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DEVELOPMENT AND VALIDATION OF RP-HPLC ANALYTICAL METHOD FOR THE QUANTITATIVE ESTIMATION OF CILNIDIPINE IN PURE FORM AND MARKETED FORMULATION

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ABSTRACT
A simple, rapid, specific and accurate reverse phase high performance liquid chromatographic method has been developed for the validated of Cilnidipine in bulk as well as in marketed pharmaceutical dosage form. This separation was performed on a Symmetry ODS (C18) RP Column, 250 mm x 4.6 mm, 5µm column with ACN : Methanol: 0.1% OPA in the ratio of 60:30:10 as mobile phase at a flow rate of 1.0 mL min−1 with UV detection at 235 nm; the constant column temperature was Ambient. The run time under these chromatographic conditions was less than 6.0 min. The retention time of Cilnidipine was found to be 2.570min. The calibration plot was linear over the concentration range of 6–14 µg mL−1 with limits of detection and quantification values of 0.8 and 0.24ng mL−1 respectively. The mean % assay of marketed formulation was found to be 99.79%, and % recovery was observed in the range of 98-102%. Relative standard deviation for the precision study was found <2%.The developed method is simple, precise, specific, accurate and rapid, making it suitable for estimation of Cilnidipine in bulk and marketed pharmaceutical dosage form dosage form.

KEYWORDS: Cilnidipine, RP-HPLC, Validation, Accuracy, Linearity, ICH Guidelines.

INTRODUCTION
Cilnidipine is a diesterified 1, 4-dihydropyridine-3, 5-dicarboxylic acid. A calcium channel blocker, it is used as an antihypertensive. It has a role as a calcium channel blocker, an antihypertensive agent and a cardiovascular drug. It is a dihydropyridine, a 2-methoxyethyl ester and a C-nitro compound. Cilnidipine is a dihydropyridine calcium antagonist.[1] It was jointly developed by Fuji Viscera Pharmaceutical Company, Japan and Ajinomoto, Japan and approved in 1995. Compared with other calcium antagonists, Cilnidipine can act on the N-type calcium channel that existing sympathetic nerve end besides acting on L-type calcium channel that similar to most of the calcium antagonists. This drug is approved in China, Japan, Korea, India, and several countries in the European Union. Cilnidipine is indicated for the management of hypertension for end-organ protection.[2] It is reported to be useful in elderly patients and in those with diabetes and albuminuria. Cilnidipine has been increasingly used in patients with chronic kidney disease. Hypertension is the term used to describe the presence of high blood pressure. The blood pressure is generated by the force of the blood pumped from the heart against the blood vessels. Thus hypertension is caused when there is too much pressure on the blood vessels and this effect can damage the blood vessel.[3] The IUPAC name of 3-O-(2-methoxy ethyl) 5-O-[(E)-3-phenyl prop-2-enyl] 2, 6-dimethyl-4-(3-nitro phenyl)-1, 4-dihydropyridine-3, 5-dicarboxylate. The Chemical Structure of Cilnidipine is shown in fig-1.

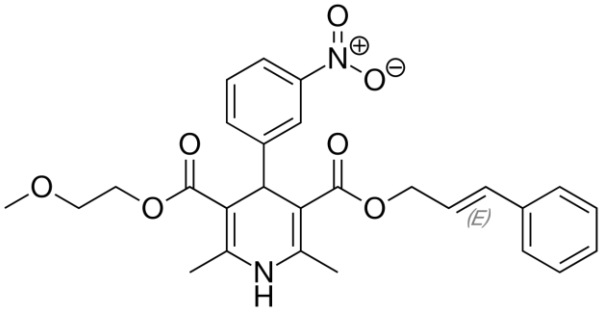

Fig-1: Chemical Structure of Cilnidipine.

MATERIALS AND METHODS

Table-1: List of Instrument Used.

