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Development And Validation Of A Rp-Hplc Method For Simultaneous Determination Of Cilnidipine And Chlorthalidone In Pure And Pharmaceutical Dosage Form

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Abstract: *A rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validated of Cilnidipine and Chlorthalidone, in its pure form as well as in tablet dosage form. Chromatography was carried out on an Altima C18 (4.6 x 150mm, 5µm) column using a mixture of ACN, Methanol and Phosphate buffer pH4.6 (10:25:65 v/v) as the mobile phase at a flow rate of 1.0ml/min, the detection was carried out at 234nm. The retention time of the Cilnidipine and Chlorthalidone was 2.088, 6.068 ±0.02min respectively. The method produce linear responses in the concentration range of 25-125ppm of Cilnidipine and 10-50ppm of Chlorthalidone. The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.*

Keywords: *Cilnidipine, Chlorthalidone, RP-HPLC, validation.*

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I. General Introduction

Analysis may be defined as the science and art of determining the composition of materials in terms of the elements or compounds contained in them. The reasons for the development of newer methods of drugs analysis are:

1. The medication or medication mix may not be authentic in any pharmacopeias.
2. An appropriate scientific strategy for the medication may not be accessible in the writing because of Patent directions.
3. Analytical methods for a drug in combination with other drugs may not be available.
4. Analytical methods for the quantification of the medicine in biotic fluids may not be accessible.
5. The existing analytical procedures may require expensive reagents and solvents.It may aside from ornate volumnious parentage and crack procedures and these may not be reliable.[1,2]

Different Methods Of Analysis

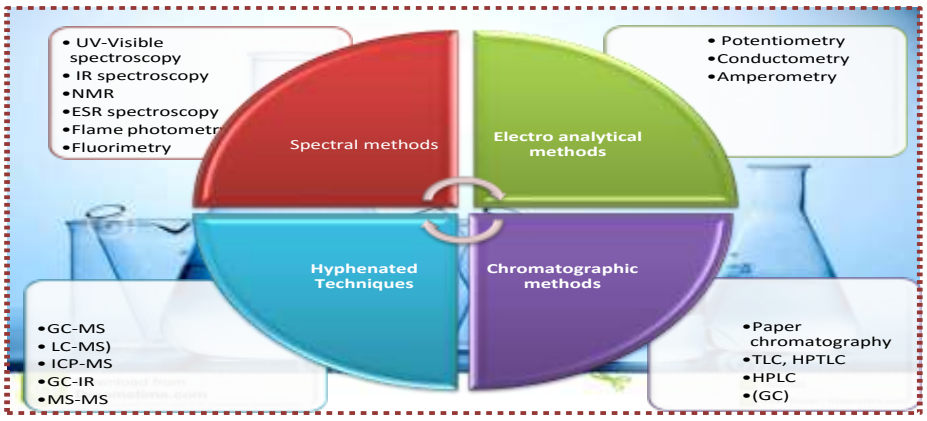


Table 1.1: Summary of Hyphenated separation techniques[2]

Separation technique	Hyphenated mode
Liquid chromatography	Liquid chromatography-mass spectrometry(LC/MS) Liquid chromatography-Fourier-transform infrared Spectrometry(LC-FTIR) Liquid chromatography-nuclear magnetic resonance spectroscopy(LC/NMR) Liquid chromatography-inductively coupled plasma mass spectrometry(LC-ICPMS)
Gas chromatography	Gas chromatography-mass spectrometry(GC/MS) Gas chromatography-Fourier-transform infrared(GC- FTIR) Gas chromatography-FTIR-MS(GC-FTIR-MS)
Capillary electrophoresis	Capillary electrophoresis-mass spectrometry(CE/MS) Capillary electrophoresis-nuclear magnetic resonance spectroscopy(CE/NMR) Capillary electrophoresis-surface enhanced Raman spectrometry (TLC-SERS)
Thin layer chromatography(TLC)	Thin layer chromatography- mass spectrometry(TLC/MS) Thin layer chromatography- surface enhanced Raman spectrometry(TLC-SERS)
Superficial fluid chromatography/ extraction(SFC/SFE)	Superficial fluid extraction-capillary gas chromatography-mass spectrometry(SFE-CGC-MS) Superficial fluid-Fourier-transform infrared(SFC-FTIR)

I.1 INTRODUCTION TO HPLC

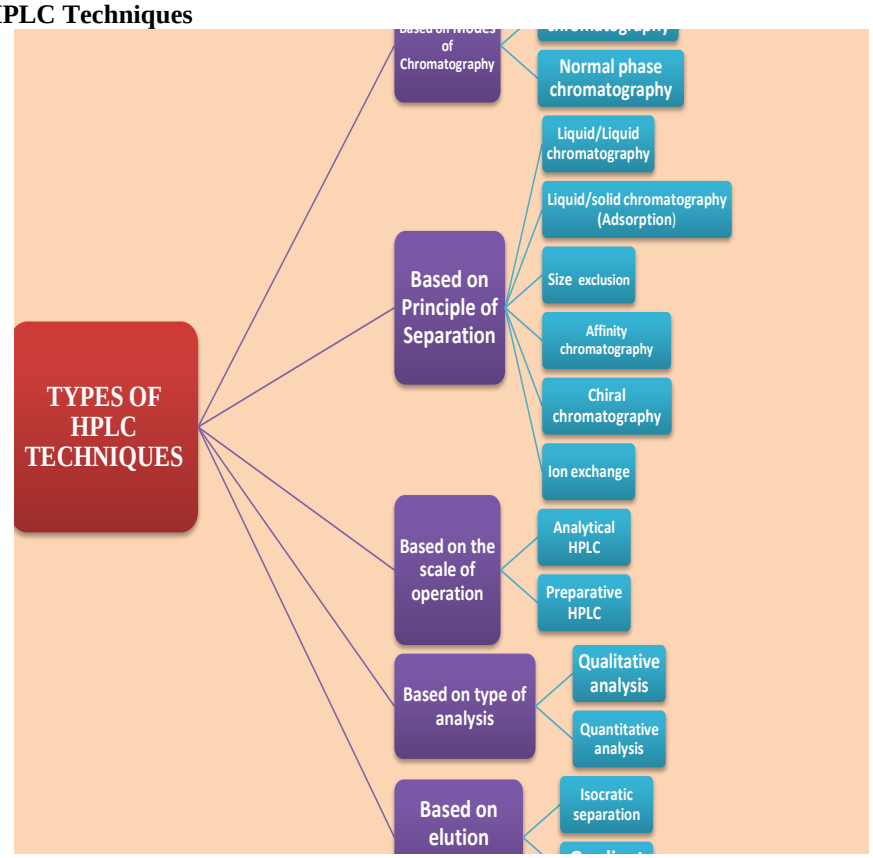
HPLC is also called as high pressure liquid chromatography since high pressure is used to increase the flow rate and efficient separation by forcing the mobile phase through at much higher rate. The pressure is applied using a pumping system. Those improvement from claiming HPLC from traditional segment chromatography might be attributed of the improvement from claiming littler molecule sizes. Smaller particle size is important since they offer more surface area over the conventional large particle sizes.

1. Improved resolution of separated substances
2. column packing with very small (3,5 and 10 µm) particles
3. Faster separation times (minutes)
4. Sensitivity
5. Reproducibility

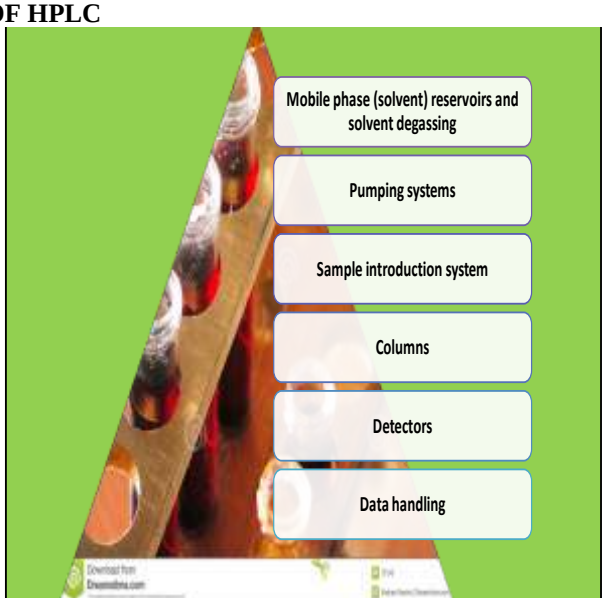
6 Constant stream detectors fit for taking care of little stream rates.

7. Not difficult example recovery, taking care of Furthermore support. [6]

I.2 Types of HPLC Techniques



I.3 IINSTRUMENTATION OF HPLC



The basic liquid chromatograph consists of six basic units. The mobile phase supply system, the pump and programmer, the sample valve, the column, the detector and finally a means of presenting and processing the results.

1.Mobile phase (solvent) reservoirs and solvent degassing

The mobile phase supply system consists of number of reservoirs (200 mL to 1,000 mL in capacity). They are usually constructed of glass or stainless steel materials which are chemically resistant to mobile phase.

2.Mobile phase

Mobile phases in HPLC are usually mixtures of two or more individual solvents. The usual approach is to choose what appears to be the most appropriate column, and then to design a mobile phase that will optimize the retention and selectivity of the system. The two most critical parameters for nonionic mobile phases are strength and selectivity. [8,24]

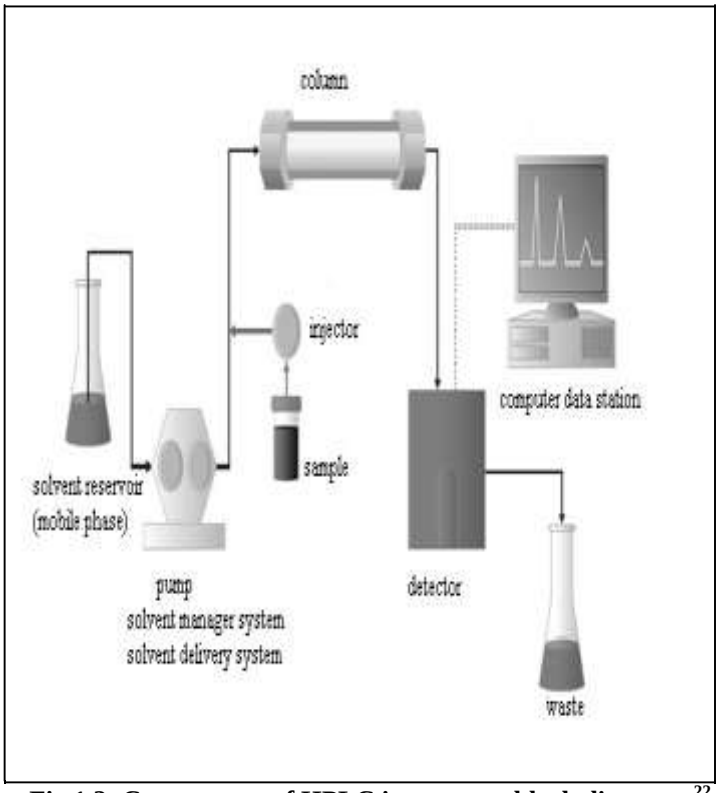


Fig.1.2: Components of HPLC instrument block diagram. [22]

II. Method Development And Validation

Literature survey: To avoid duplication of the method and invention of new method.Information about the solubility, the structure of the drugs and their Physico- chemical properties for the known compounds and can be applied for the new compounds.

Selection of chromatographic method: First reversed phase should be tried. If not successful, normal phase should be taken into consideration. For ion exchange or ion pair chromatography, first ion suppression by pH control and reversed phase chromatography should be tried.

Selection of stationary phase: Matching the polarity of sample and stationary phase and using a mobile phase of different polarity achieves a successful separation.

Selection of mobile phase: Mobile phase may be either single liquid or combination of liquids, which are compatible with sample, column and instrument.

Selection of suitable detector: There are essentially two kinds of detectors: property detectors and bulk property detectors;Solute..

2.1. INTRODUCTION TO HPLC METHOD DEVELOPMENT:

A decent technique advancement methodology must require just whatever number trial keeps running as are important to accomplish the coveted last outcome .it must be straightforward as could reasonably be expected, yet it must permit the utilization of complex instruments. For ex. PC modeling.[7]

2.1.1.Nature of sample

Before start of method advancement, we have to audit about the specimen, keeping in mind the end goal to characterize objectives of partition. The sorts of sample related data that can be critical are:

The different sorts of sample related data that might be important for strategy improvement was condensed in Table 2.1.1.

S.No	Sample requirements
1.	Molecular weights of compounds
2.	pK_a values of compounds
3.	UV spectra of compounds
4.	Concentration range of compounds in samples of interest
5.	Sample solubility

Table 2.1.1: Information Concerning Sample[7]

2.1.2. Method requirements

The goals of the analytical method that want to be remain advanced are considered. The defined parameters estimation limits, selectivity, linearity, range, accuracy and precision

2.1.3. Separation objectives

The objectives of HPLC partition should be determined clearly, that include:

1. The utilization of HPLC to separate cleansed sample components for special distinguishing proof or quantitative examination.
2. It might be important to isolate all degradants or polluting influences from an item for solid substance measure or not.
3. In quantitative investigation, the required levels of exactness and accuracy must be known (an accuracy of ± 1 to 2 % is typically achievable).
4. Regardless of whether a single HPLC process is adequate for crude material or at least one unique systems are sought for plans.

At the point when the quantity of test samples for investigation at one time is more prominent than 10, a run time of under 20 minutes frequently will be vital.[7]

2.2. OPTIMIZATION

Reversed-Phase HPLC offers multiple parameters for optimizing a separation. To plan separation by RP-HPLC, the analyst must select both a stationary phase and a mobile phase appropriate to the analyte under investigation. In addition the analyst must identify chromatographic conditions that will maintain the sharpness

of analyte bands as the sample moves through the stationary phase column to the detector. The primary attempt at optimization of partition requires choice of a promising arrangement of conditions.[23]
To optimize the solvent strength, one approach is to begin with a mobile phase that is probably too strong and reduce solvent strength to increase k' (capacity factor, is the measure of retention) between successive runs. When all the peaks fit within the range 0.5< k' < 20, the mobile phase is close ideal from the viewpoint of dissolvable quality. The solvent properties are useable with regards to choosing at least one solvents.

Separation Variable	Preferred Initial Choice
Column	
Dimensions(length,ID)	15 × 0.46 cm
Particle size	5 µm [a]
Stationary phase	C_8 or C_{18}
Mobile phase	
Solvents A and B	Buffer – acetonitrile
%B	80-100 % [b]
Buffer (compound,P^H, concentration)	25Mmpotassium phosphate, 2.0 <Ph< 3.0 [c]
Additives (eg., amine modifiers, ion pair reagents)	Do not use initially
Flow rate	1.5 -2.0 mL/min
Temperature	35-45 ^{0}C
Sample size	
Volume	<25 µL
Weight	<100 µg

Table 2.2.1: Preferred experimental conditions for the intial HPLC separation.[7]

A) 3.5mm particles are an alternative, using a 7.5 cm column.
B) For an initial isocratic run; an initial inclination run is favored.
C) No cushion required for impartial test samples ; for pH <2.5, pH-stable columns suggested.Once the solvent strength has been properly adjusted for the sample, the next selection parameter that can be explored is α (band spacing). Much of the time, it is possible to get a satisfactory detachment basically by changing solvent quality. This is usually sufficient for simple and easily resolved samples such as less polar and medium polar non-ionic solutes, but for samples containing ionic and ionisable compounds, apart from changes in solvent strength, several other separation variables are to be altered to optimize band spacing, retention or peak shape.

