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Development and Validation of Stability Indicating RP-HPLC Method for Simultaneous Estimation of Nebivolol Hydrochloride and Cilnidipine in Tablet Dosage Form

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ABSTRACT:

A simple, specific, accurate and stability-indicating reversed phase high performance liquid chromatographic method was developed for the simultaneous determination of Nebivolol hydrochloride and Cilnidipine in tablet dosage form. A Spheri-5-RP-18 column having 250×4.6 mm i.d., with mobile phase composed of Methanol: 20 mM Ammonium acetate (85:15, v/v; pH 4.0 adjusted with Formic acid. The retention times of Nebivolol hydrochloride and Cilnidipine were found to be 3.4 min and 8.4 min, respectively. Linearity was established for Nebivolol hydrochloride and Cilnidipine in the range of 5-30 µg/ml and 10-60 µg/ml respectively. The percentage recoveries for Nebivolol hydrochloride and Cilnidipine were found to be in the range of 99.22-99.91% and 99.81-100.82% respectively. The limit of detection for Nebivolol hydrochloride and Cilnidipine were found to be 0.80 µg/ml and 1.73 µg/ml respectively. The limit of quantitation for Nebivolol hydrochloride and Cilnidipine were found to be 2.43 µg/ml and 5.26 µg/ml respectively. Both the drugs were subjected to acid, alkali, oxidation, dry heat and photolytic degradation. The degradation studies indicated, Nebivolol hydrochloride to be susceptible to oxidation while Cilnidipine showed degradation in oxidative and photolytic condition. The degraded products peaks were well resolved from the pure drug peak with significant differences in their retention time values. The proposed method was validated and successfully applied to the simultaneous estimation of Nebivolol hydrochloride and Cilnidipine in bulk drugs and formulations.

KEY WORDS: Nebivolol hydrochloride, Cilnidpine, degradation, reversed phase high performance liquid chromatography, stability indicating method, Validation.

INTRODUCTION:

Nebivolol hydrochloride (NEB), 1-(6-fluoro-3,4-dihydro-2*H*-chromen-2-yl)-2-[[2-(6-fluoro-3,4-dihydro-2*H*-chromen-2-yl)-2-hydroxyethyl]amino]ethanol, is white to off-white powder, sparingly soluble in dimethyl formamide, soluble in methanol. Nebivolol hydrochloride is a highly selective β1-blocker with nitric oxide mediated vasodilatory actions and it is used in the treatment of hypertension. It is official in Indian Pharmacopoeia [1], which recommends a liquid chromatography method for its analysis. The structure of Nebivolol hydrochloride is shown in **FIGURE.1.**

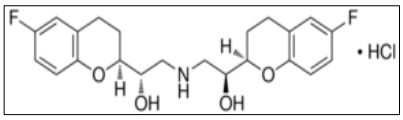

FIGURE.1: NEBIVOLOL HYDROCHLORIDE

Cilnidipine (CIL), 3-O-(2-methoxyethyl) 5-O-[(E)-3-phenylprop-2-enyl] 2,6- dimethyl-4-(3-nitrophenyl)-1,4dihydropyridine-3,5-dicarboxylate, is light yellow crystalline powder, soluble in DMSO, ethanol, water, and methanol. Cilnidipine is a dihydropyridine calcium-channel blocker. It inhibits cellular influx of calcium, thus causing vasodilatation. It has greater selectivity for vascular smooth muscle. It has little or no action at the SA or AV nodes and -ve inotropic activity is rarely seen at therapeutic doses. The structure of Cilnidipine is shown in **FIGURE.2.**

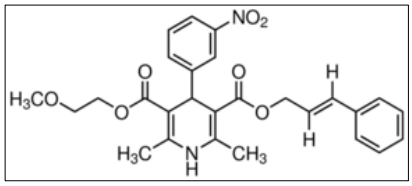

FIGURE.2: CILNIDIPINE

Nebivolol hydrochloride (NEB) and Cilnidipine (CIL) combination available as tablet dosage form for the treatment of hypertension in Indian market. The literature survey of Nebivolol hydrochloride and Cilnidipine reveals that number of methods are available for individual estimation of these drugs[2-7] and in combination with other drugs[8-11], but no stability indicating RP-HPLC method is reported for simultaneous estimation of these drugs in combined dosage form. However, one RP-HPLC[12] and two first order derivative UV spectrophotometric[13] methods are available for estimation of these drugs in combination.

