REVERSE PHASE HPLC METHOD FOR DETERMINATION OF NEBIVOLOL IN PHARMACEUTICAL PREPARATIONS

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ABSTRACT
A simple and reliable high-performance liquid chromatography (HPLC) method was developed and validated for determination of nebivolol in pharmaceutical preparations. The method was developed on Ace C_{18} column using a mobile phase of methanol-water (70:30, v/v). The eluent was monitored by UV detection at 282 nm. The analysis was performed in less than 6 min with a flow rate of 1.0 mL min\(^{-1}\). Calibration curve was linear over the concentration range of 0.25-8.0 µg mL\(^{-1}\). Intra- and inter-day precision values for nebivolol were less than 4.82, and accuracy (relative error) was better than 3.00%. The mean recovery of nebivolol was 99.8% for pharmaceutical preparations. The limits of detection (LOD) and quantification (LOQ) were 0.10 and 0.25 µg mL\(^{-1}\), respectively. Also, the developed and validated HPLC method was successfully applied for the quality control of commercial nebivolol dosage forms to quantify the drug and to check the formulation content uniformity.

Keywords: Nebivolol, HPLC, Pharmaceutical preparation, Validation.

INTRODUCTION
β-blockers constitute one of the most frequently prescribed groups of cardiovascular drugs. They are competitive antagonists at β-adrenergic receptor sites and are used in the management of cardiovascular disorders, such as hypertension, angina pectoris, cardiac arrhythmias and myocardial infarction.

Nebivolol hydrochloride is chemically known as α-α-[iminobis(methylene)] bis[6-flouro-3,4-dihydro-2H-1-benzopyran-2-methanol]hydrochloride (Figure 1).

![Figure 1. Chemical structure of nebivolol.](image)

It is a highly selective β_{1}-blocker with nitric oxide-mediated vasodilatory actions and beneficial effects on vascular endothelial function. Nebivolol is used in the management of hypertension. It is given by mouth as the base. The usual dose is 5 mg daily. An initial dose of 2.5 mg daily is employed in the elderly and in patients with renal impairment [1].

Various analytical methods have been reported for determination of nebivolol including UV spectrophotometry [2], first-order derivative spectrophotometry [3], radioimmunoassay (RIA) [4] high-performance liquid chromatography (HPLC) [5], liquid chromatography-mass spectrophotometry (LC-MS) [6] and LC-MS-MS [1, 7].

The purpose of this investigation was to develop and validate a method using a simple, rapid, sensitive, precise, accurate and specific reversed phase HPLC assay. The method uses a simple mobile phase composition and the rapid run time of 6 min. Hence, this method can be used for the analysis of large number of samples.

MATERIALS AND METHODS
Chemicals and reagents
Nebivolol was obtained from Department of Cardiology, Faculty of Medicine, Ataturk University (Erzurum, Turkey). Methanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Vasoxen tablet containing 5.0 mg of nebivolol was obtained from Ulagaylar Pharmaceutical Industry (Istanbul, Turkey). HPLC-grade organic solvents were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade. Distilled water was prepared as required by using aquaMAX™ ultra, Young instrument (Korea) ultrawater purification system.

Instrumentation
A Perkin Elmer series 200 HPLC system equipped with programmable UV/Vis detector and Total Chromatography Data System software was used (Perkin Elmer Life and Science, Shelton, CT, USA). The HPLC mobile phase of methanol-water (70:30, v/v). Separation was achieved using an Ace C_{18} column (5 µm, 4.6×250 mm i.d.) with a guard column (4 mm x 3 mm i.d., Phenomenex) packed with the same material at a flow rate of 1.0 mL min\(^{-1}\). The eluent was monitored by UV detection at 282 nm.

Preparation of the standard and quality control solutions
The stock standard solution of nebivolol was prepared with methanol to a concentration of 50 µg mL\(^{-1}\) and stored at 4°C under refrigeration. The six standard solutions from 0.25 to 8.0 µg mL\(^{-1}\) (0.25, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 µg mL\(^{-1}\)) in methanol were made by a serial dilution. Three quality control (QC) samples at the concentrations of 0.75, 3.0 and 7.0 µg mL\(^{-1}\) were prepared from the stock standard solution.
Procedure for pharmaceutical preparations

The average tablet mass was calculated from the mass of tablets of Vasoxen (5 mg nebivolol tablet, which was composed of nebivolol and some excipients). They were then finely ground, homogenized and portion of the powder was weighed accurately, transferred into a 10 mL brown measuring flask and diluted to scale with methanol. The mixture was sonicated for at least 30 min to aid dissolution and then filtered through a Whatman No 42 paper. An appropriate volume of filtrate was diluted further with methanol so that the concentration of nebivolol in the final solution was within the working range and then analyzed by HPLC (figure 3).

