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Development and Validation of High-Performance Thin-Layer Chromatography Method for Determination of Nebivolol and Amlodipine besylate in Combined Dosage Forms

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ABSTRACT

The manuscript describes validated High-Performance Thin-Layer Chromatography (HPTLC) method for the estimation of Nebivolol (NEB) and Amlodipine (AML) in combined dosage form. The HPTLC separation was achieved on an aluminium-backed layer of silica gel 60F254 using chloroform-methanol-toluene-ammonia (8+ 1.8+ 1+ 0.3, v/v/v/v/) as mobile phase. Quantitation was achieved with UV detection at 273 nm over the concentration range 400-1000 ng/spot and 200 – 800 ng/spot for Nebivolol and Amlodipine, respectively with mean recovery of 100.3 ± 0.76 and 100.5 ± 0.92 % for Nebivolol and Amlodipine by HPTLC method. These method were found to be Simple, sensitive, accurate, precise, reproducible, and economical can be applicable for the simultaneous determination of Nebivolol and Amlodipine in combined dosage form.

Key words: Nebivolol, Amlodipine besylate, HPTLC

INTRODUCTION

Nebivolol (NEB), a, a1–(imino bis (methylene)) bis (6-fluoro-3, 4-dihydro-2H -1-benzopyran-2-methanol), is an antihypertensive drug and a selective ß1 -receptor antagonist without partial agonist activity (1). Amlodipine (AML), 3-ethyl 5-methyl 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1, 4-dihydropyridine-3, 5- dicarboxylate, is a long-acting calcium channel blocker used as an anti-hypertensive in the treatment of angina (2). Nebivolol is not official in any pharmacoepia. Amlodipine besylate is official in IP and BP. HPLC (3, 4) method describe in IP and BP for its estimation. Literature survey reveals various methods like HPLC (5, 6), HPTLC (7), LC-MS (8) and Spectrophotometry (9) for determination of NEB in dosage forms as well as biological fluids. Literature survey reveals spectrophotometry (10) HPLC (11, 12) Spectrofluorometry (13) Differential-Pulse Voltammetry (14), LC/MS/MS (15) and HPTLC (16) methods for determination of AML in dosage forms as well as in biological fluids. Till now there is only one reported UV Spectrophotometry method (17) for simultaneous determination of NEB and AML is reported in combined dosage forms. But there is no reported HPTLC method for simultaneous determination of NEB and AML in combined dosage forms and this combination is not official in any pharmacopoeia so no official method is available for estimation of these two drugs in combined dosage form. Hence, the aim was to develop simple, accurate, precise, reproducible and economical HPTLC method for simultaneous determination of NEB and AML in pharmaceutical dosage form.

EXPERIMENTAL

Apparatus and chromatographic conditions

A Linomat V autosprayer, TLC scanner III with wincats-4 software, twin-trough flatbottom TLCdevelopment chambers, and viewing cabinet with UV lamps (camag, muttenz, Switzerland) were used. HPTLC plates used were 10×10 cm silica gel with an indicator fluorescing at 254 nm, layer thickness 0.2 mm, a Shimadzu (UV-1700), Sartorius CP224S analytical balance (Gottingen, Germany), ultra sonic cleaner (Frontline FS 4, Mumbai, India) and corning volumetric flasks were used during the study. Solution of AML and NEB were applied to silica gel 60F254 HPTLC plates (10×10 cm) by means of a Linomat V automatic spotter equipped with a 100 µL syringe and operates with settings of band length, 6mm; distance between bands, 5mm; distance from bottom of plate, 10mm; and distance from the bottom of plate, 10mm.The plate was developed in a twin-trough chamber previously saturated for 30 min with the mobile phase, chloroform-methanol-toluene-ammonia (8+1.8+1+0.3,v/v/v/v/) , for a distance of 8 cm. The spots on the air-dried plate were scanned with the scanner 3 at 273 nm using the deuterium source.

Reagents and Materials

NEB and AML besylate powders with 99.94 and 99.96 % purity, respectively, supplied by Torrent Research Centre (Ahmedabad, India), were used as standard. The tablets (5 mg NEB and 5 mg AML per tablet) were procured from the local pharmacy; Chloroform and toluene (AR grade) were purchased from S.D. Fine-Chem Ltd., Mumbai. Methanol and Ammonia (AR grade) were purchased from Finar Chemicals Ltd, Ahmedabad India, and nylon 0.45 µm – 47 mm membranes filter (Gelman Laboratory, Mumbai, India).

Preparation of NEB and AML Standard Stock Solutions

A mixed stock solution of NEB ((400 µg/mL) and AML (200µg/mL) was prepared by accurately weighing NEB (40 mg) and AML besylate (27.72 mg) equivalent to AML (20 mg) dissolving in methanol and diluted to 100 mL with methanol in the same volumametric flask.