Decision of organic solvent A change from methanol to acetonitrile or THF frequently brings about vast changes in partition. solvent quality An adjustment in percent organic frequently gives significantchanges in maintenance and detachment.

Temperature The temperature can be shifted between 0 to 70ºC with the end goal of controlling band dispersing; in any case, temperatures of 25-60 ºC are more typical.

The prescribed additives added to the reversed-phase mobile phases on account of samples that contain acids, bases or salts are given in Table 1.7.[7]

Sample characteristics	Additives
Basic compounds (ex: amines)	50mM Phosphate buffer, 30 Mm triethylamine (buffer pH 3.0)
Acidic compounds (ex: carboxylic acids)	50mM Phosphate buffer, 1% acetic acid (buffer pH 3.0)
Mixture of acids and bases	50mM Phosphate buffer, 30 mMtriethylamine, 1% acetic acid
Cationic salts (ex: tetralkylquarternary ammonium compounds)	30 mMtriethylamine, 50mM sodium nitrate
Anionic salts	1% acetic acid, 50mM sodium nitrate

Table 2.2.2: Recommended substances for reversed-phase mobile phases.[7]

A temperature of 40-600C appears to be convenient in reversed phase-LC, when sample component permits. Contrasted with surrounding conditions operation at these temperatures generally doubles the column effectiveness as far as number of theoritical plates.Different concentrations of the different organic solvents are required to keep up steady solvent strenght. Fluctuating the pH of mobile phase may radically modify division

specifically in RP-HPLC, if the acids or bases are sample component.. When dealing with a sample component of weak base, pKa 8 the ion suppression technique can be used by adding an acidic bufferIf it is not effective, the ion pair reagent, for example, alkyl sulfonic acid might be included.And ion pairs are more efficacious than ion suppression for strong bases, pKa>8,. Band spacing in Mobile phase composition is majorly affected ; no other variable will for the most part demonstrate as intense for controlling values of partition. The partition goals in HPLC method improvement are compressed in Table 2.2.3.

Goal	Comment
Resolution	Precise and rugged quantitative analysis requires that R_s be greater than 1.5.
Separation time	<5-10 min is desirable for routine procedures
Quantitation	□ 2% for assays; □ 5% for less demanding analyses □ 15% for trace analyses.
Pressure	<150 bar is desirable, <200 bar is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal/noise ratios.
Solvent consumption	Minimum mobile-phase use per run is desirable.

Table 2.2.3: Separation goals in HPLC method development.[7]

2.3. **ANALYTICAL METHOD VALIDATION**

As Per ICH method validation can be defined as "Documented establishing evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result predetermined specifications and quality characteristics".[22,20,26]

2.3.1. Objective of validation

There are two important reasons for validating assays in the pharmaceutical industry.The very much important matter was assay validation is an integral part of the quality control process.The second is that current good manufacturing practice regulation requires assay validation. In industry it would be difficult to confirm that the product being manufactured is uniform and that meet the standards set to assure fitness for use. The varying nature of the differences between the analytical development laboratory and quality control laboratory is a good reason for validation program.

Method validation study circle are being inluded with Specificity/Selectivity, Linearity, Accuracy, Precision, Limit of location, Limit of Quantitation, Robustness, System appropriateness and Stability criteria.[22]

2.3.2.Accuracy

It is defined as the closeness of the measured value to the true value. In a method with high accuracy.
Accuracy is represented and determined by recovery studies.
There are three ways to determine accuracy:
1. Comparison to a reference standard
2. For each determination fresh samples were prepared and assay value is calculated.[22] Acceptance criteria: The accuracy should be within 98-102%.

2.3.3Precision

Precision can be defined as "the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample".

2.3.4. Repeatability: Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision and involves multiple measurements of the same sample (different preparations) by the same analyst under the same conditions.

- 5 or 6 determinations
- At two or three different concentrations

Should be done and the relative standard deviations were calculated.
The % RSD can be calculated by

$$\% \, \mathbf{RSD} = \frac{\mathbf{SD} \times \mathbf{100} \, \%}{\mathbf{MEAN}}$$

Where, RSD = relative standard deviation SD = standard deviation
The standard deviation SD is given by,

2.3.5. Intermediate precision: Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment etc. In the same laboratory, when the same method is applied many times.

2.3.6.Reproducibility: The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.[22]Reproducibility expresses the precision between laboratories.

Acceptance criteria: The % RSD must be less than 2.

2.3.7.Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results directly proportional to the concentration (amount) of analyte in the sample.
The results are being proceeded utilising a linear least squares
regression. [22]
Acceptance criteria: Correlation coefficient(R2)>0.998.

2.3.8.Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present; these include impurities, degradants and matrix etc. Due to lack of specificity of individual analytical process might be remunerated by other supporting analytical processes
Purity Tests: To guarantee that all the analytical processes done permit an exact articulation of the substance of contaminations of an analyte, i.e. related substances test, heavy metals, residual solvents content etc.
Assay : To give an accurate result that makes an exact statement in the content or potency of the analyte in a sample.[22]
Acceptance criteria: No interference should be present.

2.3.9.Limit of Detection

The limit of detection of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily can be quantified exactly. LOD can be defined as the smallest level of analyte that gives a measurable response.
Where, σ = the standard deviation of the response S = the slope of the calibration curve.
Acceptance criteria: S/N should be 3:1

2.3.10.Robustness

It is defined as 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. It includes various method parameters that are changed withina practical range and the quantitative impact of the factors is resolved. [22]
The typical variations are:

- Influence of variations of pH in a mobile phase.
- Influence of variations in mobile phase composition.
- Different columns (different lots and/or suppliers).
- Temperature.
- Flow rate.

2.3.11. System Suitability Testing

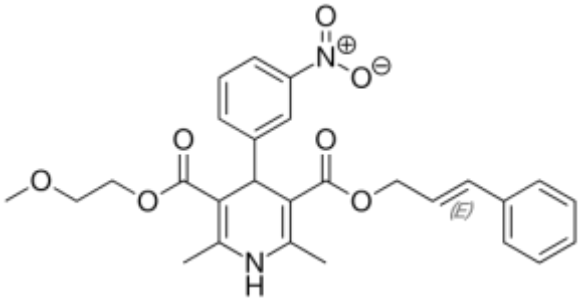
This is refined with framework system suitability tests, that can be characterized as tests to guarantee that the technique can create after effects of acceptable accuaracy and precission.
The parameters that can be used to determine system suitability prior to analysis, includes Plate number (N), Tailing factor, k and/or α, Resolution (Rs)and Relative standard deviation (RSD) of peak height or peak area for repetitive injections. Typically at least two of these criteria are required to demonstrate system suitability for any method.[22]

S.No	Characteristics	Acceptance criteria
1	Accuracy	98-102%
2	Precision	RSD<2
3	Specificity	No interference
4	Detection limit	S/N >3:1
5	Quantitation limit	S/N > 10:1
6	Linearity	R^2> 0.99

Table 2.3: Acceptance criteria of validation for HPLC.[22]

III. DRUG PROFILE

Drug **:** Cilnidipine
Drug category **:** Anti hypertension
Structure **:**

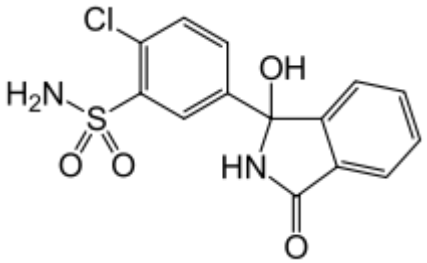


Molecular Formula : C27H28N2O7
Molecular Weight :492.52gm/mole.

PHARMACODYNAMICS:
Mechanism of action:
Cilnidipine is a dihydropyridine calcium-channel blocker. Cilnidipine inhibits cellular influx of calcium, causing vasodilatation. vascular smooth muscle has greater selectivity by it. It has null action at the SA or AV nodes and -ve inotropic activity is poorly seen at therapeutic doses.
Therapeutic efficacy/ Indications: Hypertension
Adverse reactions: Dizziness; headache , flushing, peripheral oedema; tachycardia;; GI disturbances; increased micturition frequency; lethargy; eye pain; depression; hypotension ,,ischaemic chest pain; cerebral or myocardial ischaemia; transient blindness, abnormal liver function, palpitations rashes; fever;; gingival hyperplasia; myalgia; tremor; impotence.
Contraindications: Cardiogenic shock; recent MI or acute unstable angina; severe aortic stenosis.

INTERACTIONS:
Drug interactions:; carbamazepine, aldesleukin; phenytoin ,antipsychotics that cause hypotension; may modify insulin and glucose responses; quinidine;;; rifampicin; cimetidine; erythromycin , Other antihypertensives.

DRUG PROFILE
Drug : Chlorthalidone
Synonym :2-chloro-5-(1-hydroxy-3-oxo-2,3-dihydro-1H-isoindol-1- yl)benzene-1-sulfonamide, Chlorphthalidolone, Chlortalidone, Phthalamodine, Chlorthalidone.
Drug category : Antihypertensive Agents
Structure :



IUPAC Name **:**2-chloro-5-(1-hydroxy-3-oxo-2,3-dihydro-1H-isoindol-1- yl)benzene-1-sulfonamide
Molecular Formula **:** C14H11ClN2O4S
Molecular Weight : 338.766

PHYSICOCHEMICAL PROPERTIES
Deblockedion **:** A benzenesulfonamide-phthalimidine that tautomerizes to a benzophenones form. It is considered a thiazide-like diuretic.

Physical State : White To Yellowish-White Crystalline Powder
Solubility : Chlorthalidone is practically insoluble in water, in ether and in chloroform; soluble in methanol; slightly soluble in alcohol.
Dosage : 25 milligrams (mg) to 100 mg.
Melting point : 179ºC
pKa(strongest basic) : -2.6
Log p value: 1.6
PHARMACOKINETICS
Half-life: 40 hours
Absorption : Absorbed relatively rapidly after oral administration.
Protein binding : High (75% [58% to albumin])
Metabolism : Liver
Excretion **:** The major portion of the drug is excreted unchanged by the kidneys.

PHARMACODYNAMICS
Mechanism of action:
Chlorthalidone represses sodium ion transport over the renal tubular epithelium in the cortical diluting fragment of the ascending loop of Henle. By expanding the conveyance of sodium to the distal renal tubule, Chlorthalidone in a indirect way builds potassium discharge by means of the sodium-potassium exchange mechanism.
Indications : For management of hypertension either as the sole therapeutic agent or to enhance the effect of other antihypertensive drugs in the more severe forms of hypertension. **Adverse reactions:**

1. Sore throat with fever
2. Unusual bruising or bleeding
3. Severe skin rash with peeling skin
4. Difficulty swallowing or breathing

Contraindications:
Chlorthalidone should not be used in pregnancy unless absolutely necessary. Large amounts of diuretics may suppress milk production, but the American Academy of Pediatrics considers thiazides to be compatible with breastfeeding.

INTERACTIONS
Drug interactions :

1. Loop diuretics, including furosemide (Lasix), bumetanide (Bumex), and torsemide (Demadex)
2. Digoxin (Lanoxin)
3. Drugs containing lithium
4. Nonsteroidal anti-inflammatory drugs (NSAIDs), including ibuprofen (Motrin or Advil), naproxen (Naprosyn), and nabumetone (Relafen) Diabetes medications

Food interactions:
Take with food (increases availability).

IV. LITERATURE REVIEW

Snehal N. Patel et al.,(2015) Development and validation of an UV spectrophotometric method for simultaneous determination of cilnidipine and chlorthalidone. A simple, accurate and precise dual wavelength spectrophotometric method was developed for simultaneous determination of Cilnidipine and Chlorthalidone in combined pharmaceutical dosage

form. The principle for dual wavelength method is the absorbance difference between two points on the mixture spectra is directly proportional to the concentration of the component of interest. The wavelengths selected for determination of Cilnidipine are 271.83 nm and 278.34 nm, whereas, the wavelengths selected for determination of Chlorthalidone are 233.83 nm and 250.0 nm. Methanol is used as a solvent. Regression analysis of Beer's plots showed good correlation in concentration range of 2-10 µg/ml for Cilnidipine and 2.5-12.5 µg/ml for Chlorthalidone. The linearity of the proposed method was investigated in the range of 2-10mg/mL (r2=0.9990) for Cilnidipine and 2.5 -12.5mg/mL (r2 = 0.9986) for Chlorthalidone. The limits of detection (LOD) were 0.4174 µg/mL and 0.068 µg/mL, and the limits of quantitation (LOQ) were 1.264 µg/ml and 0.206 µg/ml, for Cilinidipine and Chlorthalidone respectively The proposed method was successfully applied to determination of these drugs in commercial tablets. Parth R. Nayak et al.,(2016) RP-HPLC Method Development And Validation For Simultaneous Estimation Of Chlorthalidone, Cilnidipine And Olmesartan In Tablet Dosage Form.. Linearity were found to be in the range of 6.25-18.75 µg/ml, 5-15 µg/ml and 20-60 µg/ml

for chlorthalidone, cilnidipine and olmesartan respectively. The percentage recoveries of all the drugs were found to be 100.07%, 98.79% and 100.09% for chlorthalidone, cilnidipine and olmesartan.

Parth R. Nayak et al.,(2016) RP-HPLC Method Development And Validation For Simultaneous Estimation Of Chlorthalidone, Cilnidipine And Olmesartan In Tablet Dosage Form. The quantification was carried out using C18 column (150 x 4.6mm, 5µm) and mobile phase comprised of Buffer, Acetonitrile and TEA in proportion of 80:20:0.1 %v/v/v The flow rate was 1.0 ml/min and the eluent was monitored at 248 nm. The selected chromatographic conditions were found to effectively separate chlorthalidone, cilnidipine and olmesartan were 4.667 min, 6.887 min and 3.807 min respectively. Linearity were found to be in the range of 6.25-18.75 µg/ml, 5-15 µg/ml and 20-60 µg/ml for chlorthalidone, cilnidipine and olmesartan respectively. The percentage recoveries of all the drugs were found to be 100.07%, 98.79% and 100.09% for chlorthalidone, cilnidipine and olmesartan.

V. AIM AND OBJECTIVE

AIM:

The main aim of the present study is development of accurate, precise, sensitive, selective, reproducible and rapid analytical technique for cost effective simultaneous estimation of cilnidipine and chlorthalidone.

