Stability Indicating HPTLC Method for Estimation of Nebivolol Hydrochloride and Amlodipine Besylate in Combination

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Abstract

A sensitive, selective, precise and stability indicating high-performance thin-layer chromatographic method of analysis of Amlodipine besylate and Nebivolol hydrochloride both as a bulk drug and in formulations containing these two in combination was developed and validated. The method employed TLC aluminum plates precoated with silica gel 60F 254 as the stationary phase. The solvent system consisted of Ethyl acetate: Methanol: Dilute ammonia (8.5:1:1, v/v/v). This system was found to give compact spots for Amlodipine besylate (Rf 0.40 ± 0.01) and Nebivolol hydrochloride (Rf 0.60 ± 0.02). Amlodipine besylate and Nebivolol hydrochloride were individually subjected to stress degradation conditions like oxidation, dry heat treatment, photo degradation and hydrolysis under different pH. The peaks of products formed during stress degradation studies were well resolved from the bulk drug peak with significantly different Rf values. Densitometric analysis of Amlodipine besylate and Nebivolol hydrochloride was carried out at 240 and 280 nm respectively. The linear regression analysis data showed good linear relationship in the concentration range 500–2000 ng spot⁻¹ for both Amlodipine besylate and Nebivolol hydrochloride. The method was validated for linearity, accuracy, specificity, LOD, LOQ, precision and robustness. The limits of quantitation for Amlodipine besylate and Nebivolol hydrochloride were found to be 313 and 277 ng spot⁻¹ respectively. The statistical analysis proved that the method is repeatable and selective for the estimation of the said drugs. As the method could effectively detect the drugs in the presence of their degradation products, it can be employed as a stability indicating one.

Keywords:
Amlodipine besylate; Nebivolol hydrochloride; HPTLC; Stability

1. Introduction

Amlodipine besylate, chemically known as 3-ethyl-5-methyl (4RS)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6- methyl-1,4-dihydropyridine-3,5-dicarboxylate benzene sulphonate. The chemical structure of amlodipine is as shown in Fig. 1. It belongs to calcium antagonists of class dihydropyridines [1-3]. Quantification of amlodipine besylate has been performed in the past [4-10] using HPTLC, HPLC coupled with fluorometric detection, UV spectrophotometry, liquid chromatography coupled with electro spray ionization mass spectrometry or UV spectrophotometry, gas chromatography, and voltammetry.

Nebivolol hydrochloride is the racemate (dl-nebivolol) of the enantiomers l-nebivolol and d-nebivolol. Chemically it is α â- [iminobis(methylene)]bis(6-fluoro-3,4-dihydro-2H-1-
benzopyran-2-methanol]) [11]. The structure of nebivolol is as shown in Fig 2. It is a competitive and highly selective beta-1 receptor antagonist with mild vasodilating properties, used in the treatment of essential hypertension. Clinical trials have shown that the frequency of adverse events is low. Quantification of nebivolol- the racemic form, has been done in past [12-18] using HPTLC and RP-HPLC method, liquid chromatography coupled with electrospray ionization mass spectrometry or with spectrophotometry.

![Fig. 1 Structure of Amlodipine besylate](image)

![Fig.2 Structure of d and l- Nebivolol](image)

To our knowledge, no study related to the stability indicating high performance thin layer chromatography (HPTLC) determination of amlodipine besylate and nebivolol hydrochloride in a combined pharmaceutical dosage forms has ever been mentioned in literature. The international conference on harmonization (ICH) guideline entitled Stability Testing of New Drug Substances and Products requires the testing to be carried out to elucidate the inherent stability characteristics of the active substances [19].

