Stability-Indicating TLC-Densitometric Determination of Nebivolol Hydrochloride in Bulk and Pharmaceutical Dosage Form

Atul A. Shirkhedkar*, Prasad M. Bugdane, and Sanjay J. Surana
R.C. Patel College of Pharmacy, Shirpur Dist: Dhule, (M.S.) 425 405 India

Abstract

A simple, selective, precise, and stability-indicating high-performance thin-layer chromatographic (HPTLC) method for densitometric determination of nebivolol hydrochloride both as a bulk drug and in formulation was developed and validated as per the international conference on harmonization guidelines (ICH). The method employed TLC aluminium plates precoated with silica gel 60F254 as the stationary phase. The solvent system consisted of toluene–methanol–triethylamine (3.8:1.2:0.2 v/v/v). Densitometry analysis of nebivolol hydrochloride was carried out in the absorbance mode at 281 nm. The system was found to give compact spot for nebivolol hydrochloride ($R_f$ value of 0.33 ± 0.02). The linear regression analysis data for the calibration plots showed good relationship with $r^2 = 0.9994 ± 0.0002$ in the concentration range 500–3000 ng/spot. The mean value ± SD of slope and intercept were 3.761 ± 0.017 and 127.39 ± 19.53 with respect to peak area. The limits of detection (LOD) and limit of quantitation (LOQ) were 63.10 ng/spot and 191.23 ng/spot, respectively. Nebivolol hydrochloride was subjected to acid and alkali hydrolysis, oxidation, thermal degradation, and photodegradation. All the peaks of degradation products were well-resolved from the standard drug with significantly different $R_f$ values. Statistical analysis proves that the method is repeatable, selective and accurate for the estimation of said drug. The proposed developed HPTLC method can be applied for identification and quantitative determination of nebivolol hydrochloride in the bulk and pharmaceutical dosage form.

Introduction

Nebivolol hydrochloride, $\alpha,\alpha'$[iminobis (methylene)] bis [6-fluoro-3, 4- dihydro-2H-1-benzopyran-2-methanol] hydrochloride is an antihypertensive (1,2). Nebivolol hydrochloride occurs in two isomeric forms (3). (+) Nebivolol acts as strong adrenergic $\beta_1$ blocker whereas (–) nebivolol as vasodilator (4,5).

In literature, various methods have been reported for the estimation of nebivolol hydrochloride from pharmaceutical dosage form and biological fluids. The first order UV-spectrophotometry (6), reverse-phase high-performance liquid chromatography (HPLC)–UV detection (7), enantiomeric resolution on normal and reversed amylase based-chiral phases using HPLC (8), enantioseparation using chiral stationary phase (9), high-speed determination in human urine by LC–tandem mass spectrometry (10), rapid quantification in human plasma by liquid chromatography coupled with electrospray ionization tandem mass spectrometry (11), are some of those. High-performance thin-layer chromatography (HPTLC) densitometric quantitative analysis from tablet formulation (12, 13) has been reported. All of these methods have their advantages and limitations. However, to our knowledge, no article related to the stability-indicating HPTLC determination of nebivolol hydrochloride has been described in literature.

The aim of this work is to develop an accurate, specific, repeatable, and stability-indicating method for the determination of nebivolol hydrochloride in the presence of its degradation products as per ICH guidelines.

Experimental

Nebivolol hydrochloride was supplied as a gift sample from Cadila Pharmaceuticals, (Ahmedabad, India). All chemicals and reagents used were of analytical-grade and purchased from Qualigens Fine Chemicals (Mumbai, India).

HPTLC instrumentation

The samples were spotted in the form of bands of width 6 mm with Camag microliter syringe on precoated silica gel aluminium Plate 60F254 (20 cm × 10 cm with 0.2 mm thickness, E. Merck, Darmstadt, Germany) using Camag Linomat V (Muttenz, Switzerland). A constant application rate of 150 nL/s was employed, and the space between two bands was 15 mm. The slit dimension was kept 6 mm × 0.45 mm. The mobile phase consisted of toluene–methanol–triethylamine (3.8:1.2:0.2, v/v/v). Linear ascending development was carried out in twin trough glass chamber saturated with mobile phase. The optimized chamber saturation time for mobile phase was 25 min at room temperature. The length of chromatogram run was approximately 80 mm. Subsequent to the development, TLC plates were dried in current of air with the help of an air dryer. Densitometric
scanning was performed using Camag TLC scanner III in the absorbance mode at 281 nm. The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum in the range of 190–400 nm.