So in present study it was decided to develop stability indicating RP-HPLC method for simultaneous estimation of Nebivolol hydrochloride and Cilnidipine in pure form as well as in pharmaceutical formulation. The method was validated in compliance with ICH guidelines[14].

MATERIALS AND METHODS:

Materials: Nebivolol hydrochloride was kindly gifted by Cadila healthcare Ltd, (Ahmedabad, Gujarat). Cilnidipine was kindly gifted by Laksh fine chem Pvt. Ltd., (Vitthal Udhyognagar, Anand, Gujarat). Methanol, water and ammonium acetate used were of HPLC grade (Lichrosolv Merck India Limited, Mumbai). The tablet formulation (LN-βeta 5, Eris life sciences Pvt. Ltd. Amingaon, North Guwahati, Assam) containing 5 mg NEB and 10 CIL was procured from local market.

Equipment: The liquid chromatographic system was of Perkin Elmer (USA), series 200, which consisted of following components: A gradient system, Quaternary LC Pump, PDA detector and Rheodyne injector with 20µl loop injector. The chromatographic analysis was performed using Total ChromNavigator version 6.3 software on a Spheri-5-RP-18 column (250×4.6 mm, 5 µm particle size).

Chromatographic conditions: The separation was achieved on Spheri-5-RP-18 Perkin Elmer Brownlee column (250×4.6 mm, 5 µm particle size) with mobile phase containing Methanol: 20 mM Ammonium acetate buffer (pH 4 adjusted with 1% formic acid) in the ratio of 85:15 v/v at a flow rate of 1.0 ml/min. The eluted compounds were monitored at the wavelength of 274nm. The sample injection volume was 20 µl and total run time was 15 min.

Preparation of Standard Solutions:

Standard stock solutions were prepared by weighing 10 mg each of NEB and CIL. The weighed drugs were transferred to two separate 10 ml volumetric flasks. Volumes were made up to the mark with mobile phase to obtain a solution containing 1000 µg/ml of NEB and CIL.

Mixed standard stock solution:

2.5 ml of NEB standard stock solution and 5 ml CIL standard stock solution were transferred to a 25 ml of volumetric flask and 0.25 ml of 1% acetic acid was added and then volume was made up to the mark with mobile phase to give a solution containing 100 µg/ml NEB and 200 µg/ml CIL.

Preparation of sample solution:

A composite of 20 tablets with label claim of 5 mg of Nebivolol hydrochloride and 10 mg of Cilnidipine were grinded into fine powder. Tablet powder equivalent to 5 mg of NEB and 10 mg of CIL was transferred into 10 ml volumetric flask containing 5 ml mobile phase, sonicated for 15 minutes and volume was made up to the mark with same solvent, the resulting solution was filtered using 0.45 µ filter. From filtrate, 0.2 ml of solution was transferred into 10 ml volumetric flask and 0.1 ml of 1% acetic acid was added and then volume was made up to mark with same solvent to

obtain the concentration of 10 µg/ml of NEB and 20 µg/ml of CIL. The solution was injected under above chromatographic conditions and peak areas were measured.

Forced degradation studies:

Forced degradation studies were performed on 30 µg/ml of NEB and 60 µg/ml of CIL concentration.

Acid degradation:

An accurately weighed quantity of tablet powder equivalent to 5 mg of Nebivolol hydrochloride and 10 mg of Cilnidipine was transferred into 100ml round bottom flask, to this 10 ml HPLC grade methanol was added to dissolve it. Sonicated for about 15 min. 5 ml of 2 M HCl was added in the flask. The mixture was refluxed at 80ºC for 6 hours. Then, solution was neutralized with 2 M KOH solution to avoid further degradation. This mixture was taken in 50 ml volumetric flask and diluted up to mark with mobile phase. The solution was filtered through 0.45µ filter discarding the first 5 ml of solution. From this stock solution, 3 ml of solution was taken into 10 ml of volumetric flask and volume was made up to the mark with mobile phase to get 30 µg/ml NEB and 60 µg/ml CIL. The forced degradation was performed in the dark to exclude the possible degradation effect of light and controls of the respective solutions were made at each stage of degradation study to eliminate possible changes due to heat and light. The solutions obtained was injected and chromatogram was recorded at the 274 nm.

