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Method Development and Validation for Simultaneous Estimation of Related Impurities of Cilnidipine and Chlorthalidone in Tablet Dosage Form By Rp-Hplc

Dr. Bhalchandra A. Kadam[1], Mr. Omkar U. Wagh[2], Mr. Kazi Abusufeeyan Ikbal[3], Mr. Shubham Sanjekar[4], Miss. Akshata Hajgude[5], Miss. Sayali Satyendra Pawar[6], Dr. Vikrant V. Nawale[7]
[1,2]Assistant Professor, ASPM's K. T. Patil College of Pharmacy, Dharashiv.
[3,5]Assistant Professor, TPCTS COE Dharashiv.
[4]PhD Research Scholar, Sinhgad College of Pharmacy, Affiliated to Savitribai Phule Pune University, Vadgaon (Bk), Pune.
[6]PhD Research Scholar, JSPM University, Wagholi, Pune – 412207, Maharashtra, India.
[7]Assistant Professor, SSJPS R.P. College of Pharmacy, V.P. Educational Campus, Dharashiv.

ABSTRACT

Chlorthalidone drug is a diuretic used to treatment of hypertension disease. The duration of action for the Chlorthalidone drug is the highest in the pharmacology of the drug. Cilnidipine drug is a calcium channel protein inhibitor and blocker. It has also shown neuroprotective effects in a rat focal brain ischemia model by removing free radicals and activating the phosphatidylinositol 3-kinase pathway. A novel HPLC method was developed and validated for the estimation of related impurities of cilnidipine and chlorthalidone in pharmaceutical formulations. The chromatographic separation was carried out by isocratic elution using an Hypersil BDS C18 column (250×4.6mm; 5µ). The mobile phase was composed of phosphate buffer at a pH of 4.0 and acetonitrile in the ratio of 80:20 (V/V) at a flow rate of 1.0 mL/min. The eluents were detected and quantified at a UV detection wavelength of 225 nm. Calibration curves of all analytes in the range of 1-15µg/mL showed a good correlation linearly (r ≥ 0.99) with recovery rate of more than 98% for each analyte. The percentage RSD in intraday, interday precision and ruggedness were found to be less than 5. Small variations in the developed conditions like mobile phase ratio, flow rate, pH and UV wavelength do not influence the results. The detailed quantitative results of this study show that this method is simple, quick, precise, accurate, sensitive, cost-effective and robust. Thus, the method development and validation result confirm that this method be successfully applied for determination and quantification of impurities for the routine quality control analysis in pharmaceutical dosage forms. Extensively limit of detection establishment and limit of quantification are determined. And gradient mode elution also checked for scope of related substances method development for impurities of Chlorthalidone and Cilnidipine drug components, however known impurities need to be monitored.

Keywords: *Method Development, Validation, RP-HPLC, Cilnidipine, Chlorthalidone.*

1.INTRODUCTION

Hypertension is the most common cardiovascular disease. As many as 50 million people in the United States have systolic and/or diastolic blood pressure above 140/90 mm Hg. Elevated arterial pressure causes pathological changes in the vasculature and hypertrophy of the left ventricle. As a consequence, hypertension is the principal cause of stroke, leads to disease of the coronary arteries with myocardial infarction and sudden cardiac death, and is a major contributor to cardiac failure, renal insufficiency, and dissecting aneurysm of the aorta. Hypertension is defined conventionally as blood pressure 140/90; this serves to characterize a group of patients who carry a risk of hypertension-related cardiovascular disease that is high enough to merit medical attention. However, from the standpoint of health promotion, it should be noted that the risk of both fatal and nonfatal cardiovascular disease in adults is lowest with systolic blood pressures of less than 120 mm Hg and diastolic less than 80 mm Hg; these risks increase progressively with higher levels of both systolic and diastolic blood pressure.

Liquid chromatography (LC) is a physical separation technique conducted in the liquid phase. A sample is separated into its constituent components (or analytes) by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column). For example, the flowing liquid can be an organic solvent forced through the column at high speed and the stationary phase can be porous silica particles packed in a column. The modern form of column chromatography has been called high performance, high Pressure, high-resolution and high-speed liquid chromatography. HPLC is a modern form of LC that uses small-particle columns through which the

mobile phase is pumped at high pressure.

