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**SIMULTANEOUS ESTIMATION OF NEBIVOLOL HYDROCHLORIDE AND S-AMLODIPINE BESYLATE BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY****ANILKUMAR SHARMA\*<sup>1</sup>, BHAVESH PATEL<sup>2</sup> AND RAKSHIT PATEL<sup>2</sup>**<sup>1</sup>Professor, Parul Institute of Pharmacy, Vaghodiya, Vadodara, Gujarat, India<sup>2</sup>K. B. Institute of Pharmaceutical Education and Research, Gandhinagar, Gujarat, India.*\*Corresponding Author*      drbhp@rediffmail.com**ABSTRACT**

A simple, rapid, and reliable HPTLC method has been established for simultaneous determination of Nebivolol and S-Amlodipine in pharmaceutical dosage forms. Identification and quantification were performed on silica gel 60 F254 HPTLC plates, prewashed with methanol, with chloroform:toluene:methanol:glacialacetic acid, (5:2:3:0.1, v/v/v/v), as mobile phase. This system furnished compact bands for Nebivolol ( $R_f$  0.48) and S-Amlodipine ( $R_f$  0.33). Calibration curves were established showing the dependence of response (peak area) on the amount chromatographed. The validated linearity ranges were 500–2500 ng /spot ( $r_2 = 0.9978$ ) and 250–1250 ng/spot ( $r_2 = 0.9972$ ) for Nebivolol and S-Amlodipine, respectively. The spots were scanned at  $\lambda = 271$  nm. The suitability of this HPTLC method for quantitative determination of the compounds was proved by validation in accordance with the requirements of the ICH guidelines. The method was used for determination of the compounds in commercial pharmaceutical dosage forms. The method is simple, reproducible, accurate and can be used as a more economical alternative to other chromatographic techniques for routine quality control.

**KEY WORDS**

HPTLC, Nebivolol and S-Amlodipine, Validation

**INTRODUCTION**

Nebivolol, chemically [(2RS,2,SR)-bis(6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl)]-2,2'-indioethanol hydrochloride, is a third-generation vasodilating cardioselective  $\beta$ -blocking agent. Its molecular formula is  $C_{22}H_{25}F_2NO_4 \cdot HCl$  and it has a molecular weight of 444.90 g/mole<sup>1,2</sup>. S-Amlodipine besylate, (4-S)-2-[(2-aminoethyl)oxy]methyl-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate

benzene sulfonate, a third-generation dihydropyridine calcium channel antagonist. Its molecular formula is  $C_{20}H_{25}ClN_2O_5 \cdot C_6H_6O_3S$ , and it has a molecular weight of 567.10 g/mole<sup>3</sup>. The antihypertensive and antianginal effects of amlodipine by the calcium channel-blocking effect are attributed to S-amlodipine, whereas R-amlodipine is regarded as an impurity that might be inactive or might have undesirable

activities<sup>4</sup>. A recent literature survey revealed that few methods were available for the determination of Nebivolol hydrochloride in pure, pharmaceutical dosage forms and/or biological fluids, it includes high-performance liquid chromatography (HPLC)<sup>5-7</sup>, high-performance thin-layer chromatographic (HPTLC), UV spectrophotometric<sup>8</sup> and liquid chromatographic tandem mass spectrometry<sup>9,10</sup>. Numerous different analytical methods have been developed for quantitative determination of Amlodipine besylate in pure, pharmaceutical dosage forms and/or biological fluids. These methods include high performance liquid chromatography<sup>11</sup>, high-performance thin layer chromatography<sup>12,13</sup>, differential-pulse voltammetry<sup>14</sup>, spectrofluorometric<sup>15</sup> and spectrophotometry<sup>16-26</sup>. However, there is no method for the simultaneous determination of these two drugs by high performance thin-layer chromatography (HPTLC). HPTLC is a more effective technique for the simultaneous determination in single samples in routine analysis.

The aim of the proposed work was to develop an HPTLC method for the simultaneous determination of Nebivolol hydrochloride and S-Amlodipine besylate. Quantitative estimation was accomplished by densitometric scanning with UV detector at 271 nm wavelength. The assay was validated in accordance with the requirements of ICH guidelines (Q2B)<sup>27</sup>. The method was confirmed by application on authentic dosage forms.