OBJECTIVES :

Following are the objectives of present work: To develop analytical method

- Selecting the HPLC separation mode.
- Selecting/ optimizing the mobile phase.
- Selecting column for analysis.
- Selecting the appropriate detector system.
- Selecting appropriate gradient/ isocratic medium.
- Selecting appropriate flow rate, temperature and pH.

 To validate different parameters.

- Specificity
- Linearity
- Precision
- Accuracy
- Limit of detection
- limit of quantitation
- Robustness

VI. EXPERIMENTAL WORK

6.1. INSTRUMENTS USED

S.No	Instruments And Glasswares	Model
1	HPLC	WATERS Alliance 2695 separation module, software: Empower 2, 996 PDA Detector.
2	pH meter	LabIndia
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

Table 6.1. : Instruments used

6.2.CHEMICALS USED:

S.No	Chemical	Brand names
1	Chlorthalidone(Pure)	Sura labs
2	Cilnidipine(Pure)	Sura labs
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)
4	Acetonitrile for HPLC	Merck

6.2. Table: chemicals used

6.3. HPLC METHOD DEVELOPMENT:

6.3.1.TRAILS

Preparation of standard solution:

Accurately weigh and transfer 10 mg of Chlorthalidone and Cilnidipine working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and

Instrument used

Waters HPLC with auto sampler ,
PDA detector 996 model.

Temperature
35ºc

Column
Altima C18 (4.6×150mm, 5µ)
Buffer

Phosphate buffer (pH-4.6)-Dissolve 0.9g of anhydrous dihydrogen phosphate and
1.298 g of Citric acid mono hydrate in sufficient water to produce 1000mL . Adjust the pH
4.6 by using ortho phosphoric acid.

pH
4.6
Mobile phase

Buffer: Methanol: ACN (65:25:10v/v)

Flow rate
1ml/min
Wavelength

234 nm
Injection volume
l□ : 10

Run time :
14 min

make volume up to the mark with the same Methanol.
0.375ml of Chlorthalidone is Pipetted out and 0.3ml of the Cilnidipine is also, into a 10ml volumetric flask and dilute up to the mark with diluents from the above stock solutions.

Procedure:

By changing the chromatographic conditions inject the samples and record the chromatograms and note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Optimization of Column:

The method was performed with various columns like C18 column, ODS and Zodiac column. Altima C18 (4.6×150mm, 5µ) found as ideal because it gave good peak shape and at 1ml/min flow.

6.3.2.OPTIMIZED CHROMATOGRAPHIC CONDITIONS

6.4. VALIDATION

6.4.1. PREPARATION OF BUFFER AND MOBILE PHASE:

Preparation of Phosphate buffer (pH-4.6):

Dissolve 0.9g of anhydrous di hydrogen phosphate and 1.298 g of Citric acid mono hydrate in sufficient water to produce 1000mL .Adjust the p H 4.6 by using ortho phosphoric acid.

Preparation of mobile phase:

Measured accurately 650 ml (65%) of Buffer and 250 ml of Methanol (25%) and 100ml (10%) of Acetonitrile were mixed and degassed in digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase used as the diluent.

6.4.2. VALIDATION PARAMETERS

6.4.2.1. SYSTEM SUITABILITY

Weigh accurately and transfer 10 mg of Chlorthalidone and 10mg of Cilnidipine working standard into a 10ml

of clean dry volumetric flasks add 7mL of the Diluents and sonicate to dissolve it completely and make volume to t he mark with the same solvent. (Stock solution) Further pipette 0.375ml of Chlorthalidone and 0.3ml of the Cilnidipine from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

6.4.2.2. Preparation of Sample Solution:

Weigh average of Tablet and crush in a mortar using pestle and weight 10 mg equivalent weight of Chlorthalidone and 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.

Pipette 0.375ml of Chlorthalidone and 0.3ml of the Cilnidipine from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

PROCEDURE:

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

PREPARATION OF DRUG SOLUTIONS FOR LINEARITY:

Accurately weigh and transfer 10 mg of Chlorthalidone and 10mg 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)

Preparation of Level – I (12.5ppm of Chlorthalidone&10ppm of Cilnidipine):

Pipette out 0.125ml of Chlorthalidone and 0.1ml of Cilnidipine stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (25ppm of Chlorthalidone&20ppm of Cilnidipine):

Pipette out 0.25ml of Chlorthalidone and 0.2ml of Cilnidipine stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (37.5ppm of Chlorthalidone&30ppm of Cilnidipine):

Pipette out 0.375ml of Chlorthalidone and 0.3ml of Cilnidipine stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (50ppm of Chlorthalidone&40ppm of Cilnidipine):

Pipette out 0.5ml of Chlorthalidone and 0.4ml of Cilnidipine stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (62.5ppm of Chlorthalidone&50ppm of Cilnidipine):

Pipette out 0.625ml of Chlorthalidone and 0.5ml of Cilnidipine stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Procedure:

Infuse each level into the chromatographic system and measure the peak region.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

6.5. PRECISION (REPEATABILITY)

Preparation of Chlorthalidone and Cilnidipine Solution:

Weigh accurately 10 mg of Cilnidipine and 10mg of Chlorthalidone 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)

Likewise pipette 0.375ml of Chlorthalidone and 0.3ml of the Cilnidipine from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

The standard solution was injected for five times and measured the area for all five injections in HPLC.The %RSD for the area of five replicate injections was found to be within the specified limits.

6.6. INTERMEDIATE PRECISION (RUGGEDNESS):

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:

DAY 1:

The standard solution was injected for six times and measured the area for all six injections in HPLC.The %RSD for the area of six replicate injections was found to be within the specified limits.

DAY 2:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:

For preparation of 50% Standard stock solution:

Weigh accurately and transfer 10 mg of Cilnidipine and 10mg of Chlorthalidone 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 0.187ml of Chlorthalidone and 0.15ml of the Cilnidipine from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

For preparation of 100% Standard stock solution:

Accurately weigh and transfer 10 mg of Chlorthalidone and 10mg 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 0.375ml of Chlorthalidone and 0.3ml of the Cilnidipine from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

For preparation of 150% Standard stock solution:

Accurately weigh and transfer 10 mg of Chlorthalidone and 10mg 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 0.562ml of Chlorthalidone and 0.45ml of the Cilnidipine from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Chlorthalidone and Cilnidipine and calculate the individual recovery and mean recovery values.

6.7. ROBUSTNESS:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results. .

For preparation of Standard solution:

Accurately weigh and transfer 10 mg of Chlorthalidone and 10mg 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 0.375ml of Chlorthalidone and 0.3ml of Cilnidipine from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Effect of Variation of flow conditions:

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded

Effect of Variation of mobile phase organic composition:

The sample was analyzed by variation of mobile phase i.e. Buffer: Methanol: ACN was taken in the ratio and 75: 15: 10, 55:35:10 instead 65:25:10, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

VII. RESULTS AND DISCUSSION

7.1. TRAILS

Trail 1: Table A

Mobile phase	Methanol: Water (80:20%v/v)
Column	ODS C18 (4.6 ×150mm, 5µm particle size) Make; waters
Flow rate	0.6ml/min
Wavelength	234 nm
Column temp	28ºC
Injection Volume	10 µl
Run time	10minutes

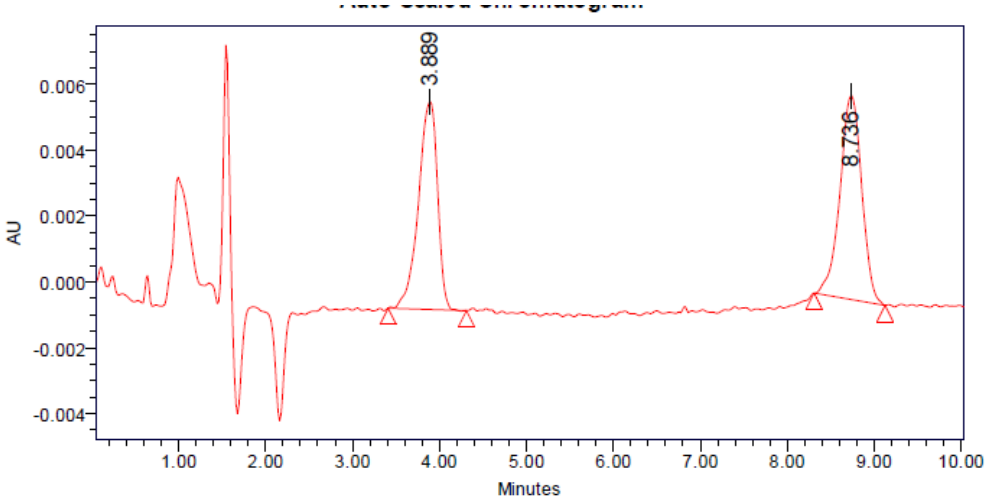


Figure 7.1.1. : chromatogram for trail 1

Table 7.1.B. peak results for trail 1

S.No	Peak Name	R_t	Area	Height	USP Resolution	USP Tailing	USP Plate count
1	Cilnidipine	3.889	96377	6320		0.81	1642
2	Chlorthalidone	8.736	107991	6192	11.26	0.94	6110

Observation:

This trial shows improper base line in the chromatogram, so more trials were required for obtaining good peaks.

Trail 2: TABLE A

Mobile phase	Water(pH 3) : Acetonitrile (65% -35%v/v)
Column	Zodiac C18 (4.6×250mm)5µ
Flow rate	0.7 ml/min
Wavelength	234 nm
Column temp	30ºC
Injection Volume	10 µl
Run time	7 minutes

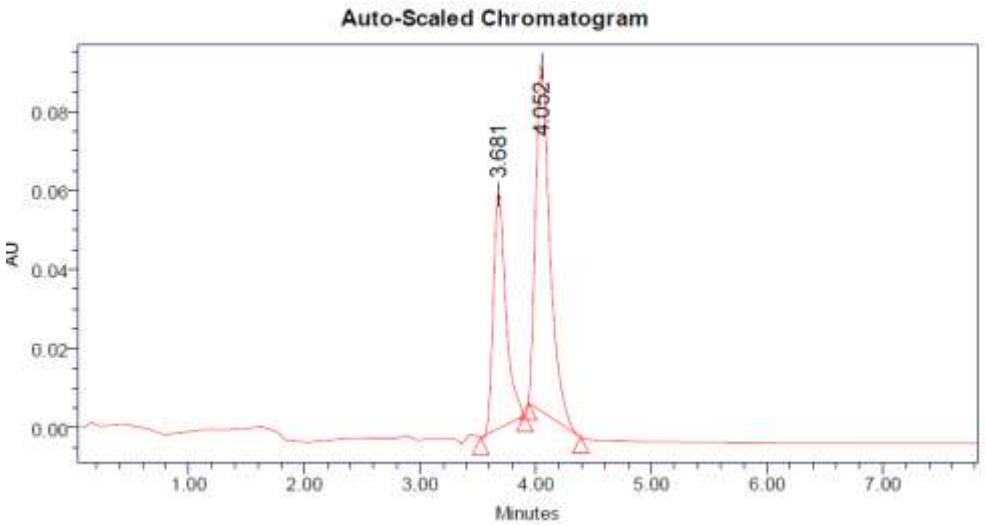


Figure7.1.2: chromatogram for trail 2

Table B: peak results for trail 2

S. No	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate Count
1	Cilnidipine	3.681	453159	59898		1.42	5843
2	Chlorthalidone	4.052	755096	87984	1.72	1.64	5346

Observation:
This trial show very less plate count, and show improper baseline in the chromatogram, so more trials were required for obtaining good peaks.

Trail 3: TABLE A

Mobile phase :	Methanol: ACN (70:30% v/v)
Column :	Zodiac C18 (4.6×250mm 5µm)
Flow rate : **Wavelength** :	0.8 ml/min 234 nm
Column temp :	30ºC
Injection Volume :	10 µl
Run time :	10 minutes

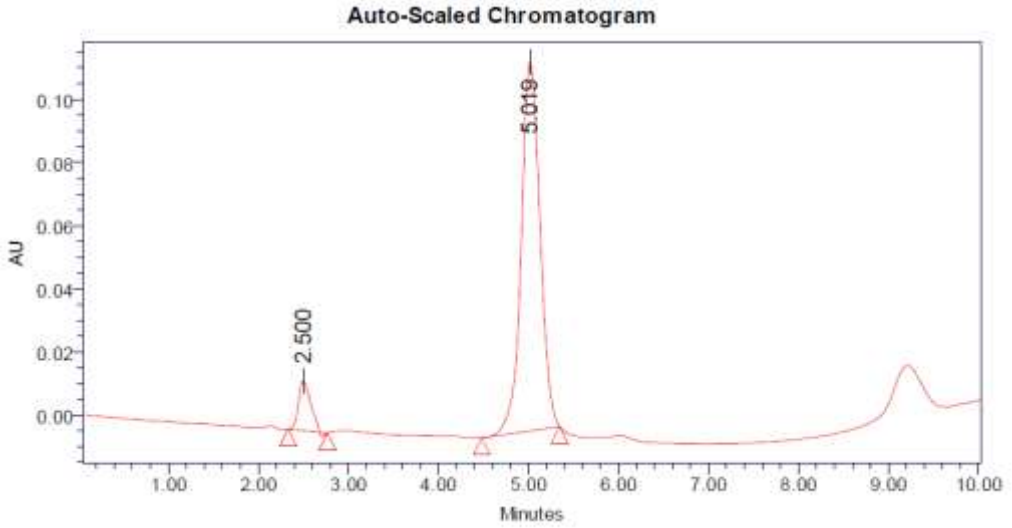


Figure:.7.1.3. - chromatogram for trail 3

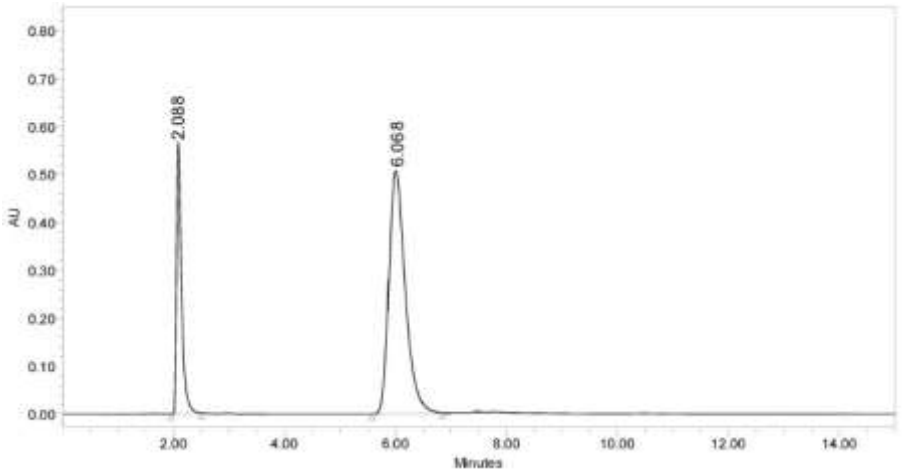
Table B: - peak results for trail 3

S. No	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate Count
1	Cilnidipine	2.500	152465	15939		1.24	1465
2	Chlorthalidone	5.019	1693743	117063	7.85	0.97	1202

Observation:
This trial shows improper baseline and show less plate count in the chromatogram, so more trials were required for obtaining peaks.