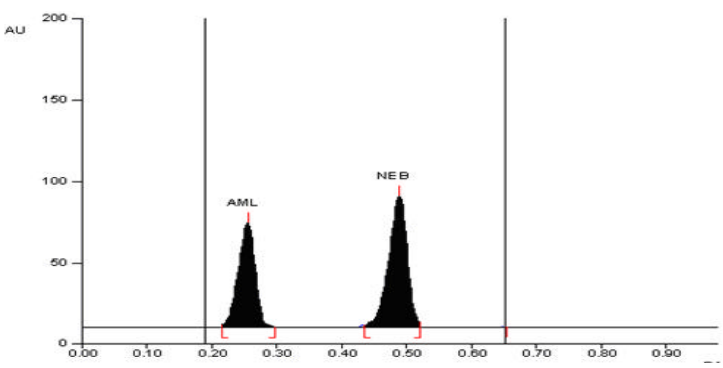
Preparation of Sample Solutions

Twenty tablets were weighed and powdered. The quantity of the powder equivalent to 5 mg of NEI and 5 mg of AML was transferred to a 50 ml volumetric flask. The content was mixed with methanol (30 mL), sonicated for 20min. to dissolve the drug as completely as possible. The solution was then filtered through a nylon 0.45 µm membrane filter. The volume was adjusted up to the mark with methanol.

RESULTS AND DISCUSSION

Several mobile phases were tried to accomplish good separation of NEB and AML. Using the mobile phase chloroform: methanol: toluene: ammonia (8 + 1.8 + 1+ 0.3, v/v/v/v) and *10*×10 cm HPTLC silica gel 60F254 aluminium-backed plates, good Separation was attained with retardation factor (Rf) values of 0.27 for AML and 0.51 for NEB. A wavelength of 273 nm was used for quantification of the drugs. Resolution of peaks with clear baseline separation was found (Figure 1).

Fig 1- HPTLC chromatogram of NEB (800 ng/spot) and AML(600ng/spot)



Stationary phase: 10×10 cm HPTLC silica gel 60F254 aluminium-backed plates, Mobile phase: chloroform: methanol: toluene: ammonia (8 + 1.8 + 1+ 0.3, v/v/v/v), Detection: UV 273 nm.

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Validation of the Proposed Method

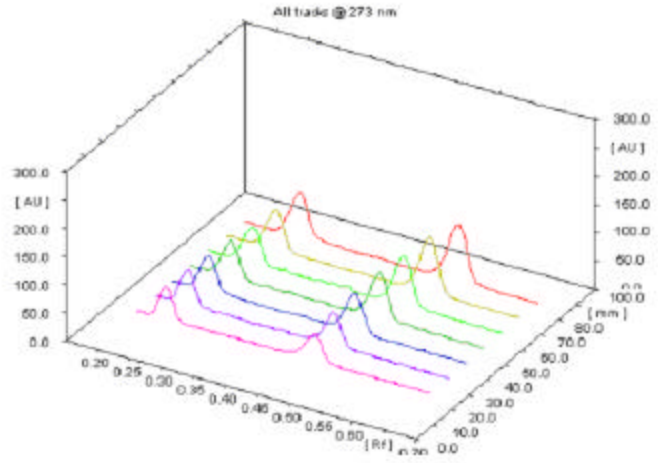
Linearity - Linear correlation was obtained between peak areas and absorbance Vs concentrations of NEB and AML in range of 400-1000nm/spot and 200-800nm/spot for HPTLC method. The linearity of the calibration curves was validated by the high value of correlation coefficients of regression (Table 1). 3-D view of calibration curve was shown in (Figure 2)

Table 1. Regression analysis data and summary of validation parameters for the proposed HPTLC method.

Parameters	HPTLC method NEB	AML
Concentration range	400-1000(ng/spot)	200-800(ng/spot)
Slope	2.1254	1.1783
Intercept	136.91	645.69
Correlation coefficient	0.9954	0.9927
LOD[a]	28.11(ng/spot)	40.26(ng/spot)
LOQ[b]	85.18(ng/spot)	122.08(ng/spot)
Accuracy (% recovery, n = 6)	101.7 ± 0.23	99.23 ± 1.57
Repeatability(% RSD[c], *n* = 6)	0.24	3.47
Precision (%RSD)		
Interday (*n* = 6)	0.45-2.26	0.27-1.63
Intraday (*n* = 6)	0.39-0.81	0.27-1.63

[a]*LOD = Limit of detection.*
[b]*LOQ = Limit of quantification.*
[c]*% RSD = Percent relative standard deviation.*

Fig 2 -3D-Chromatogram of Nebivolol and Amlodipine at 273 nm.



