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Original Article

# Simultaneous Estimation of Amlodipine Besylate and Nebivolol Hydrochloride in Pharmaceutical Tablets Formulation by RP-HPLC Using PDA Detector

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## Abstract

The present work was undertaken with the aim to develop and validate a rapid and consistent RP-HPLC method in which the peaks will be appear with short period of time as per ICH Guidelines.The separation was achieved on a stainless steel analytical column, Eclipse XDB plus $C_{18}$ column (4.6 X 150 mm; 5 µm) in an isocratic mode. The mobile phase was composed acetonitrile and 0.01 M ammonium acetate (pH adjusted to 4.5 using glacial acetic acid), which were mixed in the ratio of 50: 50. The flow rate was monitored at 1.0 mL/min. The wavelength selected for detection was 265 nm. The retention time found for amlodipine besylate and nebivolol hydrochloride was 2.967 and 3.510 min, respectively. The % recovery was 100.20- 100.86 for amlodipine and 100.20 - 100.78 for nebivolol. The linearity was established in the range of 5-25 µg/mL for amlodipine and 10-50 µg/mL for nebivolol. The slope, intercept, and correlation coefficient were found to be 314.2x, +162.4, and 0.999 for amlodipine besylate and 248x, -305.7, and 0.9998 for nebivolol hydrochloride, respectively. The limits of detection for amlodipine besylate and nebivolol hydrochloride obtained by the proposed method was 0.07 and 0.20 µg/ml and the limits of quantification for amlodipine besylate and nebivolol hydrochloride obtained by the proposed method was 0.23 and 0.61 µg/mL, respectively. The method was found to be suitable for the quality control test of amlodipine besylate and nebivolol hydrochloride simultaneously in a bulk drugs as well as in a formulations.

*Keywords*: Amlodipine besylate, isocratic separation, validation, nebivolol hydrochloride, RP-HPLC, $C_{18}$ column.

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## 1. Introduction

Amlodipine besylate (Figure 1) is chemically defined as (RS)-3-ethyl-5-methyl-2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate benzenesulfonate (Merck Index, 1996), which is a dihydropyridine analog, a long-acting calcium channel blocker i.e. anti-hypertensive activity and inhibits the influx of extracellular calcium across the myocardial and vascular smooth muscle cell membranes. It acts on peripheral arterial vasodilator which acts directly on the vascular smooth muscle to cause a reduction in peripheral vascular resistance and in blood pressure. It is an official drug which is included in Indian Pharmacopoeia, British Pharmacopoeia as well as in European Pharmacopoeia [1–5].

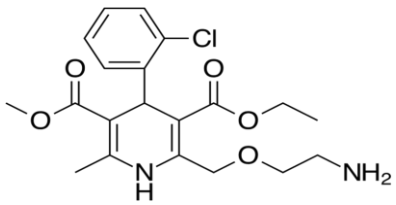


**Figure 1.** Chemical structure of amlodipine besylate.

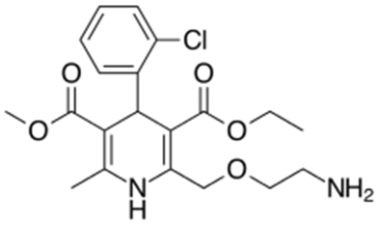


**Figure 2.** Chemical structure of nebivolol hydrochloride.

Nebivolol hydrochloride (Figure 2) is chemically defined as α, α' [Iminobis (methylene) bis [6-fluoro-3, 4-dihydro-2H-1-benzopyran-2-methanol] (Merck Index, 1996), which is a $\beta_1$-Blocker (i.e., Anti-hypertensive drug), which reduces peripheral vascular resistance as well as significantly increases the stroke volume, which preserves the cardiac output [1-2].