S. No.	Instruments/Equipments/Apparatus
1.	**HPLC** with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	**T60-LAB INDIA** UV – Vis spectrophotometer
3.	Electronic Balance **(SHIMADZU ATY224)**
4.	Ultra Sonicator **(Wensar wuc-2L)**
5.	Thermal Oven
6.	Symmetry ODS RP C_{18},5µm, 15mm x 4.6mm i.d.
7.	P^H Analyzer **(ELICO)**
8.	Vacuum filtration kit **(BOROSIL)**

Table 2: List of Chemicals Used.

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
3.	Dipotassium hydrogen orthophosphate	96%	A.R.	Sd fine-Chem ltd; Mumbai
4.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
5.	Potassium dihydrogen orthophosphate	99.9%	A.R.	Sd fine-Chem ltd; Mumbai
6.	Sodium hydroxide	99.9%	A.R.	Sd fine-Chem ltd; Mumbai
7.	Hydrochloric acid	99.9%	A.R.	Loba Chem; Mumbai.
8.	Hydrogen Peroxide	99.9%	A.R.	Loba Chem; Mumbai.

Selection of Wavelength

The Standard & Sample Stock Solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis.[4] It scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Cilnidipine, so that the same wave number can be utilized in HPLC UV detector for estimating the Cilnidipine. The scanned UV spectrum is attached in the following page.

Sample & Standard Preparation for the UV-Spectrophotometer Analysis

25 mg of Cilnidipine standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase.

Further dilution was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

Optimization of Chromatographic Conditions

The chromatographic conditions were optimized by different means.[5] (Using different column, different mobile phase, different flow rate, different detection wavelength & different diluents for sample preparation etc.

Table 3: Summary of Process Optimization.

Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
Develosil $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Methanol : Acetonitrile = 40 : 60	1.0ml/min	235nm	Very Low response	Method rejected
Inertsil $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Methanol : Acetonitrile = 55 : 45	1.0ml/min	235nm	Low response	Method rejected
Phenomenex Luna $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Acetonitrile : Water = 50:50	1.0ml/min	235nm	Tailing peaks	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	Methanol : Water = 70:30	1.0ml/min	235nm	Resolution was not good	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	ACN : Methanol: 0.1% OPA = 70:25:5	1.0ml/min	235nm	Tailing peak	Method rejected
Symmetry $C_{18,}$ ODS, Reverse Phase, 250 mm x 4.6 mm, 5µm, Column.	ACN : Methanol: 0.1% OPA = 60:30:10	1.0ml/min	235nm	Nice peak	Method accepted

Preparation of 0.1% OPA Solution
0.1% OPA was prepared by taking 1 ml of OPA in 1000 ml HPLC grade water.

Preparation of Mobile Phase
600ml of HPLC Grade Acetonitrile, 300ml of HPLC Grade Methanol and 100ml 0.1% OPA were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration.

Method Validation
Specificity
As per ICH guideline Q2 (R1)[17,25,30], "Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matrix, etc."

Method Precision
As per ICH guideline[17,25,30] Q2 (R1), "The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions."

Further, the precision[6-9] may be considered at three levels: repeatability, intermediate precision, and reproducibility.

Repeatability: Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Intermediate Precision: Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

Reproducibility: Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

Detection Limit: The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.[10] Several approaches for determining the detection limit are possible, depending on whether the procedure is non-instrumental or instrumental.

Quantitation Limit: The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices and is used particularly for the determination of impurities and/or degradation products.[11] Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

Linearity
The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample. Refer to below table to understand the meaning of the above statement.[12]

Range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.[13]

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.[14]

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. To design the robustness experiment, you must have better understanding of analytical method that you are using.[15] For example, if you are using an HPLC technique for your method- then what are the likely weaknesses of an HPLC technique? Is it flow rate, temperature of column oven or mobile phase composition? What are likely challenges during sample preparation procedure that you are using? It can be sonication time, solution stability or filtration. So, based on scientific rational, one has to decide on robustness study parameters.

