Temperature: Ambient
Sample size: 20 Ml

2. Preparation of calibration curve:

i. Preparation of standard stock solution:

ACE standard stock solution: Accurately weighed quantity 10 mg of NEB was dissolved in methanol and volume was made up to 100 ml mark (100µg/ml).

AML standard stock solution: Accurately weighed quantity 10 mg of AML was dissolved in methanol and volume was made up to 100 ml mark (100µg/ml).

ii.**Procedure:** The mobile phase was allowed to equilibrate with the stationary phase until steady baseline was obtained. The series of concentration from 2-20 µg/ml for NEB and 2-20 µg/ml of AML solutions were injected and peak area was recorded.

3. System suitability test:

Preparation of standard drug solution

NEB standard solution: Accurately weighed quantity 10 mg of NEB was dissolved in mobile phase and volume was made up to 100 ml mark.

AML standard solution: Accurately weighed quantity 10mg of AML was dissolved in mobile phase and volume was made up to 100 ml mark.

B) Procedure: Filtered mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. A 20 µL std. drug solution was injected was made five replicates and the system suitability parameters were recorded.

4. Application of proposed method for estimation of NEB and AML Laboratory mixture:

Preparation of laboratory mixture (standard): Accurately weighed quantity of NEB 10 mg was transferred to 100 ml volumetric flask, shaken vigorously for five minutes and volume was made up to mark with mobile phase. And accurately weighed quantity of 10 mg was transferred to 100 ml volumetric flask, shaken vigorously for five minutes and volume was made up to mark with mobile phase.

Preparation of laboratory mixture (sample): Three different laboratory mixtures of NEB and AML were prepared by appropriately weighing the quantities of drug samples so as to get the concentration of 05 µg/ml of NEB and 05 µg/ml of AML. The peak area of standard laboratory mixture and sample laboratory mixture as compared to obtain the concentration.

5. Application of proposed method for estimation of NEB and AML in formulation

Standard stock solution:

Accurately weighed quantity of NEB 10 transferred to 100 ml volumetric flask, shaken vigorously flask, shaken vigorously foe five minutes and volume was made up to mark with mobile phase. And accurately weighed quantity of AML 10 mg was transferred 100 ml volumetric flask, shaken vigorously for five minutes and volume was made up to mark with mobile phase.

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Sample solution preparation: The quantity of drug in 1 ml is calculated with the help of density bottle on this the drug is taken and diluted with mobile phase. The solution was filtered through whatman filter paper No. 42.

Procedure: Equal volumes (20L) of standard and sample solution were injected separately after equilibrium of stationary phase. The chromatograms were recorded and the response i.e. peak area of major peaks were measured .The content of NEB and AML was calculated by comparing a sample peak with that of standard.

6. **Validation parameters:**

a. **Accuracy:** It was ascertained on the basis of recovery studies performed by standard addition method.

Preparation of standard solution: An accurately weighed quantity of tablet formulation was taken in 10 ml volumetric flask; to standard solution of NEB and AML was added in different proportions. Then volume was adjusted up to the mark with the mobile phase. The solution was diluted with mobile phase to get final concentration & filtered through whatman filter paper No. 41.

b. **Precision:** Precision of an analytical method is expressed as S.D. or R.S.D of series of measurements. It was ascertained by replicate estimation of the drugs by proposed method.

c. **Ruggedness:** In the present HPLC method, ruggedness was assessed by analyzing the same homogeneous sample under different experimental conditions, including analysis by different analysts with different days while maintain identical chromatographic parameters. The assay results obtained were carried out under two different conditions-

1. Days
2. Analyst

d. **Specificity:** Specificity was measured as ability of the proposed method to obtain well separated peak for NEB and AML without any interference from the component of matrix.