As per the literature review, there were various methods developed for the estimation of amlodipine besylate and nebivolol hydrochloride in individual dosage form or in combination with different drugs such as for amlodipine besylate UV spectrophotometry [6-8], HPLC [9] and for nebivolol hydrochloride UV spectrophotometric method [10-12], HPLC [13], and HPTLC [14-16] were reported. However, to the best of our knowledge no one has reported RP-HPLC method for the simultaneous estimation of these drugs. Hence, the aim of our presented study is to develop and validate the simultaneous estimation of amlodipine and nebivolol in tablet dosage forms.

## 2. Materials and Methods

### *2.1. Instrumentation*

A Waters Alliance 2695 separation module equipped with a 2487 UV detector was employed throughout this study. Column that was employed in the method was Eclipse XDB

plus $C_{18}$ column (4.6 X 150 mm; 5 µm). The samples were injected with an automatic injector. The 20 µL volume of sample was injected. The input and output operations of the chromatographic system were monitored by Waters Empower software. The flow rate selected was 0.8 mL per min. The detection was done at 265 nm. The temperature and run time was monitored at 25 ± 2°C and 10.0 min, respectively.

The ultra violet spectra of the drugs used for the investigation were taken on a Lab India UV 3000 spectrophotometer for finding out their $\lambda_{max}$ values.

Solubility of the compounds was enhanced by sonication on an ultra sonicator (Power Sonic 510, Hwashin Technology).

All the weighings in the experiments were done with an Afcoset electronic balance. The Hermle microlitre centrifuge Z100 (model no 292 P01) was used for the centrifugation process and Remi equipments (model no-CM101DX) Cyclomixer was used.

*2.2. Reagents and Materials*

The reference sample of amlodipine besylate and nebivolol hydrochloride was supplied by M/s Pharma Train, Hyderabad, Telangana**.** HPLC grade water (prepared by using 0.45 Millipore Milli-Q) was procured from Standard Reagents, Hyderabad. HPLC grade acetonitrile and methanol were purchased from Merck, Mumbai. The chemicals used for preparation of buffer include ammonium acetate (Finar Chemicals, Ahmedabad), glacial acetic acid (Standard Reagents, Hyderabad).

0.45 µ membrane filters (Advanced Micro Devices Pvt. Ltd., Chandigarh, India) were used for filtration of various solvents and solutions intended for injection into the column.

*2.3. Glassware*

All the volumetric glassware used in the study was of Grade A quality Borosil.

*2.4. Optimization of the chromatographic conditions*

Several modifications in the mobile phase were made by changing proportions of acetonitrile, methanol, and water. Various modifiers were used such as chloroform, Tetrahydrofuran (THF), ethanol, Isopropyl alcohol (IPA), n-Hexane, and dichloromethane, with a 5 µ particle size column, used for separation initially. However, the best resolution of 2.76 was observed by using an acetonitrile with 0.01 M ammonium acetate buffer (pH 4.5) in the ratio of 50: 50, much above the desirable limit of USP resolution 2.0. The retention time obtained for amlodipine besylate and nebivolol hydrochlorides are 2.967 and 3.510 min., respectively.

*2.5. Preparation of Ammonium Acetate Buffer*

The buffer solution was prepared by dissolving 7.7 grams of ammonium acetate in 900 mL HPLC grade water in a 1000 mL clean and dry flask. The mixture was stirred well

until complete dissolution of the salt. Further 100 mL of water was added and the pH was adjusted to 4.5 using glacial acetic acid.

*2.6. Preparation of Mobile Phase*

The mobile phase was prepared by mixing 500 mL of HPLC garde acetonitrile and 500 mL 0.01 M ammonium acetate buffer (pH 4.5) in a clean and dry flask. The mixture was degassed in ultra sonicator for 5 minutes. The resultant mobile phase was filtered through 0.45 µ membrane filter (Advanced Micro Devices Pvt. Ltd., Chandigarh, India) under vacuum.

*2.7. Diluent Preparation*

The diluent was prepared by mixing HPLC grade acetonitrile and ammonium acetate buffer (pH 4.5) in the ratio of 50:50 (v/v). This solution was used for diluting the drug solutions in the study.