OPTIMIZED CHROMATOGRAM (STANDARD)
TABLE A FOR TRAIL 4

Mobile phase	Buffer: Methanol: ACN (65:25:10v/v)
Column	Altima C18 (4.6×150mm, 5.0 µm)
Flow rate	1 ml/min
Wavelength	234 nm
Column temp	35ºC
Injection Volume	10 µl
Run time	14 minutes



Optimized Chromatogram FIGURE 7.1.4

Table B: - peak results for trail 4

S.No	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate Count
1	Cilnidipine	2.088	3425413	567933		1.0	5565.5
2	Chlorthalidone	6.068	1629854	517733	2.5	1.1	5355.2

Observation:
From the above chromatogram it was observed that the CILNIDIPINE and Chlorthalidone peaks are well separated and they shows proper retention time, resolution, peak tail and plate count. So it's optimized trial.

Optimized Chromatogram (Sample)

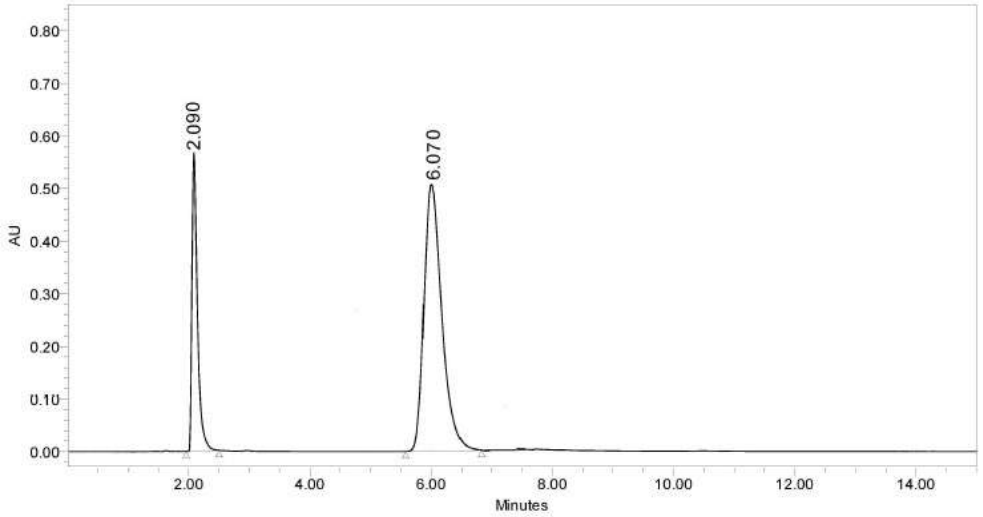


Figure: Optimized Chromatogram (Sample) Table: Optimized Chromatogram (Sample)

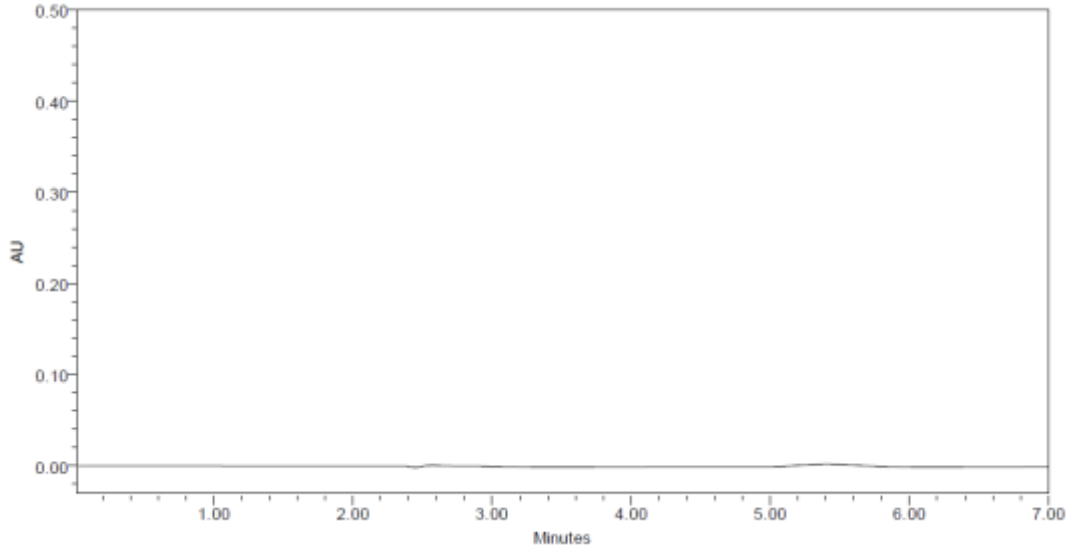
S. No	Peak name	R_t	Area	Height	USP Resolution	USP Tailing	USP plate count
1	Cilnidipine	2.088	3425413	567933		1.0	5565.5
2	Chlorthalidone	6.068	1629854	517733	2.5	1.1	5355.2

Acceptance criteria:

- Resolution between two drugs must be not less than 2
- Theoretical plates must be not less than 2000
- Tailing factor must be not less than 0.9 and not more than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

7.2. VALIDATION

Blank:



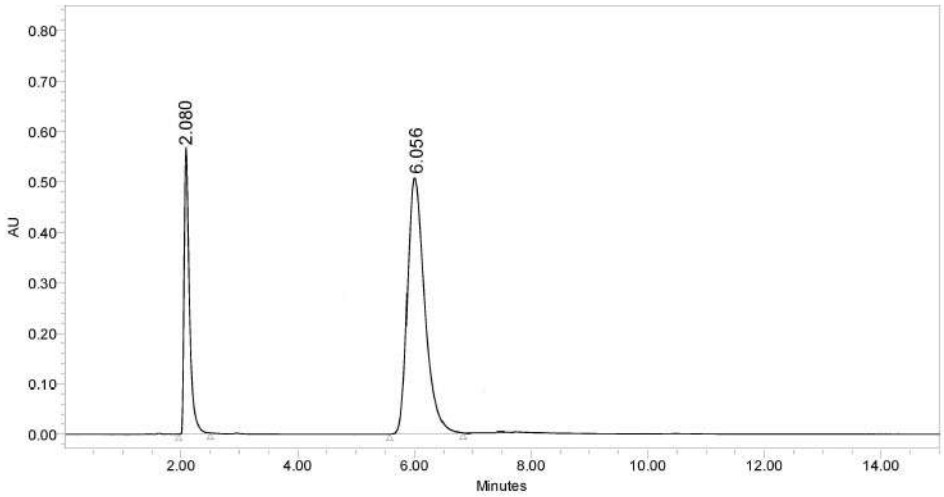


Fig.7.2. : Chromatogram showing blank (mobile phase preparation) System suitability:

Fig: Chromatogram showing injection -1

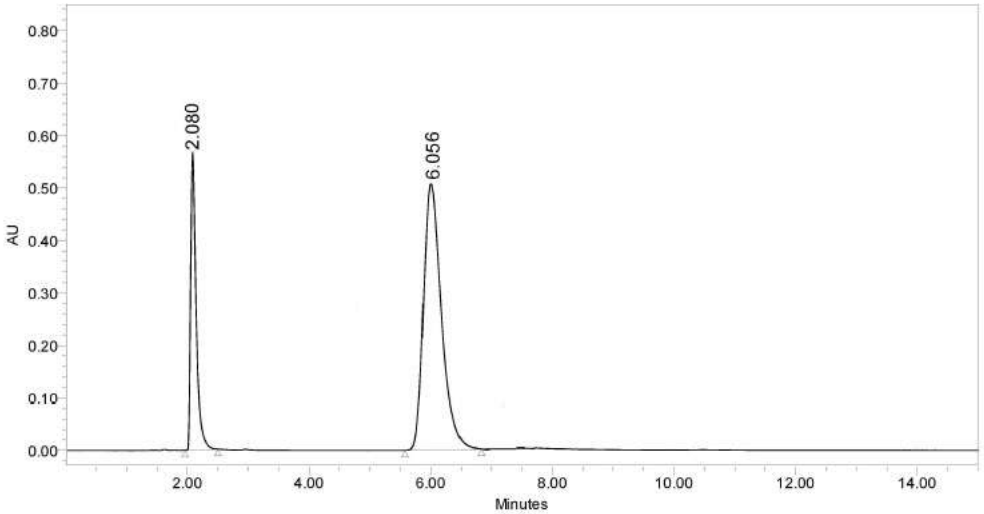


Fig: Chromatogram showing injection -2

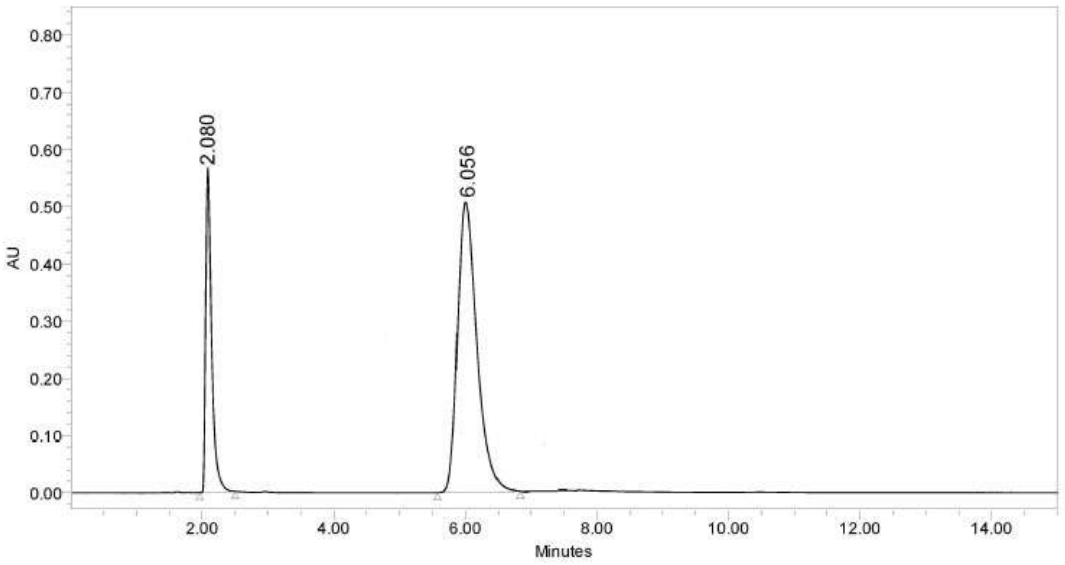


Fig: Chromatogram showing injection -3

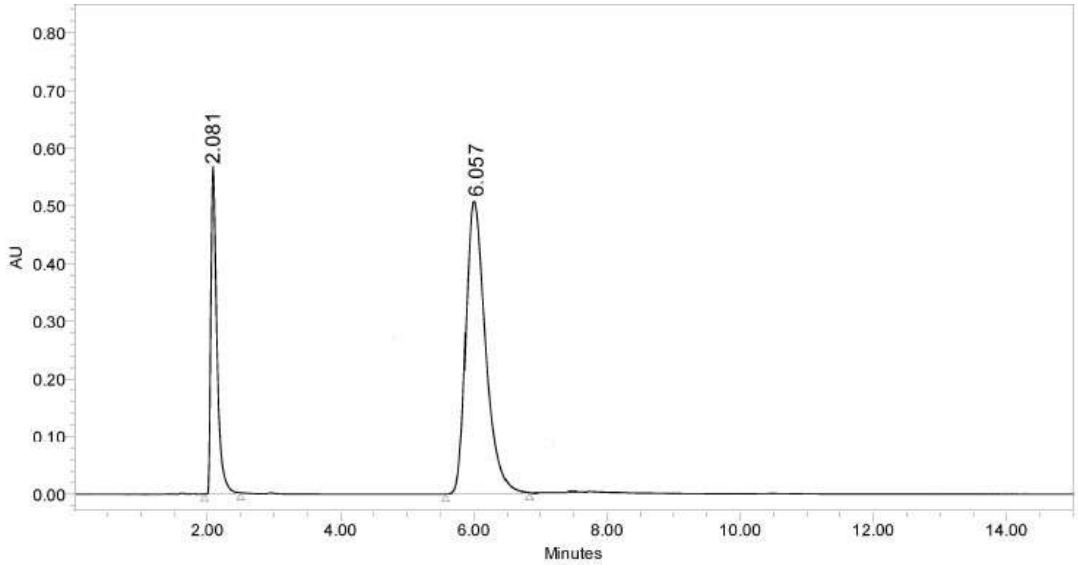


Fig: Chromatogram showing injection -4

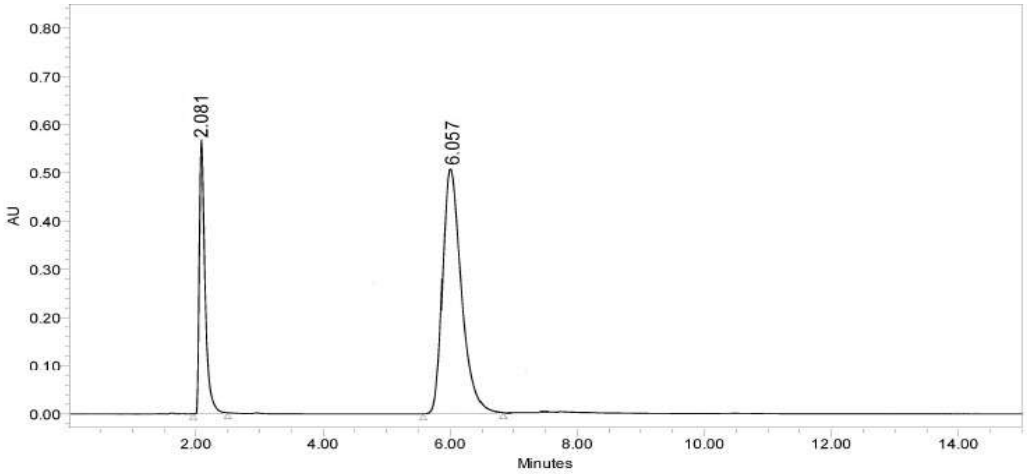


Fig:Chromatogramshowinginjection5

Table: Results of system suitability for Cilnidipine

S.No	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Cilnidipine	2.080	3569412	567917	5568.0	1.0
2	Cilnidipine	2.080	3465125	517719	6359.2	1.1
3	Cilnidipine	2.080	3598154	567933	5565.5	1.0
4	Cilnidipine	2.081	3586491	517733	5355.2	1.1
5	Cilnidipine	2.081	3582694	567917	6348.0	1.0
Mean			3560375			
Std. Dev			54225.61			
% RSD			1.523031			

Acceptance criteria:

- %RSD of five different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is suitable.

