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Research Article

DEVELOPMENT AND VALIDATION OF HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY METHOD FOR SIMULTANEOUS ESTIMATION OF NEBIVOLOL HYDROCHLORIDE AND CILNIDIPINE

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ABSTRACT

Objective: The proposed method describes method development and validation of nebivolol hydrochloride and cilnidipine in combined pharmaceutical tablet dosage form by high-performance thin-layer chromatography (HPTLC) having adequate specificity, sensitivity, and reproducibility.

Methods: Nebivolol hydrochloride and cilnidipine drug combination is used for the treatment of hypertension. Precoated aluminum plates with silica gel 60 F_{254} (E-Merck, Germany) were used for the chromatographic separation which was carried using chloroform:glacial acetic acid:methanol, in 8.5:1:0.5 (v/v/v) as a mobile phase. HPTLC separation of two drugs was carried by densitometric measurement at 270 nm.

Reults: The drugs were satisfactorily resolved with retardation factor values of 0.0.29±0.008 and 0.69±0.007 for nebivolol hydrochloride and cilnidipine, respectively. The method was found to be linear in the range of 100–1000 ng/spot and 50–500 ng/spot for nebivolol hydrochloride and cilnidipine, respectively. The correlation coefficient was found to be 0.989 and 0.996 for nebivolol hydrochloride and cilnidipine, respectively. Limit of detection and limit of quantitation values were 16.395 ng/band and 49.681 ng/band and 31.788ng/band and 96.328 ng/band, respectively. The mean recovery was found to be 100.570–101.936 and 100.269–101.333 for nebivolol hydrochloride and cilnidipine, respectively. The intra- and inter-day precision was found to be within the limit.

Conclusion: A simple, accurate, precise, and sensitive HPTLC method has been developed and validated in combined pharmaceutical tablet dosage form for simultaneous estimation of nebivolol hydrochloride and cilnidipine.

Keywords: Method development high-performance thin-layer chromatography, Nebivolol hydrochloride, Cilnidipine, International Council for Harmonisation.

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INTRODUCTION

Hypertension is a disease characterized by abnormal elevation of blood pressure (BP) in the arteries. It is broadly classified as primary and secondary. About 90–95% of cases are termed as primary (idiopathic) hypertension, which refers to high BP for which no exact cause can be found. The remaining 5–10% of secondary hypertension can be caused by conditions that affect your kidneys, arteries, heart or endocrine system. Prevention and control of high BP are the main focuses for reducing the severity of cardiovascular diseases [1].

Combination of drugs such as nebivolol HCl and cilnidipine has been prescribed to manage hypertension [2]. Nebivolol HCl (1RS,1′RS)-1,1′-[(2RS,2′SR)-bis(6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl)]- 2,2′-iminodiethanol hydrochloride [3] is a highly selective β1-blocker with nitric oxide-mediated vasodilatory actions and beneficial effects on vascular endothelial function.

Cilnidipine is a dihydropyridine calcium channel blocker. It is a dual blocker of L-type voltage-gated calcium channels in vascular smooth muscle and N-type calcium channels in sympathetic nerve terminals that supply blood vessels [4]. This combination is launched in the market under brand name Ln Beta, manufactured by Eris Lifesciences Pvt. Ltd., Ahmedabad.

It was found that UV spectroscopic, stability-indicating RP-HPLC, HPLC, and bioanalytical methods have been reported for analysis of these two drugs in combination [5-12]. However, there is only one paper reported for the analysis of the combination by high-performance thin-layer chromatography (HPTLC) [13].

In view of the need for a suitable method for routine analysis in combined formulations, attempts are being made to develop simple, precise, accurate, rapid, economical, and reproducible analytical methods for the simultaneous estimation of nebivolol HCl and cilnidipine and extend it to their determination in the formulation. The work deals with development and validation of HPTLC method for the estimation of nebivolol HCl and cilnidipine by HPTLC in accordance with the International Council for Harmonisation (ICH) guidelines for analytical application.

Nebivolol hydrochloride and cilnidipine were a generous gift by Actavis Pharmaceuticals Ltd., Mumbai, India and Prayosha Healthcare Pvt. Ltd., Ankleshwar, India, respectively. Chloroform, glacial acetic acid, methanol, and all other chemicals used were of analytical reagent grade.