*2.8. Preparation of Standard Solution*

About 30 mg amlodipine besylate was weighed accurately and transferred into a 100 mL clean and dry volumetric flask. Initially, the drug was dissolved with 70 mL of diluent. The solution was sonicated for 15 min for complete dissolution of the drug. The final volume was made up with the same solvent. From the above prepared solution 1.0 mL transferred to a 10 mL clean and dry volumetric flask and it was diluted up to the mark with the same diluent. This stock solution contains 30 µg/mL of amlodipine besylate.

Similarly, about 100 mg nebivolol hydrochloride was weighed accurately and transferred into a 100 mL clean and dry volumetric flask. Initially, the drug was dissolved with 70 mL of diluent. The solution was sonicated for 15 min for complete dissolution of the drug. The final volume was made up with the same solvent. From the above prepared solution 1.5 mL transferred to a 100 mL clean and dry volumetric flask and it was diluted up to the mark with the same diluent. This stock solution contains 15 µg/mL of nebivolol hydrochloride.

*2.9. Preparation formulation (Tablet) Solution*

A commercial brand of tablet NEBICARD-SM, (manufactured by Torrent) was employed for this study. Each tablet contained 2.5 mg of amlodipine besylate and 5 mg of nebivolol hydrochloride. Twenty tablets were weighed, finely powdered and an accurately weighed sample of powdered tablets equivalent to 25 mg of amlodipine besylate and nebivolol hydrochloride was extracted with small amount of diluent in a 25 mL clean and dry volumetric flask. The solution was shaken well and allowed to stand for 15 min with intermittent sonication to ensure complete solubility of drugs. The contents are made up to the mark with the diluent and filtered through a 0.45µ membrane filter.

**Table 1.** Recovery of amlodipine besylate and nebivolol hydrochloride from formulation.

| Sr. No. | Formulation | Label claim (mg) | Amount found (mg) (n=3) | % Amount found |
|---|---|---|---|---|
| 1. | NEBICARD-SM (manufactured by Torrent.) | Amlodipine besylate (2.5 mg) | 2.46 mg | 98.40 |
| | | Nebivolol hydrochloride (5.0 mg) | 4.90 mg | 98.00 |