Table: Results of method precession for Chlorthalidone:

S.No.	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Chlorthalidone	2.080	3582264	567917	5568.0	1.0	2.5
2	Chlorthalidone	2.080	3586491	517719	5359.2	1.1	2.5
3	Chlorthalidone	2.080	3598154	567933	5565.5	1.0	2.5
4	Chlorthalidone	2.081	3564125	517733	5355.2	1.1	2.5
5	Chlorthalidone	2.081	3569412	562173	5568.0	1.0	2.5
Mean			3580089				
Std. Dev			13609.81				
% RSD			0.380153				

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

SPECIFICITY

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

Analytical method tested for the specificity to measure accurately quantitate CILNIDIPINE and Chlorthalidone in drug product.

Assay (Standard):

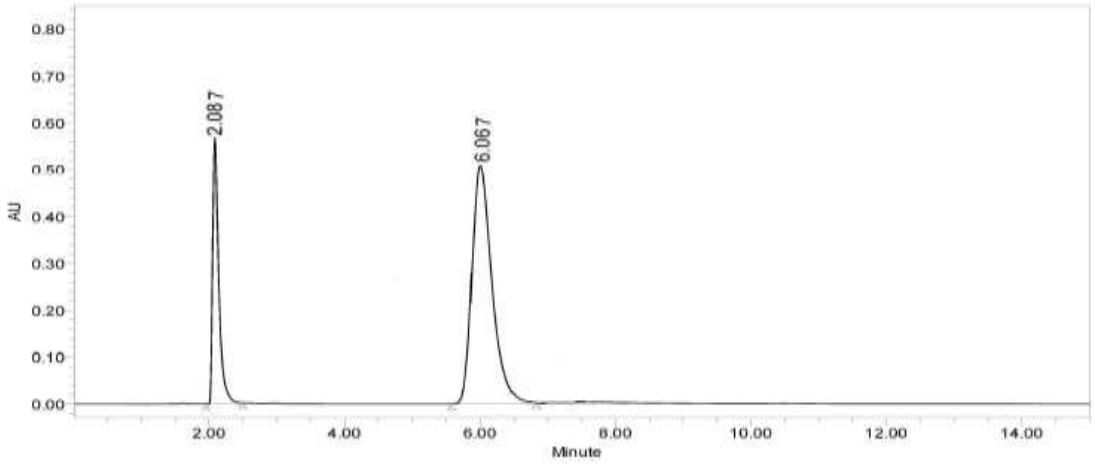


Fig: Chromatogram showing assay of standard injection -1

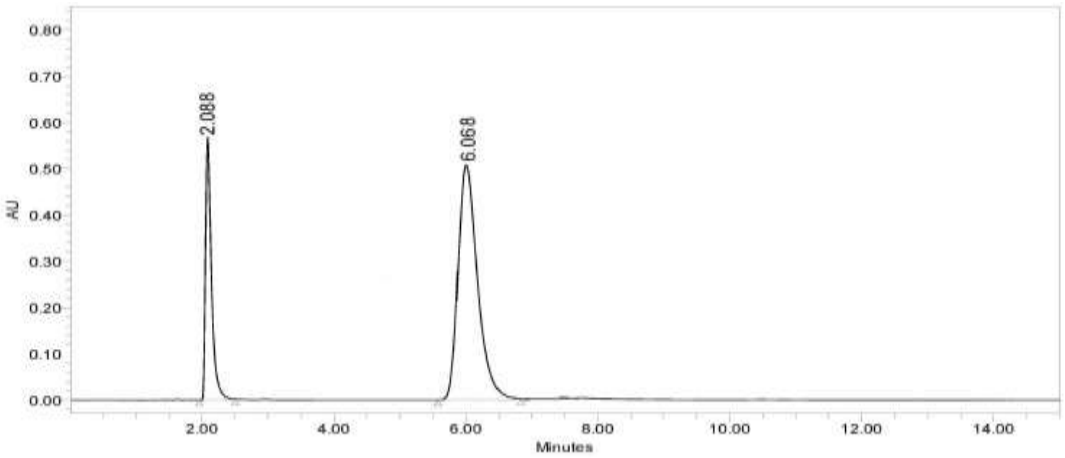


Fig. Chromatogram showing assay of standard injection -2

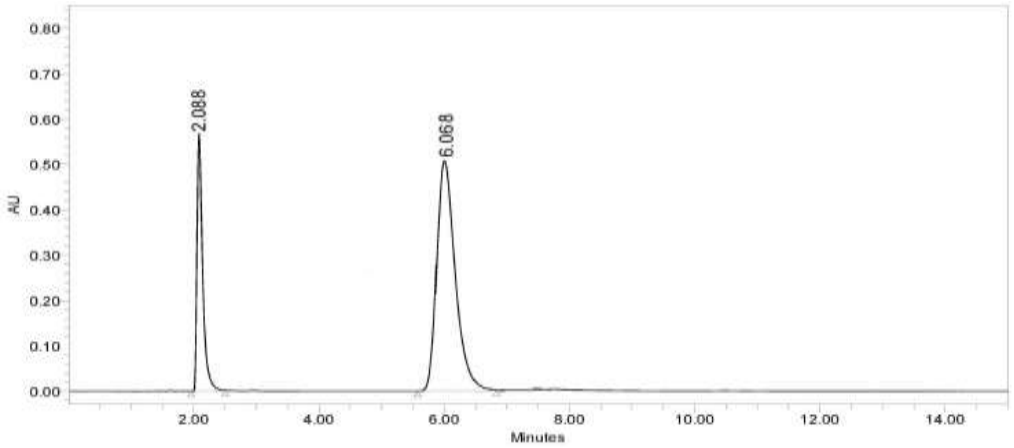


Fig. Chromatogram showing assay of standard injection -3

Table: Peak results for assay standard

S.No	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Cilnidipine	2.087	3465681	567917		1.0	5568.0	1
2	Chlorthalidone	6.067	16235984	517719	2.5	1.1	5359.2	1
3	Cilnidipine	2.088	3465413	567933		1.0	5565.5	2
4	Chlorthalidone	6.068	16298543	517733	2.5	1.1	5355.2	2
5	Cilnidipine	2.088	3465423	567933		1.0	5545.5	3
6	Chlorthalidone	6.068	16265213	517733	2.5	1.1	5352.1	3

Assay (Sample):

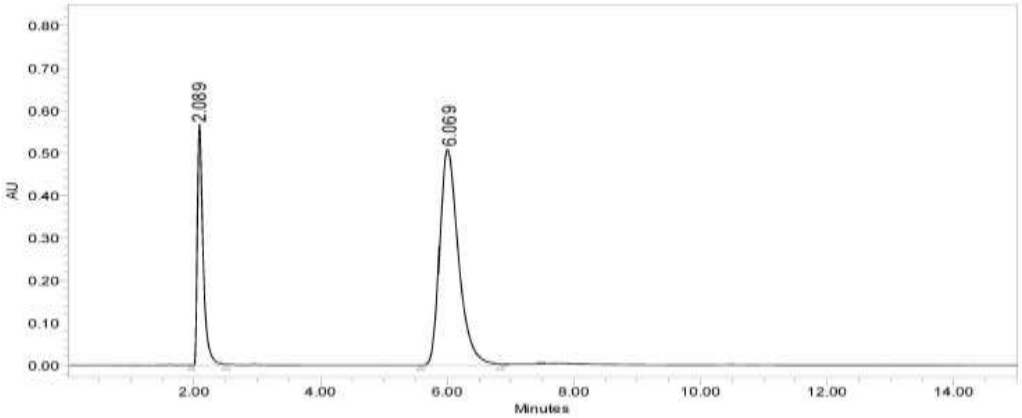


Fig: Chromatogram showing assay of sample injection-1

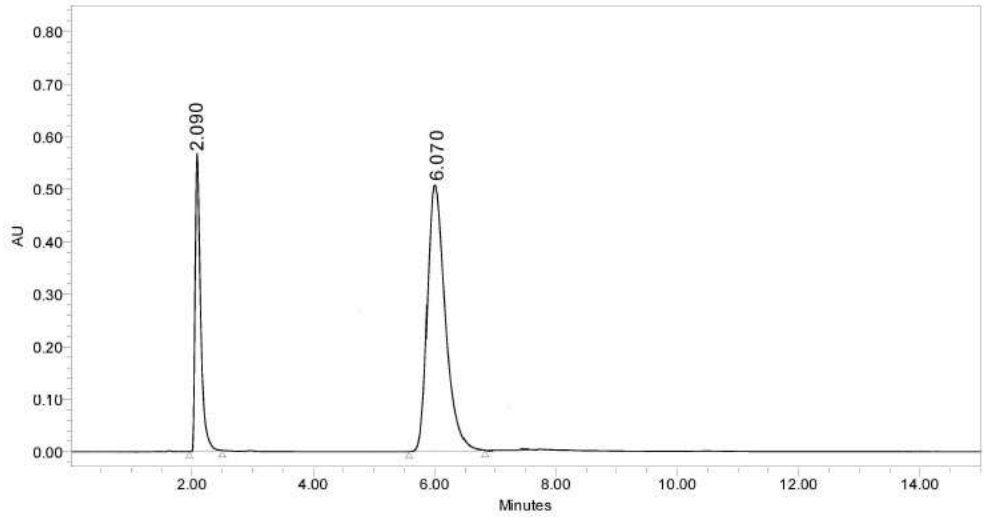


Fig. Chromatogram showing assay of sample injection-2

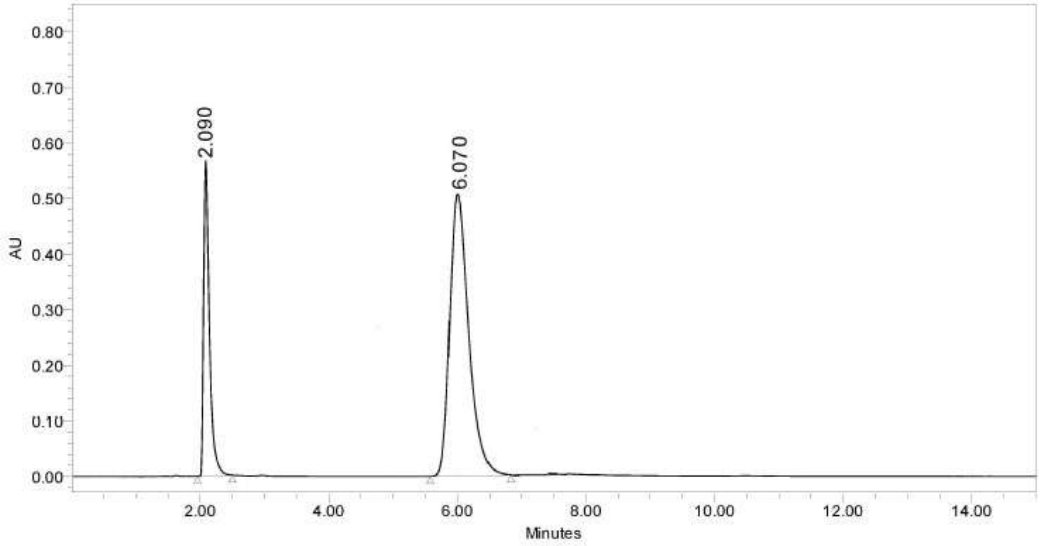


Fig: Chromatogram showing assay of sample injection-3

Table: Peak results for Assay sample

S.No	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Cilnidipine	2.089	3469821	567917		1.0	6568.0	1
2	Chlorthalidone	6.069	16259845	517719	2.5	1.1	5359.2	1
3	Cilnidipine	2.090	3468547	567933		1.0	5565.5	2
4	Chlorthalidone	6.070	16287531	517733	2.5	1.1	5355.2	2
5	Cilnidipine	2.090	3468143	567813		1.0	5391.1	3
6	Chlorthalidone	6.070	16282431	517623	2.5	1.1	5564.0	3

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of CILNIDIPINE and Chlorthalidone in pharmaceutical dosage form was found to be 99.6%.