Now-a days HPTLC is becoming a routine analysis technique due to advantages of low operating cost, high sample throughput and need for minimum sample clean up. The major advantage of HPTLC is that several samples could be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis. [20]

The aim of the present work is to develop an accurate, specific, repeatable and stability-indicating HPTLC method for the determination of amlodipine besylate and nebivolol hydrochloride in presence of their degradation products for assessment of purity of bulk drug and stability of their combined dosage forms. The proposed method was validated as per ICH guidelines [21]

2. Experimental

2.1. Materials

M/S Lupin research park, Pune, India and M/S Torrent Pharmaceutical Ltd., Mehsana, India kindly supplied bulk drug sample of Amlodipine besylate and Nebivolol hydrochloride, respectively as a gift samples. Those were used without further purification. All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, India.
2.2 Instrumentation

The samples were spotted in the form of bands of width 6 mm with a Camag 100 microlitre sample syringe (Hamilton, Bonaduz, Switzerland) aluminum TLC plate precoated with silica gel 60 F254, (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany) using a Camag Linomat 5 sample applicator. The plates were prewashed by methanol and activated at 110ºC for 5 min prior to chromatography. The slit dimension was kept at (5mm x 0.45mm) and 10 mm/s scanning speed was employed. The monochromator bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of ethyl acetate: methanol: dilute ammonia (8.5:1:1 v/v/v) and 10.5 mL of mobile phase was used per chromatography. Linear ascending development was carried out in twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25ºC ± 2). The length of chromatogram run was 8 cm. Subsequent to the development; TLC plates were dried in air. Densitometric scanning was performed on Camag TLC scanner 3, operated by Win CATS software (V1.4.3, Camag). The source of radiation utilized was deuterium lamp emitting continuous UV spectrum between 200 and 400 nm. Evaluation was via peak areas with linear regression analysis.

2.3 Forced Degradation Studies

A stock solution containing 100 mg Amlodipine besylate and Nebivolol hydrochloride in 100 mL methanol was prepared. This solution was used for forced degradation to provide an indication of the stability indicating property and specificity of proposed method. In all degradation studies, 1000 ng spot⁻¹ was applied in five replicates. In order to study the degradation products of Amlodipine besylate and Nebivolol hydrochloride using HPTLC method, most of the study was carried out by single development of TLC plate in order to prevent the movement of the non-polar degradates to extreme end of the plate.

2.3.1. Hydrolytic degradation studies

The drug solution was prepared in the methanol. The studies were carried out by using 9ml of this solution and 0.5ml of 5N HCl or 5N NaOH and keeping it overnight. The resultant solutions were neutralized and then applied on TLC plate in such a way that final concentration achieved was 1000 ng.band⁻¹ the chromatogram was developed as described in section 2.2.

2.3.2. Oxidative degradation studies

The drug solution was prepared in the methanol. The studies were carried out by using 9ml of this solution and 0.5ml of 30% hydrogen peroxide and keeping at room temperature for a period of 72 h. For HPTLC study, volume was made upto 10 ml and resultant solutions were applied on TLC plate in such a way that final concentration achieved was 1000 ng.band⁻¹ and the chromatogram was developed as described in section 2.2.

2.3.3. Thermal degradation studies

The standard drug in solid form was placed in oven at 80ºC for 24 hours to study dry heat degradation.

2.3.4. Photolytic Degradation studies

The photochemical stability of the drug was studied by exposing the bulk drug to following conditions as per ICH guideline:
U.V light- 200 Watt hrs.m$^{-2}$ followed by Cool Fluorescent light upto illumination of 1.2 million Lux hrs.

**2.4. Optimization of stability indicating HPTLC method**

The HPTLC procedure was optimized with a view to develop stability indicating assay method. Solutions of both the bulk drugs and drugs exposed to stress conditions were spotted on TLC plates and run in different solvent systems. Initially, ethyl acetate: methanol: dilute ammonia were tried in different ratios. Ethyl acetate was used to obtain significantly good $R_f$ value. Initially, toluene, acetone and ethanol in the ratio of 5:1:1 v/v/v was selected but it was found that spot had high $R_f$. Then volume of dilute ammonia was decreased and $R_f$ was found to be 0.50, but compactness of spot was lacking, as a result considerable amount of peak tailing was observed. Therefore, to reduce the tailing and improve compactness of the spot 0.2 mL dil ammonia solution was added. Ultimately the mobile phase consisting of ethyl acetate: methanol: dilute ammonia (8.5:1:1 v/v/v) was optimized. In order to reduce the neckless effect TLC chamber was saturated for 20 min using saturation paper. The mobile phase was run upto distance of 8 cm; which takes approximately 20 min for development of TLC plate. Representative densitogram is shown Fig. 3.