Mean retention time for-
NEB – 5.177
AML – 2.987

e. **Linearity and range:** According to the USP tablet powder equivalent to 80, 90, 100, 110, 120% of label claim was taken and dissolved & diluted appropriately with mobile phase to obtain a concentration in the range of 80% - 120% of the test concentration. The chromatograms of the resulting solutions were recorded. NEB and AML marketed formulation was found to be linear in the range ± 20% of the test concentration of the respective drug.

f. **Robustness and ruggedness:** The ability of an analytical method to remain unaffected by small variations in method parameters and influential environmental factors and characterize its reliability during normal usage is called as ruggedness. No change of the detected amount of the analyte in a certain sample in spite of the variation of the method parameter. The robustness study indicated that the factors selected remained unaffected by small variation of organic composition of mobile phase, wavelength and the flower rate.

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g. **Limit of Detection (LOD) and Limit of Quantitation (LOQ):** Limit of detection is the amount of analyte in a sample which can be detected but not necessarily quantitated an exact value. Limit of quantitation is the lowest amount of analyte in a sample which can be quantatively determined with suitable precision accuracy. As per ICH guideline both LOD & LOQ were performed on the basis of standard deviation of the slope.

3. Result & Discussion

A fixed dose combination containing Nebivolol (NEB) & Amlodipine (AML) is recently available in market as tablet dosage form.

Percent purity of above mentioned drugs were reported by Supplier Company as follows-

1. Nebivolol (NEB) - 99.6 %
2. Amlodipine (AML) - 99.3 %

Primarily this was not analyzed in our study and the % purity stated by the suppliers was taken as standard for comparison studies. The various tools used for characterization of the drug molecules including melting point, UV spectroscopy, solubility study, etc. The solubility study, melting point analysis, UV spectroscopy of the drug was done.

1. Solubility study

a. Nebivolol

Table No. 01: Result of solubility study of Nebivolol

Sr. No	Solvent/ Medium	Solubility Observation
1	Water	Slightly soluble
2	Methanol	Freely soluble
3	Ethanol	Freely soluble
4	Acetonitrile	Soluble
5	Chloroform	Sparingly soluble
6	Dimethyl sulfoxide (DMSO)	Freely soluble

Nebivolol hydrochloride exhibits moderate solubility in polar organic solvents and limited solubility in water. The drug is slightly soluble in water but freely soluble in organic solvents such as methanol and ethanol. It is also soluble in acetonitrile and dimethyl sulfoxide, whereas it shows sparing solubility in chloroform. This solubility profile is important for selecting suitable solvents during analytical method development, particularly in reverse-phase high-performance liquid chromatography (RP-HPLC) for drug analysis.

b. Amlodipine

Table No. 02: Result of solubility study of Amlodipine

Sr. No.	Solvent	Solubility of Amlodipine
1	Water	Slightly soluble
2	Methanol	Freely soluble
3	Ethanol	Freely soluble
4	Acetonitrile	Soluble
5	Chloroform	Sparingly soluble
6	Dimethyl sulfoxide (DMSO)	Freely soluble

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Amlodipine besylate exhibits moderate solubility characteristics depending on the polarity of the solvent such as methanol and ethanol. It is also soluble in acetonitrile and dimethyl sulfoxide, whereas it shows sparing solubility in chloroform.

2. **Melting point range determination:** Melting point of drug was determined by placing small amount of sample in capillary tube closed at one end and holds the capillary on melting point apparatus. The melting point of NEB and AML was found to be 223 ºC and 199 ºC, respectively.

3. **UV spectroscopy Analysis:** The ultraviolet absorption spectrum of NEB & AML was obtained using Shimadzu 1800- UV visible spectrophotometer and 1 cm quartz cells, over a wavelength range of 400 to 200 nm. Drug wavelength maxima (λ max) of NEB and AML were observed to be 276 nm and 236 nm, respectively. From the overlain spectrum the wavelengths selected foe estimation of drugs is 265 nm.

4. **Selection of mobile phase:** Each mobile phase was filtered through Whatman filter paper No. 42. Peak, well resolved peaks with symmetry within limits and significant Based on sample solubility & stability, various mobile phase compositions were evaluated to achieve acceptable separation using selected chromatographic conditions.