Calibration curve of nebivolol hydrochloride
A stock solution of nebivolol hydrochloride (1000 µg/mL) was prepared in methanol. Different volume of stock solution 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 µL were spotted in six replicates on TLC plates to obtain concentration of 500, 1000, 1500, 2000, 2500, and 3000 ng/spot of nebivolol hydrochloride, respectively. The data of peak area versus drug concentration were treated by linear least square regression.

Method validation (14–18)

Precision
Repeatability of sample application and measurement of peak area were carried out using six replicates of the same spot (2000 ng/spot of nebivolol hydrochloride). The intra-day and inter-day variation for the determination of nebivolol hydrochloride was carried out at three different concentration levels of 1000, 1500, and 2000 ng/spot.

Robustness of the method
By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phase having different composition like toluene–methanol–trimethylamine (3.6:1.4:0.2, v/v/v), (4.0:1.0:0.2, v/v/v) were tried, and chromatograms were run. The amount of mobile phase was varied as 5.02 and 10.02 mL. Development distance was varied as 7, 7.5, and 8 cm. Plate was developed at relative humidity 55% and 65%. The duration of saturation was also varied from 20, 25, and 30 min. The effects on results were examined.

Limit of detection and limit of quantification
In order to determine detection and quantification limit, concentrations in the lower part of the linear range of the calibration curve were used. Stock solution of nebivolol hydrochloride (1000 µg/mL) was prepared and different volume of stock solution 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 µL were spotted in triplicate. The amount of nebivolol hydrochloride by spot versus average response (peak area) was graphed and the equation for this was determined. The standard deviations (SD) of responses were calculated. The average of standard deviations was calculated (ASD). Detection limit was calculated by (3.3 × ASD)/b and quantification limit was calculated by (10 × ASD)/b, where b corresponds to the slope obtained in the linearity study of method.

Specificity
The specificity of the method was ascertained by analyzing standard drug and sample. The spot for nebivolol hydrochloride in sample was confirmed by comparing the Rf values and spectra of the spot with that of standard. The peak purity of nebivolol hydrochloride was accessed by comparing the spectra at three different levels, i.e., peak start (S), peak apex (M), and peak end (E) positions of the spot.

<table>
<thead>
<tr>
<th>Table I. Linear Regression Data for the Calibration Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (ng/spot)</td>
</tr>
<tr>
<td>Coefficient of Correlation r² ± SD</td>
</tr>
<tr>
<td>Slope ± SD</td>
</tr>
<tr>
<td>Intercept ± SD</td>
</tr>
</tbody>
</table>
Recovery studies

The pre-analyzed sample was over spotted with extra 80, 100, and 120% of the standard nebivolol hydrochloride, and it was analyzed by the proposed method. At each level of the amount, three determinations were performed. This was done to check the recovery of drug at different levels in the formulation.

Analysis of nebivolol hydrochloride in prepared formulation

To determine the concentration of nebivolol hydrochloride in tablets (label claim: 5 mg per tablet), twenty Nebicard tablets were weighed, mean weight determined, and ground to a fine powder. The powder equivalent to 10 mg of Nebivolol was weighed. The drug from the powder was extracted by methanol. To ensure the complete extraction of the drug, it was sonicated for 20 min, and the volume was made up to the 50 mL. The 5 µL of the previously mentioned solution (1000 ng/spot) was applied on TLC plate followed by development and scanning as described previously. The analysis was repeated in six times. The possibility of excipients interferences in the analysis was studied.

Forced degradation of nebivolol hydrochloride (19–20)

In all degradation studies the average peak area of nebivolol hydrochloride after application (1000 ng/spot) of seven replicates was obtained. The plate was developed and scanned under the previously established chromatographic conditions. Peak area was recorded for each concentration of degraded drug.

Table II. Intra-Day and Inter-Day Precision*

<table>
<thead>
<tr>
<th>Amount ng/spot found</th>
<th>Intra-day precision</th>
<th>Inter-day precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>993.82</td>
<td>999.15</td>
</tr>
<tr>
<td>1500</td>
<td>1507.24</td>
<td>1498.03</td>
</tr>
<tr>
<td>SD</td>
<td>8.47</td>
<td>3.40</td>
</tr>
<tr>
<td>%RSD</td>
<td>0.85</td>
<td>0.34</td>
</tr>
</tbody>
</table>

* Mean of three estimations at each level.