![Fig. 3](image.png)

**Fig. 3** Representative Densitogram of standard amlodipine besylate and nebivolol hydrochloride 1000 ng.band$^{-1}$ each; at 280 nm.

**2.5. Validation of the method**

Validation of optimized HPTLC method was done with respect to following parameters:

**2.5.1. Linearity and range**

The standard solutions of Amlodipine besylate and Nebivolol hydrochloride were prepared to obtain a concentration of 500 ng.$\mu$L$^{-1}$. Appropriate volume from each standard solution was spotted on the TLC plate to obtain final concentration250-2000 ng.spot$^{-1}$. Each concentration was spotted five times on the TLC plate. The plate was developed using previously described mobile phase. The peak areas were plotted against the corresponding concentrations to obtain the calibration graphs.

**2.5.2. Precision**

Precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analysis of three different concentration of
the drugs in triplicate on the same day. Intermediate precision of the method was checked by repeating studies on three different days.

2.5.3. Limit of detection and limit of quantitation

The LOD and LOQ were calculated by the method based on the standard deviation (σ) of the lowest responses and the slope (S) of the linear calibration plot, using the formula LOD = 3.3σ / S and LOQ = 10σ / S. The LOD and LOQ were experimentally verified by diluting known concentrations of standard solution of amlodipine besylate and nebivolol hydrochloride.

2.5.4. Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spot for Amlodipine besylate and Nebivolol hydrochloride in sample was confirmed by comparing the Rf and spectra of the spot with that of standard. The peak purity of Amlodipine besylate and Nebivolol hydrochloride was assessed by comparing the spectra at three different levels i.e. peak start (S), Peak maxima (M) and peak end (E) position.

2.5.5. Accuracy

Accuracy of the developed method was tested by standard addition method. Drug corresponding to 80, 100 and 120% was added separately to preanalysed sample. At each level, the amount recovered was calculated.

2.6. Analysis of marketed formulation

To determine the content of Amlodipine besylate and Nebivolol hydrochloride in conventional tablets (Brand name: Nebicard SM, label claim: 2.5 mg Amlodipine besylate and 5 mg Nebivolol hydrochloride per tablet), twenty tablets were weighed, their average weight was determined and then they were finely powdered. An equivalent quantity of the drug was weighed and transferred into a 100 mL volumetric flask containing 50 mL methanol, sonicated for 10 min and diluted to 100 mL with methanol. The resulting solution was centrifuged at 3000 rpm for 5 min and supernatant was analyzed for drug content (1000 μg.mL⁻¹). The above filtered solution was diluted appropriately to produce 100 μg.mL⁻¹ 10 microlitre of the above solution (1000 ng./band) was applied on the TLC plate followed by development and scanning as described in Section 2.2. The analysis was performed in triplicate. The possibility of excipient interference in the analysis was studied by spotting commonly used excipients like starch, magnesium stearate.

3. Results and discussion

3.1. Stability indicating property

HPTLC studies of samples obtained on stress testing of amlodipine besylate and nebivolol hydrochloride under different conditions suggested the following degradation behavior.

3.1.1. Stress degradation results (Refer Table 1)

3.2. Validation of the Stability Indicating Method

The results of validation studies on the stability indicating method developed for Amlodipine besylate and nebivolol hydrochloride in the current study involving ethyl acetate: methanol: dilute ammonia (8.5:1:1v/v/v) as the mobile phase for HPTLC are given below.
3.2.1. Linearity

The response for both the drugs was linear in the concentration range between 500-2000 ng.band\(^{-1}\). The mean values of slope, intercept and correlation coefficient were 3.1605 and 101.67 and 0.9983, for amlodipine besylate and 4.8864, 480.68, 0.9989 for nebivolol hydrochloride respectively.

