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Analytical method development and validation for simultaneous estimation of nebivolol hydrochloride and cilnidipine in combined dosage form

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ABSTRACT

A Simple, Accurate and Precise RP-HPLC method and spectrophotometric (First order derivative) method was developed and validated for simultaneous determination of Nebivolol Hydrochloride and Cilnidipine in bulk and Pharmaceutical dosage form In RP-HPLC, separation was achieved on Reversed-Phase C_8 column (250 mm × 4.6 mm, 5µm) using Water : Acetonitrile (pH-3.5 adjusted with 0.2 % Ortho Phosphoric Acid) (50:50 v/v) as a mobile phase with flow rate 1.5 ml/min and spectrophotometric UV detection at 290 nm based on peak area with linear calibration curves at concentration ranges 160-240µg/ml for Nebivolol Hydrochloride and 320-480 µg/ml for Cilnidipine. The Retention times of Nebivolol Hydrochloride and Cilnidipine were found to be 4.645 min, 6.915 min respectively. The mean recoveries of Nebivolol Hydrochloride and Cilnidipine were found to be 99.85-100.07 % and 99.89-100.12 % respectively. In First order derivative method Linearity was carried out by using concentration range 2-10 µg/ml for Nebivolol Hydrochloride (269.15 nm ZCP OF Cilnidipine) and 4-20 µg/ml for (281.85nm ZCP OF Nebivolol Hydrochloride) .At Zero crossing point (ZCP) of Cilnidipine (269.15nm)Nebivolol Hydrochloride shows a measurable absorbance value, whereas at Zero crossing point (ZCP) of Nebivolol Hydrochloride (281.85nm) Cilnidipine shows measurable absorbance value. The mean recoveries of Nebivolol Hydrochloride and Cilnidipine were found to be 99.14-100.01 % and 99.55 - 99.76 % respectively. The result of analysis has been validated as per ICH Q2(R1) guideline. Both method have been applied successfully for determination of NEBI and CIL in its Pharmaceutical formulation.

Key words: Nebivolol Hydrochloride (NEBI), Cilnidipine (CIL), RP-HPLC, First order derivative Method, Validation.

INTRODUCTION

❖Nebivolol Hydrochloride (NEBI) is a β –adrenergic blocking agent used in the treatment of Hypertension, Angina pectoris, Cardiac arrhythmias. It is chemically known as 1-(6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl)-2-{[2-(6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl)-2-hydroxyethyl]amino}ethan-1-ol

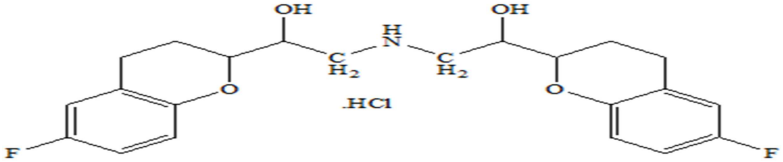

Figure 1: structure of NEBI

❖Cilnidipine (CIL) is a Calcium channel blocker Cilnidipine is the novel calcium antagonist accompanied with L-type and N-type calcium channel blocking function. Chemically Cilnidipine is O3-(2-methoxyethyl) O5-[(E)-3-phenylprop-2-enyl] 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine- 3,5-dicarboxylate

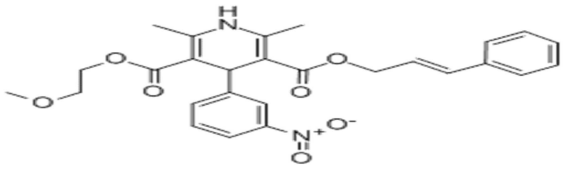

Figure 2: Structure of CIL

Based on literature review, there are number of UV spectrophotometric and chromatographic methods available for estimation of both the drugs either alone or in combination with other drugs. But there is no method available for estimation Of these two drugs simultaneously. Hence the present study was aimed to develop a simple, economical, accurate and precise UV spectrophotometric and HPLC methods for the simultaneous estimation of both the drugs and to validate the developed methods.