Table III. Recovery Studies*

<table>
<thead>
<tr>
<th>Initial amount (ng)</th>
<th>Excess drug added to analyte (%)</th>
<th>Amount recovered (ng)</th>
<th>Recovery (%)</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>0</td>
<td>997.33</td>
<td>99.73</td>
<td>1.80</td>
</tr>
<tr>
<td>1000</td>
<td>80</td>
<td>798.11</td>
<td>99.76</td>
<td>1.59</td>
</tr>
<tr>
<td>1000</td>
<td>100</td>
<td>994.12</td>
<td>99.41</td>
<td>1.62</td>
</tr>
<tr>
<td>1000</td>
<td>120</td>
<td>1196.94</td>
<td>99.74</td>
<td>0.87</td>
</tr>
</tbody>
</table>

* Mean of three determinations at each level.

Figure 4. A typical HPTLC chromatogram of Nebivolol hydrochloride from pharmaceutical dosage form (Rf = 0.33) (A). HPTLC chromatogram of acid treated Nebivolol hydrochloride (B). HPTLC chromatogram of base treated Nebivolol hydrochloride (C). HPTLC chromatogram of dry heat degraded Nebivolol hydrochloride (D). HPTLC chromatogram of hydrogen peroxide (3% v/v) degraded Nebivolol hydrochloride (E). HPTLC chromatogram of Photo degraded Nebivolol hydrochloride (F).

Acid- and base-induced degradation

The 10 mg of nebivolol hydrochloride was separately dissolved in 10 ml methanolic solution of 0.1 M HCl and 0.1 M NaOH. These solutions were kept for 8 h at room temperature in the dark in order to exclude the possible degradative effect of light. 1 mL of the described solution was taken, neutralized, and diluted up to 10 mL with methanol. The resultant solution were applied on TLC plates, (10 µL each, of 1000 ng/spot). The chromatograms were run as described earlier.

Hydrogen peroxide-induced degradation

The 10 mg of nebivolol hydrochloride was separately dissolved in 10 mL of methanolic solution of hydrogen peroxide (3.0%, v/v). The solution was kept for 8 h at room temperature in the dark in order to exclude the possible degradative effect of light. 1 mL of described solution was taken and diluted up to 10 mL with methanol. The resultant solution was applied on TLC plate, (10 µL each, of 1000 ng/spot). The chromatograms were run as described in section 2.1.

Dry heat degradation products

Nebivolol hydrochloride 10 mg was stored at 55°C for 3 h in oven. It was transferred to 10-mL volumetric flask containing methanol and volume was made up to the mark. 1 µL
(1000 ng/spot) was applied on TLC plate and chromatogram were run as described earlier.

**Light heat degradation products**

The 10 mg of nebivolol hydrochloride was dissolved in 10 mL of methanol. The solution was kept in the sun light for 8 h. 1 mL of the described solution was taken and diluted up to 10 mL with methanol. The resultant solution was applied on TLC plate (10 µL each of 1000 ng/spot). The chromatograms were run as described earlier.

Nebivolol hydrochloride in acid, base, hydrogen peroxide, photo, and dry heat degraded sample solutions and in standard drug solutions were spectral scanned to access the peak purity.

**Results and Discussion**

**Development of optimum mobile phase**

TLC procedure was optimized with a view to develop a stability-indicating assay of method. Initially, mobile phase consisted of toluene–methanol (4:2, v/v) gave good resolution with Rf value of 0.33 for nebivolol hydrochloride, but a typical peak nature was missing. Finally, the mobile phase consisting of toluene–methanol–triethylamine (3.8:1.2:0.2 v/v/v) gave a sharp and well-defined peak at Rf value of 0.33. Well-defined spots were obtained when the chamber was saturated with the mobile phase for 25 min at room temperature.

**Calibration curve**

The linear regression data for the calibration curve (n = 6) as shown in Table I showed a good linear relationship over the concentration range 500–3000 ng/spot with respect to peak area.

No significant difference was observed in the slopes of standard curves.