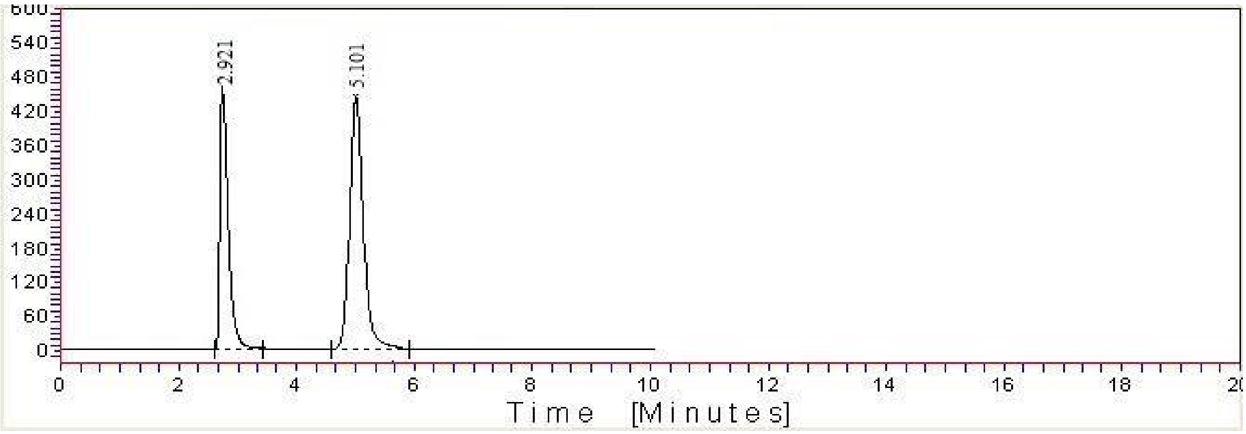

Fig no. 01: final chromatogram obtained by using Acetonitrile: Buffer (60:40) pH4 as mobile phase

Chromatographic conditions:
Column: Intersil 4.6(id) x 250 mm
Particle size packing: 5µm
Stationary phases: C18 Intersil
Mobile phase: Acetonitrile: Buffer (60:40) pH4
Detection wavelength : 265 nm
Flow rate: 1ml/min
Temperature: Ambient
Sample size: 20 µL

5. **Preparation of calibration curve:** The series of concentration from µg/ml for 2-20 µg/ml NEB and 2-20 µg/ml for AML drugs solutions were injected and peak area was recorded.

6. **System suitability test:** System suitability is a pharmacopoeial requirement and is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis.

7. **Application of proposed method for estimation of NEB and AML Laboratory Mixture:** The peak area of standard laboratory mixture and sample laboratory mixture was compared to obtain the concentration.

8. **Application of proposed method for estimation of NEB and AML in formulation:** Equal volumes (20µL) of standard and sample solution were injected separately after equilibrium of stationary phase. The chromatograms were recorded and the responses were measured. The content of NEB and AML was calculated by comparing a sample peak with that of standard.