EXPERIMENTAL SECTION

Materials and Reagents

➢ Nebivolol Hydrochloride and Cilnidipine were procured as a Gift samples from Torrent Pharmaceuticals, Ahemdabad and Laksh Fine chem Anand, respectively. All Chemicals and Reagents used were of analytical or Pharmaceutical grade. HPLC grade Water, Methanol and Acetonitrile (Merck). And Ortho Phosphoric Acid, Triethyl Amine (Spectrochem)**.** The Pharmaceutical formulation used in this study was LN-βeta 5 Tablet procured from the local market and labeled to contain 5mg NEBI and 10 mg CIL per Tablet.

Diluent: Water : Acetonitrile (50:50) v/v

Instrumentation

Analytical Technologies HPLC System consist of S-1122 Solvent delivery system (pump), 2203 UV- Visible detector, Rheodyne injector with 20µl loop injector. Alchrome A 2000 Software system controller. Whatman filter paper no. 41, pH meter Systronic model no. 335 were used. A reverse phase C_8 (250 mm× 4.6 mm, 5µm) analytical column was used. Weighing was done on Swisser.

Chromatographic condition

Table 1: Optimization of Chromatographic Condition

Parameters	Optimized condition
Column	C_8 (250 mm× 4.6 mm,5 µm)
Wavelength detection	290 nm
Mobile Phase composition	0.2% OPA + 0.2% TEA (pH-3.5) Water: Acetonitrile (50 : 50 v/v)
Column Temperature	Ambient
Injection Volume	20µl
Flow rate	1.5 ml/min

Preparation of Standard stock and working standard Solution:

In HPLC Accurately weighed about 100 mg of NEBI and 200 mg of CIL in 50 ml of volumetric flask. Dilute it up to the maek with mobile phase to get concentration 2000 µg / ml solution of NEBI and 4000 µg /ml of CIL. Take 5 ml sample solution in 50 ml of volumetric flask diluted with mobile phase to get concentration 200 µg /ml of NEBI and 400 µg /ml of CIL. In First order derivative method Accurately weighed about 20 mg of NEBI and 40 mg of CIL in 200 ml of volumetric flask. Dilute it up to the make with methanol to get concentration 100 µg / ml solution of NEBI and 200 µg /ml of CIL. Take 2 ml sample solution in 100 ml of volumetric flask diluted with methanol to get concentration 2 µg /ml of NEBI and 4 µg /ml of CIL.

Selection of Analytical wavelength (first order derivative method)
Working Standard solution of NEBI 6 µg/ml and 12 µg/ml were scanned separately in the range of 200-400 nm. Convert these spectra into first order derivative spectra. Data was obtained by overlay spectra of both drugs. Data was obtained at 269.15 nm shows absorbance of NEBI at which CIL shows zero absorbance and at 281.85 nm shows absorbance of CIL at which NEBI shows Zero Absorbance.

Method Validation:
Specificity:
Specificity is ability to measure specifically the analyte of interest without any interference from excipient and mobile phase component. For the determination of specificity 200 µg/ml solution of the standard NEBI and 400 µg/ml solution of the standard CIL was injected. Marketed formulation of same concentration was also injected. Both chromatograms were compared.

Linearity:
In HPLC, Linearity was evaluated by analysis of working standards of NEBI and CIL of five different concentrations. The ranges of linearity were from 160 µg/ml to 240 µg/ml for NEBI and 320 µg/ml to 480 µg/ml for CIL. The peak area and concentration of each drug was subjected to regression analysis to calculate the calibration equations and correlation coefficients. In first order derivative Linearity was evaluated by analysis of working standards of NEBI and CIL of five different concentrations. The ranges of linearity were from 2 µg/ml to 10 µg/ml for NEBI and 4 µg/ml to 20 µg/ml for CIL.The peak area and concentration of each drug was subjected to regression analysis to calculate the calibration equations and correlation coefficients

Accuracy:
The Standard was spiked with formulation at these concentration levels of 50%, 100%, 150% and the mixture were analyzed by the proposed method. The experiment was conducted in triplicate for both methods.