Equipment

The instruments used in the study were Camag HPTLC system comprising Linomat-5 applicator, Camag thin-layer chromatography scanner 3, Win Computer-Aided Transcription System software (Version 1.4.3, Camag), aluminum plates precoated with silica gel 60 F_{254} (E-Merck, Germany), Hamilton syringe (100 µl), and Deuterium lamp which was used as a radiation source. Shimadzu balance model AY-120, hot air oven (Kumar Laboratory Oven), photostability chamber (Make Newtronic. Model IC DAC version 1.2), and calibrated glass wares were used for the study.

HPTLC analysis

Standard solution preparation and selection of analytical wavelength

Stock solution for cilnidipine and nebivolol hydrochloride was prepared by dissolving 10 mg of drug in 10 ml methanol. Further dilution of nebivolol hydrochloride and cilnidipine stock solution was made using methanol to get the standard solution having 100 µg/mL and 50 µg/mL, respectively.

From the standard stock solution, further dilutions were done using methanol and scanned over the range of 200–400 nm, and the spectra were obtained. It was observed that both drugs showed considerable absorbance at 270 nm.

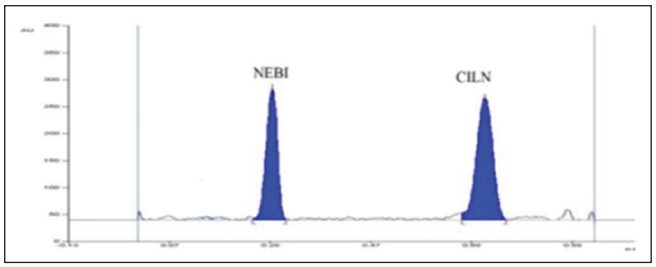

Fig. 1: Densitogram of nebivolol HCl (100 ng/band) and cilnidipine (200 ng/band)

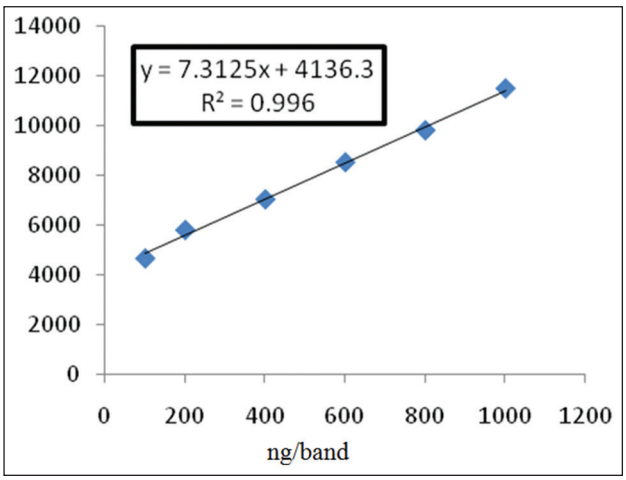

Fig 2: Calibration curve for nebivolol HCl

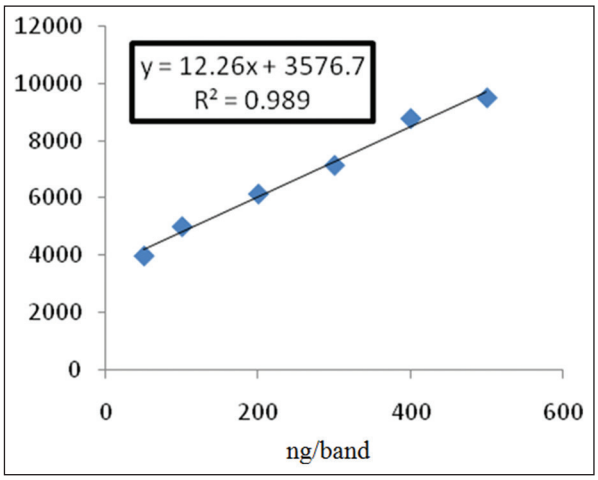

Fig 3: Calibration curve for cilnidipine

Chromatographic conditions

Samples were applied on the 10 cm × 10 cm plate as a band with a width of 6 mm and slit dimensions were kept as 4.00 × 0.45 mm. Chamber saturation time was 20 min and the migration distance was 80 mm. Mobile phase used was chloroform:glacial acetic acid:methanol, in 8.5:1:0.5 (v/v/v) ratio.