Accuracy - The recovery experiments were carried out by the standard addition method. The mean recoveries obtained was 101.7 ± 0.23 % and 99.2 ± 1.57 % for NEB and AML by HPTLC method (Table 2).The high values indicate that method are accurate.

Table 2. Results of recovery study for NEB and AML by the proposed HPTLC method (n=3)

Method	Drug	Amount of sample taken (ng/mL)	Amount of standard spiked (%)	Mean % Recovery ± SDa*
HPTLC	NEB	300	50	99.51 ± 0.75
		300	100	100.5 ± 1.39
		300	150	100.4 ± 1.62
	AML	300	50	99.42 ± 0.91
		300	100	100.7 ± 1.79
		300	150	99.98 ± 1.45

[a]*S.D = Standard deviation.*

Method precision - The % RSD values for NEB and AML were found to be 0.24 and 3.46 using HPTLC method (Table1). The low values of RSD indicate the proposed method is repeatable.

Intermediate precision - The low RSD values of interday (0.45-2.26 % and 0.27-1.63%) And intraday (0.39-0.81 % and 0.27-1.63 %) variations for NEB and AML, by HPTLC method reveal that the proposed method are precise (Table 1).

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

LOD and LOQ - LOD for NEB and AML were found to be 28.1 ng/spot and 40.3 ng/spot, by HPTLC method. LOQ for NEB and AML were found to be 85.2ng/spot and 122.1 ng/spot, by HPTLC method (Table 1). These data show that the method is sensitive for the determination of NEB and AML.

Assay of the pharmaceutical formulation

The proposed validated method were successfully applied to determine NEB and AML in their combined dosage form (brand A and B).The spectra of sample is shown in figure 3. The results obtained for NEB and AML were comparable with the corresponding labeled amounts (Table 3).

Fig 3- Chromatogram of sample solution of Nebivolol and Amlodipine at 273 nm

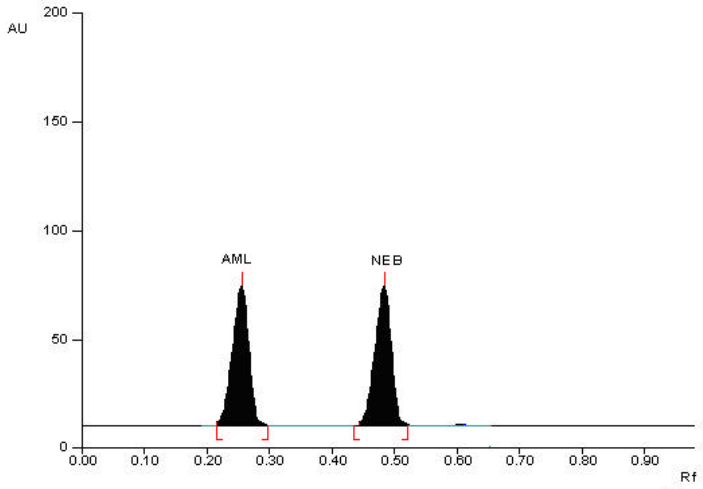


Table 3. Assay results for the combined dosage form using the proposed HPTLC

Parameters	HPTLC method NEB ± S.D[a] (*n*[b] = 6)	AML± S.D[a] (*n*[b] = 6)
Brand A	99.56 ± 2.11	99.54 ± 1.48
Brand B	98.12 ± 1.10	101.8 ± 0.86

[a]*S.D = Standard deviation.*
[b]*n = Number of determinations.*

Comparison of the proposed methods with reported method

The assay results for NEB and AML in their combined dosage forms obtained using HPTLC and reported method were compared with each other by applying paired t-test. The calculated t value 1.32 for NEB and 1.18 for AML were less than the tabulated t-value (2.31) at 95 % confidence interval. Statistical comparison of the results obtained by proposed HPTLC method with the results obtained by reported method shows good agreement and indicates no significant difference in the content of NEB and AML by the proposed HPTLC and reported method (Table 4).

Table 4. Comparison between results obtained by the proposed HPTLC method and reported method

Parameters	HPTLC method NEB	AML	Reported Method NEB	AML
Assay results ± S.D[a]	99.56 ± 2.11	99.54 ± 1.48	99.80 ± 1.21	99.89 ± 1.75
n[b]	6	6	6	6
t-value (2.31)[c]	1.32	1.18	_	_

[a]*S.D = Standard deviation*
[b]*n = number of determinations*
[c]*Figures in the parentheses represent corresponding to t-tabulated value at 95% confidence interval*

CONCLUSION

The results of the analysis of pharmaceutical formulation by the proposed method are highly reproducible and reliable and are in good agreement with the label claim of the drug. The additives usually present in the pharmaceutical

formulations of the assayed samples did not interfere with determination of NEB and AML. The method can be routinty used for the analysis of the NEB and AML in combined dosage form.

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