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Simultaneous Estimation of Nebivolol Hydrochloride and Hydrochlorothiazide in Tablets by TLC-Densitometry

Keyur B. Ahir, Emanual M. Patelia* and Falgun A. Mehta

Department of Pharmaceutical Chemistry and Analysis, Indukaka Ipcowala College of Pharmacy, New Vallabh Vidyanagar – 388121, Gujarat, India

Abstract

A simple, rapid and accurate thin-layer chromatography (TLC)–densitometric method has been established and validated for the simultaneous determination of nebivolol hydrochloride and hydrochlorothiazide in tablets. The method is based on TLC separation of the two drugs followed by densitometric measurements of their spots at 281 nm. The separation was carried out on Merck TLC aluminium plate of silica gel 60F 254 using toluene:ethyl acetate:methanol:ammonia (3:2.7:1.7:0.1 v/v/v) as a mobile phase. Nebivolol hydrochloride and hydrochlorothiazide gave sharp and well defined peak at R_f 0.68 and 0.38, respectively. Calibration curves for nebivolol hydrochloride and hydrochlorothiazide were linear in range 500-3000 ng/spot and 1000-6000 ng/spot, respectively. Method was successively applied to tablet formulation. No chromatographic interferences from the tablet excipients were found. The method was validated in accordance with the requirements of ICH guidelines.

Keywords: Nebivolol hydrochloride; Hydrochlorothiazide; TLC-densitometry; Validation, ICH guidelines

Introduction

Nebivolol hydrochloride (NEB), α, α' [iminobis (methylene)] bis [6-fluro-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 β_1 blocker whereas (-) nebivolol as vasodilator [4,5].

Hydrochlorothiazide (HCTZ), 6- chloro-3, 4-dihydo-2H-1, 2, 4-benzothizdiazine-7-sulfonamide1, 1 dioxide is a popular thiazide diuretics acts on the kidneys to reduce sodium (Na) reabsorption in the distal convoluted tubule [6].

Both these drugs are used in the treatment of arterial hypertension. The chemical structures of drugs are shown in Figure 1. A combination of both drugs is recently launched in the market.

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 [7], reverse-phase HPLC/UV detection [8], enantiomeric resolution using HPLC [9], enantioseparation using chiral stationary phase [10], liquid chromatography/tandem mass spectrometry [11], liquid chromatography coupled with electro spray ionization tandem mass spectroscopy [12], are some of those. HPTLC densitometric quantitative analysis from tablet formulation [13,14] has been reported. Also several analytical methods are studied for determination of hydrochlorothiazide alone and in combinations with other drugs [15-21]. RP-HPLC method has been found for simultaneous determination of NEB and HCTZ in combined dosage form [22]. NEB is not official in I.P., B.P or U.S.P. while HCTZ is official in I.P. B.P and U.S.P.

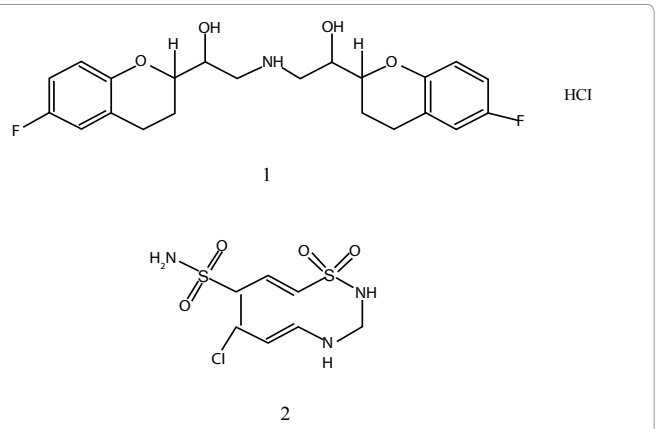

Figure 1: Chemical structures of nebivolol hydrochloride (1) and hydrochlorothiazide (2).

However, to our knowledge, no information related to the TLC-densitometry determination of NEB and HCTZ in pharmaceutical dosage forms has ever been mentioned in literature.