RESULTS AND DISCUSSION

Development of Analytical Method

Selection of Wavelength

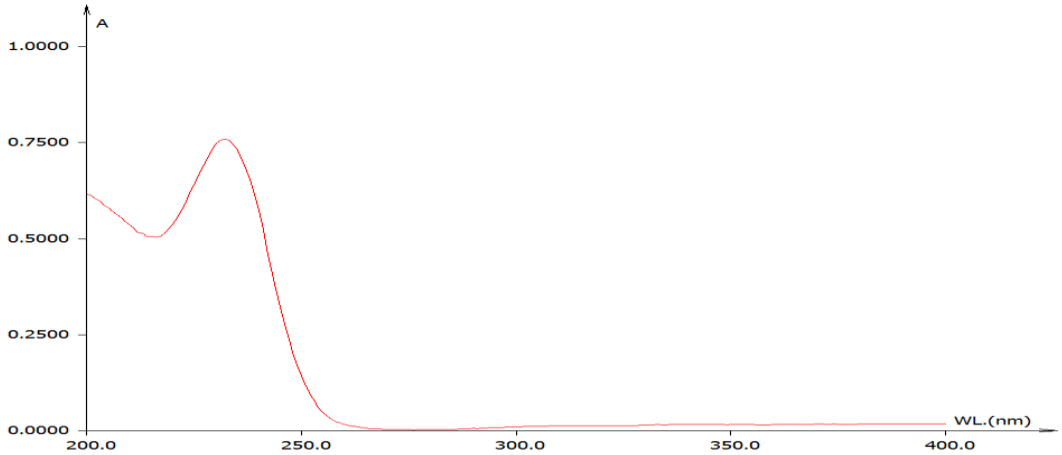

Fig-2: UV Spectrum for Cilnidipine.

Observation: While scanning the Cilnidipine solution we observed the maxima at 235nm. The UV spectrum has been recorded on T60-LAB INDIA make UV – Vis spectrophotometer model UV-2450.

Summary of Optimized Chromatographic Conditions

The Optimum Chromatographic conditions obtained from experiments can be summarized as below.[16]

Table 4: Summary of Optimised Chromatographic Conditions.

Mobile phase	ACN : Methanol: 0.1% OPA = 60:30:10% v/v/v
Column	Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm
Column Temperature	Ambient
Detection Wavelength	235 nm
Flow rate	1.0 ml/ min.
Run time	06 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	10µl
Type of Elution	Isocratic
Retention time	2.631 minutes

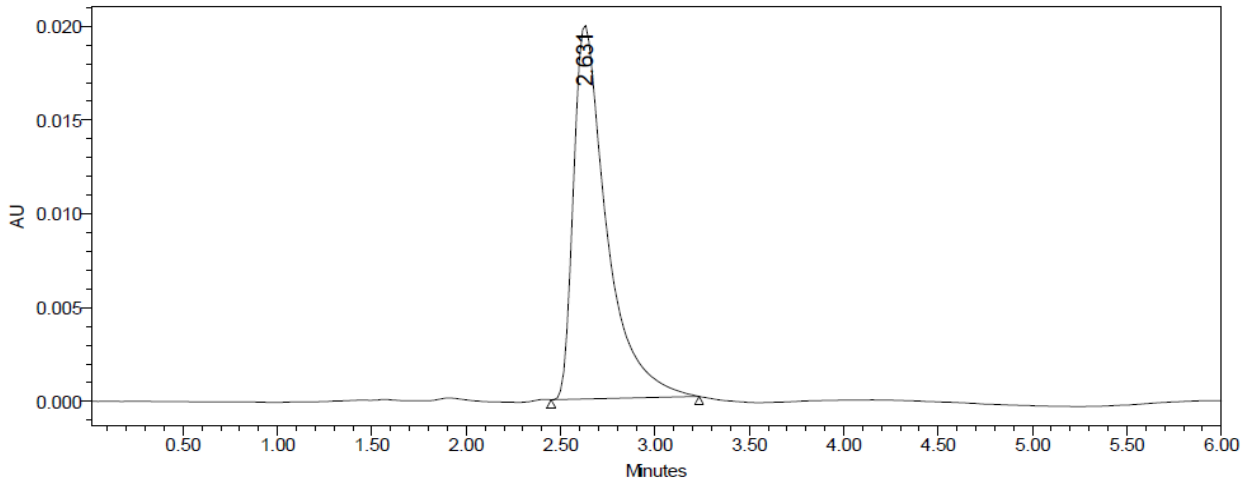

Fig-3: Chromatogram of Cilnidipine in Optimized Condition.