Precision:
Pure samples of NEBI and CIL were analyzed over different days to obtain inter-day (intermediate precision, n = 3) and within the same day to obtain intra-day precision (repeatability, n = 3), then the RSD % values were calculated.

Robustness:
Method robustness was evaluated by changing the pH, flow rate, wavelength and mobile phase composition to evaluate the impact on the performance of the method and the results will be expressed in terms of %RSD.

System Suitability:
System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as Theoretical Plates, Tailing factors, Resolution (% RSD, retention time and area for six repetitions) were determined and compared against the specifications set for the method.

RESULTS AND DISCUSSION

A simple, accurate Reverse Phase High Performance Liquid Chromatographic method and First order Derivative method have been developed and validated as per ICH guide line for the simultaneous estimation of Nebivolol Hydrochloride (NEBI) and Cilnidipine (CIL) in Tablet formulation.

In RP-HPLC, Chromatographic separation was achieved on Reversed-Phase C_8 column (250 mm × 4.6 mm, 5µm) in isocratic mode using Water : Acetonitrile (pH-3.5 adjusted using 0.2 % Ortho phosphoric acid) (50:50 v/v) as the mobile phase at a flow rate 1.5 ml/min and spectrophotometric UV detection at 290 nm. The method was validated for Specificity, Linearity, Precision, Accuracy and Robustness. Linearity of NEBI and CIL was in the range of 160-240 µg/ml and 320 -400 µg/ml, respectively. The % Recoveries obtained for both drugs were 99.85-100.07 % (NEBI) and 99.89-100.12 % (CIL), respectively. Percentage RSD was found to be less than 2.0 for all parameters for both drugs. LOD and LOQ for NEBI in this method were found 4.93 µg/ml and 14.94 µg/ml respectively. For CIL, they were 4.6 µg/ml and 14.51 µg/ml. Assay of marketed formulations were also done by this HPLC and they were found 99.22-100.94 % and other system suitability parameters were found in given limit.

In First order derivative method, NEBI and CIL were estimated on UV-visible double beam spectrophotometer (Systronics - 2203) using Methanol as a solvent and detection was carried out at 269.15 nm for NEBI and 281.85 nm for CIL. The linearity range was found to be 2-10 µg/ml for NEBI and 4-20 µg/ml for CIL. The co-relation coefficients were found to be 0.999 for NEBI and 0.997 for CIL on their wavelength maxima.% RSD of linearity, repeatability, intraday and intermediate precision was found to be less than 2 %.So the developed method was validated and parameters of validation were within the range. LOD and LOQ for NEBI in this method were found 1.782 µg/ml and 5.4 µg/ml respectively. For CIL, they were 0.0033 µg/ml and 0.01 µg/ml. Assay of marketed formulations were also done by this First order derivative and they were found 98.95-102 % of labelled claim.

Specificity:

The Standard and Sample were identical to each other as shown in figure.3 and figure. 4.