Data analysis

All statistical calculations were performed with the Statistical Product and Service Solutions (SPSS) for Windows, version 10.0. Correlations were considered statistically significant if calculated P values were 0.05 or less.

RESULTS AND DISCUSSION

Method development and optimization

The development of the RP-HPLC method for determination of drugs has received considerable attention in recent years because of its importance in routine quality control analysis. A RP-HPLC method was proposed as a suitable method for the estimation of nebivolol in pharmaceutical dosage form. A good separation was achieved using an Ace C18 column (5 µm, 4.6×250 mm i.d.). The chromatographic conditions were adjusted in order to provide a good performance of the assay. The method involved a mobile phase consisting of methanol-water (70:30, v/v) accomplished at 282 nm. The retention time was 3.3 min at a flow-rate of 1 mL min⁻¹ and the injection volume was 10 µL. The total run time for an assay was approximately 6 min. The integrator attenuation was 10 and the chart speed was 0.2 cm min⁻¹. The mobile phase was chosen after several trials with other solvent combinations. Mobile phase selection was based on peak parameters (symmetry, tailing), run time, ease of preparation and cost. Figure 2 show a typical chromatogram obtained from the analysis of a standard nebivolol using the proposed method. As shown in Figure 2, nebivolol was eluted forming symmetrical peak and well separated from the solvent front. Observed retention time allowed a rapid determination of the drug.

Validation of the method

System suitability

A system suitability test of the chromatography system was performed before each validation run. Five replicate injections of a system suitability/calibration standard and one injection of a check standard were made. Area relative standard deviation, tailing factor and efficiency for the five suitability injections were determined. The check standard was quantified against the average of the five suitability injections. For all sample analyses, the tailing factor was ≤ 1.08, efficiency ≥ 1950 and %RSD ≤ 1.84%.

Figure 2. HPLC-UV chromatograms of nebivolol (0.25, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 µg mL⁻¹).

Linearity

Calibration curve was constructed for nebivolol standard by plotting the concentration of compound versus peak area response. Standard solutions containing 0.25, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 µg mL⁻¹ of nebivolol were prepared and 10 µL was injected into the HPLC column (Figure 2). The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method. The regression equations were calculated from the calibration graphs, along with the standard deviations of the slope (Sb) and intercept (Sa) on the ordinate (Table 1).

Accuracy and precision

Accuracy of the assay method was determined for both intra-day and inter-day variations using the six times analysis of the QC samples. Precision of the assay was determined by repeatability (intra-day) and intermediate precision (interday). Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time that was evaluated by assaying the QC samples during the same day. Intermediate precision was assessed by comparing the assays on different days (3 days). Accuracy and precision calculated for the QC samples during the intra- and inter-day run are given in Table 2. The accuracy ranged from 1.33% to 3.00% and precision from 2.64% to 4.82%. All the values were within the acceptance criteria of 5.0 %.

Sensitivity

Limits of detection (LOD) and quantification (LOQ) were estimated from the signal-to-noise ratio. The detection limit was defined as the lowest concentration level resulting in a peak height of three times the baseline noise. The quantitation limit was defined as the lowest.
concentration level that provided a peak height with a signal-to-noise ratio higher than 10, with precision (% RSD) and accuracy (% bias) within ±10%. LOD and LOQ values of HPLC method were determined to be 0.10 and 0.25 µg mL⁻¹, respectively (Table 1).

**Stability**

Stability studies indicated that the samples were stable when kept at room temperature, 4 and -20 °C refrigeration temperature for 8 h (short-term) and refrigerated at 4 and -20 °C for 72 h (long-term). The results of stability studies were given in Table 3 and no significant degradation was observed.

**Recovery**

Recovery studies by spiking different concentrations of pure drug in the preanalyzed tablet samples within the analytical concentration range of the proposed method. The added quantities of the individual drugs were estimated by above method. The results of recovery studies were found to be satisfactory and the results are presented in Table 4.