Observation: The selected and optimized mobile phase was ACN: Methanol: 0.1% OPA = 60:30:10 and conditions optimized were flow rate (1.0 ml/minute), wavelength (235nm), Run time was 06 mins. Here the peaks were separated and showed better resolution, theoretical plate count and symmetry.[18] The proposed chromatographic conditions were found appropriate for the quantitative determination of the drug.

Method Validation

The optimized analytical method was validated for system suitability, linearity and range, precision, limit of detection [LOD], limit of quantitation [LOQ] and accuracy in accordance with ICH guidelines.[17,25,30] for analytical procedures Q2[R1].

System Suitability Parameter: System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters[19] were established. The data are shown in Table-5.

Table 5: Data of System Suitability Parameter.

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	8.47
2	Asymmetry	T ≤ 2	Cilnidipine=0.23
3	Theoretical plate	N > 2000	Cilnidipine=2987
4	Tailing Factor	T<2	Cilnidipine=1.17

Specificity

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Cilnidipine working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.1ml of the above Cilnidipine stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution

Weight 10 mg equivalent weight of Cilnidipine sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 0.1ml of Cilnidipine above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure

Inject the three replicate injections of standard and blank solutions and check the interferences in both solutions.

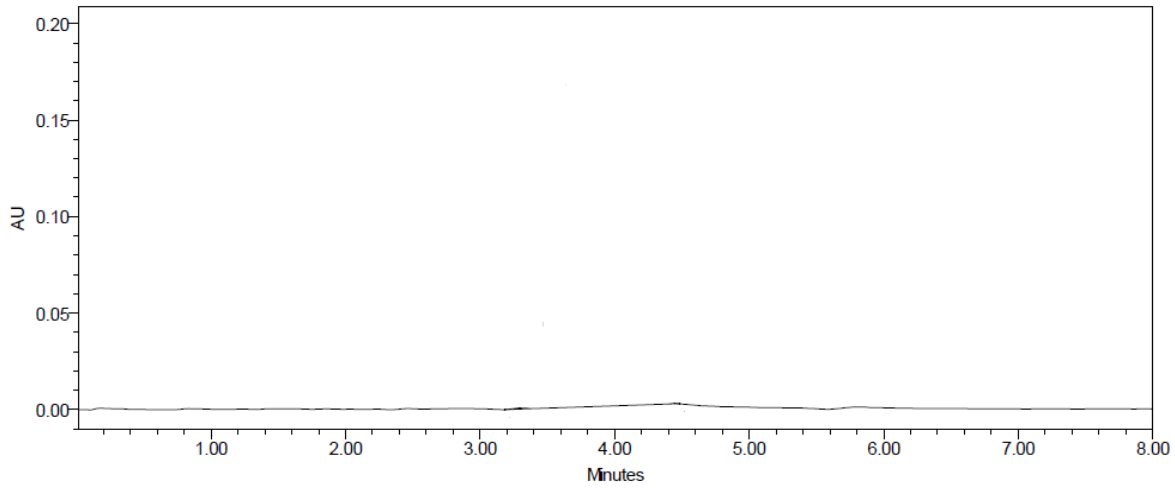

Fig-4: Chromatogram of Blank Solution.