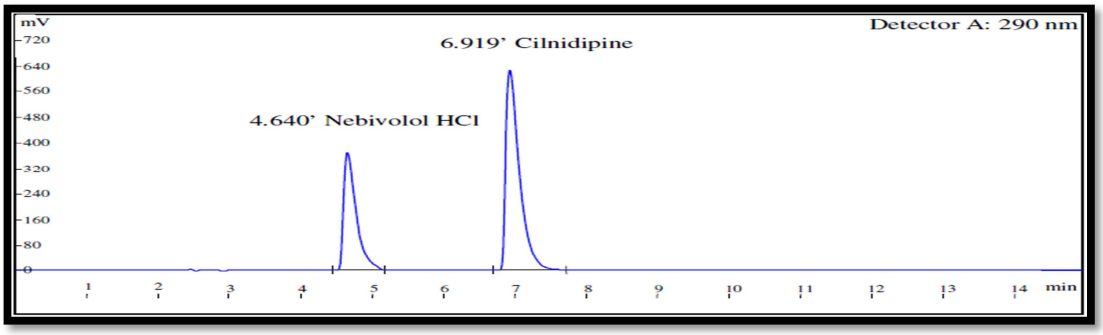

Figure.3: Chromatogram of Standard

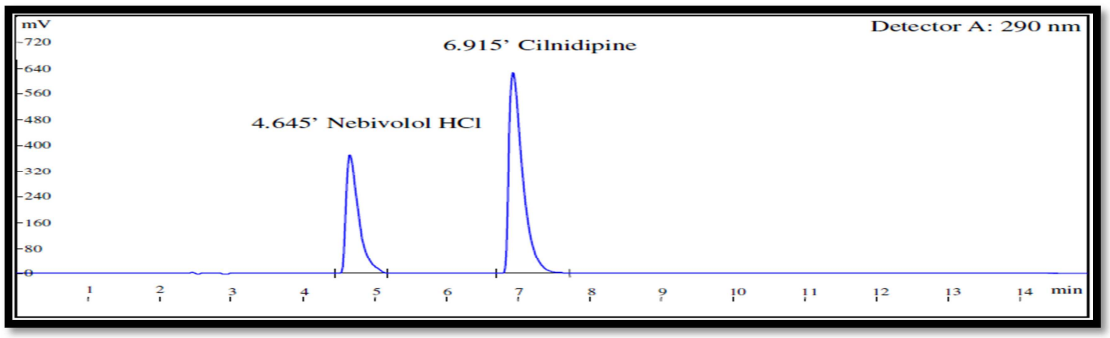

Figure.4: Chromatogram of Sample

Linearity:

The Calibration Curve found to be linear over the Concentration ranges of 160-240 µg/ml for NEBI (as shown in fig. 5) and 320-480 µg/ml for CIL(As shown in fig. 6).The correlation coefficient was found to be 0.999 for both NEBI and CIL.

Table no:2 Linearity Results

Conc. (µg/ml)		Mean Area ± SD		% RSD	
NEBI	CIL	NEBI	CIL	NEBI	CIL
160	320	1436277 ± 1922.87	2535971 ± 34157.34	0.133	1.34
180	360	1606805 ± 8728.88	2886242.4 ± 9440.0	0.543	0.32
200	400	1794548.6 ±2064.82	3204855.8 ± 7114.24	0.115	0.22
220	440	1977354.8 ± 5250.94	3520590.2 ± 21488.75	0.265	0.610
240	480	2155357.8 ± 997.60	3855676 ± 42938.03	0.046	1.11