The present paper describes a reliable, rapid and accurate method for determination of NEB and HCTZ using TLC-densitometry.

Experimental

Materials and reagents

Nebivolol hydrochloride and hydrochlorothiazide were kindly supplied as a gift sample by Cadila Pharmaceuticals Ltd., Dholka, and Ahmadabad India. All the reagents used were of analytical reagent grade (S.D. Fine Chemicals, Mumbai, India) and used without further purification.

Instrumentation and chromatographic conditions

The samples were spotted on TLC aluminium plate 60 F254 (20 cm×10 cm) with 200 µm thickness; (E MERCK, Darmstadt, Germany)

***Corresponding author:** Emanual M. Patelia, Department of Pharmaceutical Chemistry and Analysis, Indukaka Ipcowala College of Pharmacy, New Vallabh Vidyanagar 388121, Gujarat, India, E-mail: ricky.emanual@gmail.com

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using a Camag Linomat V (Switzerland).The samples were streaked in the form of narrow bands of width 6 mm with a constant application rate of 150 nl/ sec using nitrogen aspirator and the space between two bands was kept constant at 15.4 mm. The slit dimension was kept at 6 mm×0.45 mm. The mobile phase consists of toluene:ethyl acetate:methanol:ammonia (3:2.7:1.7:0.1 v/v/v). Linear ascending development was carried out in 20 cm×10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). The optimized chamber saturation time for mobile phase was 20 min, at temperature (25°C ± 2°C); the relative humidity (60% ± 5%); the length of chromatogram run was 8 cm and TLC plates were air dried. Densitometric scanning was performed on Camag TLC Scanner 3 equipped with winCATS software version 1.3.0 at 281 nm. The source of radiation utilized was deuterium lamp.

Preparation of standard solutions and calibration graphs

The working standard solution 500 µg/mL of NEB and 1000 µg/mL of HCTZ were separately prepared in methanol. Different volumes in the range 1–6 µL of NEB and HCTZ solutions were over spotted on TLC plates with the help of microlitre syringe, using Linomat V sample applicator. The plate was developed and scanned in above established chromatographic conditions. Peak area was recorded for each concentration of drugs and curves (concentration *vs.* peak area) were constructed.

Method validation

Mixed standard solution was prepared by dissolving 10 mg of NEB and 25 mg of HCTZ in 50 ml methanol and used for validation study [23-25].

Precision: System precision of the method was assessed as repeatability and performed by spotting 1000 ng /spot of NEB and 2500 ng/spot of HCTZ.

Method precision was assessed as intra-day and inter-day variations. Intra-day and inter-day variations for the determinations of NEB and HCTZ was carried at three different concentration levels 1000, 1500, 2000 ng of NEB and 2500, 3750, 5000 ng of HCTZ, respectively.

Accuracy: Recovery experiment was checked by over spotting 80%, 100% and 120% of the mixed standard drug solution of NEB and HCTZ and the spots were reanalyzed by the proposed method. The experiment was conducted in triplicate. This was performed to check the recovery of the drug at different levels in formulation

Specificity: Specificity of the method was ascertained by analysing standard drug and sample solution. The mobile phase resolved both the drugs very efficiently, as shown in Figure 2. The spot for NEB and HCTZ was confirmed by comparing the R_f and spectra of the spot with that of standard. A typical absorption overlain spectrum of NEB and HCTZ shown in Figure 3**;** wavelength 281 nm was selected for densitometric scanning. Peak purity of NEB and HCTZ was assessed by comparing the spectra of sample with that of standard at three different levels, i.e., peak start (S), peak apex (M) and peak end (E) positions of the spot, as shown in Figure 4.

Robustness: Robustness was studied in six replicate at the concentration 1000 ng/spot of NEB and 2500 ng/spot of HCTZ. In this study, seven parameters (mobile phase composition, mobile phase volume, development distance, relative humidity, duration of saturation, time from spotting to chromatography and chromatography to spotting) were studied and the effects on the results were examined.