High-performance liquid chromatography (HPLC), sometimes called high-pressure liquid chromatography, is a separation technique based on a solid stationary phase and a liquid mobile phase.

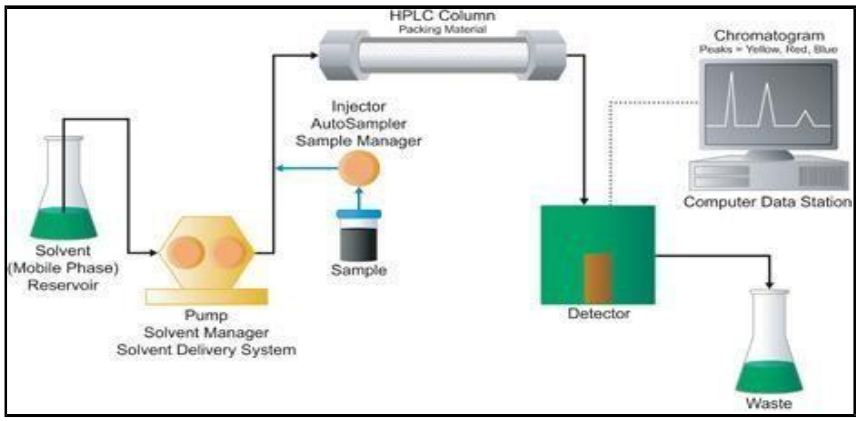

Fig.: Block diagram of HPLC

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice. Analytical methods need to be validated or revalidated.

- Before their introduction into routine use.
- Whenever the conditions change for which, the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix).
- Whenever the method is changed and the change is outside the original scope of the method. The USP has published specific guidelines for method validation for compound evaluation.

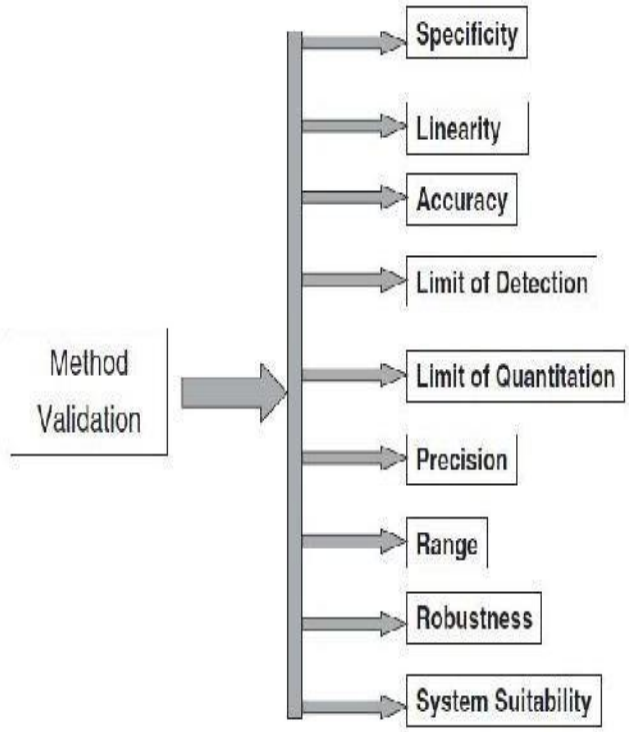

Fig.: Analytical Method Validation Flow Chart

Infrared spectroscopy

For IR spectroscopy analysis, prepared a plate of KBr with each drug by applying the pressure of about 7-10 tones. This KBr plates figure scanned in IR spectroscopy instrument at 400-4000 cm^{-1} to determine IR spectrum of each drug components.