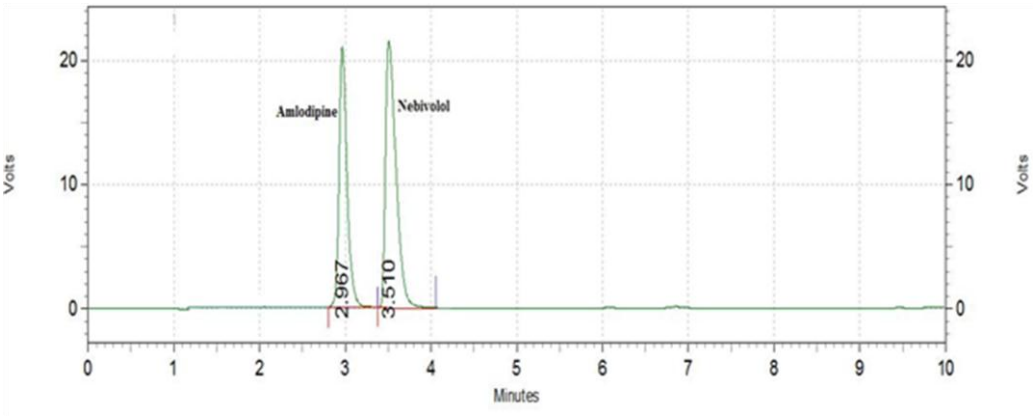


**Figure 3 A.** A typical chromatogram of amlodipine besylate and nebivolol hydrochloride (Sample).

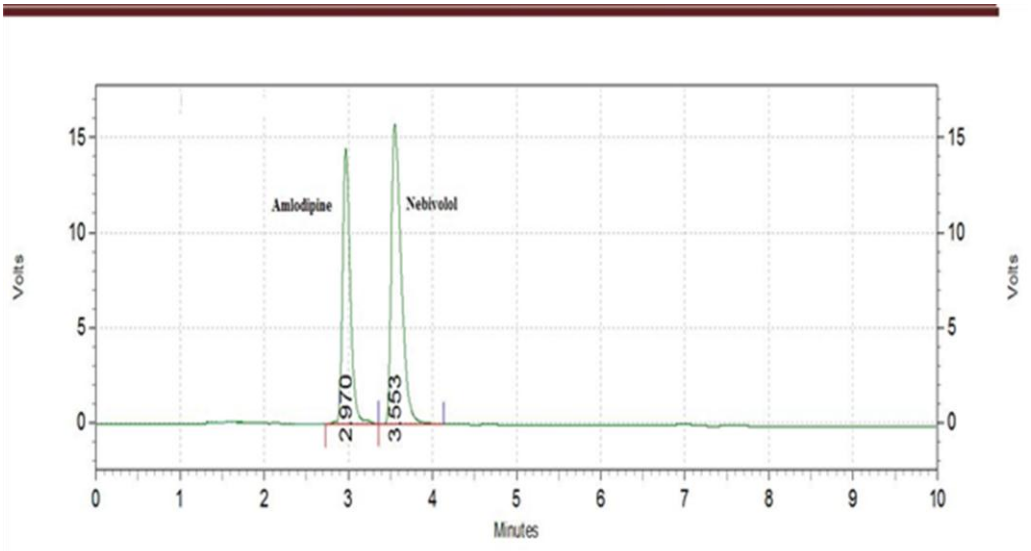


**Figure 3 B.** A typical chromatogram of amlodipine besylate and nebivolol hydrochloride (Standard).

From this filtrate, pipetted out 0.2 mL of the filtrate and transferred to a clean and dry 10 mL volumetric flask. Further the volume was made upto the mark to with the diluent to get a concentration of 30 µg/mL of amlodipine besylate and 15 µg/mL of nebivolol hydrochloride. Now the sample of 20 µL was

injected and chromatographed. The average of the peak areas was calculated.

### *2.10. Method Suitability*

The commercial tablet formulation of amlodipine besylate and nebivolol hydrochloride namely NEBICARD-SM (manufactured by Torrent) was analyzed by the proposed method (Table 1). The values were found to be in good agreement with the labeled amounts, which confirms the suitability of the method for the analysis of the drugs in pharmaceutical dosage forms.

## 3. **Results and Discussion**

### *3.1. Specificity and Selectivity*

An aqueous mixture of amlodipine besylate and nebivolol hydrochloride (15.0 and 30.0 µg/mL concentration respectively) was prepared and injected into the column and the retention time was checked and any interference at the retention time was checked by comparing the response in the blank. No interference was observed at the retention time

**Table 2.** Linearity range for amlodipine besylate

| Sr. No. | Concentration (µg/mL) (n=6) | Mean Peak area (mV*min) |
|---|---|---|
| 1. | 5 | 1657.314 |
| 2. | 10 | 3374.494 |
| 3. | 15 | 4926.184 |
| 4. | 20 | 6439.408 |
| 5. | 25 | 7979.980 |
| | (Correlation coefficient) $R^2$ | 0.999 |

**Table 3.** Linearity range for nebivolol hydrochloride

| Sr. No. | Concentration (µg/ml) (n=6) | Mean Peak area (mV*min) |
|---|---|---|
| 1. | 10 | 2172.570 |
| 2. | 20 | 4760.503 |
| 3. | 30 | 7019.417 |
| 4. | 40 | 9532.054 |
| 5. | 50 | 12186.890 |
| | (Correlation coefficient) $R^2$ | 0.999 |

for the respective drug. The method was found to be precise and specific. (Figure 3 (A & B)).