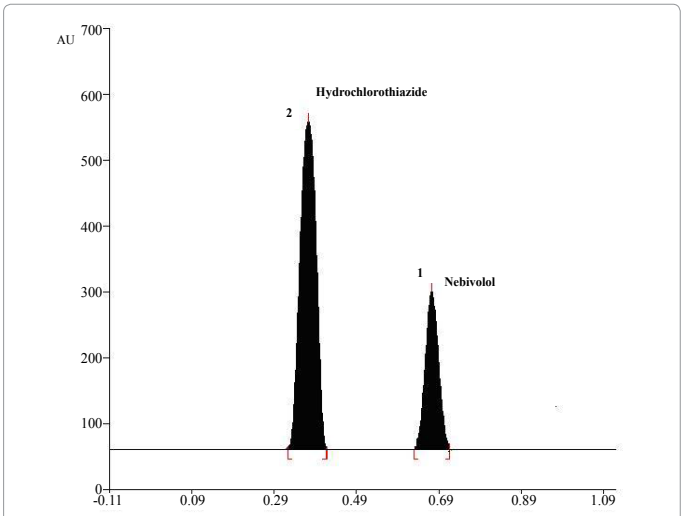

Figure 2: Densitogram obtained from nebivolol (1) and hydrochlorothiazide (2) in mobile phase toluene : ethyl acetate methanol: ammonia (3:2.7:1.7:0.1, v/v/v).

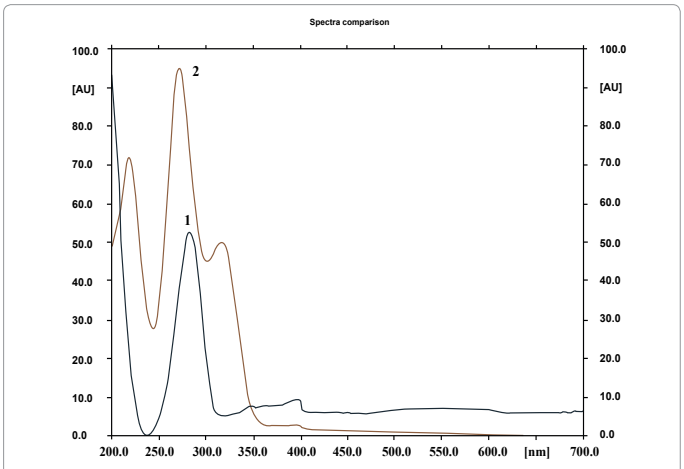

Figure 3: A typical overlain spectrum of standard nebivolol hydrochloride (1) and hydrochlorothiazide (2).

Ruggedness: The ruggedness of the proposed method was evaluated by two different analysts.

Limit of detection (LOD) and limit of quantification (LOQ): 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 NEB and HCTZ was prepared and different volume of stock solution in the range 500 to 1000 ng for NEB and 1000 to 2000 ng for HCTZ were over spotted in triplicate. The amount of both the drugs by spot *versus* average response (peak area) was graphed and the equation for this was determined. The standard deviations (S.D.) of responses were calculated. The average of standard deviations was calculated (A.S.D.). LOD was calculated by (3.3×A.S.D.)/*b* and LOQ calculated by (10×A.S.D.)/*b*, where "*b*" corresponds to the slope obtained in the linearity study of method.

Analysis of nebivolol and hydrochlorothiazide marketed formulation

To determine the content of NEB and HCTZ simultaneously in conventional tablets (label claim 5 mg nebivolol and 12.5 mg hydrochlorothiazide); twenty tablets were accurately weighed, average

weight determined and ground to fine powder. A quantity of powder equivalent to 5 mg nebivolol and 12.5 mg of hydrochlorothiazide was transferred into 25 ml volumetric flask containing 15 ml methanol, sonicated for 10 min and diluted to mark with same solvent and filtered using 0.45 µm filter (Millifilter, MA). Appropriate volume 5 µL was applied on TLC plate followed by development and scanning as described in section 2.2. The analysis was repeated for six times. Nebivolol and hydrochlorothiazide gave sharp and well defined peaks at R_f 0.68 and 0.38, respectively, when scanned at 281 nm. The results are shown in Table 1 indicate that there was no interferences from the excipients commonly present in the tablets.