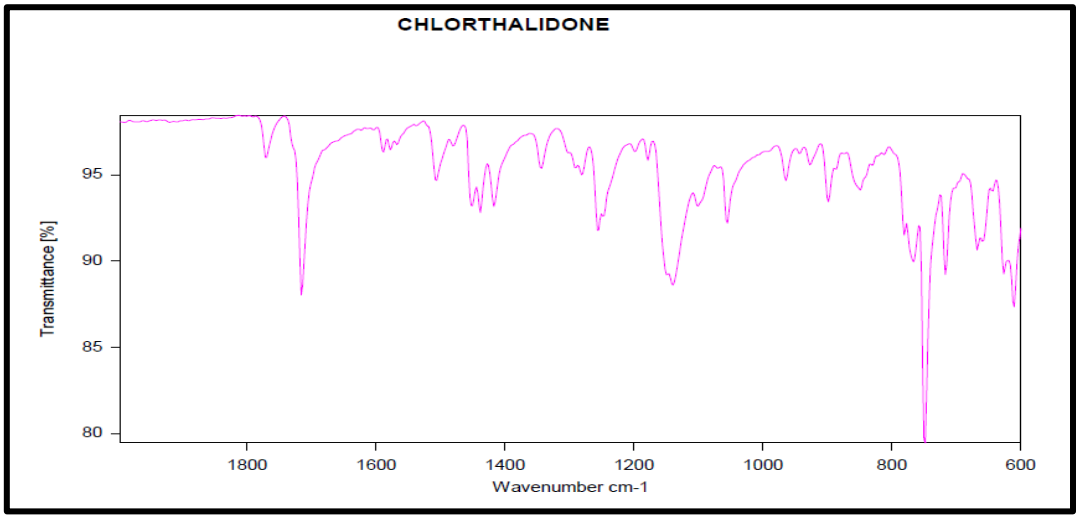

Fig.: IR Spectra of sample Chlorthalidone drug

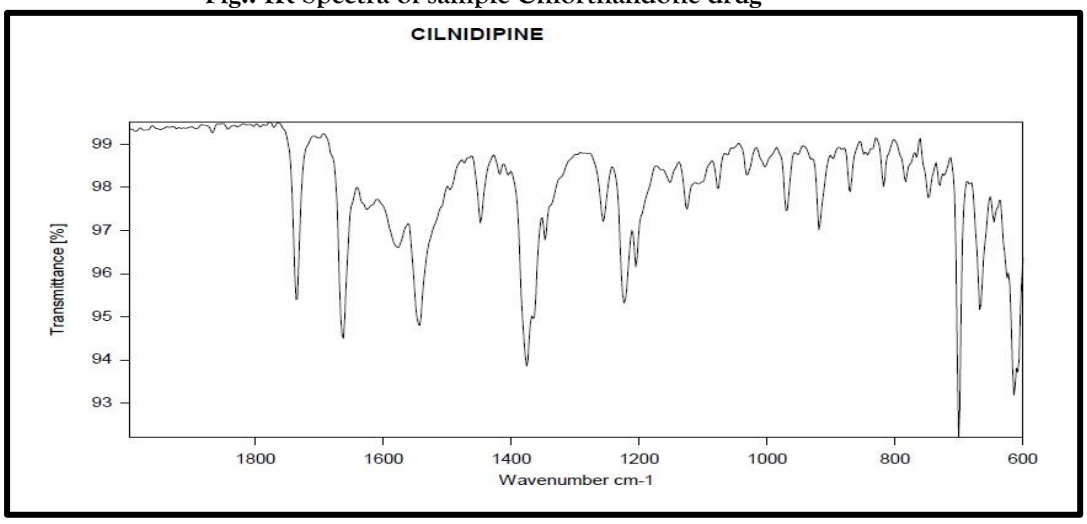

Fig.: IR Spectra of sample Cilnidipine drug

Functional Group Frequency cm^{-1}

Solubility Study

Table: Solubility of Cilnidipine and Chlorthalidone drugs

Solvents	Solubility of Drug	
	Chlorthalidone	Cilnidipine
Water	Insoluble	Soluble
0.1 N NaOH	Very slightly soluble	Very slightly soluble
0.1 N HCl	Very slightly soluble	Soluble
Methanol	Freely soluble	Freely soluble

Method development for simultaneous estimation of related impurities of cilnidipine and chlorthalidone in it's pharmaceutical dosage form by RP-HPLC Elution Mode selection:

The elution mode selected for estimation of all drug components cilnidipine and Chlorthalidone as reverse phase high pressure liquid chromatography system. As all drug components are polar in nature, henceforth reverse phase liquid chromatography methodology is suitable for these drug components. In reverse phase chromatography system mobile phase is polar in nature and stationary phase is non-polar

in nature. As compared to normal phase liquid chromatography system, reverse phase liquid chromatography is less expensive, convenient and simple.