Analysis of tablet formulation

Twenty tablets were weighed accurately and finely powdered. A quantity of powder equivalent to 10 mg of cilnidipine (5 mg of Nebivolol hydrochloride) was weighed and transferred to a 10 ml volumetric flask containing about 5 ml of methanol and shaken for 5 min, and volume was made up with the methanol. The solution was filtered using Whatman paper No. 41, and 1 ml filtrate was further diluted to 10 ml with methanol to get sample stock solution of 100 ng/µl of cilnidipine and 50 ng/µl of nebivolol hydrochlordie. 2 ml of sample solution was applied on HPTLC plate to obtain a final concentration of 200 ng/band of cilnidipine and 100 ng/band of nebivolol. After chromatographic development, peak areas of the bands were measured at 270 nm and concentration of drug in the sample was estimated from the calibration curves. Procedure was repeated 6 times for the analysis of homogenous sample.

METHOD VALIDATION [14]

The method was validated as per the ICH guidelines.

Linearity

The standard stock solution of cilnidipine (100 ng/µl) and nebivolol hydrochloride (50 ng/µl) was applied as over spotting on HPTLC plate

Table 1: Calibration curve of nevibolol hydrochloride and cilnidipine (*n*=6)

Sr. No	Nebivolol hydrochloride		Cilnidipine	
	Concentration (ng/band)	Peak area	Concentration (ng/band)	Peak area
1	100	4686	50	3955.4
2	200	5825.4	100	4981.6
3	400	7061	200	6126.4
4	600	8553.1	300	7132.2
5	800	9839.8	400	8770.6
6	1000	11521.5	500	9496.6

Table 2: Regression analysis of calibration curves of nebivolol HCl and cilnidipine

Parameter	Cilnidipine	Nebivolol HCl
Detection Wavelength (nm)	270	270
Linearity range (ng/band)	100-1000	50-500
Correlation coefficient (r)	0.996	0.989
Linear regression equation[a] (y=mx+c)		
Intercept (c)	4136.3	3576.7
Slope (m)	7.312	12.26

HCl: Hydrochloride

Table 3: Statistical validation of intra- and inter-day precision studies

Component	Precision	% of Label claim	SD	% RSD
Cilnidipine	Intraday (*n*=6)	101.128	0.961	0.009
	Interday (n=3×3)	100.003	1.115	0.011
Nebivolol HCl	Intraday (n=6)	101.260	1.517	0.014
	Interday (n=3×3)	100.675	1.046	0.010

RSD: Reflex sympathetic dystrophy, SD: Standard deviation, HCl: Hydrochloride

Table 4: Recovery studies of nebivolol HCl and cilnidipine (n=3)

Drug	Amount taken (ng per band)	Amount added (ng/band)	Total amount (ng/band)	% Recovery*	% RSD*
Nebivolol hydrochloride	100	50	150	100.696	1.396
	100	100	200	101.936	1.137
	100	150	250	100.570	1.744
Cilnidipine	200	100	300	100.269	0.869
	200	200	400	101.333	1.685
	200	300	500	100.849	1.445

RSD: Reflex sympathetic dystrophy, HCl: Hydrochloride

Table 5: LOD and LOQ

Parameters	Nebivolol hydrochloride	Cilnidipine
LOD (ng/band)	16.395	31.788
LOQ (ng/band)	49.681	96.328

LOD: Limit of Detection, LOQ: Limit of Quantitiation

Table 6: Robustness data in terms of % RSD (n=3)

Sr. No	Parameters	Variation	% RSD* NEB	% RSD* CIL
1	Time from application to development	0, 30, and 60 min	0.099	0.054
2	Time from development to scanning	0, 30, and 60 min	0.057	0.028

RSD: Reflex sympathetic dystrophy

Table 7: Analysis of tablet formulation by HPTLC

Drug	Label claim (mg/tablet)	% of label claim	SD*	% RSD*
Cilnidipine	10	101.417	1.799	0.017
Nebivolol hydrochloride	5	100.955	1.565	0.015

*Average of six determinations. HPTLC: High-performance thin-layer chromatography, RSD: Reflex sympathetic dystrophy

in range of 1–10 µl with the help of CAMAG 100 µL sample syringe, using Linomat 5 sample applicator to obtain final concentration 100–1000 ng/band for cilnidipine and 50–500 ng/band for nebivolol hydrochlordie. The plate was developed and scanned under the above established chromatographic conditions. Each standard in six replicates was analyzed, and peak areas were recorded. Calibration curves of cilnidipine and nebivolol hydrochloride were plotted of peak area versus concentration.