*3.2. Linearity*

In order to find out the linearity range of the proposed HPLC method, the curves were constructed by plotting peak areas obtained for the analyte against their concentrations. A good linear relationship ($r^2$ = 0.999) was observed between the concentration of amlodipine besylate and nebivolol hydrochloride and their corresponding peak areas. The relevant regression equation was y = 314.2x + 162.4 ($r^2$= 0.999) for amlodipine besylate and nebivolol hydrochloride y = 248x - 305.7 ($r^2$ = 0.999) (where y is the peak area and x is the concentrations of amlodipine besylate and nebivolol hydrochloride (µg/mL)) (Table 2 and 3) (Figure 4 and 5).

*3.3. Precision*

Precision is the level of reproducibility of the results as reported between sample analyzed on the same day (intra-day) and

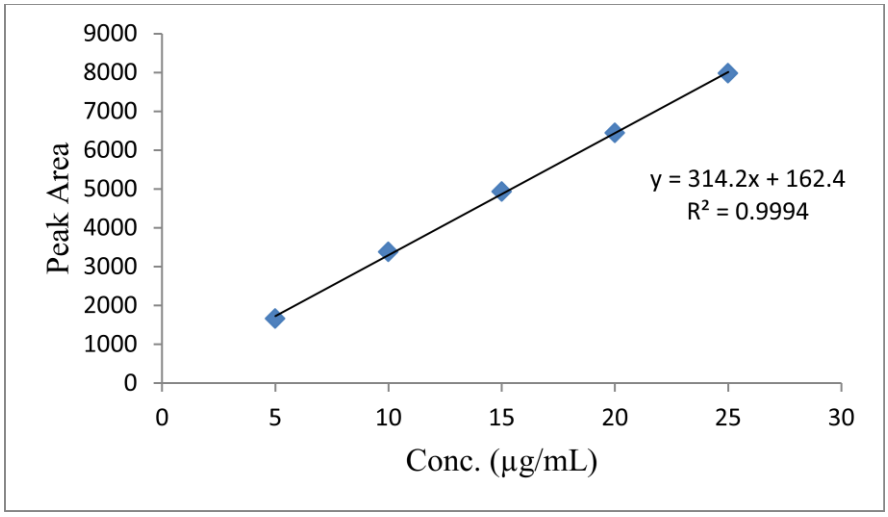


**Figure 4.** Calibration curve for amlodipine besylate.

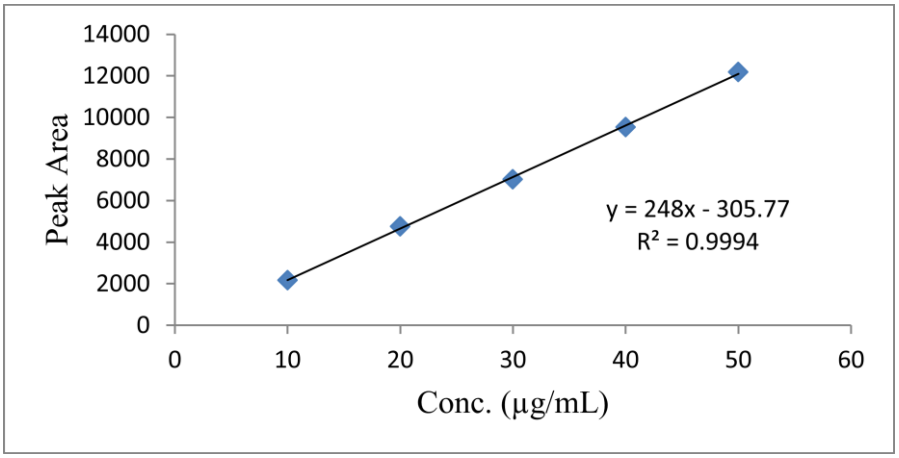


**Figure 5.** Calibration curve for nebivolol hydrochloride.

**Table 4.** Intra-day precision of the proposed method for amlodipine besylate and nebivolol hydrochloride.

| Injection | Peak area for amlodipine besylate | Peak area for nebivolol hydrochloride |
|---|---|---|
| Injection-1 | 3319.572 | 4724.173 |
| Injection-2 | 3372.572 | 4710.595 |
| Injection-3 | 3402.279 | 4699.750 |
| Injection-4 | 3332.712 | 4700.723 |
| Injection-5 | 3360.175 | 4725.173 |
| Injection-6 | 3410.670 | 47134.987 |
| **Average** | 3356.982 | 4712.083 |
| **Standard Deviation** | 33.09 | 12.25 |
| **%RSD** | 0.98 | 0.26 |

