Method Development and Validation for the Simultaneous Estimation of Nebivolol hydrochloride and Amlodipine besylate in Tablet Dosage Forms by RP-HPLC

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ABSTRACT
A simple, specific and precise high performance liquid chromatographic method was developed and validated for Nebivolol HCl and Amlodipine besylate in a combined tablet dosage form. Nebivolol hydrochloride, a long acting, cardioselective β-blocker and Amlodipine besylate, a calcium channel blocker are considered in combination for the treatment of hypertension. The chromatographic separation was performed using Eclipse XDB plus C18 Column (4.6 × 150 mm, 5μm particle size). The mobile phase consisted of combination of 0.01 M Ammonium acetate buffer (pH 4.5) and Acetonitrile in the ratio of 50:50 v/v at a flow rate of 0.8 ml/min with the detection wavelength at 265 nm. Both the drugs showed good linearity in the concentration range of 10-50 μg/ml and 5-25 μg/ml respectively with a correlation coefficient of 0.999 for both drugs. The retention time for Nebivolol HCl and Amlodipine besylate were found to be 3.553 and 2.970 min respectively. The developed analytical method was validated for linearity, precision, accuracy, ruggedness, robustness, specificity and system suitability, which were within the acceptance limits according to ICH guidelines. The developed method can successfully be employed for routine quality control of Nebivolol HCl and Amlodipine besylate in combined tablet dosage forms.

Keywords: HPLC, Hypertension, method development, validation.

INTRODUCTION
Nebivolol HCl is an antihypertensive, which is a competitive and selective β1 receptor antagonist. [1] It is chemically known as α; α’ – [iminobis(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 [Fig. 1]. Amlodipine besylate is chemically 2 – [(2-aminoethoxy) methyl] – 4-(2-chlorophenyl) – 1, 4 – dihydro – 6 – methyl – 3, 5 – pyridine carboxylic acid 3 – ethyl 5 – methyl ester. It is a dihydropyridine calcium antagonist used in the treatment of hypertension [Fig. 2]. It is a long acting calcium channel blocker, which inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. [2]

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Research Article
The literature survey revealed that several HPLC [1-8] and UV [9-14] methods have been reported for the simultaneous estimation of Nebivolol HCl and Amlodipine besylate in pharmaceutical dosage forms. However, HPLC is the most commonly used analytical method for the estimation of these drugs, either individually or in combination with other drugs. The present work was intended to develop and validate a simple, sensitive and rapid analytical method with optimum chromatographic conditions for simultaneous quantification of Nebivolol HCl and Amlodipine besylate in pharmaceutical dosage forms. The developed method was validated as per ICH guidelines [15-17], and can be applied successfully in routine quality control of Nebivolol HCl and Amlodipine besylate in bulk and in combined dosage forms.

MATERIALS AND METHODS

Equipment
An Agilent model - 1220 Infinity LC – HPLC system having Agilent openLAB CDS (EZ Chrome) software “version A.04.05” equipped with a variable wavelength detector (VWD) and a manual injector was used. It was manufactured by Agilent Technologies, USA. An Eclipse XDB plus C18 Column (4.6 × 150 mm, 5μm particle size) was used for the analytical separation and quantification of the mixtures. An ELICO (LI 120) pH meter was used for adjusting the pH of the buffer.

Reagents and Chemicals
Pure samples of Nebivolol HCl and Amlodipine besylate were obtained from Micro Labs, Bangalore. The commercial tablet (NEBISTAR-SA) containing 5 mg of Nebivolol HCl and 2.5 mg of Amlodipine besylate was employed in the study.

HPLC grade acetonitrile and millipore water were purchased from Thermo Fisher Scientific India Pvt. Ltd., Mumbai. Analytical grade glacial acetic acid and ammonium acetate were purchased from Merck Specialty, Mumbai and Finar chemicals Limited, Ahmedabad respectively.

Chromatographic Conditions
A freshly prepared 50:50 v/v mixture of 0.01 M Ammonium acetate buffer (pH 4.5) and acetonitrile was used as the mobile phase. Both ammonium acetate buffer and acetonitrile were filtered through a 0.45μ membrane filter and sonicated before use. The flow rate was adjusted to 0.8 ml/min, the injection volume was 20μL and the detection wavelength was 265 nm.

Preparation of Analytical Solutions

Preparation of Mobile Phase
A mixture of Acetonitrile and Ammonium acetate buffer was mixed in the ratio of 50:50 v/v. Then the resulting solution was filtered through 0.45μ nylon membrane filter, degassed and used as the mobile phase.