Base degradation:

An accurately weighed quantity of tablet powder equivalent to 5 mg of Nebivolol hydrochloride and 10 mg of Cilnidipine was transferred into 100 ml round bottom flask, to this 10 ml HPLC grade methanol was added to dissolve it. Sonicated for about 15 min. 5 ml of 2 M KOH was added in the flask. The mixture was refluxed at 80ºC for 6 hours. Then, solution was neutralized with 2 M HCl solution to avoid further degradation. This mixture was taken in 50 ml volumetric flask and diluted up to mark with mobile phase. The solution was filtered through 0.45µ filter discarding the first 5 ml of solution. From this stock solution, 3 ml of solution was taken into 10 ml of volumetric flask and volume was made up to the mark with mobile phase to get 30 µg/ml NEB and 60 µg/ml CIL. The forced degradation was performed in the dark to exclude the possible degradation effect of light and controls of the respective solutions were made at each stage of degradation study to eliminate possible changes due to heat and light. The solutions obtained was injected and chromatogram was recorded at the 274 nm.

Oxidative degradation:

An accurately weighed quantity of tablet powder equivalent to 5 mg of Nebivolol hydrochloride and 10 mg of Cilnidipine was transferred into 100 ml round bottom flask, to this 10 ml HPLC grade methanol was added to dissolve it. Sonicated for about 15 min. 5 ml of 6% w/v H_2O_2 was added in the flask. The mixture was refluxed at 80ºC for 6 hours. This mixture was taken in 50 ml volumetric flask and diluted up to mark with mobile phase. The solution was filtered through 0.45µ filter discarding the first 5 ml of solution. From this stock solution, 3 ml of solution was taken into 10 ml of volumetric flask and volume was made up to the mark with mobile phase to get 30 µg/ml NEB and 60 µg/ml CIL. The forced degradation was performed in the dark to exclude the possible degradation effect of light and controls of the respective solutions were made at each stage of degradation study to eliminate possible changes due to heat and light. The solutions obtained was injected and chromatogram was recorded at the 274 nm.

Thermal degradation:

An accurately weighed quantity of tablet powder equivalent to 5 mg of Nebivolol hydrochloride and 10 mg of Cilnidipine was taken in porcelain dish and exposed to a temperature of 80ºC for 48 hours in hot air oven. After 48 hours sample powder was transferred to a 25 ml volumetric flask, dissolved in HPLC grade methanol and diluted up to the mark with mobile phase to produce 200 µg/ml NEB and 400 µg/ml CIL. From this solution, 1.5 ml of solution is taken into 10 ml of volumetric flask and volume was made up to the mark with mobile phase to produce 30 µg/ml NEB and 60 µg/ml CIL. 20 µl of the resulting solution was injected and chromatogram was recorded at the 274 nm.

Photolytic degradation:

An accurately weighed quantity of tablet powder equivalent to 5 mg of Nebivolol hydrochloride and 10 mg of Cilnidipine was taken in petri-dish and exposed to a UV light (UV=200 W h/m^2) (ICH Q1B, Option II) in a photo-stability chamber for 48 hours. After 48 hours sample powder was transferred to a 25 ml volumetric flask, dissolved in HPLC grade methanol and diluted up to the mark with mobile phase to produce 200 µg/ml NEB and 400 µg/ml CIL. From this solution, 1.5 ml of solution is

taken into 10 ml of volumetric flask and volume was made up to the mark with mobile phase to produce 30 µg/ml NEB and 60 µg/ml CIL. 20 µl of the resulting solution was injected and chromatogram was recorded at the 274 nm.