Wavelength selection:

All two drugs Cilnidipine and Chlorthalidone in methanol show reasonably good →response at 225 nm wavelength.

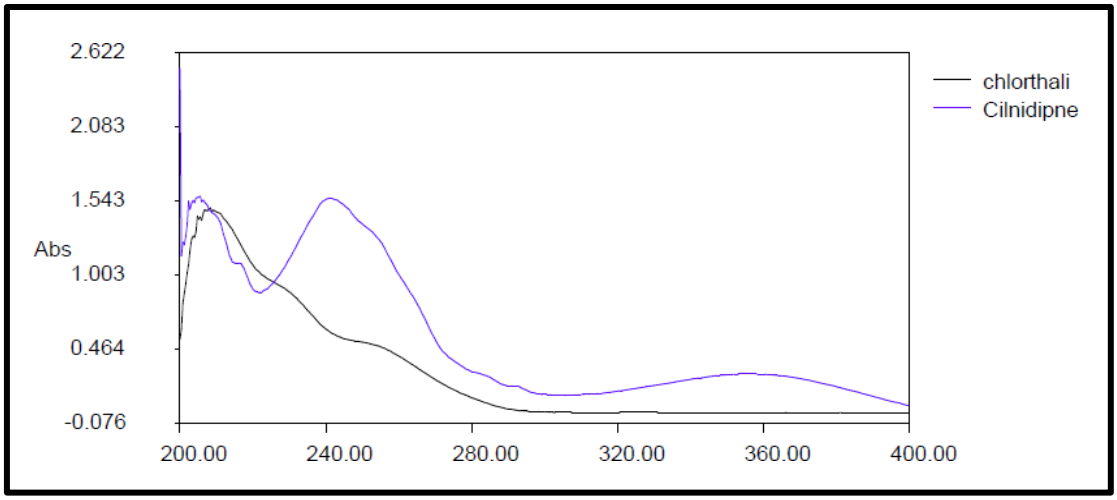

Fig.: Overlay UV Spectrum of Cilnidipine and Chlorthalidone drugs for selection of wavelength detection

Method development trial-1 Objective of trial-1:

To optimize mobile phase for simultaneously elution of peak of Cilnidipine and Chlorthalidone in single run.

Constant parameters for trial-1:

Chromatography system for trial-1: Reverse phase high pressure liquid chromatography (RP-HPLC)

Stationary phase (Column) for trial-1: Hypersil BDS C18 (250 X 4.6) mm, 5µm. Flow rate for trial-1: 1.0 ml/minute flow rate of mobile phase selected for retention and elution of Cilnidipine and Chlorthalidone peaks in chromatography system.

Injection volume for trial-1: 20µl injection volume selected for sample and standard solution containing all two components of Cilnidipine and Chlorthalidone.

Column oven temperature (Column compartment) for trial-1: 25°C or temperature for column compartment can keep off as ambient temperature selected for chromatography system

Sample temperature (Auto sampler) for trial-1: 25°C or temperature for sample compartment can keep off as ambient temperature selected for chromatography. Detection Wavelength for trial-1: 225 nm wavelength selected for detection of all two components of Cilnidipine and Chlorthalidone simultaneously by considering UV spectrum of respective components.

Elution mode for trial-1: Isocratic elution: The single channel is used for retention of peaks of all two components of Cilnidipine and Chlorthalidone without using any gradient program.

Sample concentration for trial-1: Standard solution and Sample solution concentration for individual components consist as Cilnidipine: 50 ppm and Chlorthalidone: 50 ppm

Diluent system for trial-1: Diluent for sample and standard solution preparation kept as methanol to make freely solubilize and elution of all components Cilnidipine and Chlorthalidone.