Preparation of buffer
0.77 g of ammonium acetate was accurately weighed and transferred to 1000 ml volumetric flask. It was dissolved by adding small quantities of distilled water and finally made up to 1000 ml with distilled water. The pH of the buffer was adjusted to 4.5 with glacial acetic acid, filtered through 0.45μ nylon membrane filter and finally degassed.

Nebivolol HCl standard solutions
Stock solution: 100 mg of Nebivolol HCl was accurately weighed and transferred to a 100 ml volumetric flask. 50 ml of mobile phase was added, sonicated for 15 minutes and cooled to room temperature. The solution was diluted and made up to 100 ml with mobile phase to get a 1000μg/ml solution.

Working standard solution: 10 ml of stock solution was diluted to 100 ml with the mobile phase to obtain a 100μg/ml solution.

Amlodipine besylate standard solutions
Stock solution: Amlodipine besylate equivalent to 50 mg of Amlodipine was accurately weighed and transferred to 100 ml volumetric flask. 50 ml of mobile phase was added, sonicated for 15 minutes and cooled to room temperature. The solution was diluted and made up to 100 ml with mobile phase to get a 500μg/ml solution.

Working standard solution: 10 ml of stock solution was diluted to 100 ml with the mobile phase to obtain a 50μg/ml solution.

Preparation of mixed standard stock solution
100 mg of Nebivolol HCl and 50 mg of Amlodipine besylate were weighed accurately into a 100 ml volumetric flask and dissolved in sufficient quantity of mobile phase and finally made up to 100 ml with the mobile phase.

Working standard solution: 10 ml of standard stock solution was taken into a 100 ml volumetric flask and diluted to 100 ml with mobile phase to get a concentration of 100μg/ml of Nebivolol HCl and 50μg/ml of Amlodipine besylate.

Calibration of the method
Standard stock solutions of Nebivolol HCl and Amlodipine besylate were diluted to get the working standard concentrations in the range of 10-50μg/ml and 5-25μg/ml respectively. Five replicates of each dilution were injected into the column and chromatograms were recorded. The corresponding calibration curve was constructed by plotting concentrations against the respective peak areas. The regression equation was obtained and the values of
slope-a, intercept-b, and correlation coefficient ($R^2$) were determined.

**Preparation of sample solutions**
20 tablets were weighed accurately and crushed to fine powder. Each tablet contained 5 mg of Nebivolol HCL and 2.5 mg of Amlodipine besylate. The quantity of powder containing the drugs equivalent to 25 mg of Nebivolol HCL and 12.5 mg of Amlodipine besylate was weighed and dissolved in sufficient quantity of mobile phase in a 25 ml volumetric flask and finally made up to volume with the mobile phase. The solution was filtered through 0.45μ nylon membrane filter. The amount of Nebivolol HCl and Amlodipine besylate present in tablet formulation was calculated by comparing the peak area of the standard with that of sample. The amount of drugs in tablet was calculated by using the formula given below:

$$
\text{Amount of drug in tablet} = \frac{\text{Peak area of standard}}{\text{Peak area of sample}} \times \text{Label Claim}
$$

**Fig 3: UV overlain absorption plot of Nebivolol HCl and Amlodipine besylate**

**Fig. 4: Calibration curve for Nebivolol HCl**

**Fig. 5: Calibration curve for Amlodipine besylate**

**HPLC-Method Development and Validation**
An analytical method for the estimation of Nebivolol HCl and Amlodipine besylate in tablets was developed and validated according to ICH guidelines. Analytical variable parameters such as linearity, precision, accuracy (percent recovery), specificity and system suitability were tested using the optimized chromatographic conditions and instruments.

**Linearity**
The calibration curves constructed by analyzing a series of concentrations of each drug ranging from 10-50μg/ml for Nebivolol HCl and 5-25μg/ml for Amlodipine besylate showed good linearity. The regression equations and correlation coefficients for Nebivolol HCl and Amlodipine besylate were found to be $y = 248x-305.7; 0.999$ and $y = 314.2x+162.4; 0.999$ respectively and the results were shown in Table 1 and Fig. 3, 4 & 5.

**Accuracy (percent Recovery)**
The accuracy study was performed on 80 %, 100 % and 120 % of the analytical method target concentrations of Nebivolol HCl and Amlodipine besylate. Standard and sample preparations were injected into HPLC system and three determinants for each concentration level were obtained. The percentage recoveries of Nebivolol HCL and Amlodipine besylate were calculated using standard at the same concentration at each concentration level as shown in Table 2.

**Precision**

*System Precision:* System precision was checked by injecting five replicate preparations of the standard drug solutions of Nebivolol HCl (20μg/ml) and Amlodipine besylate (10μg/ml). The corresponding peak areas were measured and % RSD calculated.