RESULTS AND DISCUSSION:

Method development and optimization:

The main objective of this stability indicating chromatographic method was to separate and quantitate Nebivolol hydrochloride and Cilnidipine in presence of degradation products. An isocratic method was employed using Methanol and Ammonium acetate buffer 20mM pH 6.5 in the ratio of 75:25 v/v as mobile phase, Spheri-5 RP-18, Perkin Elmer Brownlee Columns with flow rate of 1.0 ml/min on HPLC equipped with Photo diode array(PDA) detector. Tailing was observed in peak for Cilnidipine, hence to reduce the tailing attempt was made with Methanol and Ammonium acetate buffer 20mM pH 6.5 in the ratio of 80:20 v/v as mobile phase. In this mobile phase tailing was observed in peak for Cilnidipine. Then the mobile phase was modified Methanol: Buffer (Ammonium acetate 20 mM pH 4 adjusted with 1% Formic acid) (85:15 v/v) (Flow rate: 1.0 ml/min). In optimized mobile phase Nebivolol hydrochloride and Cilnidipine peaks were well resolved from their degradation products. The retention time of Nebivolol hydrochloride and Cilnidipine were about 3.4 and 8.4 respectively. **(FIGURE. 3)**

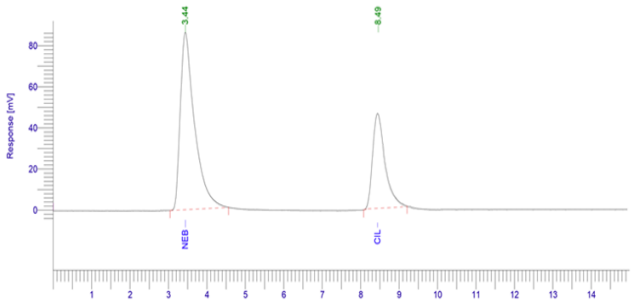

FIGURE. 3: CHROMATOGRAM OF STANDARD SOLUTION CONTAINING 100 MG/ML OF NEB AND 200 MG/ML OF CIL+ 0.1 ML OF 1% ACETIC ACID USING MOBILE PHASE COMPOSED OF METHANOL: BUFFER (AMMONIUM ACETATE 20 MM PH 4 ADJUSTED WITH 1% FORMIC ACID) (85:15) (FLOW RATE: 1.0 ML/MIN)

Forced Degradation:

The degradation study indicated that NEB was susceptible to oxidation while it was stable to acid, base, UV radiation and dry heat under experimental conditions. The chromatogram of the H_2O_2 degraded sample of NEB showed one additional peak at t_R 5.85 min **(FIGURE 4).**

CIL was found to be susceptible to H_2O_2 and UV radiation while it was stable to acid, base and dry heat under experimental conditions. CIL gets degraded into one degradation product in oxidative stress condition and photolytic exposure. The chromatogram of the H_2O_2 degraded sample of CIL showed one additional peak at t_R 5.04 min **(FIGURE 4)** and chromatogram of photo induced degraded sample of CIL showed one additional peak at t_R 5.24 min **(FIGURE 5).** Summary of degradation studies of both the drugs is given in **Table 7.**

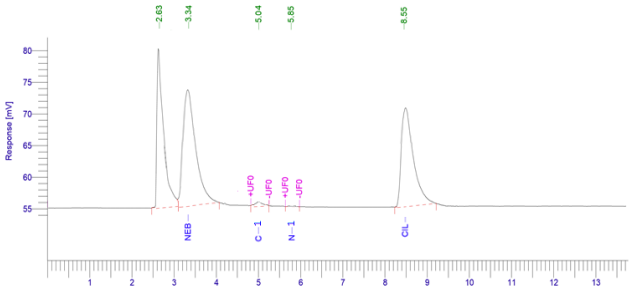

FIGURE 4: CHROMATOGRAM OF FORMULATION SAMPLE CONTAINING NEBIVOLOL HYDROCHLORIDE AND CILNIDIPINE ITS DEGRADATION PRODUCTS IN OXIDATIVE CONDITION