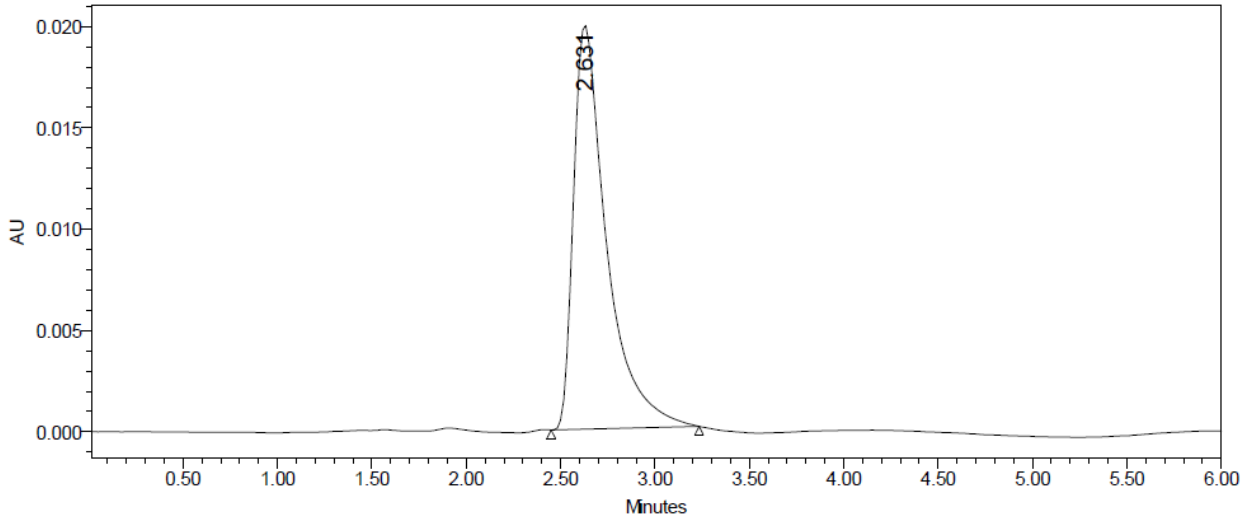

Fig-5: Chromatogram of Cilnidipine Standard Solution.

Observation: In this test method blank, standard solutions were analyzed individually to examine the interference. The above chromatograms show that the active ingredient was well separated from blank and their excipients and there was no interference of blank with the principal peak. Hence the method is specific.

Linearity & Range

The calibration curve[20] showed good linearity in the range of 6 – 14 µg/ml, for Cilnidipine (API) with correlation coefficient (r^2) of 0.999 (Fig-6). A typical calibration curve has the regression equation of y = 19423x + 5444 for Cilnidipine.

Table 6: Results of Linearity Data.

CONC.(µg/ml)	MEAN AUC (n=6)
0ppm	0
6ppm	129013
8ppm	166523
10ppm	198315
12ppm	234151
14ppm	275819