*Method Precision:* The method precision study was performed for five replicate sample preparations of marketed formulation containing Nebivolol HCl (20μg/ml) and Amlodipine besylate (10μg/ml). The corresponding peak areas were measured and % RSD calculated as exhibited in Table 3.

**Robustness**
Robustness of the developed analytical method was tested by evaluating the affect of small variations in analytical method parameters such as change in flow rate from 0.8 ml/min by ±0.1 ml/min, change in wavelength by ±5 nm and change in the mobile phase B ratio by ± 5 %. The chromatograms were recorded and the results are shown in Table 4.

**Ruggedness**
Ruggedness of the proposed method was determined by spiking five replicates of tablet formulation containing Nebivolol HCl (30μg/ml) and Amlodipine besylate (15μg/ml) prepared by another analyst into another HPLC system. The retention time and peak areas were obtained. The mean and % RSD were found to be within the acceptance criteria as shown in Table 5.

**Limit of Detection (LOD) and Limit of Quantification (LOQ)**
The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The LOQ is the smallest concentration of the analyte which gives response that can be accurately quantified (signal to noise ratio of 10). LOD and LOQ...
were calculated as 3.3δ/S and 10δ/S, respectively as per ICH guidelines, where δ is the standard deviation of the response and S is the slope of the calibration plot. The results are presented in Table 1.

Table 1: Optimized chromatographic conditions of the proposed method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nebivolol HCl</th>
<th>Amlodipine besylate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration range (µg/ml)</td>
<td>10-50</td>
<td>5-25</td>
</tr>
<tr>
<td>Retention time (min)</td>
<td>3.553</td>
<td>2.970</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>258.79</td>
<td>343.43</td>
</tr>
<tr>
<td>Intercept (a)</td>
<td>-305.7</td>
<td>+162.4</td>
</tr>
<tr>
<td>LOD</td>
<td>0.20</td>
<td>0.07</td>
</tr>
<tr>
<td>LOQ</td>
<td>0.61</td>
<td>0.23</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>Theoretical plates</td>
<td>3889</td>
<td>5108</td>
</tr>
<tr>
<td>Symmetry factor</td>
<td>0.98</td>
<td>1.09</td>
</tr>
<tr>
<td>Resolution</td>
<td>2.76</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Recovery studies and Assay results of the proposed method

<table>
<thead>
<tr>
<th>Drug</th>
<th>Amount of standard drug added to pre-analyzed formulation (µg/ml)</th>
<th>Amount recovered</th>
<th>Mean % recovery ± S.D (n=5)</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebivolol HCl</td>
<td>20</td>
<td>19.99</td>
<td>100.86±0.23</td>
<td>99.69</td>
</tr>
<tr>
<td>Amlodipine besylate</td>
<td>30</td>
<td>30.11</td>
<td>100.76±0.31</td>
<td>99.19</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>40.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20.08</td>
<td>100.76±0.31</td>
<td>99.19</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>29.98</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3: System and Method Precision of the proposed method

<table>
<thead>
<tr>
<th>Drug</th>
<th>Spiked level (µg/ml) (n=5)</th>
<th>System precision</th>
<th>Method precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S.D</td>
<td>R.S.D</td>
<td>S.D</td>
</tr>
<tr>
<td>Nebivolol HCl</td>
<td>20</td>
<td>12.25</td>
<td>0.26</td>
</tr>
<tr>
<td>Amlodipine besylate</td>
<td>10</td>
<td>33.09</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Table 4: Robustness of the proposed method

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Set values</th>
<th>RT (min)</th>
<th>Peak Area (mV*min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nebivolol HCl</td>
<td>Amlodipine besylate</td>
</tr>
<tr>
<td>Flow rate (mL/min)</td>
<td>0.7</td>
<td>3.545</td>
<td>2.949</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>3.509</td>
<td>2.925</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>3.512</td>
<td>2.950</td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td>260</td>
<td>3.540</td>
<td>2.959</td>
</tr>
<tr>
<td></td>
<td>265</td>
<td>3.544</td>
<td>2.942</td>
</tr>
<tr>
<td></td>
<td>270</td>
<td>3.510</td>
<td>2.935</td>
</tr>
<tr>
<td>Change in Mobile phase B</td>
<td>45 %</td>
<td>3.505</td>
<td>2.920</td>
</tr>
<tr>
<td></td>
<td>50 %</td>
<td>3.503</td>
<td>2.955</td>
</tr>
</tbody>
</table>