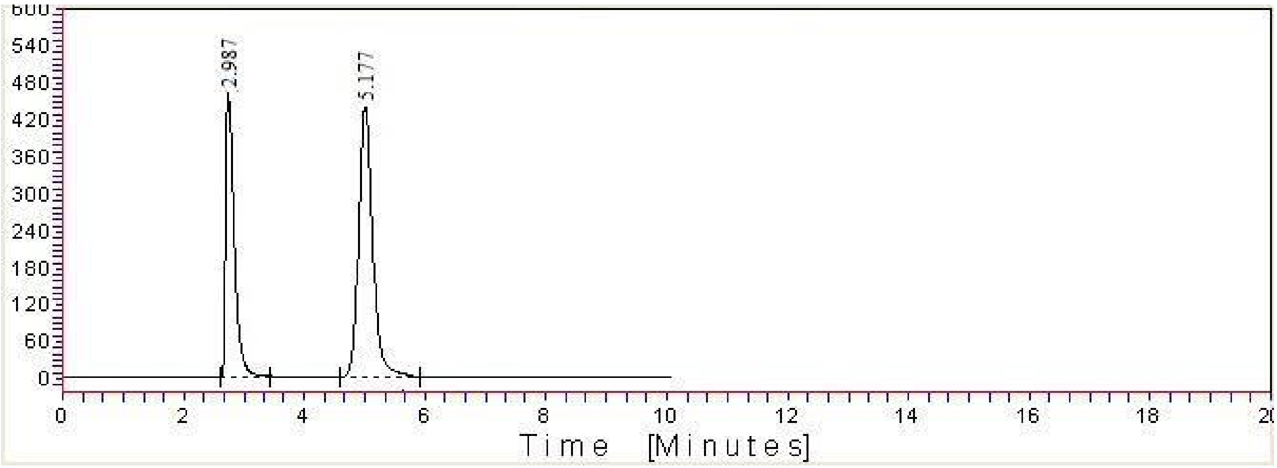

Fig. No. 02: Chromatogram obtained by formulation of NEB and AML

9. **Validation parameters:**

a. **Accuracy:** It was ascertained on the basis of recovery studies performed by standard addition method.

b. **Precision:** Precision of an analytical method is expressed as S.D. or R.S.D of series of measurements. It was ascertained by replicate estimation of the drugs by proposed method.

c. **Ruggedness:** The studies of ruggedness were carried out under two different conditions-

1. Days
2. Analyst

a. **Interday (Different days):** Same procedure was performed as under marketed formulation analysis on different days. The % label claim was calculated. Data was obtained for day 1. Day 2, and day 3.

b. **Interday:** It was performed using same procedure as under marketed formulation analysis and absorbance recorded at 3 hrs. Interval within a day.

c. **Different analyst:** The sample solution was prepared by two different analysts and same procedure was followed as described earlier. The % label claim was calculated as done in marketed formulation estimation.

d. **Specificity:** Specificity was measured as ability of the proposed method to obtain well separated peak for NEB and AML without any interference from component of matrix. Mean retention time for –

NEB – 5.177
AML – 2.987

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e. **Linearity and range:** According to USP tablet powder equivalent to 80, 90, 100, 110, 120 % of label claim was taken and dissolved & diluted appropriately with mobile phase to obtain a concentration IN THE RANGE OF 80%-120% of the test concentration. NEB and AML marketed formulation was found to be linear in the range ±20% of the test concentration of the respective drug