## EXPERIMENTAL CONDITIONS

### Materials:

Nebivolol hydrochloride and S-Amlodipine besylate used as working standards were gifted from Torrent Research Centre (Gandhinagar, India). All chemicals were of analytical reagent grade (S.D. Fine Chemicals, Mumbai, India). Two brands of tablets, NEBICARD-SM (Torrent Ltd) and NEBISTAR-SA (Lupin Ltd), were procured from the local market.

### Instrumentation:

A Camag Linomat V fully automatic sample applicator, a Camag TLC Scanner III, a Camag twin trough flat-bottom TLC developing chambers, and viewing cabinet with UV lamps (Camag, Muttenz, Switzerland) were used. Merck HPTLC plates coated with silicagel 60 F 254 (0.2 mm thickness) on aluminium sheets were used as the stationary phase (E. Merck KGaA, Darmstadt, Germany). Ultrasonicator used for homogenizing of test and standard solutions.

### Chromatographic conditions:

Chromatography was performed on 10 cm × 10 cm aluminum HPTLC plates coated with 0.2 mm layers of silica gel 60 F 254 (E. Merck, Darmstadt, Germany). The plates were prewashed with methanol and activated at 50 °C for 5 min prior to chromatography.

Samples were applied as 6-mm bands, 10.0 mm apart and 10 mm from the lower edge of the plate, by means of a 100-μL Hamilton (Reno, Nevada, USA) micro syringe, mounted on a Linomat V applicator (Camag, Muttenz Switzerland); the spraying rate was 15 μL<sup>-1</sup>.

The mobile phase consisted of chloroform:toluene:methanol: glacial acetic acid (5:3:2:0.1, v/v/v/v). Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 20 min at room temperature (25°C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 8 cm. The average development time was 20 min. After development the plate was dried in an oven at 50°C for 5 min. Densitometric scanning at λ = 271 nm, using a deuterium light source, was then performed with a Camag TLC Scanner equipped with win CATS Software Version 1.3.4. The slit dimensions were 5.00 mm × 0.45 mm and 10 mm/s scanning speed was employed.

### Preparation of Standard Stock Solutions:

Accurately weighed Nebivolol (50 mg) and S-Amlodipine (25 mg) were transferred to a 100 mL volumetric flask and dissolved in, and then diluted to the mark with, methanol to obtain a standard stock solution of Nebivolol (500 µg/mL) and S-Amlodipine (250 µg/mL).

#### **Preparation of Test Sample Solutions:**

To determine the content of Nebivolol and S-Amlodipine simultaneously in conventional tablets (label claim: 5mg Nebivolol and 2.5 mg S-Amlodipine per tablet, combination tablet containing both analytes), the twenty tablets were weighed, their mean weight determined and they were finely powdered and powder equivalent to 5 mg Nebivolol and 2.5 mg S-Amlodipine was weighed. Then equivalent weight of the drug was transferred into a 10 ml volumetric flask containing 5 ml methanol, sonicated for 15 min and diluted to 10 ml with methanol to obtain solution of Nebivolol (500 µg/mL) and S-Amlodipine (250 µg/mL). The mixture was filtered using 0.45 µm nylon membrane filter.

## **METHOD VALIDATION**

The method was validated in accordance with ICH guidelines (Q2B) (55).

#### **Linearity:**

Calibration curves were plotted over a concentration range of 500-2500 ng/spot and 250-1250 ng/spot for Nebivolol and S-Amlodipine, respectively. Accurately prepared standard solutions of Nebivolol and S-Amlodipine (1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 µL) were applied to the plate. Calibration plots were constructed by plotting peak area against the Corresponding amount of each drug. Each reading was the average of 3 determinations.

#### **Accuracy:**

The accuracy of the method was determined by use of standard additions at three different levels, i.e. multiple level recovery studies. Sample stock solution of tablet formulation

containing 1000 ng mL<sup>-1</sup> and 500 ng mL<sup>-1</sup> for Nebivolol and S-Amlodipine was prepared. This solution was spiked with 75%, 100%, and 125% of the standard drug solutions and percentage recoveries were determined.

#### **Method precision (Repeatability):**

The repeatability of sample application and measurement of peak area were expressed in terms of %R.S.D. and were found to be 0.2209 and for 0.2461 Nebivolol and S-Amlodipine respectively.