Variable parameters for trial-1:

The variable parameters for mobile phase optimization of this trial are as Mobile phase for trial-1: The mobile phase used as composition of water and methanol Mobile phase composition for trial-1: The composition of Water and methanol is kept as 50:50

Run time for trial-1: For elution of all components Cilnidipine and Chlorthalidone run time kept as 15 minutes

Observation:

Only one peak observed in chromatogram which have retention time as about

2.7 minutes.
The peak shape found is not good in terms of tailing factor and theoretical plate count

RESULT:
Method is capable only for one component as single peak eluted. The results are not found as per objective.

Inference:
Only one component eluted in chromatogram, rest peaks are not observed in the chromatography system in the selected run time. It may be due difference between polarity of each component.
Stationary phase is capable for retention and elution of single component and may be capable other two components by alteration of mobile phase in chromatography.
Run time is not sufficient for elution of components in chromatography system. Need to identify which component eluted as single peak in chromatogram and which are not eluted.

Changes required for next trial:
Individual component Cilnidipine and Chlorthalidone solution need to prepare in diluent.
Individual component solution needs to inject in chromatography system for confirmation of eluted peak.

Method development trial-2

Objective of trial-2:
To Identify the eluted peak in mobile phase (water: methanol) due to Cilnidipine in chromatography system.

Constant parameters for trial-2:
Chromatography system for trial-2: Reverse phase high pressure liquid chromatography (RP-HPLC)
Stationary phase (Column) for trial-2: Hypersil BDS C18 (250 X 4.6) mm, 5µm Flow rate for trial-2: Mobile phase flow rate 1.0 ml/minute selected for retention and elution of Cilnidipine and Chlorthalidone peaks in chromatography.
Injection volume for trial-2: Injection volume 20µl selected for sample and standard solution containing cilnidipine and Chlorthalidone.
Column oven temperature (Column compartment) for trial-2: Ambient (25°C) or temperature for column compartment can keep off as ambient temperature selected for chromatography system
Sample temperature (Auto sampler) for trial-2: Ambient (25°C) or temperature for sample compartment can keep off as ambient temperature selected for chromatography system
Detection Wavelength for trial-2: 225 nm wavelength selected for detection of Cilnidipine and Chlorthalidone simultaneously in chromatography system. Elution mode for trial-2: Isocratic elution mode selected. The single channel is used for retention of peaks of Cilnidipine and Chlorthalidone without using any gradient program.
Sample concentration for trial-2: Standard solution and Sample solution concentration for individual components as: Cilnidipine: 50 ppm and Chlorthalidone: 50 ppm
Diluent system for trial-2: For sample and standard solution preparation diluent kept as methanol to make freely solubilize and elution of all components of Cilnidipine and Chlorthalidone.

Variable parameters for trial-2:
The variable parameter for method development to mobile phase optimization of this trial are as Cilnidipine drug solution of 50 PPM prepared and injected in chromatography. Mobile phase for trial-2: The composition of mobile phase used as water and methanol as same as trial -1
Mobile phase composition for trial-2: The mobile phase composition Water and methanol is kept as 50:50 as same as trial-1

Observation:
Only one peak observed in chromatogram which have retention time as about
2.7 minutes.
The peak shape of single peak is not found to be good in terms of tailing factor and theoretical plate count

Result:
The single peak observed due to cilnidipine. Chromatographic method is capable only for one component as single peak eluted.

Inference: Only one component eluted in chromatogram that is cilnidipine, rest peaks are not observed in the chromatography system in the selected run time due to their polarity difference from each other.

Column (stationary phase) is capable for retention and elution of single component and may be capable other two components by alteration of mobile phase in chromatography system.

Run time is not sufficient for retention and elution of all the components in chromatography system need to check interference of Chlorthalidone drug at the retention time of peak

Changes required for next trial:

Chlorthalidone drug solution need to prepare in diluent same concentration as per methodology.

Chlorthalidone component solution need to inject in chromatography system for confirmation of eluted peak.

Method development trial-3

Objective of trial-3:

To check the interference of Chlorthalidone at retention time of cilnidipine in chromatography system.

Constant parameters for trial-3:

Chromatography system for trial-3: Reverse phase high pressure liquid chromatography.

Stationary phase (Column) for trial-3: Hypersil BDS C18 (250 X 4.6) mm, 5µm column used.