LINEARITY

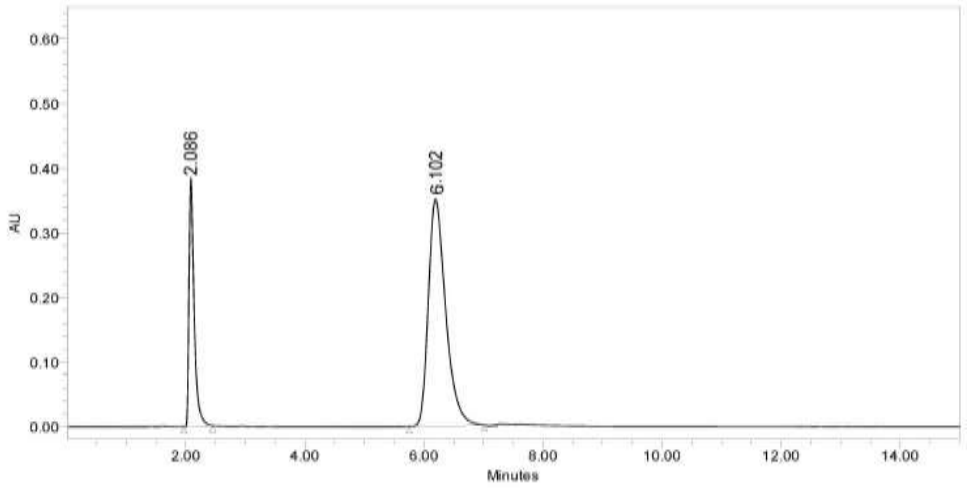


Figure: Linearity level-1

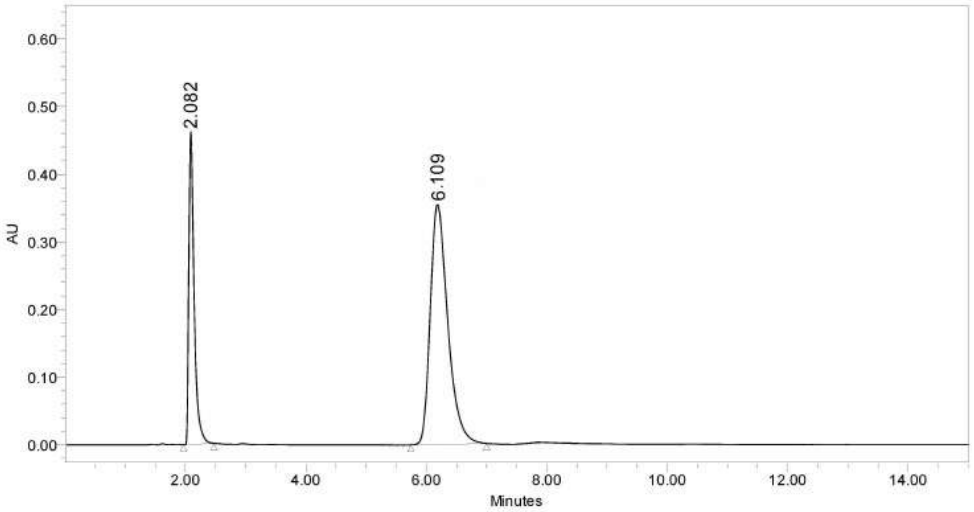


Figure: Linearity level-2

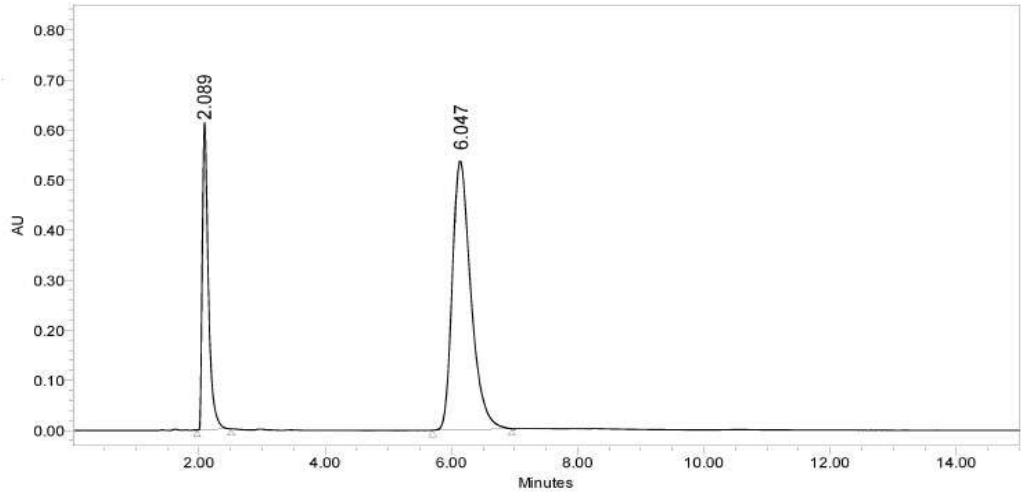


Figure: Linearity level-3

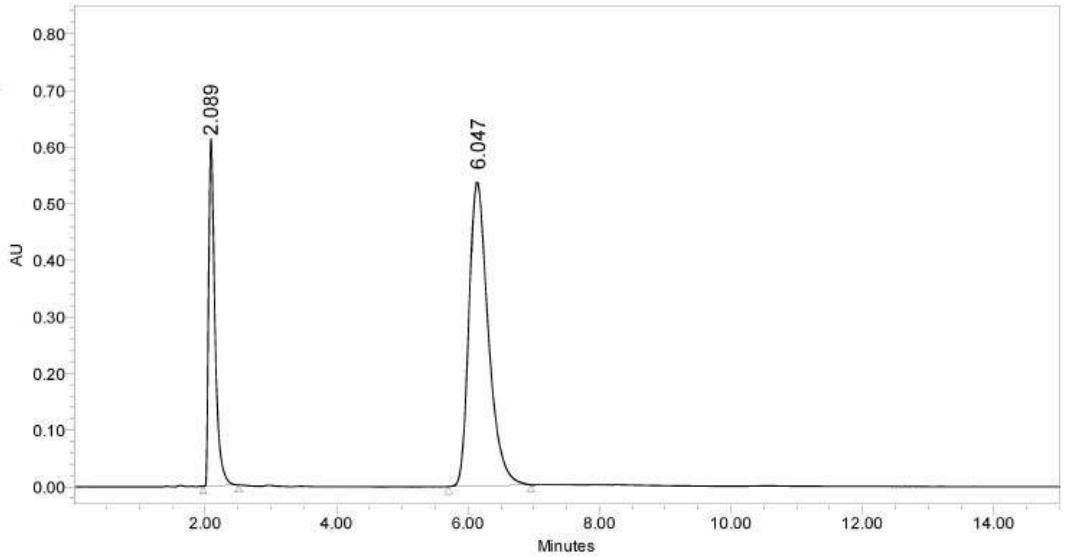


Figure: Linearity level-4

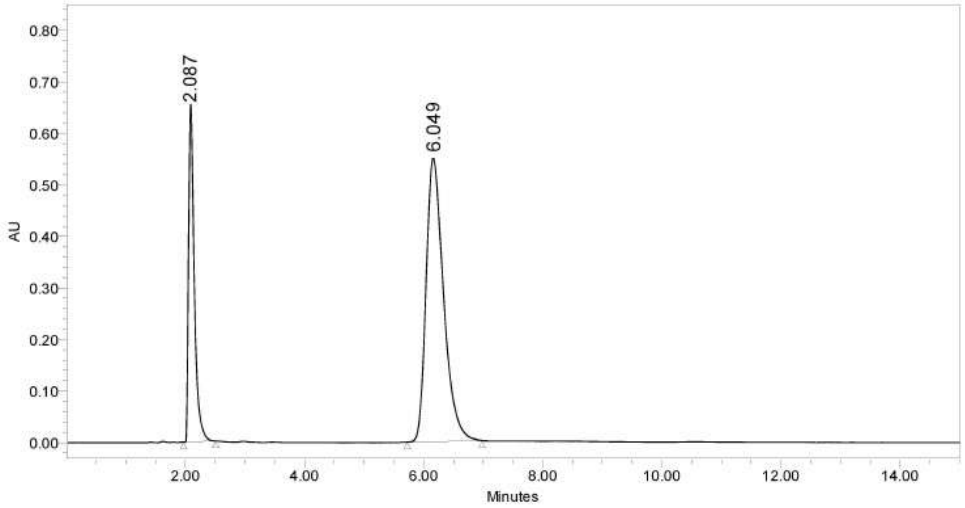


Figure: Linearity level-5

CHROMATOGRAPHIC DATA FOR LINEARITY STUDY:
CILNIDIPINE:

Concentration Level (%)	Concentration g/ml	Average Peak Area
33.3	25	1010252
66.6	50	2049374
100	75	3072706
133.3	100	3921068
166.6	125	4952813

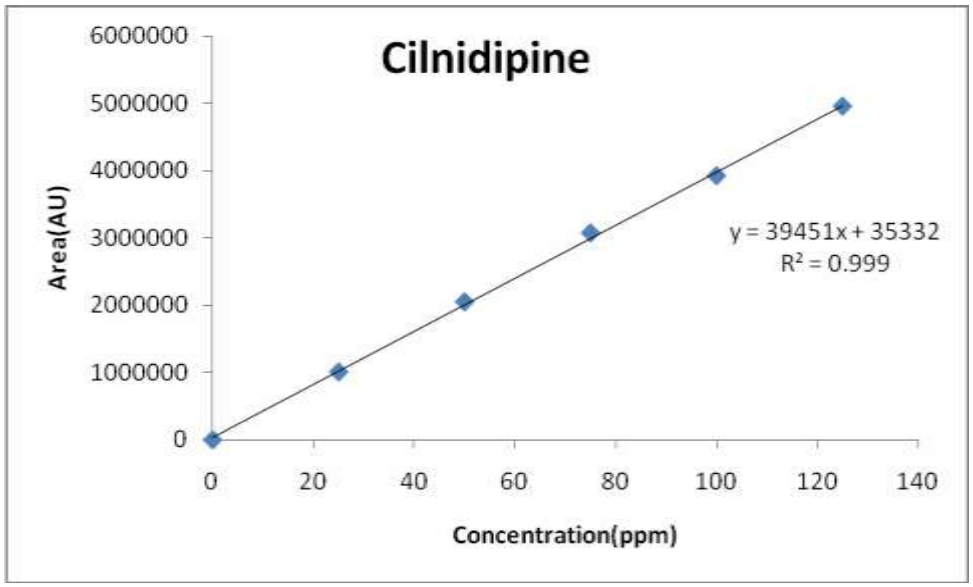


Figure 6.3.4 calibration graph for Cilnidipine

LINEARITY PLOT:
The plot of Concentration (x) versus the Average Peak Area (y) data of Cilnidipine is a straight line.
Y = mx + c
Slope (m) = 39451 Intercept (c) = 35332 Correlation Coefficient (r) = 0.999
VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

CONCLUSION:
Correlation Coefficient (r) is 0.99, and the intercept is 35332. These values meet the validation criteria.
Chlorthalidone

Concentration Level (%)	Concentration g/ml	Average Peak Area
33	10	8040807
66	20	14318417
100	30	21087985
133	40	27913928
166	50	34584741

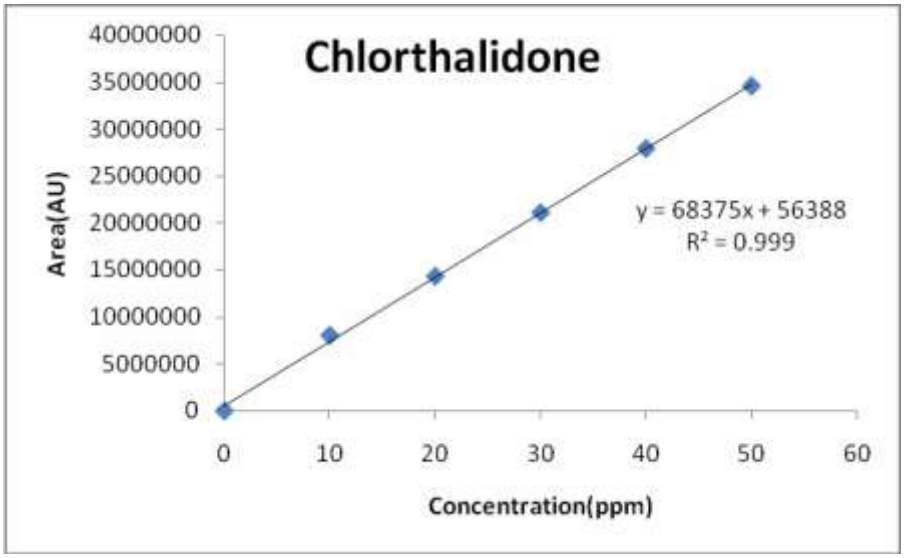


Figure 6.3.4 calibration graph for Chlorthalidone

LINEARITY PLOT:
The plot of Concentration (x) versus the Average Peak Area (y) data of Chlorthalidone is a straight line.
Y = mx + c
Slope (m) = 68375 Intercept (c) = 56388 Correlation Coefficient (r) = 0.999

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.
CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 56388. These values meet the validation criteria.

PRECISION:
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.