Result and Discussion

Optimization of HPTLC method

TLC procedures were optimized with a view to separate and quantify NEB and HCTZ from tablet formulation. Initially, mobile phase consisting of toluene, ethyl acetate and methanol was tried in the ratio of 3:2.5:1 (v/v/v), but the typical peak nature of chromatogram was missing. Therefore, the ratio of toluene, ethyl acetate and methanol was changed to 3:2.7:1.7 v/v/v. HCTZ showed symmetrical but tailing was observed with NEB. The peak tailing was overcome by adding 0.1 ml of ammonia to the mobile phase. Finally, the mobile phase consisting of toluene:ethyl acetate:methanol:ammonia (3:2.7:1.7:0.1 v/v/v) gave symmetric peak with good resolution. The R_f of NEB and HCTZ was found to be 0.68 ± 0.02 and 0.38 ± 0.02, respectively.

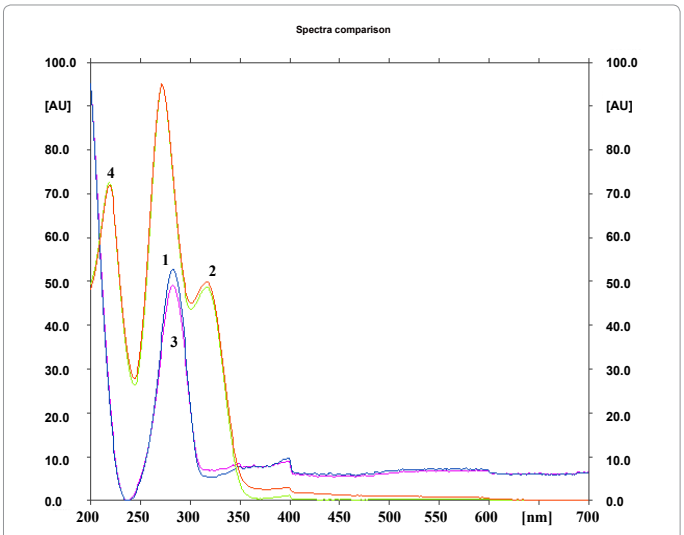

Figure 4: Peak Purity specta of standard nebivolol hydrochloride (1), hydrochlorothiazide (2) and nebivolol (3) and hydrochlorothiazide (4) extracted from tablets were scanned at the peak- start, peak-apex r(s, m) and peak- apex -peak end r(m, e) positions of the spot (correlation > 0.99).

Component	Label Claim (mg)	% Label Claim*	% R.S.D.
Nebivolol	5	99.99	1.51
Hydrochlorothiazide	12.5	99.54	1.65

*mean of six estimations

Table 1: Assay of tablet formulation.

Drug	Amount applied ng/spot	Intra-day precision [%R.S.D., n=3]	Inter-day precision [% R.S.D., n=3]
NEB	1000	1.77	1.53
	1500	1.30	0.76
	2000	1.25	1.24
HCTZ	2500	0.39	0.34
	3750	0.52	0.38
	5000	0.65	0.63

Table 2: Result of precision study.

Component	Initial Amount (ng/spot)	% Amount of standard drug added	% Drug recovered *	% R.S.D.
NEB	1000	0	100.19	1.60
		80	99.90	1.78
		100	100.52	1.42
		120	100.17	1.01
HCTZ	2500	0	100.03	1.79
		80	100.49	1.54
		100	100.48	1.39
		120	100.19	1.78

*mean of three estimations at each level

Table 3: Results of recovery studies.

Linearity

Linearity responses for NEB and HCTZ were assessed in the concentration ranges 500-3000 ng/spot and 1000- 6000 ng/spot, respectively. The linear equations for the calibration plots were Y=3.7582X+157.12 and Y=1.0178 X+8947.5, with correlation coefficient (r) being 0.9995 and 0.9991 for NEB and HCTZ, respectively. Calibration range was established with six replicate readings of each concentration.