Flow rate for trial-3: Mobile phase flow rate set as 1.0 ml/minute for retention and elution of Cilnidipine and Chlorthalidone peaks in chromatography.

Injection volume for trial-3: Injection volume 20µl selected for sample and standard solution containing Chlorthalidone in HPLC method

Column oven temperature (Column compartment) for trial-3: Ambient (25°C) or temperature for column compartment can keep off as ambient temperature selected for liquid chromatography system

Sample temperature (Auto sampler) for trial-3: Ambient (25°C) or temperature for sample compartment can keep off as ambient temperature selected.

Detection Wavelength for trial-3: 225 nm detection wavelength selected for detection of all components of Cilnidipine and Chlorthalidone simultaneously in chromatography system.

Elution mode for trial-3: Isocratic elution mode. The single channel is used for retention of peaks of components Cilnidipine and Chlorthalidone without using any gradient program.

Sample concentration for trial-3: Concentration of standard solution and sample solution for individual components as : Cilnidipine: 50 ppm, and Chlorthalidone: 50 ppm, Diluent system for trial-3: Diluent for sample and standard solution preparation kept as methanol to make freely solubilize and elution of Cilnidipine and Chlorthalidone.

Variable parameters for trial-3:

The variable parameter for analytical method development to mobile phase optimization of this trial are as

Chlorthalidone drug solution of 50 PPM prepared and injected in chromatography.

Mobile phase for trial-3: The composition of mobile phase used as water and methanol as same as trial -2

Mobile phase composition for trial-3: The mobile phase composition Water and methanol is kept as 50:50 as same as trial-2

Observation:

No any peak observed in chromatogram. The clear base line observed in chromatography system.

Result:

There is no any peak observed in chromatogram. Chlorthalidone peak not eluted in chromatogram.

Inference:

There is no any interference of Chlorthalidone at the retention time of cilnidipine.

The first peak would be cilnidipine as high polarity of drug component after that remaining peak will elute.

Mobile phase composition may lead the elution of other omponents in liquid chromatography.

Column (stationary phase) is capable for retention and elution of single component and may be capable other two components by changing composition of mobile phase in chromatography

system.

Run time 20 minutes is not sufficient for retention and elution of all the components in current chromatography conditions.

Need to check the effect of higher concentration of organic solvent in mobile phase in chromatography system.

Changes required for next trial:

Mobile phase composition needs to vary.

Organic proportion of mobile phase need to decrease for elution of other components in chromatography system.

Linearity and Range:

The linearity for Cilnidipine, Chlorthalidone drug, impurity-1 and impurity-2 were performed by analysis of standard solution combined in range of LOQ & 5-15 µg/ml drug concentration respectively. The Correlation co-efficient value for all drug components of linearity curve was achieved as 0.99. Regression line equation for Cilnidipine drug and Chlorthalidone drug and impurity-1 and impurity-2 components are as following:

For Cilnidipine drug: **y = 44.79x + 44.3** and for Chlorthalidone drug: **y = 23.38x + 10.83 and** for impurity-1 **y = 19.13x + 17.60,** for impurity-2= **y = 14.59x + 12.88**

Table : Linearity data for Cilnidipine drug.

S. No.	Concentration (µg/ml)	Peak Area
1	1	69.508
2	5	198.885
3	7.5	289.558
4	10	396.117
5	12.5	487.404
6	15	591.841

Table: Linearity data for Chlorthalidone drug

S. No.	Conc. (µg/ml)	Peak Area
1	1	41.351
2	5	122.982
3	7.5	179.267
4	10	245.324
5	12.5	302.072
6	15	366.765

Repeatability:

Repeatability data of peak area for Cilnidipine, Chlorthalidone drug, impurity-1 and impurity-2, basis on six measurements of same solution of Cilnidipine, Chlorthalidone drug, impurity-1 and impurity-2 are depicted in table. The % RSD for Cilnidipine, Chlorthalidone drug, impurity-1 and impurity-2 components were found to be 1.62, 1.48,1.72 and 1.51 respectively**.**

Table: Data of repeatability for Cilnidipine drug

Cilnidipine drug				
S.No.	**Conc (µg/ml)**	**Peak Area**	**Mean ± SD**	**% R.S.D**
1.	10	403.247	391.83 ±6.34	1.62
		390.212		
		385.905		
		391.394		
		393.746		
		386.457		