**Table 1.** Linearity of of nebivolol by HPLC method.

<table>
<thead>
<tr>
<th>Method</th>
<th>Range (µg mL⁻¹)</th>
<th>LRa</th>
<th>Sa</th>
<th>Sb</th>
<th>R</th>
<th>LOD (µg mL⁻¹)</th>
<th>LOQ (µg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>0.25 - 8.0</td>
<td>y=6843.4x+106.1</td>
<td>11.87</td>
<td>16.76</td>
<td>0.9997</td>
<td>0.10</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*a* Based on three calibration curves, LR: Linear regression, Sa: Standard deviation of intercept of regression line, Sb: Standard deviation of slope of regression line, R: Coefficient of correlation, y: peak area, x: nebivolol concentration, LOD: Limit of detection, LOQ: Limit of quantification

**Table 2.** Precision and accuracy of nebivolol by HPLC method.

<table>
<thead>
<tr>
<th>Method</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added (µg mL⁻¹)</td>
<td>Found ± SD</td>
</tr>
<tr>
<td>HPLC</td>
<td>0.75</td>
<td>0.73 ± 0.023</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>2.96 ± 0.078</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>6.88 ± 0.184</td>
</tr>
</tbody>
</table>

SD: Standard deviation of six replicate determinations, RSD: Relative standard deviation, 
aAverage of six replicate determinations, Accuracy: (%relative error) (found-added)/addedx100

**Table 3.** Stability of nebivolol in solution.

<table>
<thead>
<tr>
<th>Stability (%)</th>
<th>Room temperature stability</th>
<th>Refrigeratory stability, +4 °C</th>
<th>Frozen stability, -20 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Recovery % ± RSD)</td>
<td>(Recovery % ± RSD)</td>
<td>(Recovery % ± RSD)</td>
</tr>
<tr>
<td>Method</td>
<td>Added (µg mL⁻¹)</td>
<td>8 h</td>
<td>24 h</td>
</tr>
<tr>
<td>HPLC</td>
<td>0.75</td>
<td>98.2 ± 4.27</td>
<td>101.3 ± 4.78</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>99.6 ± 5.84</td>
<td>99.2 ± 3.49</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>98.4 ± 3.43</td>
<td>98.6 ± 4.09</td>
</tr>
</tbody>
</table>

**Table 4.** Recovery of nebivolol in pharmaceutical preparations by HPLC method.

<table>
<thead>
<tr>
<th>Method</th>
<th>Pharmaceutical preparation</th>
<th>Added (µg mL⁻¹)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Found ± SD</td>
<td>Recovery (%)</td>
<td>RSDa (%)</td>
</tr>
<tr>
<td>HPLC</td>
<td>Vasoxen tablet (1.5 µg mL⁻¹)</td>
<td>0.5</td>
<td>0.49 ± 0.021</td>
<td>98.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5</td>
<td>2.53 ± 0.134</td>
<td>101.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.5</td>
<td>6.52 ± 0.188</td>
<td>100.3</td>
</tr>
</tbody>
</table>

SD: Standard deviation of six replicate determinations, RSD: Relative standard deviation, aAverage of six replicate determinations, Accuracy: (%relative error) (found-added)/addedx100
Comparison of the methods

The method was validated according to either USP 26 [8] or the ICH guidelines [9] for validation of analytical procedures in order to determine the linearity, sensitivity, precision and accuracy for the analyte. The proposed method is very effective for the assay of nebivolol in tablets. The validity of the proposed method was presented by recovery studies using the standard addition method. For this purpose, a known amount of reference drug was spiked to formulated tablets and the nominal value of drug was estimated by the proposed methods. Each level was repeated six times. The results (Table 4) were reproducible with low SD and RSD. No interference from the common excipients was observed.

The proposed method was compared with spectrophotometric methods. There are two studies for determination of nebivolol by zero [2] and first-order derivative spectrophotometry method [3] in literature. In these studies, linearity ranges were determined as 5.0-50 μg mL⁻¹ and 10-70 μg mL⁻¹, respectively. In this present work, developed HPLC method has small linearity range (0.25-8.0 μg mL⁻¹). As the LOQ of the proposed HPLC methods is lower than the earlier reported work [2,3]. Therefore, the method can be adopted for routine quality testing and dissolution studies.

CONCLUSION

A rapid and simple isocratic HPLC method for determination of nebivolol has been developed and validated. This chromatographic assay fulfilled all the requirements to be identified as a reliable and feasible method, including accuracy, linearity, recovery and precision. The chromatographic run time of 6 min allows the analysis of a large number of samples in a short period of time. Therefore, the method is also suitable for analysis of sample during accelerated stability studies, routine analysis of formulations and raw materials.

REFERENCES