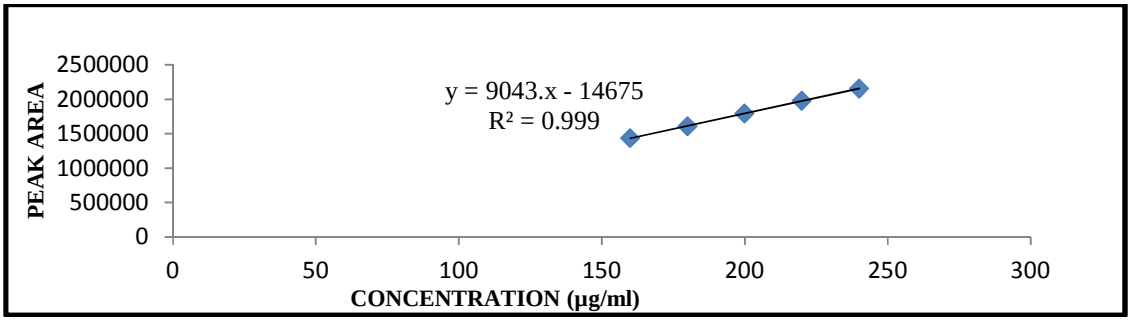

Figure 5: Calibration curve of NEBI

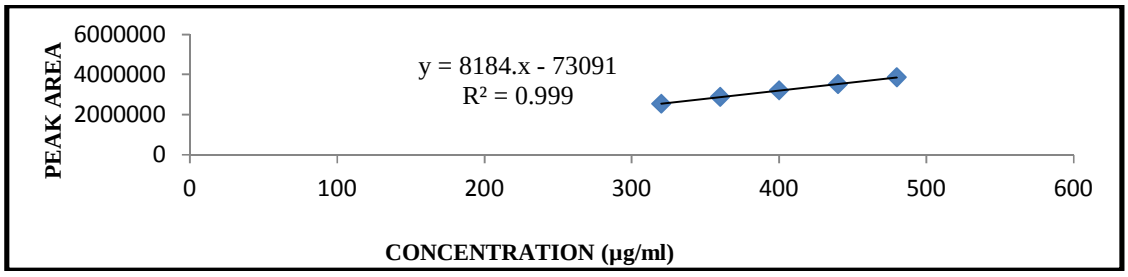

Figure 6: Calibration curve of CIL

Accuracy:

Table no: 3 Accuracy data for NEBI at 160 µg/ml

AMOUNT OF NEBI (µg/ml)	% OF STD NEBI ADDED	AMOUNT ADDED (µg/ml)	AMOUNT FOUND (µg/ml)	% RECOVERY (MEAN ± SD)	% RSD
160	50	80	239.82	100.04 ± 0.141	0.14
			240.49		
			240.06		
	100	160	321.19	100.07 ± 0.266	0.265
			319.56		
			319.96		
	150	240	398.32	99.85 ± 0.4590	0.45
			398.38		
			401.54		

Table no: 4 Accuracy data for CIL at 320 µg/ml

AMOUNT OF CIL (µg/ml)	% OF STD CIL ADDED	AMOUNT ADDED (µg/ml)	AMOUNT FOUND (µg/ml)	% RECOVERY (MEAN ± SD)	% RSD
320	50	160	478.52	99.89 ± 0.29	0.29
			478.88		
			481.11		
	100	320	642.72	100.03 ± 0.32	0.32
			638.83		
			639.25		
	150	480	800.43	100.12 ± 0.180	0.18
			799.94		
			802.66		

Precision:

Table no: 5 Precision Data

NEBI			CIL		
Intraday					
Conc µg/ml	**Mean area** ± SD	**%RSD**	**Conc µg/ml**	**Mean area** ± SD	**%RSD**
160	1446358.63± 1438.1	0.98	320	2560206 ± 6179.79	0.24
200	1793900 ± 5130.26	0.28	400	3205889.66 ± 9281.14	0.28
240	21567262 ± 3759	0.17	480	3837983.6 ± 36187.6	0.94
Interday					
160	1451180.66 ±12513.9	0.84	320	2561712 ± 7683.66	0.2
200	1793858 ±5450.62	0.3	400	3205013 ± 22157.41	0.69
240	2159394.33± 7148.92	0.33	480	3843025.6 ±30387.9	0.82

Robustness:

Table no: 6 Result of Robustness study

Parameters	**Variation**	**NEBI**		**CIL**	
		Mean area ± SD	**% RSD**	**Mean area ± SD**	**%RSD**
Flow rate ml/min	1.35	1929106 ± 7049.17	0.36	3477108 ± 13045.43	0.37
	1.5	1753822 ± 3871	0.22	3156677 ± 7699.46	0.24
	1.65	1585395 ± 13601.76	0.85	2878721 ± 12309.08	0.42
Mobile Phase pH	3.3	1763363 ± 8026.02	0.45	3162205 ± 9268.09	0.29
	3.5	1753822 ± 3871	0.22	3156677 ± 7699.46	0.24
	3.7	1766756 ± 5706.82	0.32	3169492 ± 9605.94	0.30
Mobile Phase Ratio	48:52	1762377 ± 8751	0.49	3162622 ± 7134	0.22
	50:50	1753822 ± 3871	0.22	3156677 ± 7699.46	0.24
	52:48	1764671 ± 18120	1.0	3136601 ± 16555	0.52