Table: Repeatability data for Chlorthalidone drug

Chlorthalidone drug				
S. No.	**Conc (µg/ml)**	**Peak Area**	**Mean ± SD**	**% R.S.D**
1.	10	249.749	243.50 ±3.60	1.48
		241.636		
		238.993		
		242.377		
		243.866		
		244.362		

Intraday precision:

The results of intraday precision for Cilnidipine and Chlorthalidone drug impurity- 1 and impurity-2 components are shown in below table 4.12. % R.S.D. for Intraday precision was found to be 1.30-3.35 for Cilnidipine drug and 1.03-2.72 for Chlorthalidone drug and 1.58-2.88 for impurity-1 and 2.46-4.38 for impurity- 2.

Table: Intraday precision data for estimation of Cilnidipine drug

	Cilnidipine drug		
S. No.	**Conc. (µg/ml)**	**Mean peak area ± SD (n=3)**	**% RSD**
1	0.25	67.92± 2.28	3.35
2	10	395.86± 5.64	1.42
3	15	582.48± 7.60	1.30

Table: Intraday precision data for estimation of Chlorthalidone drug

	Chlorthalidone drug		
S. No.	**Conc. (µg/ml)**	**Mean peak area ± SD (n=3)**	**% RSD**
1	0	40.34± 1.10	2.72

2	10	245.41± 3.92	1.60
3	15	361.53± 3.83	1.06

Interday precision:
The results for intraday precision for Cilnidipine and Chlorthalidone drug impurity-1 and impurity-2 are shown in table. % R.S.D for interday precision was found to be 0.236-0.978 for Cilnidipine drug and 0.198-0.805 for Chlorthalidone drug and for impurity-1 and for impurity-2 0.384-1.029 for for impurity-2.

Table: Interday precision results for estimation of of Cilnidipine drug

Cilnidipine drug			
S. No.	**Conc. (µg/ml)**	**Mean peak area ± SD (n=3)**	**% RSD**
1	0.25	68.34± 0.94	1.37
2	10	385.01±5.45	1.42
3	15	586.73±6.32	1.08

Table: Interday precision results for estimation of Chlorthalidone drug

Chlorthalidone drug			
S. No.	**Conc. (µg/ml)**	**Mean peak area ± SD (n=3)**	**% RSD**
1	0	41.22± 1.04	2.52
2	10	241.33± 1.95	0.81
3	15	365.42 ± 6.98	1.91

LOD and LOQ:
Calibration curve is repeated for five times and the standard deviation (SD) of intercepts was computed. Then the LOD and LOQ value were calculated as follows: LOD = 3.3 * SD/slope value of calibrationcurve.
LOQ = 10 * SD/slope value of calibration curve.
Where, SD = Standard deviation (SD) of intercepts.

Table: Limit of Detection results for Cilnidipine drug and Chlorthalidone impurity-1 and impurity-2

Cilnidipine drug	**Chlorthalidone drug**	**Impurity-1**	**Impurity-2**
LOD = 3.3 * (SD / Slope) = 3.3 * (4.553/39.350) 0.382 µg/ml	LOD = 3.3 * (SD / Slope) = 3.3 * (2.782/24.415) = 0.376µg/ml	LOD = 3.3 * (SD / Slope) = 3.3 * (0.514/20.601) = 0.082µg/ml	LOD = 3.3 * (SD / Slope) = 3.3 * (0.406/15.726) = 0.085µg/ml

Table: Limit of Quantitation results for Cilnidipine drug and Chlorthalidone impurity-1 and impurity-2

Cilnidipine drug	Chlorthalidone drug	Impurity-1	Impurity-2
LOQ = 10 * (SD / Slope) = 10 * (4.553/39.350) = 1.157µg/ml	LOQ = 10 * (SD / Slope) = 10 * (2.782/24.415) = 1.139 µg/ml	LOQ = 10 * (SD / Slope) = 10 * (0.514/20.601) = 0.249 µg/ml	LOQ = 10 * (SD / Slope) = 10 * (0.406/15.726) = 0.258µg/ml

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