Table 5: Ruggedness of the proposed method

<table>
<thead>
<tr>
<th>S. No</th>
<th>parameter</th>
<th>Nebivolol HCl</th>
<th>Amlodipine besylate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Analyst 01</td>
<td>100.21% w/w</td>
<td>101.43% w/w</td>
</tr>
<tr>
<td>2</td>
<td>Analyst 02</td>
<td>99.97% w/w</td>
<td>99.72% w/w</td>
</tr>
<tr>
<td>3</td>
<td>Acceptance criteria</td>
<td>90-110% w/w</td>
<td>90-110% w/w</td>
</tr>
</tbody>
</table>

Specificity
The specificity of the method was determined to check whether there is any interference due to presence of excipients, impurities or other components with the retention time of analytical peaks which may affect the specificity of the analytical method. The HPLC chromatograms were recorded for the drug-matrix (mix of the drug and excipient) which showed almost no interfering peaks within retention time ranges.

System suitability
Five replicates of working mixed standard solution were injected and the parameters like theoretical plate number (N), symmetry factor (K) and resolution were calculated to check the system suitability. The results are presented in Table 1.

RESULTS AND DISCUSSION
The present study was aimed to develop a sensitive, precise and accurate method for simultaneous estimation of Nebivolol HCl and Amlodipine besylate in combined tablet dosage forms by RP-HPLC. An Eclipse XDB plus C18 Column (4.6 × 150 mm, 5µm particle size) was chosen as the stationary phase for the separation and determination of Nebivolol HCl and Amlodipine besylate. For the optimization of the mobile phase, various mixtures consisting of acetonitrile, methanol and 0.01 M Ammonium acetate buffer were examined at different ratios. The choice of the optimum composition is based on chromatographic response factor. A composition of 50:50 v/v of 0.01 M Ammonium acetate buffer and Acetonitrile provided an efficient separation of Nebivolol HCl and Amlodipine besylate with sufficient retention times. The injection volume was 20µL and the variable wavelength detector was set at 265 nm. The run time was 10 min. A flow rate of 0.8 ml/min was found to be optimum from the studied range of 0.5-2.0 ml/min, which gave optimum retention time, base line stability and noise. The retention times for Nebivolol HCl and Amlodipine besylate were found to be 3.553 min and 2.970 min respectively.

The chromatograms for the validation studies were recorded and shown in Fig. 6-9. The developed method was applied to the simultaneous estimation of Nebivolol HCl and Amlodipine besylate in commercial tablets. Satisfactory results were obtained for each compound showing good agreement with label claim, as shown in Table 2. The linear dynamic range was 10-50µg/ml and 5-25µg/ml for Nebivolol HCl and Amlodipine besylate respectively. The regression coefficients were found to be about 0.999 for both the drugs indicating good linearity of the method as exhibited in Table 1. The recovery study was performed on 80 %, 100 % and 120 % of the target concentrations of Nebivolol HCl and Amlodipine besylate sample preparations and their percentage recovery was found to be 100.86 % and 100.76 % respectively as a mean % recovery of all determinants at the three concentration levels as shown in Table 2. It indicates that the method is accurate and the commonly used excipients present in the tablet formulation did not interfere with the proposed method. The precision of the method was...
determined from the peak areas of five homogeneous sample preparations. The system and method precisions obtained for Nebivolol HCl and Amlodipine besylate were found to be less than 2 % (% RSD) as shown in Table 3, indicating that the method is quite precise.

The LOD and LOQ for Nebivolol HCl and Amlodipine besylate were found to be 0.20μg/ml; 0.61μg/ml and 0.07μg/ml; 0.23μg/ml respectively. The optimum HPLC conditions like detection wavelength, ratio of mobile phase and flow rate set for the proposed method have been slightly modified as a means to evaluate the robustness of the method. The results indicated that the selected factors remained unaffected by small variations in these quantities, as shown in Table 4. The result for method ruggedness was found to be 100.21 % for Nebivolol HCl and 101.43 % for Amlodipine besylate as shown in Table 5.
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The absence of additional peaks in the chromatograms showed non-interference of the common excipients used in the tablets indicating that the method is specific for the drugs under study. System suitability results such as theoretical plates, tailing factor and resolution were found to be 3889 & 5108 (theoretical plates), 0.98 & 1.09 (symmetry factor) and 2.76 (resolution) respectively for Nebivolol HCl and Amlodipine besylate.

The validation of the developed method for the required parameters as per ICH guidelines showed that all the parameters were within the acceptance criteria. The proposed method was applied for the simultaneous determination of Nebivolol hydrochloride and Amlodipine besylate in marketed tablet dosage form. The assay results confirmed with the label claim of the formulation. It can be concluded that the proposed HPLC method is sufficiently sensitive and reproducible for simultaneous estimation of Nebivolol HCl and Amlodipine besylate in tablet dosage forms.

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