Analysis of marketed formulation

Table no: 7 Analysis of marketed formulation

NEBI			CIL		
Amt taken (µg/ml)	**Amt found (µg/ml)**	**% Assay**	**Amt taken (µg/ml)**	**Amt found (µg/ml)**	**% Assay**
200	198.44	99.22	400	400.85	100.21
	198.67	99.33		402.59	100.66
	201.88	100.94		398.16	99.54
MEAN	199.66	99.83	MEAN	400.53	100.13
SD	1.92	0.96	SD	2.23	0.56
% RSD	0.961	0.96	% RSD	0.55	0.55

System suitability:

Table no: 8 system Suitability

SYSTEM SUITABILITY PARAMETERS	**DRUG**	
	NEBI	**CIL**
Retention time (min) ± SD	4.626 ± 0.012	6.91 ± 0.006
Tailing factor (T) ± SD	1.28 ± 0.04	1.2 ± 0.043
Number of theoretical plates (N) ± SD	7816 ± 33.20	11329 ± 346.73
Resolution (R)	4.2 ± 0.040	

Summary of validation parameters

Table no:9 validation parameters

PARAMETER		NEBI	CIL
Linearity (µg/ml)		160-240	320-480
Precision (% RSD)			
Repeatability % RSD (n=6)		0.30	0.50
Intraday % RSD (n=3)		0.17-0.98	0.24-0.94
Interday% RSD (n=3)		0.3-0.88	0.2-0.8
Accuracy (%Recovery)		99.85-100.07 %	99.89-100.12 %
Robustness (%RSD)	Flow rate	0.36-0.85	0.24-0.42
	Mobile phase	0.49-1.0	0.22-0.52
	pH	0.32-0.45	0.29-0.30
LOD (µg/ml)		4.93	4.6
LOQ (µg/ml)		14.94	14.51

First order Derivative method:

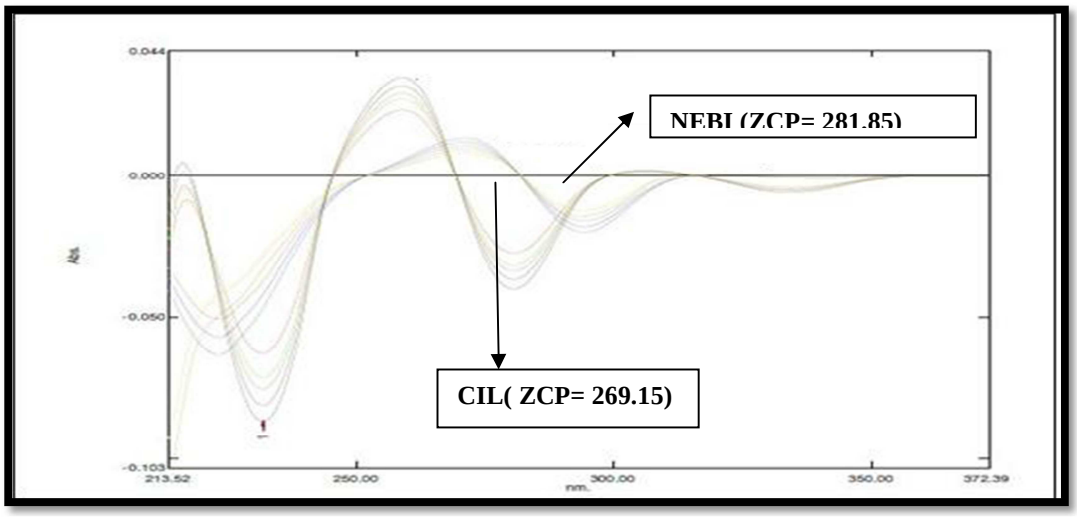

Figure 7: Derivative spectra of NEBI (6 µg/ml) and CIL (12 µg/ml)