REPEATABILITY
Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

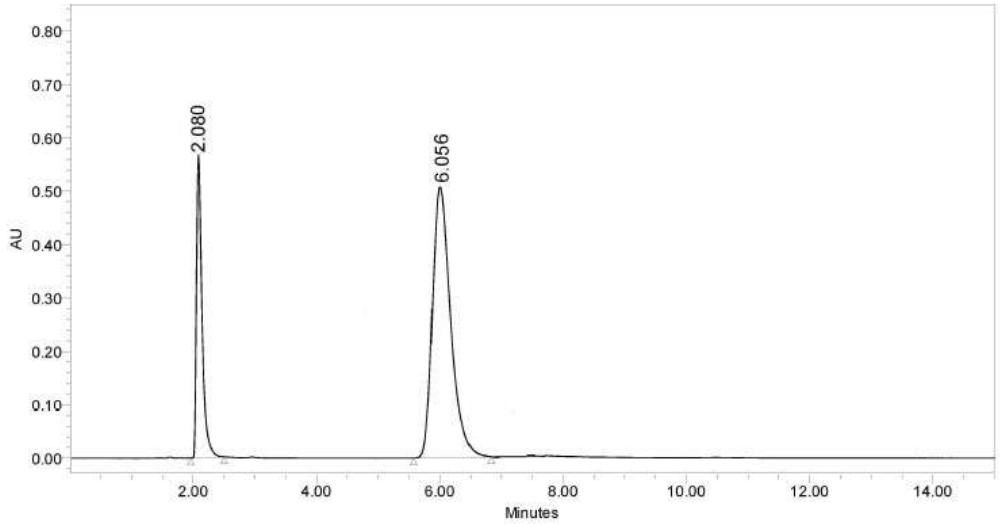


Fig. Chromatogram showing precision injection -1

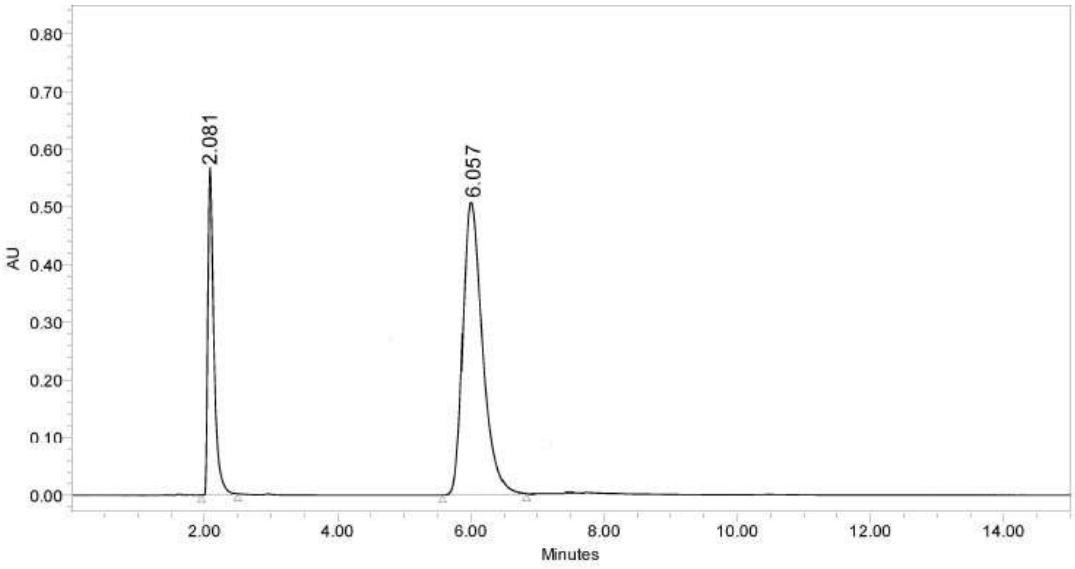


Fig. Chromatogram showing precision injection -2

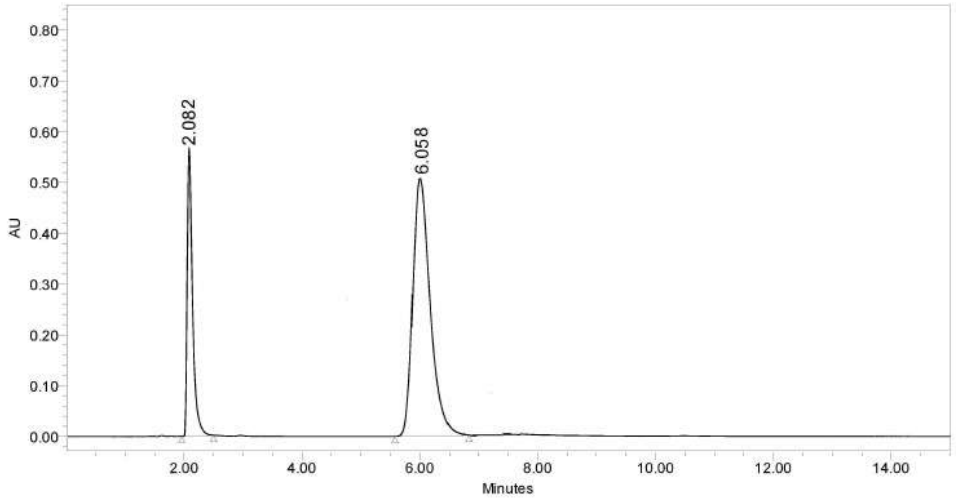


Fig. Chromatogram showing precision injection -3

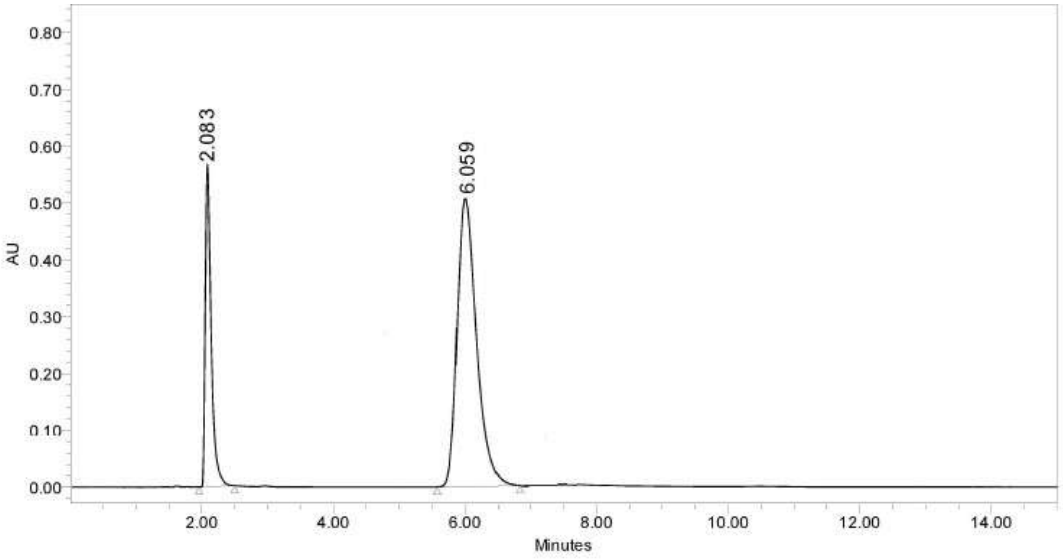


Fig. Chromatogram showing precision injection -4

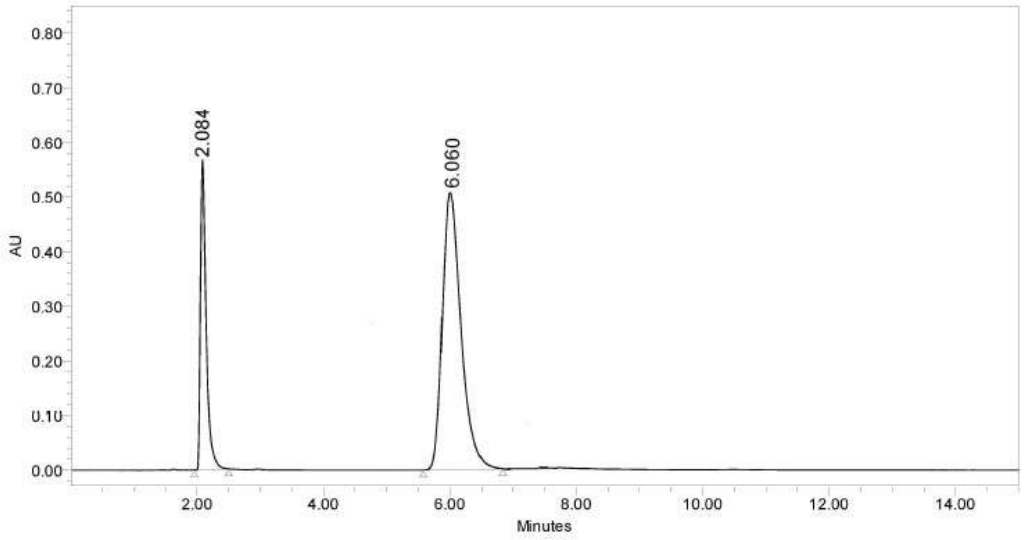


Fig. Chromatogram showing precision injection -5

Table: Results of repeatability for Cilnidipine:

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Cilnidipine	2.084	3569412	567917	5568.0	1.0
2	Cilnidipine	2.083	3465125	517719	5359.2	1.1
3	Cilnidipine	2.082	3598154	567933	5565.5	1.0
4	Cilnidipine	2.081	3586491	517733	5355.2	1.1
5	Cilnidipine	2.080	3582694	567917	5568.0	1.0
Mean			3560375			
Std. Dev			54225.61			
% RSD			1.523031			

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Table: Results of method precision for Chlorthalidone:

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Chlorthalidone	6.056	1582264	567917	5568.0	1.0	2.5
2	Chlorthalidone	6.057	1586491	517719	5359.2	1.1	2.5
3	Chlorthalidone	6.058	1598154	567933	5565.5	1.0	2.5
4	Chlorthalidone	6.059	1564125	517733	5355.2	1.1	2.5
5	Chlorthalidone	6.060	1569412	562173	5568.0	1.0	2.5
Mean			1580089				
Std. Dev			13609.81				
% RSD			0.861332				

Acceptance criteria:

- %RSD for sample should be NMT 2
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Intermediate precision: Day 1:

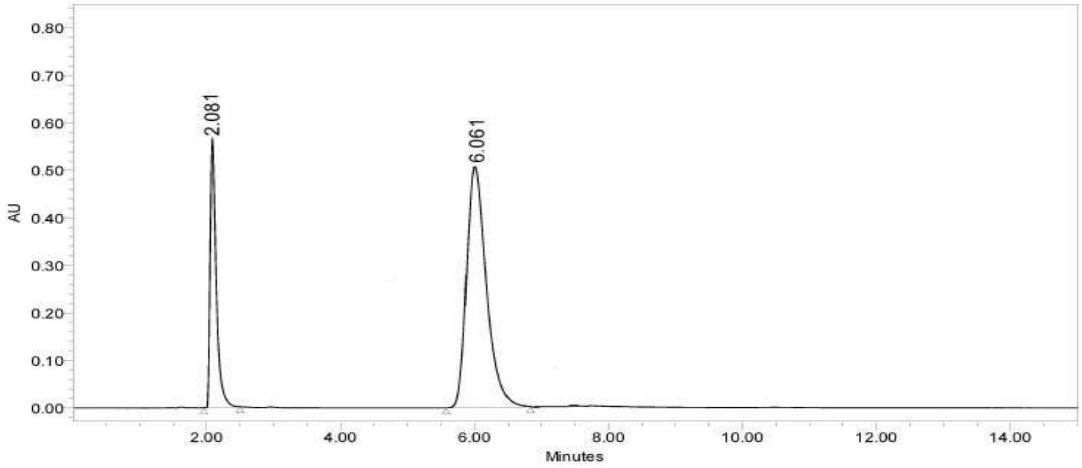


Fig: Chromatogram showing Day1 injection -1

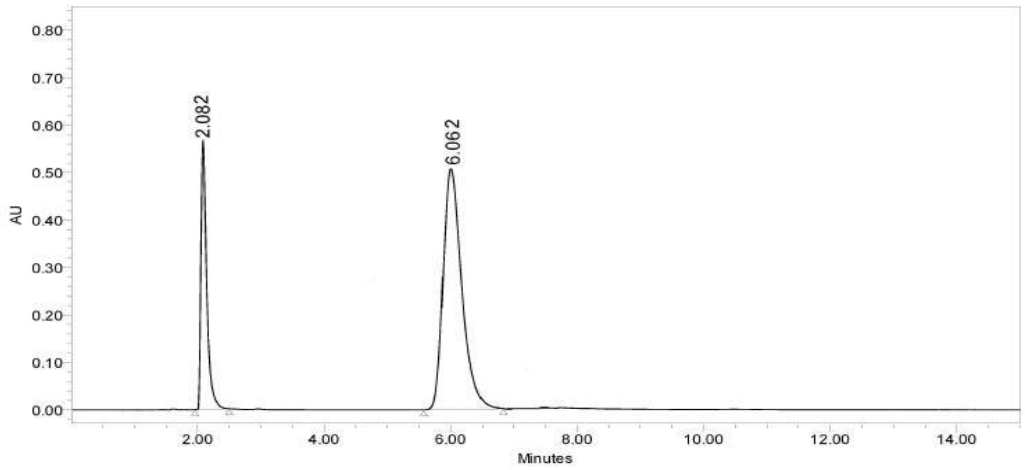


Fig: Chromatogram showing Day1 injection -2

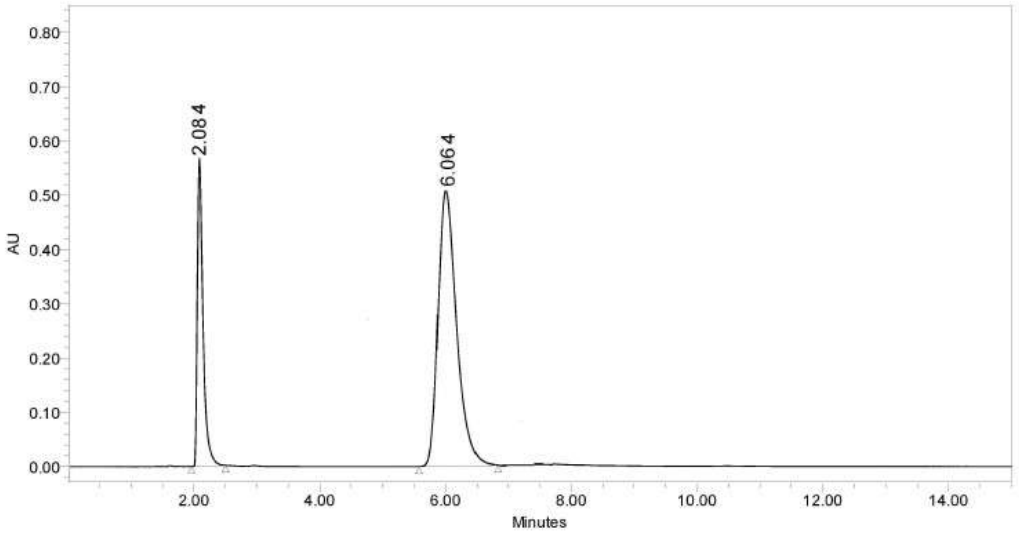


Fig: Chromatogram showing Day1 injection -3

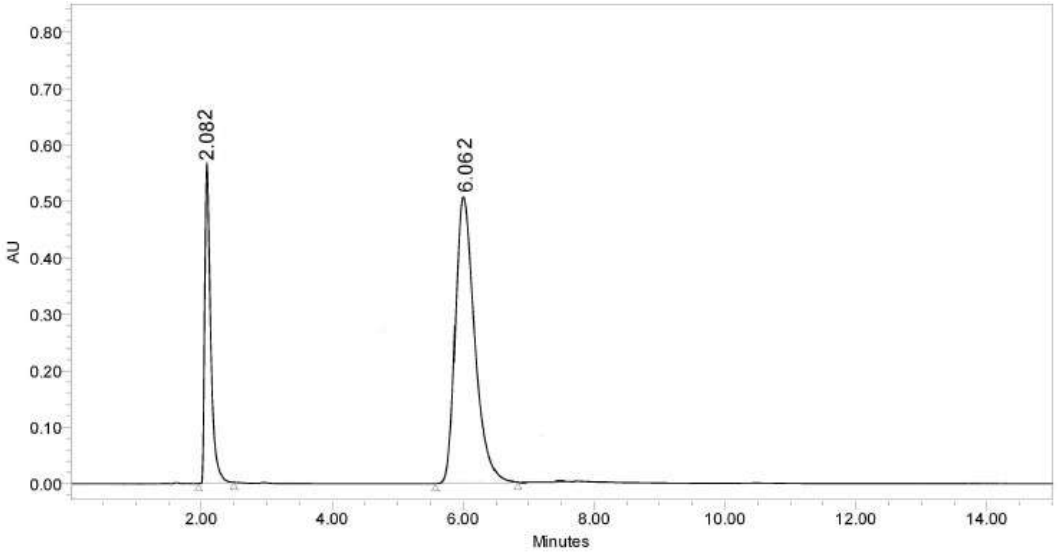


Fig: Chromatogram showing Day1 injection -4

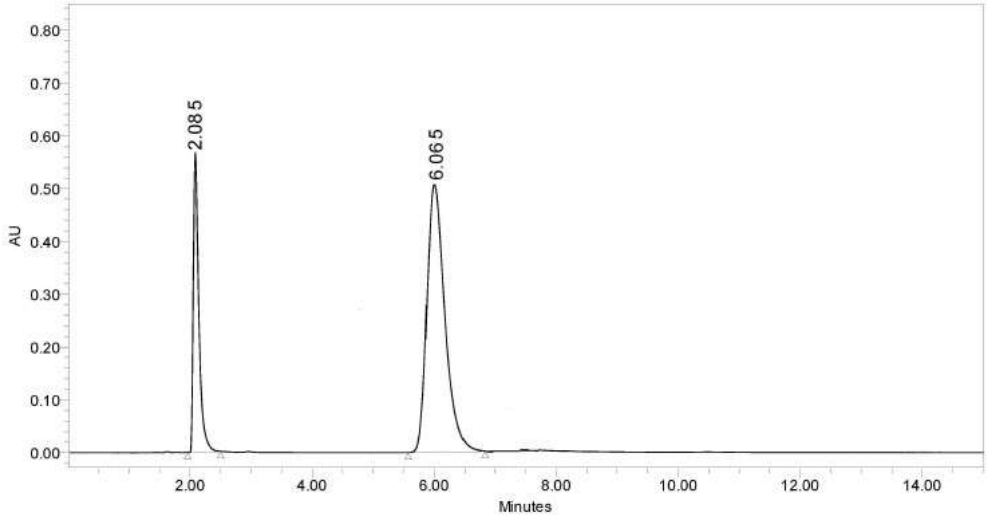


Fig: Chromatogram showing Day1 injection -5

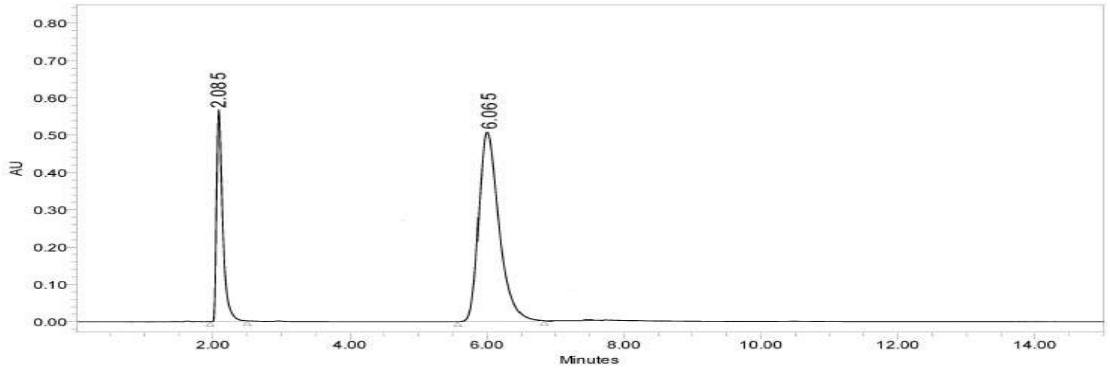


Fig: Chromatogram showing Day1 injection -6

Table: Results of Intermediate precision for CILNIDIPINE

S.No	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Cilnidipine	2.081	3481579	567917	5568.0	1.0
2	Cilnidipine	2.082	3458121	517719	5359.2	1.1
3	Cilnidipine	2.083	3426581	567933	5565.5	1.0
4	Cilnidipine	2.084	3465712	517733	5355.2	1.1
5	Cilnidipine	2.084	3451476	567917	5568.0	1.0
6	Cilnidipine	2.085	3452106	567514	5359.2	1.1
Mean			3455929			
Std.Dev			18188.92			
%RSD			0.5			