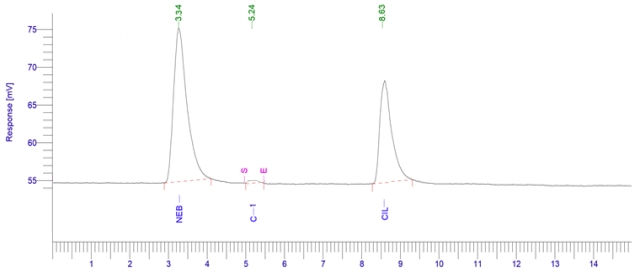

FIGURE 5: CHROMATOGRAM OF FORMULATION SAMPLE CONTAINING NEBIVOLOL HYDROCHLORIDE AND CILNIDIPINE ITS DEGRADATION PRODUCT IN PHOTOLYTIC CONDITION

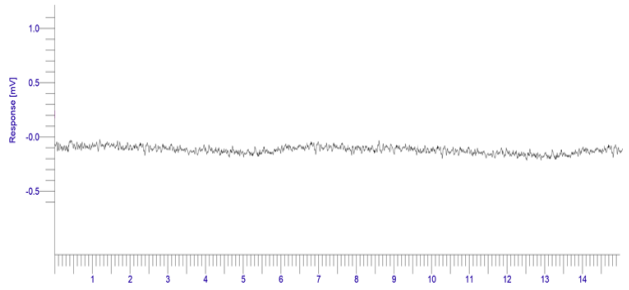

FIGURE 6: CHROMATOGRAM OF PLACEBO USING MOBILE PHASE COMPOSED OF METHANOL: BUFFER (AMMONIUM ACETATE 20 MM PH 4 ADJUSTED WITH 1% FORMIC ACID) (85:15) (FLOW RATE: 1.0 ML/MIN)

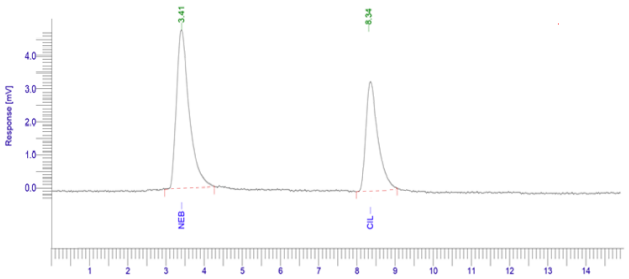

FIGURE 7: CHROMATOGRAM OF SOLUTION CONTAINING 10 MG/ML OF NEB AND 20 MG/ML OF CIL OF MARKETED TABLET FORMULATION+ 0.1 ML OF 1% ACETIC ACID USING MOBILE PHASE COMPOSED OF METHANOL: BUFFER (AMMONIUM ACETATE 20 MM PH 4 ADJUSTED WITH 1% FORMIC ACID) (85:15) (FLOW RATE: 1.0 ML/MIN)

Method Validation

The method of analysis was validated as per the recommendations of ICH[11] for the parameters like accuracy, linearity, precision, detection limit, quantitation limit and robustness.

System Suitability:

System suitability shall be checked for the conformance of suitability and reproducibility of chromatographic system for analysis. System suitability was measured by Number of theoretical Plates, Resolution, Retention time and tailing factor **(Table 1).**

TABLE 1:SYSTEM SUITABILITY TEST PARAMETERS FOR NEB AND CIL BY RP-HPLC

System suitability parameters	Result of proposed method		Acceptance criteria
	NEB	CIL	
Retention time (min.)	3.439	8.495	--
Theoretical plate number	2029	16287	>2000
Resolution	9.242		>2
Tailing factor	1.502	1.094	<2

Specificity:

Commonly used excipients in tablet preparation were spiked in a pre-weighed quantity of drugs and then area was measured and calculations carried out to determine the quantity of the drugs.