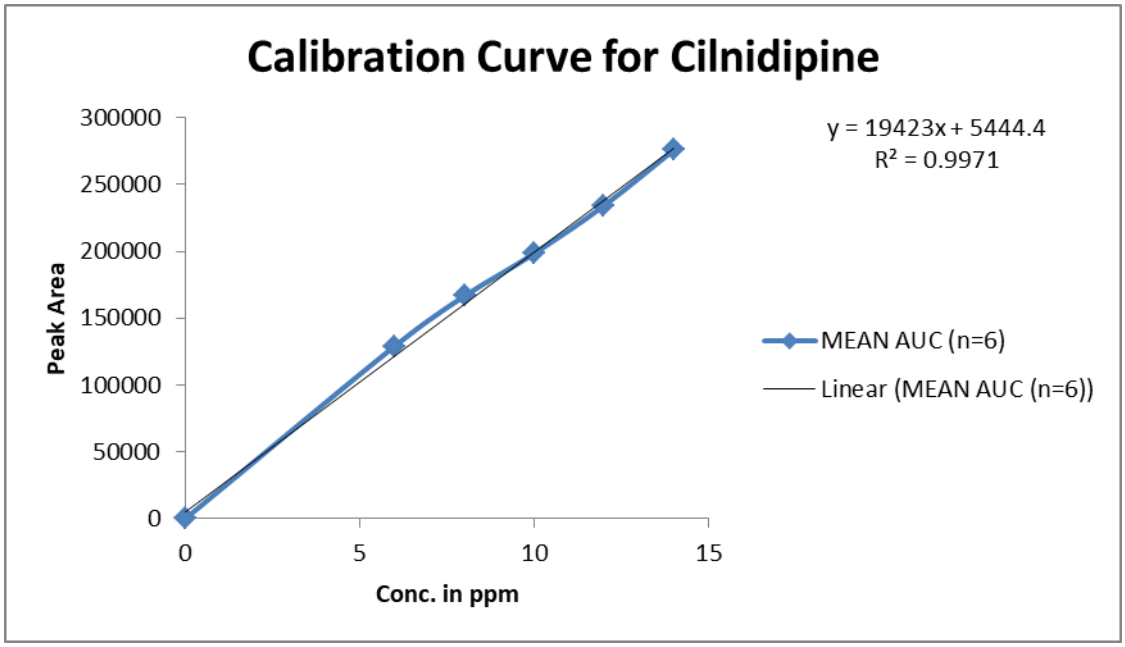

Fig-6: Calibration Curve of Cilnidipine (API).

Precision

Repeatability

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug.[21] Cilnidipine (API). The percent relative standard deviation was calculated for Cilnidipine are presented in the table-7.

Table 7: Readings of Repeatability.

HPLC Injection Replicates of Cilnidipine	Retention Time (Minutes)	Peak Area (AUC)
Replicate – 1	2.572	197236
Replicate – 2	2.570	197762
Replicate – 3	2.573	195969
Replicate – 4	2.570	194724
Replicate – 5	2.574	198327
Replicate – 6	2.573	198711
Average		**197121.5**
Standard Deviation		**1515.213**
% RSD		**0.768667**

Intermediate Precision

Intra-Assay & Inter-Assay

The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Cilnidipine revealed that the proposed method is precise.[22]

Table 8: Results of Intra-Assay & Inter-Assay.

Conc. of Cilnidipine (API) (µg/ml)	Observed Conc. of Cilnidipine (µg/ml) by the Proposed Method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	7.46	0.62	8.05	0.96
10	10.87	0.85	9.43	0.71
12	11.81	0.92	12.04	0.65

Accuracy

Recovery Study

To decide the exactness of the proposed strategy, recuperation thinks about were done by including diverse sums (80%, 100%, and 120%) of unadulterated -9. medication of CILNIDIPINE were taken and added to the pre-broke down plan of fixation 10µg/ml.[23] From that rate recuperation esteems were computed. The outcomes were appeared in table.

Table 9: Readings of Accuracy.

Conc. In ppm	Conc. Found	Peak Area		% Recovery
8	8.035	161523		100.437
8	8.153	163815		101.912
8	8.061	162023		100.762
			Avg.	101.037
			S.D	0.775
			%RSD	0.767046
Conc. In ppm	Conc. Found	Peak Area		% Recovery
10	9.930	198315		99.30
10	10.033	200320		100.33
10	10.044	200540		100.44
			Avg.	100.0233
			S.D	0.628835
			%RSD	0.628688
Conc. In ppm	Conc. Found	Peak Area		% Recovery
12	11.981	238151		99.841
12	12.066	239819		100.55
12	12.215	242712		101.791
			Avg.	100.7273
			S.D	0.987021
			%RSD	0.979894

LOD & LOQ

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.08 & 0.24µg/ml respectively.[24]

Method Robustness

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Wavelength of detection (±2nm) & organic phase in mobile phase (±5%) studied to determine the robustness of the method are also in favour of (Table-10, % RSD < 2%) the developed RP-HPLC method for the analysis of Cilnidipine (API).[26]

Table 10: Result of Method Robustness Test.