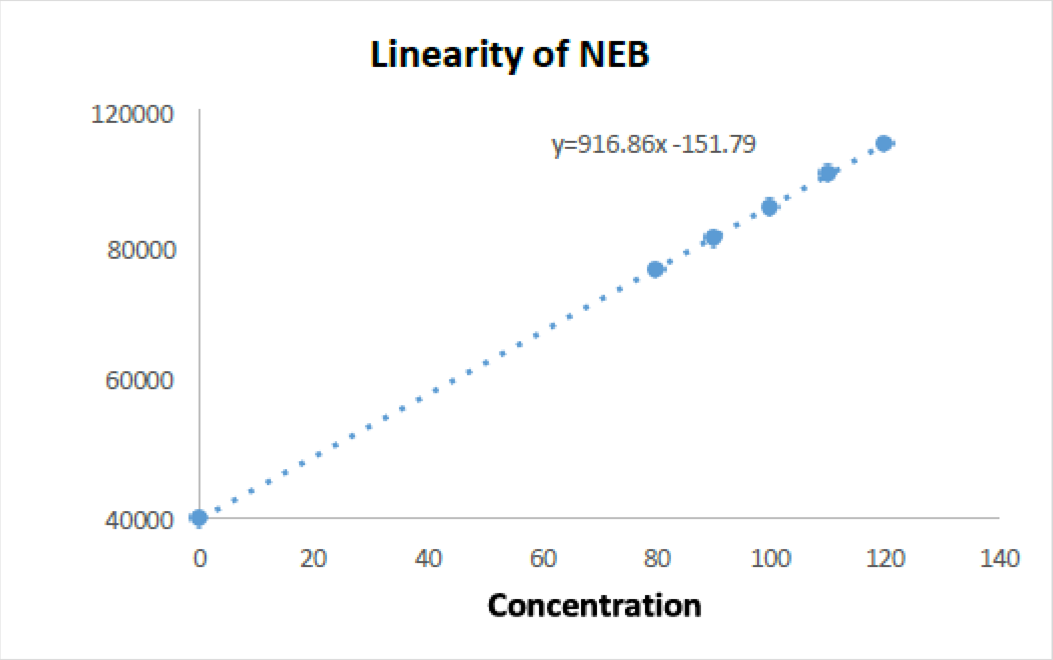

Fig. No. 03: - Plot of linearity and range study for NEB

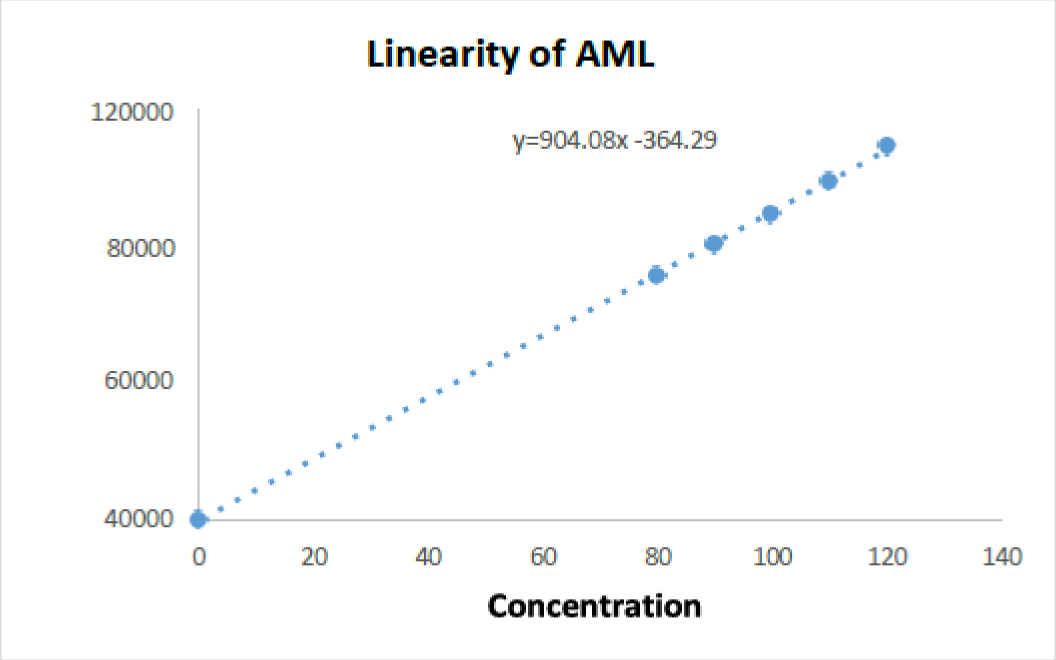

Fig. No. 04:- Plot of linearity and range study for AML

Table No. 03: Observation of Linearity and range study for NEB and AML.

Sr. No.	%Label claim	Peak area	
		NEB	AML

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1	80	72962.8	71560.96
2	90	82083.15	80506.08
3	100	91203.5	89451.2
4	110	101323.9	98996.32
5	120	109944.2	109341.4

f. **Robustness: -** Robustness is a measure of how well analytical method can produce reproducible results when there are small changes to the experimental conditions.

g. **Limit of Detection (LOD) and Limit of Quantitation (LOQ):** Limit of detection is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Limit of quantitation is the lowest amount of sample which can be quantitatively determined with suitable precision and accuracy.

4. Summary & Conclusion

RP-HPLC Method development and validation for the simultaneous Determination of Nebivolol & Amlodipine in Bulk and its Pharmaceutical Dosage Form

HPLC technique was employed in the present investigation for simultaneous estimation of Nebivolol (NEB) & Amlodipine (AML) in the tablet dosage form. IN order to establish RP-HPLC method for the following parameters were studied. HPLC with Intersil 4.6 (id) x 250 mm column and UV detector was used for the study. The standard and sample solution of NEB and AML were prepared in mobile phase.