Limit of Detection (LOD) & Limit of Quantitation (LOQ):

Calibration curve was repeated for 6 times and the standard deviation (SD) of the intercepts was calculated then LOD and LOQ were calculated as follows from the formula.

$$LOD = (3.3*SD)/Slope$$

$$LOQ = (10*SD)/Slope$$

Where,

SD = the standard deviation of the Y-intercept of 6 calibration curves.
Slope = the mean slope of the 6 calibration curves.
The limit of detection and limit of quantitation values are reported in **Table 2.**

TABLE 2: LOD AND LOQ VALUES OF NEB AND CIL BY RP-HPLC

DRUG	LOD (µg/ml)	LOQ (µg/ml)
NEB	0.802	2.431
CIL	1.736	5.262

Linearity:

The linearity of NEB and CIL was estimated in the concentration range of 5-30 µg/ml and 10-60 µg/ml respectively. Each sample solution was chromatographed six times i.e. (n=6) and the mean peak area for NEB and CIL were calculated. The calibration curve for Nebivolol hydrochloride and Cilnidipine are shown in **FIGURE 7** and **FIGURE 8.**

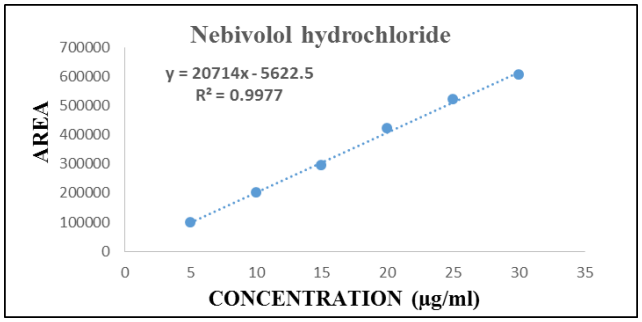

FIGURE 8: CALIBRATION CURVE FOR NEBIVOLOL HYDROCHLORIDE

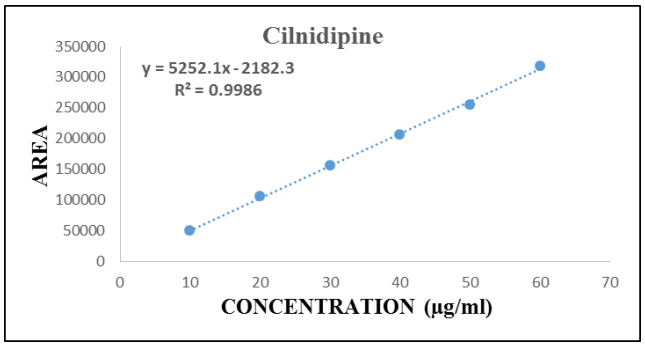

FIGURE 9: CALIBRATION CURVE FOR CILNIDIPINE

Precision:

Precision studies included the following studies:

1. Repeatability

The precision of the analytical method was studied by analysis of multiple samplings of homogeneous sample. Precision was estimated by repeatability by analyzing six trials of a homogeneous sample of 10 µg/ml of NEB and 20 µg/ml of CIL and % RSD was calculated. **(Table 3)**

2. Intra-day Precision

Standard solutions containing 10 µg/ml, 15 µg/ml and 20 µg/ml of NEB and 20 µg/ml, 30 µg/ml and 40 µg/ml of CIL were analyzed 3 times on the same day as per procedure. Chromatogram of each sample was taken. %RSD was calculated. **(Table 4)**

3. Inter-day Precision

Standard solutions containing 10 µg/ml, 15 µg/ml and 20 µg/ml of NEB and 20 µg/ml, 30 µg/ml and 40 µg/ml of CIL were analyzed on three different days as per the procedure and % RSD was calculated. **(Table 4)**

TABLE 3: REPEATABILITY DATA FOR NEB AND CIL BY RP-HPLC METHOD

Drug	Target conc. (µg/ml)	Peak Area	Mean ± SD	%RSD
NEB	10 µg/ml	200643.12 205322.28 201053.56 202023.03 200564.21	201838.32 ± 1789.508	0.886
CIL	20 µg/ml	201423.72 105450.33 105213.08 103685.71 106358.16 105623.25 105350.11	105280.10 ± 878.682	0.834