Intra- and inter-day precision
The precision of the method was demonstrated by intra- and inter-day variation studies. In the intraday studies, six replicates of standard solution (200 ng/band for cilnidipine and 100 ng/band for nebivolol hydrochloride – Assay Concentration) were analyzed in a day and percentage reflex sympathetic dystrophy (RSD) was calculated. For the interday variation studies, three replicates of standard solutions (200, 400, and 600 ng/band for cilnidipine and 100, 200, and 300 ng/band for nebivolol) were analyzed on 3 consecutive days and percentage RSD was calculated.

Accuracy
To check the accuracy of the method, recovery studies were carried out by over spotting standard drug solution to pre-analyzed sample solution at three different levels of 50, 100, and 150%. The basic concentration of sample chosen was 200 ng/band for cilnidipine and 100 ng/band for nebivolol hydrochloride. The areas were noted after the development of plate. The drug concentration was calculated using regression equations.

Limit of detection (LOD)
LOD was calculated from the following formula:

$LOD = 3.3\ \sigma/S$

Where
σ = Standard deviation of the response (y-intercept)
S = Slope of the calibration curve

Limit of quantitation (LOQ)
LOQ was calculated from the following formula:

$LOQ = 10\ \sigma/S$

Where
σ= Standard deviation of the response (y-intercept)
S = Slope of the calibration curve

Robustness
The robustness of the method was studied, during method development, by small but deliberate variations in time from application to development (0, 30, and 60 min) and time from development to scanning (0, 30, and 60). One factor at a time was changed at a concentration level of 200 ng/band for cilnidipine and 100 ng/band for nebivolol hydrochloride to study the effect on the peak area of the drugs.

RESULTS AND DISCUSSION

The standard densitograms of nebivolol hydrochloride and cilnidipine are shown in Fig. 1.

Nebivolol retardation factor (Rf) = 0.29±0.008

Cilnidipine Rf = 0.69±0.007

Linearity
The linearity data for calibration curve is shown in Table 1, the calibration curve for nebivolol hydrochloride and cilnidipine is shown in Figs. 2 and 3, respectively, and the regression data are shown in Table 2.

The results for precision studies and accuracy are shown in Tables 3 and 4.The results for LOD and LOQ for nebivolol hydrochloride and cilnidipine are shown in Table 5.The robustness data are shown in Table 6.

Analysis of tablet formulation
After chromatographic development, peak areas of the bands were measured at 270 nm and concentration of drug in the sample was estimated from the calibration curves. The procedure was repeated 6 times for the analysis of homogenous sample. The results for tablet analysis are shown in Table 7.

DISCUSSION

HPTLC method for the determination of nebivolol hydrochloride and cilnidipine was developed. Linearity for nebivolol hydrochloride and cilnidipine was found in the range of 100–1000 ng/band and 50–500 ng/band and regression coefficient (r^2) = 0.989 and 0.986,

respectively. It indicates that the proposed method found to be linear. LOD and LOQ values were 16.395 ng/band and 49.681 ng/band and 49.681 ng/band and 96.328 ng/band, respectively, and this low value of LOD and LOQ indicates that the proposed method is sensitive. The RSD values for intra- and inter-day precision studies were found to be <2%; this low value of RSD indicates that the proposed method is precise.

CONCLUSION

A new, simple, and sensitive HPTLC method has been successfully developed and validated for simultaneous determination of nebivolol hydrochloride and cilnidipine in bulk and pharmaceutical dosage form. The method was found to be accurate, precise, and economic and hence can be used for routine analysis of the drugs in combination.

AUTHOR'S CONTRIBUTION

Authors declare that this study was performed by all mentioned in this article. All liabilities relating to claims of the contents of this article will be borne by the authors.

CONFLICTS OF INTEREST

The are no conflicts of interest by the authors regarding the publication of this.

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