**Table 5.** Inter-day precision of the proposed method for amlodipine besylate and nebivolol hydrochloride (on six consecutive days n = 6).

| Days | Peak area for amlodipine besylate | Peak area for nebivolol hydrochloride |
|---|---|---|
| Day-1* | 3274.261 | 4651.753 |
| Day -2* | 3214.805 | 4632.269 |
| Day -3* | 3279.270 | 4597.611 |
| Day -4* | 3275.384 | 4627.581 |
| Day -5* | 3250.416 | 4660.032 |
| Day -6* | 3285.651 | 4656.056 |
| **Average** | 3262.827 | 4633.849 |
| **Standard Deviation** | 31.92 | 24.29 |
| **% RSD** | 0.97 | 0.52 |

*Average of Six injections

samples run on three different days (inter-day). To check the intra and inter-day variations of the method, the solutions containing 15.0 and 30.0 µg/mL of amlodipine besylate and nebivolol hydrochloride respectively, were subjected to the proposed HPLC method of analysis and the results obtained were noted. The precision of the proposed method i.e. the intra and inter-day variations in the peak areas of the drugs solutions were calculated in terms of percent RSD (Table 4 and 5). A statistical evaluation revealed that the relative standard deviation of the drug at linearity level for 6 injections was less than 2.0.

*3.4. Accuracy*

Accuracy is expressed as the closeness of the results obtained from standard samples to that of the actual known amounts. To determine the accuracy of the proposed method, the recovery studies were carried out by analyzing recovery amount (5.0 µg of amlodipine besylate and nebivolol hydrochloride) of pure drugs at different linearity level (10.0, 15.0 and 20.0 µg/mL of amlodipine besylate and 20.0, 30.0 and 40.0 µg/mL nebivolol hydrochloride) was added. Then each dilution was injected thrice (n=3). The percent recoveries of the drugs were calculated (Table 6 and 7).

**Table 6.** Accuracy data of the proposed method for amlodipine besylate

| Amount of sample taken (µg/mL) | Active drug added (µg/mL) | Amount recovered (µg/mL) | % Recovery | Mean recovery | SD | % RSD |
|---|---|---|---|---|---|---|
| 20 | 5 | 24.99 | 99.96 | 100.20 | 0.22 | 0.21 |
| | | 25.10 | 100.4 | | | |
| | | 25.06 | 100.2 | | | |
| 30 | 5 | 35.11 | 100.31 | 100.22 | 0.102 | 0.10 |
| | | 35.04 | 100.11 | | | |
| | | 35.09 | 100.25 | | | |
| 40 | 5 | 45.91 | 102.02 | 100.86 | 1.02 | 1.01 |
| | | 45.27 | 100.6 | | | |
| | | 45.01 | 100.02 | | | |

**Table 7.** Accuracy data of the proposed method for nebivolol hydrochloride

| Amount of sample taken (µg/mL) | Active drug added (µg/mL) | Amount recovered (µg/mL) | % Recovery | Mean recovery | SD | % RSD |
|---|---|---|---|---|---|---|
| 10 | 5 | 15.20 | 101.30 | 100.78 | 0.48 | 0.47 |
| | | 15.11 | 100.73 | | | |
| | | 15.05 | 100.33 | | | |
| 15 | 5 | 20.08 | 100.40 | 100.33 | 0.208 | 0.20 |
| | | 20.10 | 100.50 | | | |
| | | 20.02 | 100.10 | | | |
| 20 | 5 | 24.98 | 99.92 | 100.20 | 0.28 | 0.27 |
| | | 25.05 | 100.20 | | | |
| | | 25.12 | 100.48 | | | |