Validation of the method

Precision: Precision of the method was determined in the terms of intra-day and inter-day variation (%R.S.D.). Intra-day precision (%R.S.D.) was assessed by analyzing standard drug solutions within the calibration range, three times on the same day.

Inter-day precision (%R.S.D.) was assessed by analyzing standard drug solutions within the calibration range on three different days over a period of week. The results are shown in Table 2.

Repeatability: Repeatability of sample application was assessed by spotting 1000 ng/spot of NEB and 2500 ng/spot of HCTZ solutions six times on a TLC, followed by development and scanning as described in section 2.2. The %R.S.D. for peak area of NEB and HCTZ was found to be 0.73 and 1.21, respectively.

Accuracy: To the preanalysed sample a known amount of standard solution of pure drug (NEB and HCTZ) was over spotted at three different levels. These solutions were subjected to re-analysis by the proposed method and results of the same are shown in Table 3.

Robustness: The standard deviation of peak areas was calculated for each parameter and %R.S.D. was found to be less than 2%. The low values of %R.S.D. shown in Table 4 proved robustness of the method.

Ruggedness: The ruggedness of the proposed method was evaluated by two different analysts. The results for NEB and HCTZ were found to be 98.66%, 99.79% and 100.03%, 100.42%, respectively.

LOD and LOQ: Detection limit and quantification limit was calculated by the method as described in section 2.4.6 The LOD and LOQ for NEB were 73.58 ng and 223.09 ng. For HCTZ, LOD and LOQ

Parameter	NEB		HCTZ	
	S.D. of Peak Area	% R.S.D.	S.D. of Peak Area	% R.S.D
Mobile Phase Composition				
Toluene: ethyl acetate: methanol: ammonia (3.2:2.9:1.9:0.1)	67.14	1.78	145.80	1.25
Toluene: ethyl acetate: methanol: ammonia (2.8:2.5:1.5:0.1)	50.08	1.34	133.44	1.16
Mobile Phase Volume				
7.5 ml	74.30	1.95	140.25	1.21
15 ml	51.43	1.37	150.05	1.29
Development distance				
8 cm	69.38	1.84	128.76	1.11
8.5 cm	50.17	1.33	64.20	0.56
9 cm	39.37	1.01	105.09	0.90
Duration of saturation				
20 min	67.75	1.72	142.84	1.24
25 min	54.69	1.38	65.65	0.56
30 min	49.57	1.28	110.06	0.95
Activation of prewashed plates				
5 min	76.97	1.98	193.34	1.66
10 min	64.52	1.71	197.03	1.70
15 min	61.81	1.69	161.87	1.39
Time from spotting to chromatography				
30 min	65.71	1.75	119.17	1.02
60 min	63.80	1.70	170.42	1.47
Time from chromatography to scanning				
30 min	47.48	1.25	131.04	1.12
60 min	71.68	1.91	145.70	1.25

Table 4: Results from robustness studies.

Parameter	NEB	HCTZ
Linearity range (ng /spot)	500-3000	1000-6000
Correlation coefficient	0.9995	0.9991
Limit of detection (ng /spot)	73.58	101.12
Limit of quantitation (ng/ spot)	223.09	309.46
% Recovery (n=9)	1.45	1.62
Ruggedness (%R.S.D.)		
Analyst I (n=6)	1.88	1.82
Analyst II (n=6)	1.85	1.65
Precision (%R.S.D.)		
Repeatability (n=6)	0.73	1.21
Inter-day (n=3)	1.25-1.77	0.39-0.65
Intra-day (n=3)	0.76-1.53	0.34-0.63
Robustness	Robust	Robust
Specificity	Specific	Specific

Table 5: Summary of validation parameter.

were found to be 101.12 ng and 309.46 ng, respectively. This indicates that adequate sensitivity of the method.

The summery of validation parameters were listed in Table 5.

Conclusion

The proposed HPTLC method provides simple, accurate and reproducible quantitative analysis for simultaneous determination of NEB and HCTZ in tablets. The method was validated as per ICH guidelines.

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