**Validation of the method**

**Precision**

The repeatability of sample application and measurement of peak area were expressed in the terms of % RSD, and the results are depicted in Table II, which revealed intra-day and inter-day variation of nebivolol hydrochloride at three different concentration levels 1000, 1500, and 2000 ng/spot.

**Robustness of the method**

The standard deviation of peak areas was calculated for each parameter and % RSD was found to be less than 2%. The low values of % RSD indicated robustness of the method.

**LOD and LOQ**

Detection limit and quantification limit was calculated by the method as described earlier and were found to be 63.10 and 191.23 ng, respectively. This indicates adequate sensitivity of the method.

**Specificity**

The peak purity of the nebivolol hydrochloride was assessed by comparing the spectra at peak start, peak apex, and peak end positions of the spot as shown in Figure 1.

**Recovery studies**

The proposed method when used for extraction and subsequent estimation of nebivolol hydrochloride from pharmaceutical dosage form after over spotting with 80, 100, and 120% of additional drug afforded recovery of 98–102 % as listed in Table III.

**Analysis of prepared formulation**

A single spot of Rf 0.33 ± 0.02 was observed in chromatogram of the nebivolol hydrochloride samples extracted from tablets. There was no interference from the excipients commonly present in the tablets. The nebivolol content was found to be as per the labeled claim. The results are shown in Table IV. It may therefore be inferred that degradation of nebivolol hydrochloride had not occurred in the formulation that were analyzed by this

---

### Table IV. Analysis of Prepared Formulation

<table>
<thead>
<tr>
<th>Component</th>
<th>Labeled claim (mg)</th>
<th>Amount Found ± SD*</th>
<th>% Found ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebivolol</td>
<td>5</td>
<td>5.00 ± 0.06</td>
<td>100.08 ± 1.36</td>
</tr>
</tbody>
</table>

* mean of six determinations

### Table V. Summary of Validation Parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (ng/spot)</td>
<td>500 – 3000</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9994 ± 0.0002</td>
</tr>
<tr>
<td>Limit of detection (ng/spot)</td>
<td>63.10</td>
</tr>
<tr>
<td>Limit of quantitation (ng/spot)</td>
<td>191.23</td>
</tr>
<tr>
<td>Recovery (% RSD)</td>
<td>0.87–1.80</td>
</tr>
<tr>
<td>Precision (% RSD)</td>
<td>1.26</td>
</tr>
<tr>
<td>Repeatability (n = 7)</td>
<td>0.47–0.85</td>
</tr>
<tr>
<td>Intra-day (n = 3)</td>
<td>0.34–0.87</td>
</tr>
<tr>
<td>Inter-day (n = 3)</td>
<td>1.74</td>
</tr>
<tr>
<td>Robustness (% RSD)</td>
<td>1.89</td>
</tr>
<tr>
<td>Specificity</td>
<td>Robust</td>
</tr>
</tbody>
</table>

### Table VI. Forced Degradation of Nebivolol Hydrochloride

<table>
<thead>
<tr>
<th>Sample exposure condition</th>
<th>No degradation product (Rf value)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M HCl, 8h, RT*</td>
<td>1 (0.68)</td>
<td>82.88</td>
</tr>
<tr>
<td>0.1M NaOH, 8h, RT*</td>
<td>4 (0.49, 0.67, 0.69, 0.75)</td>
<td>76.07</td>
</tr>
<tr>
<td>3 % H2O2, 8h, RT*</td>
<td>2 (0.65, 0.68)</td>
<td>96.42</td>
</tr>
<tr>
<td>Dry heat, 3 h, 55°C</td>
<td>2 (0.63, 0.70)</td>
<td>91.90</td>
</tr>
<tr>
<td>Light heat, 8 h</td>
<td>3 (0.47, 0.54, 0.68)</td>
<td>86.69</td>
</tr>
</tbody>
</table>

* RT = Room temperature.
method. The low % RSD value indicated the suitability of this
method for routine analysis of nebivolol hydrochloride in phar-
maceutical dosage form. The typical chromatogram of nebivolol
is as shown in Figure 2.

The summary of validation parameters are listed in Table V.