#### **Intermediate Precision (Reproducibility):**

The proposed method was determined by estimating the corresponding responses of Nebivolol and S-Amlodipine at three different concentration levels 500, 1500 and 2250 ng/spot and 250, 750 and 1125 ng/spot respectively. The % R.S.D. for within and day-to-day analysis was found to be <2% in all the cases.

#### **LOD and LOQ:**

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were calculated using the following equations as per International Conference on Harmonization (ICH) guidelines (55).

$$\text{LOD} = 3.3 \times [\sigma]/S$$

$$\text{LOQ} = 10 \times [\sigma]/S$$

Where  $\sigma$  = the standard deviation of the response and S = the standard deviation of y-intercept of regression lines.

#### **Specificity:**

The specificity of the method was ascertained by analyzing standard drug and sample. The spot for Nebivolol and S-Amlodipine in sample was confirmed by comparing the R<sub>f</sub> and spectra of the spot with that of standard. The peak purity of Nebivolol and S-Amlodipine was assessed by comparing the spectra at three different levels, i.e. peak start, peak apex and peak end positions of the spot.

**Robustness:**

By introducing small deliberate changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition of chloroform: toluene: methanol: glacial acetic acid (4.5:3.2:2:0.1 and 5.2:2.8:2:0.1 v/v/v/v) was tried at two different concentration level of 500 and 750 ng/spot for Nebivolol and 250 and 375 ng/spot for S-Amlodipine.

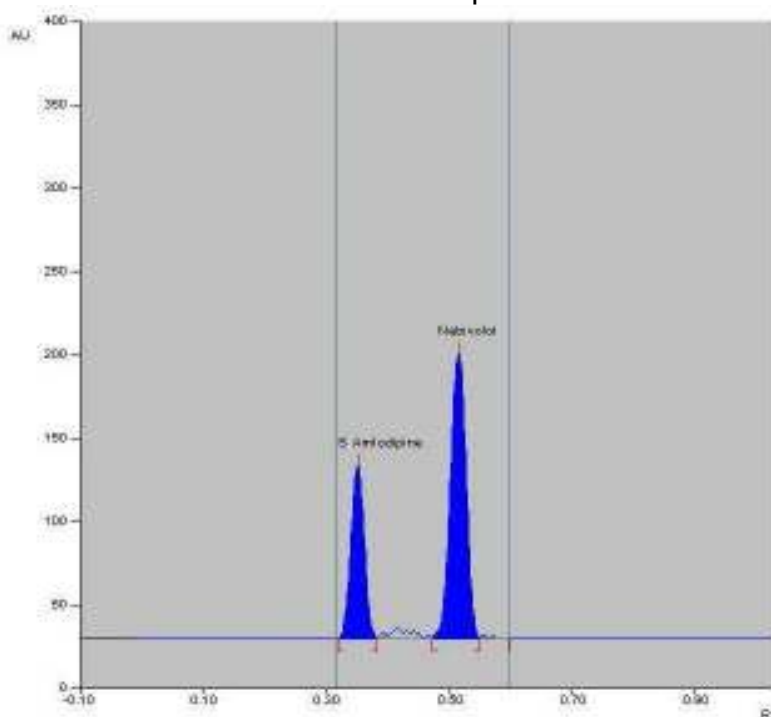
**Analysis of the marketed formulation:**

The responses of sample solutions were measured at 271 nm for quantitation of Nebivolol and S-Amlodipine by using HPTLC method as described for tablets. The amounts of Nebivolol and S- Amlodipine present in sample solution were determined by applying values of peak area

to the regression equations of the calibration graph.

**RESULT AND DISCUSSION****Development of the optimum mobile phase:**

Several mobile phases were tried to achieve good separation of Nebivolol and S-Amlodipine. Ultimately mobile phase consisting of chloroform: toluene: methanol: glacial acetic acid (5: 3: 2: 0.1, v/v/v/v) gave good resolution with  $R_f$  values of 0.33 and 0.49 for S-Amlodipine and Nebivolol respectively. Both the peaks were symmetrical in nature and no tailing was observed when plates were scanned at 271 nm (Fig. 1). Well-defined spots were obtained when plate was activated at 500C for 5 min. and the chamber was saturated with the mobile phase for 20 min at room temperature.