Change in Parameter	% RSD
Flow (1.1 ml/min)	0.68
Flow (0.9 ml/min)	0.39
More Organic	0.54
Less Organic	0.63
Wavelength of Detection (237 nm)	0.91
Wavelength of detection (233 nm)	0.93

Estimation of Cilnidipine in Pharmaceutical Dosage Form (Assay)

Label claim: 10mg

Twenty pharmaceutical dosage forms were taken and the I.P. strategy was taken after to decide the normal weight. Above measured tablets were at last powdered and triturated well. An amount of powder proportionate to 25 mg of medications were exchanged to 25 ml volumetric flagon, make and arrangement was sonicated for 15 minutes, there after volume was made up to 25 ml with same dissolvable. At that point 10 ml of the above arrangement was weakened to 100 ml with versatile stage. The arrangement was separated through a layer channel (0.45 µm) and sonicated to degas. The arrangement arranged was infused in five reproduces into the HPLC framework and the perceptions were recorded.[27]

A copy infusion of the standard arrangement was additionally infused into the HPLC framework and the peak regions were recorded. The information is appeared in Table-11.

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:

AT = Peak Area of medication acquired with test arrangement

AS = Peak Area of medication acquired with standard arrangement

WS = Weight of working standard taken in mg

WT = Weight of test taken in mg

DS = Dilution of Standard arrangement

DT = Dilution of test arrangement

P = Percentage virtue of working standard

Table 11: Recovery Data for Estimation Cilnidipine in Cilaheart-10 Tablet.

Brand Name of Cilnidipine	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the Proposed Method (n=6)	Assay % (± SD)
Cilaheart-10 Tablet (Mankind)	10mg	9.524 (± 0.635)	99.574 (± 0.258)

Observation: The amount of drug in Cilaheart-10 Tablet was found to be 9.524 (± 0.635) mg/tab for Cilnidipine & % assay was 99.574 %.

Stability Studies

Following protocol was strictly adhered to for forced degradation of Cilnidipine Active Pharmaceutical Ingredient (API). The API (Cilnidipine) was subjected to stress conditions in various ways to observe the rate and extent of degradation that is likely to occur in the course of storage and/or after administration to body. This is one type of accelerated stability studies that helps us determining the fate of the drug that is likely to happen after long time storage, within a very short time as compare to the real time or long term stability testing. The various degradation pathways studied are acid hydrolysis, basic hydrolysis, thermal degradation, photolytic degradation and oxidative degradation.[28]

Results of Stability Studies: The results of the stress studies indicated the **specificity** of the method that has been developed. Cilnidipine was stable in thermal and photolytic stress conditions. The result of forced degradation studies[29] are given in the following table-12.

Table 12: Results of Forced Degradation Studies of Cilnidipine API.

Stress Condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	81.36	18.64	100.0
Basic Hydrolysis (0.1 M NaOH)	24Hrs.	83.37	16.63	100.0
Thermal Degradation (50 ^{0}C)	24Hrs.	98.92	1.08	100.0
UV (254nm)	24Hrs.	96.33	3.67	100.0
3 % Hydrogen peroxide	24Hrs.	89.41	10.59	100.0

SUMMARY AND CONCLUSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Cilnidipine, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Symmetry ODS RP C_{18}, 5µm, 15mmx4.6mm i.d. Column was preferred because using this column peak shape, resolution and absorbance were good.

Discovery wavelength was chosen in the wake of examining the standard arrangement of medication more than 200 to 400nm. From the U.V range of Cilnidipine it is apparent that a large portion of the HPLC works can be proficient in the wavelength scope of 210-300 nm helpfully. Further, a stream rate of 1 ml/min and an infusion volume of 10µl were observed to be the best investigation. The outcome demonstrates the created technique is amazingly, one more reasonable strategy for measure and dependability related debasement examines which can help in the investigation of Cilnidipine in various details.

A sensitive & selective RP-HPLC method has been developed & validated for the analysis of Cilnidipine. Encourage the proposed RP-HPLC technique has astounding affectability, accuracy and reproducibility. The outcome demonstrates the created technique is amazingly, one more appropriate strategy for test, immaculateness and solidness which can help in the examination of Cilnidipine in various definitions.

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