Stability-indicating property

Acid and base induced degradation product

The chromatogram of the acid degraded sample for nebivolol
hydrochloride showed one peak at Rf value 0.68 (Figure 2). The
chromatogram of the base degraded sample showed four peaks at
Rf value of 0.49, 0.67, 0.69, and 0.75, respectively (Figure 2). The
areas of degraded peaks were found to be lesser than the area of
standard drug concentration (1000 ng/spot), indicating that
nebivolol hydrochloride undergoes degradation under acidic and
basic conditions.

Dry heat degradation product

The samples degraded under dry heat conditions (Figure 2)
showed additional peaks at Rf values of 0.63 and 0.70. The spots
of degraded products were well resolved from the drug spot.

Hydrogen peroxide induced degradation product

The sample degraded with hydrogen peroxide (Figure 2) showed
additional peaks at Rf values of 0.65 and 0.68. The spots
of degraded products were well-resolved from the drug spot.

Photochemical degradation product

The photo degraded sample showed three additional peaks at
Rf values of 0.47, 0.54, and 0.68 when drug solution was left in
day light for 8 h (Figure 2). From the stability-indicating studies,
this proves that the drug is susceptible to acid-base hydrolysis, oxida-
tion, dry heat degradation, and photo degradation. The results
are listed in Table VI.

Conclusion

The developed HPTLC technique is precise, specific, accurate,
and stability-indicating. The developed method was validated
based on ICH guidelines. Statistical analysis proves that the
method is repeatable and selective for the analysis of nebivolol
hydrochloride as bulk drug and in pharmaceutical dosage form.
The method can be used to determine purity of drug available
from the various sources by detecting various impurities. As the
method separates the drug from its degradation products, it can
be employed as a stability indicating method.

References

1. S. Budavari. The Merck Index, 14th ed. Merck and Co., INC., White
House Station, NJ, 2006, pp. 1112.
2. E.F Reynolds and James. Martindale, The extra pharmacopoeia,
4. P.J. Pauwels, W. Gommersen, G. Van Lommen, P.A. Janssen and
J.E. Leysen. The Receptor binding profile of the new antihyperten-
sive agent nebivolol and its stereoisomers compared with various
(1988).
5. J.R. Cockcroft, P.J. Chowienczyk, S.E. Brett, C.P. Chen, A.G. Dupont,
L. Van Nueten, S.J. Wooding and J.M. Ritter. Nebivolol vasodilates
human forearm vasculature: evidence for an L- arginine/ NO-
6. A.A. Shirkhedkar, P.M. Bugdane, S.J. Surana. First order UV-spe-
trophotometric determination of Nebivolol in bulk and tablets.
Seshgiri Rao. RP-HPLC method for estimation of nebivolol in bulk
8. Y. Hassen, Aboul-Enein and Imran Ali. HPLC enantiomeric resolu-
tion of nebivolol on normal and reversed amylose based chiral
9. Aboul-Enein, H.X. Enantioseperation of some clinically used drugs
by HPLC using chiral stationary phase. Biomed. Chromatogr. 17:
of beta blocker blocking agent in human urine by liquid chro-
matography / tandem mass spectrometry. Biomed. Chromatogr. 15:
11. N.V.S. Ramakrishna. Rapid quantification of nebivolol in human
plasma by liquid chromatography coupled with electrospray ion-
12. T. Sheshashena Reddy, P. Sita Devi. Validation of a high-perfor-
mance thin-layer chromatographic, with densitometric detection,
for quantitative analysis of nebivolol hydrochloride in tablet formu-
13. L. J. Patel, B.N. Suhagia, P.B. Shah. RP-HPLC and HPTLC methods
for the estimation of nebivolol hydrochloride in tablet dosage form.
Q1A, Stability testing of new drug substances and products, ICH,
15. International Conference on Harmonisation. ICH/CPMP guidelines
Q2(R1), Validation of analytical procedures: Text and methodology.
16. Reviewer Guidance, Validation of chromatographic methods, Food
and Drug Administration, Centre for Drug Evaluation and Research.
17. Monika Baksi, Saranjit Singh. Development of validated stability-
18. The United States Pharmacopeia. USP 28, NF 19, United States
Pharmacopoeial Convention, Rockville, MD., Asian edition, 1982,
pp. 1225.
to determine inherent stability of drugs. Pharmaceutical Tech.
Online–April-2000, pp. 1–14.
HPTLC determination of imitanib mesylate in bulk and pharmaceu-

Manuscript received March 17, 2008;
Revision received May 26, 2008.