Method validation

Linearity

The Calibration Curve found to be linear over the Concentration ranges of 160-240 µg/ml for NEBI (as shown in fig. 5) and 320-480 µg/ml for CIL(As shown in fig. 6).The correlation coefficient was found to be 0.999 for both NEBI and CIL.

Table no:10 Linearity Results

Conc. (µg/ml)		Mean Absorbance ± SD		% RSD	
NEBI	CIL	NEBI at 269.15	CIL at 281.85	NEBI	CIL
2	4	0.0248 ± 0.0004	-0.027 ± 0.0004	1.8	1.7
4	8	0.0504 ± 0.0005	-0.053±0.0007	1.1	1.3
6	12	0.0744 ± 0.0005	-0.081±0.00054	0.7	0.7
8	16	0.1008 ± 0.0008	-0.104±0.00141	0.8	1.4
10	20	0.1234 ± 0.0013	-0.129 ±0.00114	1.1	0.9

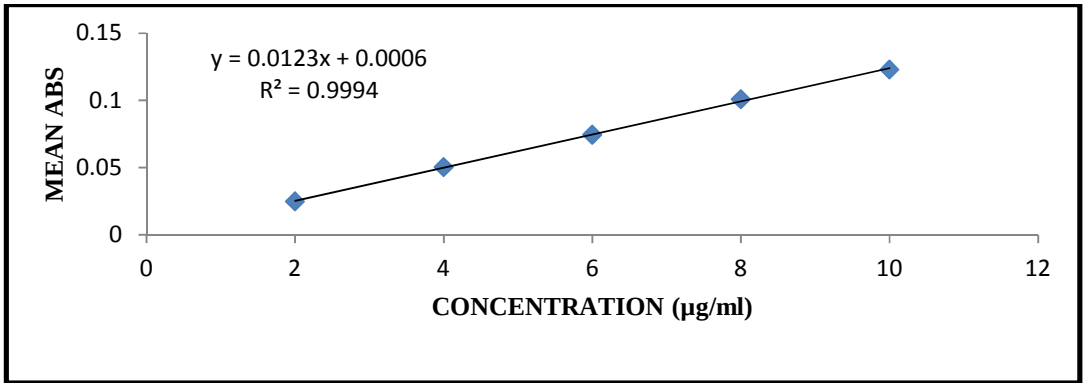

Figure 8: Calibration curve of NEBI

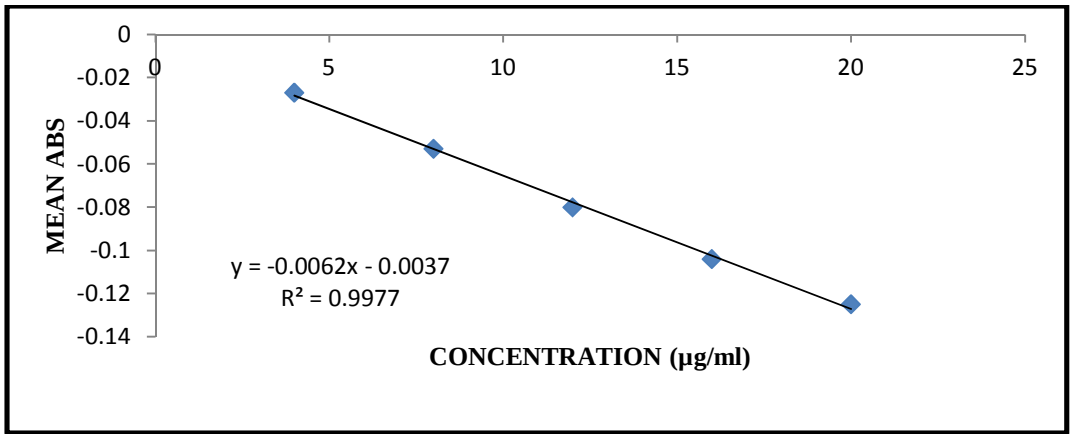

Figure 9: Calibration curve of CIL

Accuracy:

Table no: 11 Accuracy data for NEBI at 4 µg/ml

AMOUNT OF NEBI (µg/ml)	% OF STD NEBI ADDED	AMOUNT ADDED (µg/ml)	AMOUNT FOUND (µg/ml)	% RECOVERY (MEAN ± SD)	%RSD
4	50	2	6.08	99.85 ± 1.40	1.4
			5.91		
			5.97		
	100	4	8.16	101.01 ± 1.00	0.98
			8.08		
			8		
	150	6	9.91	99.14 ± 0.83	0.83
			10		
			9.8		

Table no: 12 Accuracy data for CIL at 8 µg/ml

AMOUNT OF CIL (µg/ml)	% OF STD CIL ADDED	AMOUNT ADDED (µg/ml)	AMOUNT FOUND (µg/ml)	% RECOVERY (MEAN ± SD)	%RSD
8	50	4	11.6	99.63 ± 1.025	1.03
			11.75		
			11.8		
	100	8	16.1	99.76 ± 1.55	1.56
			15.67		
			15.8		
	150	12	20.33	99.55 ± 1.27	1.26
			20.16		
			19.8		

Precision:

Table no: 13 Precision Data