*3.5. Limit of Detection (LOD) and Limit of Quantitation (LOQ)*

LOD is defined as the smallest level of analyte that gives a measurable response. LOD is based on S/N ratio (signal/noise) typically for HPLC methods. Six replicates of the analyte were measured. The LOQ is the lowest concentration that can be quantified reliably with a specified level of accuracy and precision. It is the lowest concentration at which the precision expressed by relative standard deviation (RSD) is less than 2 % and accuracy expressed by relative difference in the measured and true value is also less than 2 %. In other words, the analyte response is 10 times greater than the noise response. Six replicates of the analyte were analyzed and quantified. The limits of detection for amlodipine besylate and nebivolol hydrochloride obtained by the proposed method was 0.07 and 0.20 µg/ml and the limits of quantification for amlodipine besylate and nebivolol hydrochloride obtained by the proposed method was 0.23 and 0.61 µg/mL.

*3.6. Robustness*

The optimized HPLC conditions were slightly modified to evaluate the robustness of

**Table 8.** Results of the robustness study for amlodipine besylate

| Sr. No. | Parameters | Amlodipine besylate | | |
|---|---|---|---|---|
| | | Retention Time (min.) | Peak Area (mV*min.) | Tailing Factor |
| 1. | Standard | 2.970 | 3557.314 | 1.39 |
| 2. | Flow rate (0.7 mL/min.) | 2.949 | 3315.725 | 1.49 |
| 3. | Flow rate (0.9 mL/min.) | 2.920 | 3346.260 | 1.38 |
| 4. | Mobile Phase (55:45 % v/v) | 2.920 | 3295.760 | 1.27 |
| 5. | Mobile Phase (45:55 % v/v) | 3.012 | 3315.260 | 1.44 |
| 6. | Wavelength (260 nm) | 2.959 | 3360.520 | 1.50 |
| 7. | Wavelength (270 nm) | 2.935 | 3325.026 | 1.41 |

**Table 9.** Results of the robustness study for nebivolol hydrochloride

| Sr. No. | Parameters | Nebivolol hydrochloride | | |
|---|---|---|---|---|
| | | Retention Time (min.) | Peak Area (mV*min.) | Tailing Factor |
| 1. | Standard | 3.553 | 4760.503 | 1.18 |
| 2. | Flow rate (0.7 mL/min.) | 3.545 | 4701.535 | 1.50 |
| 3. | Flow rate (0.9 mL/min.) | 3.512 | 4638.623 | 1.43 |
| 4. | Mobile Phase (55:45 % v/v) | 3.505 | 4618.757 | 1.35 |
| 5. | Mobile Phase (45:55 % v/v) | 3.711 | 4629.352 | 1.23 |
| 6. | Wavelength (260 nm) | 3.540 | 4701.225 | 1.45 |
| 7. | Wavelength (270 nm) | 3.510 | 4681.360 | 1.52 |

the method. Small variations were made in the mobile phase ratio and flow rate. From the results, it was indicated that the selected factors remained unaffected by small variations in these quantities as well as the method was robust even by change in the mobile phase ± 5 % , flow rate ± 0.1 mL/min and change in detection wavelength ± 5nm (Table 8 and 9).

## 4. Conclusion

It can be concluded that the proposed RP-HPLC method developed for the quantitative determination of amlodipine besylate and nebivolol hydrochloride in bulk samples and in its formulations is simple, selective, sensitive, accurate, precise, and rapid. The method was proved to be superior to most of the reported methods. The mobile phases are simple to prepare and economical. The sample recoveries in the formulation were in good agreement with their respective label claims and they suggested non-interference of formulation excipients in the estimation. Hence, this method can easily be adopted as an alternative method to reported ones for the routine determination of amlodipine besylate and nebivolol hydrochloride depending upon the availability of chemicals and nature of other ingredients present in the sample. The method also finds use in clinical, biological and pharmacokinetic studies of amlodipine besylate and nebivolol hydrochloride.

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