Table 1. Percent decrease in area of amlodipine besylate and nebivolol hydrochloride under various stress conditions

<table>
<thead>
<tr>
<th>No</th>
<th>Stress conditions</th>
<th>% decrease in peak area</th>
<th>Amlodipine besylate (at 240 nm)</th>
<th>Nebivolol hydrochloride (at 280nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1 N HCl</td>
<td>negligible</td>
<td>negligible</td>
<td>negligible</td>
</tr>
<tr>
<td>2</td>
<td>5 N HCl</td>
<td>48.93</td>
<td>52.58 (with 10NHCL)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.1 N NaOH</td>
<td>negligible</td>
<td>negligible</td>
<td>negligible</td>
</tr>
<tr>
<td>4</td>
<td>5 N NaOH</td>
<td>42.10</td>
<td>11.12</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>3% H(_2)O(_2)</td>
<td>negligible</td>
<td>negligible</td>
<td>negligible</td>
</tr>
<tr>
<td>6</td>
<td>30% H(_2)O(_2)</td>
<td>10.74</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Photolytic</td>
<td>Less than 2%</td>
<td>Less than 1%</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Thermal</td>
<td>Less than 1%</td>
<td>Less than 1%</td>
<td></td>
</tr>
</tbody>
</table>

3.2.2. Precision

The developed method was found to be precise as the RSD values for repeatability and intermediate precision studies were < 2 %, respectively as recommended by ICH guideline.

3.2.3. LOD and LOQ

The LOD and LOQ were found to be 103.11ng Spot\(^{-1}\) and 312.48 ng.Spot\(^{-1}\), respectively for amlodipine besylate and 91.54ng.Spot\(^{-1}\) and 277.42ng.Spot\(^{-1}\), respectively for nebivolol hydrochloride.

Table 2. Summary of validation parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range</td>
<td>500-2000 ng.spot(^{-1})</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9983</td>
</tr>
<tr>
<td>Limit of detection</td>
<td>103.11ng.Spot(^{-1})</td>
</tr>
<tr>
<td>Limit of quantitation</td>
<td>312.48 ng.Spot(^{-1})</td>
</tr>
<tr>
<td>% Recovery (n=6)</td>
<td>Within 98.5 to 99.1%</td>
</tr>
<tr>
<td>Precision (%RSD)</td>
<td>Less than 2%</td>
</tr>
<tr>
<td>Repeatability (n=6)</td>
<td>Less than 1.5%</td>
</tr>
<tr>
<td>Inter day (n=6)</td>
<td>1.8%</td>
</tr>
<tr>
<td>Specificity</td>
<td>Specific</td>
</tr>
</tbody>
</table>
3.2.4. Robustness of the method

This method is very sensitive to ratio of mobile phase component and saturation time so they are necessary to be controlled meticulously. Slight variations in R_f values can be observed if these two parameters are not controlled.

3.2.5. Specificity

The peak purity of amlodipine besylate and nebivolol hydrochloride was assessed by comparing their respective spectra at peak start, apex and peak end positions of the spot i.e., r (S, M) = 0.9994 and r (M, E) = 0.999 for amlodipine besylate and r (S, M) = 9972 and r (M, E) = 0.9991 for nebivolol hydrochloride. Good match was obtained between standard and sample spectra of Amlodipine besylate and nebivolol hydrochloride respectively.

3.2.6. Recovery Studies

Recovery of the amlodipine besylate was in the range from 98.5 to 99.1% and for nebivolol hydrochloride it was in the range from 99.5 to 100.1%, which was obtained at various added concentrations, when the drug was fortified to a marketed sample solution.

3.3. Analysis of marketed formulation

Experimental results of the amount of amlodipine besylate and nebivolol hydrochloride in tablets, expressed as percentage of label claim were in good agreement with the label claims thereby suggesting that there is no interference from any excipients, which are normally present in tablets. The content of amlodipine besylate was found to be 98.5% ± 0.56, whereas that of nebivolol was found to be 99.2% ± 0.49.

4. Conclusion

The developed HPTLC technique is precise, specific, accurate and stability indicating. Statistical analysis proves that the method is suitable for the analysis of amlodipine besylate and nebivolol hydrochloride as bulk drug and in pharmaceutical formulation without any interference from the excipient. This study is a typical example of stability indicating assay, established following the recommendations of ICH guidelines. After exposing the drugs to different stress condition, the drug peak area was observed to decrease but no peak for degradation product could be located in spite of multi wavelength scanning. This indicates that the products of degradation do not absorb strongly in U.V or are below the LOD values. This method may be proposed for the analysis of drug content in the presence of degradation products in stability samples in industry.

References