**Fig: 01**

**Chromatogram obtained from HPTLC of Nebivolol (500 ng/spot) and S-Amlodipine (250 ng/spot) standard drug solution**

**Linearity:**

Calibration plots were constructed by plotting peak area against the corresponding amount of each drug (ng /spot). Good linear correlation was obtained between peak areas and

concentrations of Nebivolol and S-Amlodipine in the range of 500-2500 and 250-1250 ng/mL, respectively. The linearity of the calibration curves was validated by the high value of correlation coefficients of regression (Table 1).

**Table 1**  
**System suitability Parameter (n=3)**

Parameter	Nebivolol	S-Amlodipine
Rf value $\pm$ S.D	0.48 $\pm$ 0.01	0.33 $\pm$ 0.007
Linearity range	500-2500 ng/spot	250-1250 ng/spot
Slope $\pm$ S.D	1.6371 $\pm$ 0.14	1.7356 $\pm$ 0.07
Intercept $\pm$ S.D	762.78 $\pm$ 13.51	213.86 $\pm$ 8.45
Correlation coefficient ( $r^2$ )	0.9978	0.9972
LOD	44.75 ng/spot	31.28 ng/spot
LOQ	135.61 ng/spot	94.80 ng/spot

**Accuracy:**

When the method was used for extraction and subsequent estimation of Nebivolol and S-Amlodipine in pharmaceutical dosage forms

after spiking with 75, 100, and 125 % of additional drug the mean recovery was  $99.79 \pm 0.08$  and  $99.48 \pm 0.11$  for Nebivolol and S-Amlodipine respectively (Table 2).

**Table 2**  
**Results from recovery studies (n = 3)**

component Initial	Label Claim (mg)	amount (ng)	Amount added (%)	Amount recovered (ng)	Recovery (%) $\pm$ S.D	%R.S.D.
Nebivolol	5	1000	0	994.04	99.40	0.52
		1000	75)	1749.22a	99.89	0.57
		1000	100	1997.42a	99.74	0.31
		1000	125	2246.93a	99.75	0.25
S-mlodipine	2.5	500	0	496.76a)	99.35	1.09
		500	75	873.26a)	99.53	0.65
		500	100	998.52	99.70	0.44
		500	125	1121.75	99.48	0.53

**Method precision:**

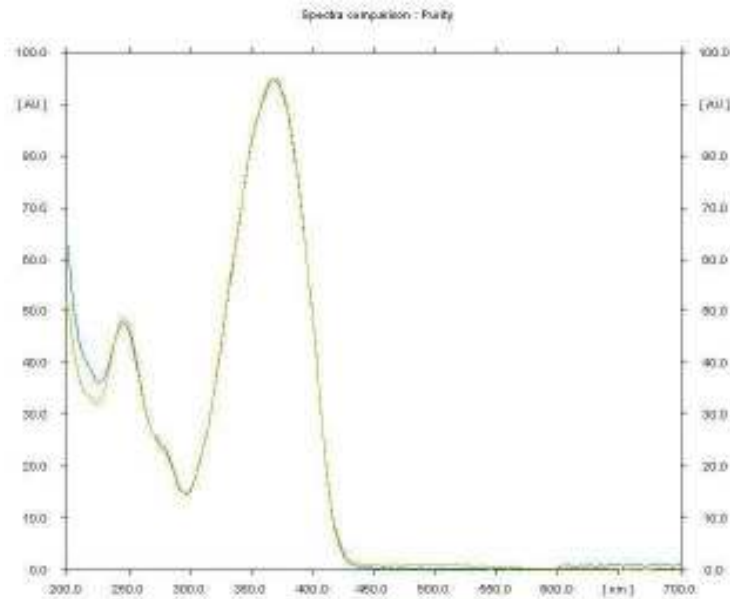
The intra-day and inter-day precision were expressed in terms of %R.S.D. and were found to be 0.54, 0.39 and 0.65, 0.70 for Nebivolol and S-Amlodipine, respectively. The %R.S.D. values shows that proposed method provides acceptable intra-day and inter-day variation of Nebivolol and S-Amlodipine.