The most suitable mobile phase was Acetonitrile: Buffer (60:40) pH4 and detection wavelength 265 nm was selected for the evaluation of the chromatogram of both drugs. The selection of the wavelength was based on the λ max obtained by scanning of the standard laboratory mixture. This system gave good resolution and optimum retention time with appropriate tailing factor (<2). The mean values of system suitability test result are depicted in Table below.

Table No. 04 Summary of system suitability test results

Sr. no.	Parameter	NEB	AML
1.	Peak area	91213.5	89458.1
2.	Retention time (min)	5.1026	2.926
3.	Asymmetry	1.845	2.1456
4.	Efficiency	116564.3	61908.11

After establishing the chromatographic conditions, standard laboratory mixture was prepared and analysed by following procedure described under experimental and results. It gave accurate, reliable results and was extended for estimation of drugs in marketed tablet formulation.

Table No. 05: Summary of laboratory mixture and marketed formulation analysis by RP-HPLC Method

Sr. no.	Sample	Statistical data	% Estimation		% Recovery	
			NEB	AML	NEB	AML
1.	Standard Laboratory mixture	**Mean**	100.07	100.03	-	-
		S.D.	0.153	0.666	-	-
		C.V.	0.002	0.007	-	-
		Mean	100.87	99.87	100.05	99.86

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2.	Nodon AM	**S.D.**	0.321	0.252	0.3205	0.33911
		C.V.	0.003	0.003	0.00320	0.00339

The above results indicate that RP- HPLC technique can be successfully applied for the estimation of above-mentioned drugs in their combined dosage formulation without prior separation.

Validation: Validation of these methods was performed as per the ICH guidelines for these following parameters

- **Accuracy** – Accuracy of the proposed method was ascertained from the recovery studies addition method.
- **Precision** – Replicate estimation of the tablet analysed by the proposed method has yielded very consistent result indicating repeatability of method. Study showed ± S.D. <2.
- **Specificity –** Studies shows that there is no interference of peak from the component of matrix.
- **Ruggedness –** Studies were carried out only for the two different parameters like different time, different day and different analyst. Results of estimation by proposed method are very much similar under variety of conditions. This study signifies the ruggedness of the method under varying condition of its performance.

Table No. 06: Summary of results of Ruggedness by RP-HPLC method

Parameter	Statistical data	% Estimation by RP-HPLC method	
		NEB	**AML**
Interday	Mean	99.80	100.03
	S.D.	0.265	0.551
	C.V.	0.003	0.006
Intraday	Mean	99.80	100.03
	S.D.	0.265	0.551
	C.V.	0.003	0.006
Different analyst	Mean	100.36	100.78
	S.D.	0.795613	0.712039
	C.V.	0.007928	0.007065

1) Linearity and Range- NEB and AML marketed formulations was found to be linear in the range of 80% to 120% of test concentration with $R^2 \approx 0.999$ for NEB and $R^2 \approx 0.9997$ for AML at selected wavelength for RP-HPLC methods. Same procedure described in USP was followed.

Conclusion

A simple, accurate, precise, and reliable reverse phase high performance liquid chromatography (RP-HPLC) method was successfully developed for the simultaneous estimation of Nebivolol and Amlodipine in pharmaceutical dosage forms. The chromatographic separation was achieved using a suitable C18 column with an optimized mobile phase composition, which provided well-resolved and symmetrical peaks for both drugs within a short run time.

Furthermore, the method was found to be robust and specific, with no significant interference observed at the retention times of the analytes. System suitability parameters such as theoretical plates, tailing factors, and retention time were within acceptable limits, confirming the reliability of the chromatographic system.

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Overall, the developed RP-HPLC method is simple, rapid, economical, and suitable for routine quality control analysis of Nebivolol and amlodipine in combined pharmaceutical formulations. This method can be effectively applied for simultaneous quantitative determination during drug analysis and quality assurance studies.

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