TABLE 4: INTRA-DAY AND INTER-DAY PRECISION STUDIES DATA FOR NEB AND CIL BY RP-HPLC

Intra-day and Inter-day Precision Studies					
DRUG	Conc. (µg/ml)	Intra-day Area Mean ± SD for n=3	%RSD	Inter-day Area Mean ± SD for n=3	%RSD
NEB	10	201849.35 ± 905.800	0.44	201713.71 ± 1219.627	0.60
	15	296811.72 ± 2250.087	0.75	295491.39 ± 3044.900	1.03
	20	415469.98 ± 4154.656	0.99	416145.28 ± 3151.580	0.76
CIL	20	105761.68 ± 939.484	0.89	105192.87 ± 681.217	0.64
	30	157199.08 ± 1439.839	0.91	155372.19 ± 1688.262	1.08
	40	206614.18 ± 1484.143	0.71	203796.14 ± 1713.840	0.84

Accuracy:

Accuracy study was determined at three different level 80 %, 100 % and 120 % of the target concentration 10 µg/ml of NEB and 20 µg/ml of CIL in triplicate. The result obtained for Nebivolol hydrochloride and Cilnidipine are shown in **Table 5.**

TABLE 5: %RECOVERY OF NEB AND CIL BY RP-HPLC METHOD

Drugs	Target Conc. (µg/ml)	Level of Spiking	Conc. of Pure API spiked (µg/ml)	Total Conc. (µg/ml)	Mean Conc. Found (µg/ml) (n=3) ± SD	% Recovery Mean (n=3) ± SD	%RSD
NEB	10µg/ml	0 %	0	10	10.03 ± 0.067	-	-
		80%	8	18	17.97 ± 0.108	99.81 ± 0.602	0.60
		100%	10	20	19.84 ± 0.153	99.22 ± 0.765	0.77
		120%	12	22	21.98 ± 0.307	99.91 ± 1.399	1.40
CIL	20µg/ml	0%	0	20	20.18 ± 0.236	-	-
		80%	16	36	36.29 ± 0.291	100.82 ± 0.810	0.80
		100%	20	40	39.92 ± 0.450	99.81 ± 1.125	1.12
		120%	24	44	43.95 ± 0.330	99.88 ± 0.751	0.75

The summary of validation parameters is shown in Table 6.

TABLE 6: SUMMARY OF VALIDATION PARAMETERS FOR NEB AND CIL BY RP-HPLC METHOD

Parameters	NEB	CIL	Remarks
Linearity (µg/ml)	5-30	10-60	Linear
% Recovery (%)	99.22-99.91%	99.81-100.82%	Accurate (98.0%-102%)
Precision(%RSD)			
Repeatability (n=6)	0.88	0.83	Precise
Intra-day (n=3)	0.44-0.99	0.71-0.91	(%RSD < 2)
Inter-day (n=3)	0.60-1.03	0.64-1.08	
LOD (µg/ml)	0.802	1.736	Sensitive
LOQ (µg/ml)	2.431	5.262	Sensitive
Specificity	Specific	Specific	Specific
Robustness	Robust	Robust	Robust

Table 7: Summary of Forced degradation Study:

Sr. No.	Condition	No. of Degradation products (R_t)(min)		%Degradation			
		NEB	CIL	NEB API	NEB Sample	CIL API	CIL Sample
1	**Acidic**	-	-	-	-	-	-
2	**Basic**	-	-	-	-	-	-
3	**Oxidative**	1 R_t 5.78	1 R_t 5.07	0.064	0.072	1.62	1.67
4	**Photolytic**	-	1 R_t 5.28	-	-	0.74	0.80
5	**Thermal**	-	-	-	-	-	-

CONCLUSION: A simple and efficient stability indicating reverse-phase HPLC method was developed and validated for quantitative determination of Nebivolol hydrochloride and Cilnidipine in pure as well as in combined tablet dosage form. . Method was found to be accurate, precise, specific and robust according to acceptance criteria and

with low level of LOD and LOQ. The developed method is suitable for simultaneous estimation of Nebivolol hydrochloride and Cilnidipine, in bulk and in pharmaceutical dosage form.

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