**LOD and LOQ:**

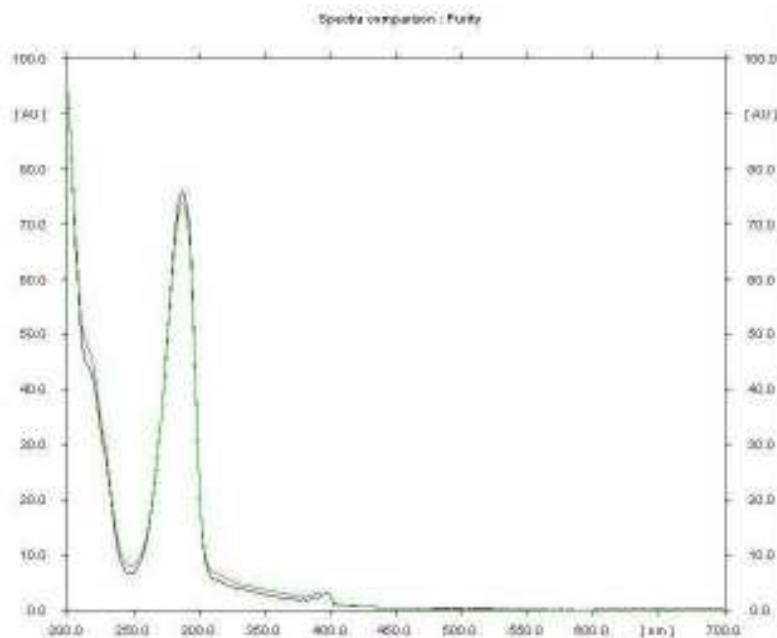
LOD for Nebivolol and S-Amlodipine was found to be 44.75 ng/spot and 31.28 ng/spot, and LOQ was found to be 135.61 ng/spot and 94.80 ng/spot respectively (Table 1).

**Specificity:**

The peak purity for Nebivolol and S-Amlodipine was tested by correlation of spectra acquired at the peak start (s), peak apex (m), and peak end (e) positions. Correlation between these spectra confirmed the purity of the Nebivolol peak (correlation  $r(s,m) = 0.999949$ ,  $r(m,e) = 0.999879$ ) (figure 2) and for S-Amlodipine peak (correlation  $r(s,m) = 0.999920$ ,  $r(m,e) = 0.999750$ ) (figure 3) respectively. Thus it can be concluded that the excipients did not interfere with the peaks from standard drug solutions.



**Fig: 02**  
**Peak purity spectra of Nebivolol**



**Fig: 03**  
**Peak purity spectra of S-Amlodipine**

**Robustness:**

The low values of %RSD obtained after introducing small changes in mobile phase composition indicated robustness of the method. There was no significant variation in the slope values.

**Assay of the marketed formulations:**

The proposed validated method successfully applied to determine Nebivolol and S-Amlodipine in their tablet dosage forms (tablets A and B). The results obtained for Nebivolol and S-Amlodipine were comparable with the



corresponding labeled amounts (Table 3). There was no interference from the excipients commonly present in the tablets. The low

%R.S.D. value indicated the suitability of this method for routine analysis of Nebivolol and S-Amlodipine in pharmaceutical dosage form.

**Table 3.**  
**Assay results for the combined dosage form using HPTLC method (n=3)**

Brand	Parameter	Component Nebivolol	S-Amlodipine
NEBICARD-SM	Label claim (mg)	5	2.5
	Drug content (%) $\pm$ S.D	99.84 $\pm$ 0.44	99.65 $\pm$ 0.73
	% RSD	0.44	0.74
NEBISTAR-SA	Label claim (mg)	5	2.5
	Drug content (%) $\pm$ S.D	99.83 $\pm$ 0.65	99.46 $\pm$ 0.98
	% RSD	0.65	0.98

## CONCLUSION

The developed HPTLC technique is simple, accurate and reproducible for simultaneous determination of Nebivolol and S-Amlodipine of in pharmaceutical dosage forms. Statistical analysis proves that the method is repeatable for the analysis of Nebivolol and S-Amlodipine as bulk drug and in pharmaceutical formulations without any interference from the excipients. The

method was validated in accordance with ICH guidelines. The method reduces analysis time compared with other methods mentioned in literature survey and seems to be suitable for routine analysis of pharmaceutical formulations in quality-control laboratories, where economy and speed are essential.

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