NEBI			CIL		
Intraday					
Conc µg/ml	**Mean area ± SD**	**%RSD**	**Conc µg/ml**	**Mean area ± SD**	**%RSD**
2	0.053667± 0.00035	0.56	4	-0.0323±0.0001	0.31
6	0.073667± 0.0005	0.66	12	-0.094± 0.0006	0.68
10	0.128333 ±0.015	1.17	20	-0.134 ±0.0005	0.37
Interday					
2	0.03216±0.0002	0.62	4	-0.04767±0.0002	0.42
6	0.105± 0.0013	1.2	6	-0.09633± 0.0015	1.56
10	0.1433 ±0.002	1.39	20	-0.14±0.0008	0.57

Robustness:

Table no: 14 Result of Robustness study

Sr no.	Drug	Wavelength (nm)	Mean Absorbance ± SD	%RSD
1	NEBI	268.15	0.071 ± 0.0004	0.57
2		269.15	0.067 ± 0.001	1.49
3		270.15	0.073 ± 0.0004	0.56
1	CIL	280.85	-0.079 ± 0.0005	0.65
2		281.85	-0.0768 ± 0.0013	1.69
3		282.85	-0.084 ± 0.0005	0.66

Analysis of marketed formulation

Table no: 15 Analysis of marketed formulation

NEBI			CIL		
Amt taken (µg/ml)	**Amt found (µg/ml)**	**% Assay**	**Amt taken (µg/ml)**	**Amt found (µg/ml)**	**% Assay**
4	4.04	101	8	8.16	102
	3.99	99.79		7.91	98.95
	3.98	99.58		8.08	101
MEAN	4.00	100.12	MEAN	8.05	100.65
SD	0.0321	0.766	SD	0.12	1.55
% RSD	0.8085	0.76	% RSD	1.49	1.53

CONCLUSION

From, this study it is concluded that the proposed RP-HPLC and First order derivative method was found to be simple, accurate, precise and useful for routine analysis of Nebivolol Hydrochloride and Cilnidipine in bulk and pharmaceutical dosage form. The obtained results were satisfactory as per ICH guidelines.

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