Acceptance criteria:

- %RSD of Six different sample solutions should not more than 2

Table: Results of Intermediate precision for Chlorthalidone

S.No	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Chlorthalidone	6.061	15481579	567917	5568.0	1.0	2.5
2	Chlorthalidone	6.062	15369852	517719	5359.2	1.1	2.5
3	Chlorthalidone	6.063	15248454	567933	5565.5	1.0	2.5
4	Chlorthalidone	6.064	15874692	517733	5355.2	1.1	2.5
5	Chlorthalidone	6.064	15236547	567933	5568.0	1.0	2.5
6	Chlorthalidone	6.064	15217547	567133	5359.2	1.1	2.5
Mean			15404779				
Std. Dev			251289.4				
% RSD			1.6				

Acceptance criteria:

- %RSD of Six different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

Day 2:

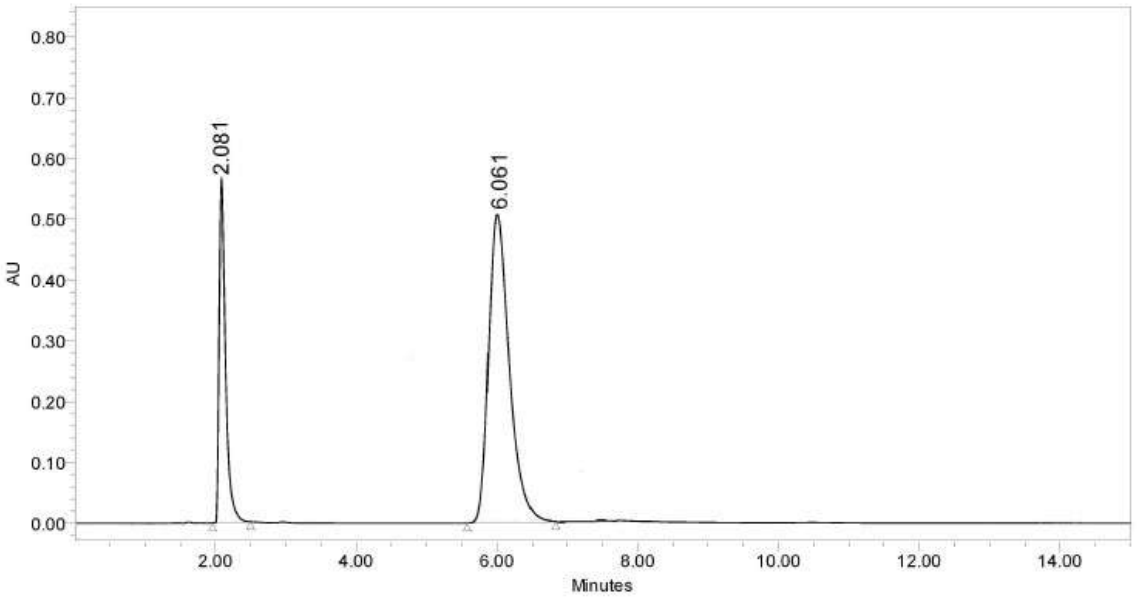


Fig: Chromatogram showing Day 2 injection -1

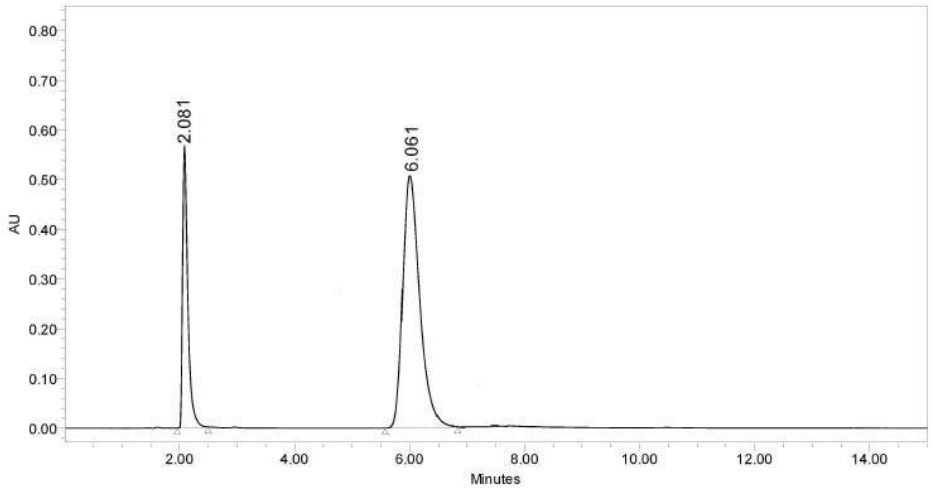


Fig: Chromatogram showing Day 2 injection -2

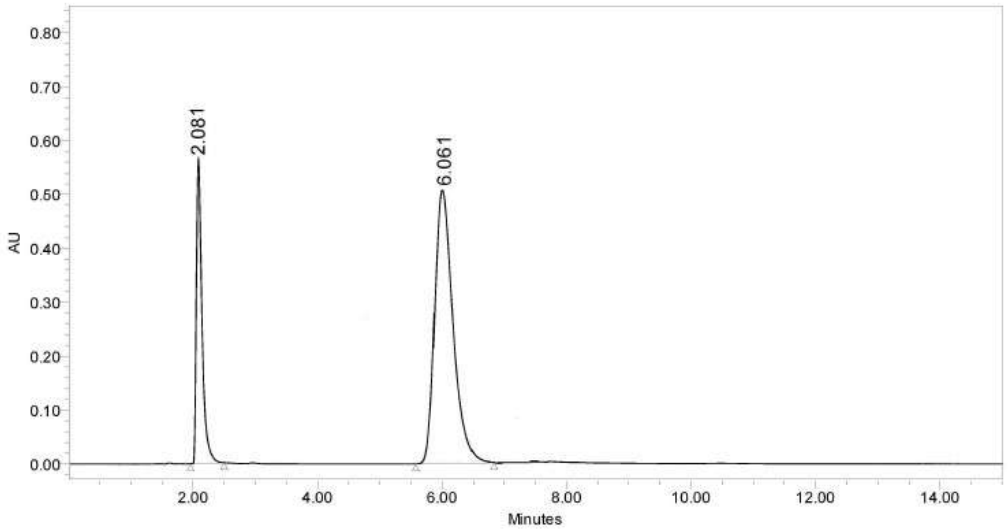


Fig: Chromatogram showing Day 2 injection -3

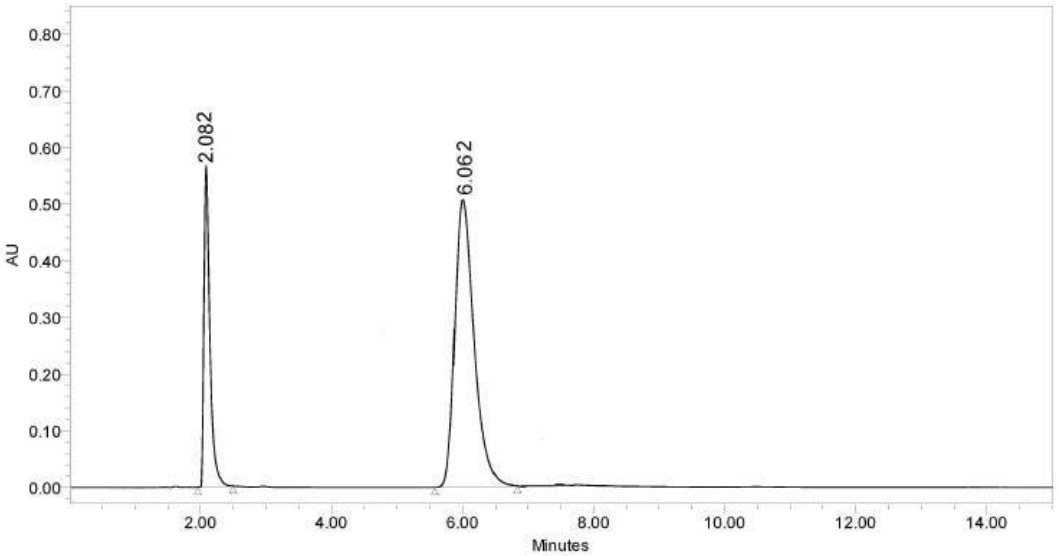


Fig: Chromatogram showing Day 2 injection -4

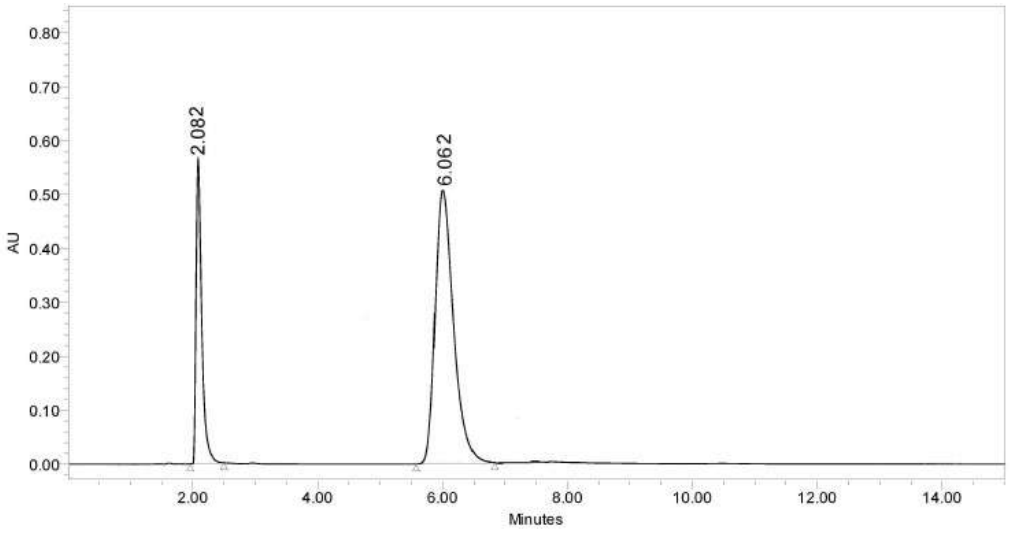


Fig: Chromatogram showing Day 2 injection -5

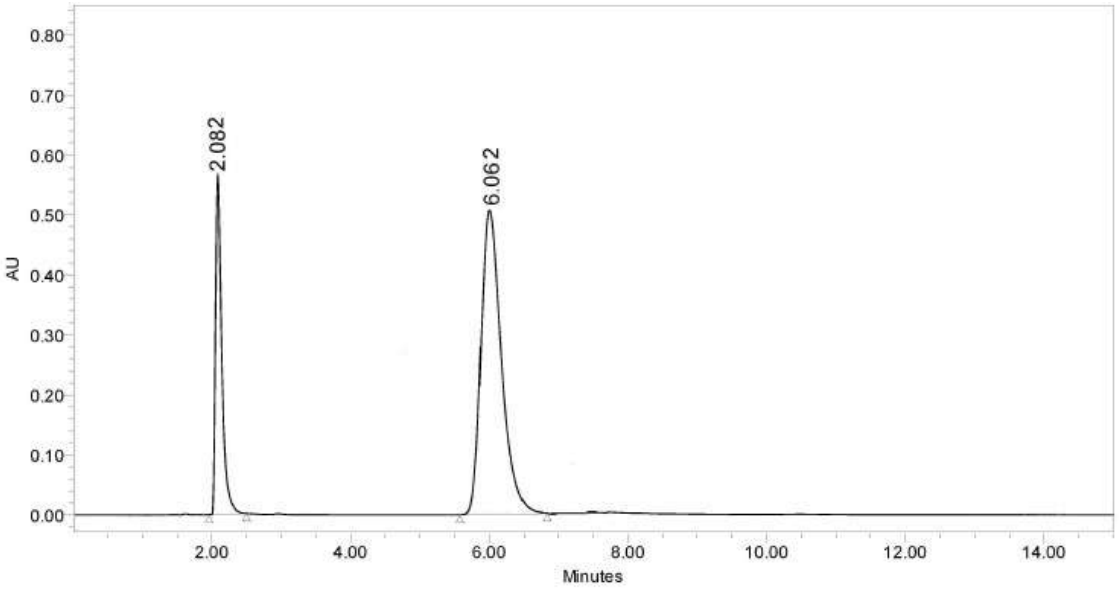


Fig: Chromatogram showing Day 2 injection -6

Table: Results of Intermediate precision Day 2 for CILNIDIPINE

S.No	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Cilnidipine	2.081	3481579	567917	5568.0	1.0
2	Cilnidipine	2.082	3458121	517719	5359.2	1.1
3	Cilnidipine	2.083	3426581	567933	5565.5	1.0
4	Cilnidipine	2.084	3465712	517733	5355.2	1.1
5	Cilnidipine	2.085	3451476	567917	5568.0	1.0
6	Cilnidipine	2.085	3452106	567514	5359.2	1.1
Mean			3455929			
Std.Dev			18188.92			
%RSD						

Acceptance criteria:

- %RSD of Six different sample solutions should not more than 2

Table: Results of Intermediate precision for Chlorthalidone

S.No	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Chlorthalidone	6.061	15481579	567917	5568.0	1.0	2.5
2	Chlorthalidone	6.062	15369852	517719	5359.2	1.1	2.5
3	Chlorthalidone	6.063	15248454	567933	5565.5	1.0	2.5
4	Chlorthalidone	6.064	15874692	517733	5355.2	1.1	2.5
5	Chlorthalidone	6.064	15236547	567933	5568.0	1.0	2.5
6	Chlorthalidone	6.064	15217547	567133	5359.2	1.1	2.5
Mean			15404779				
Std. Dev			251289.4				
% RSD			1.6				

Acceptance criteria:

- %RSD of Six different sample solutions should not more than 2
- The %RSD obtained is within the limit, hence the method is rugged.

6.3.4: ACCURACY:

Accuracy at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated.

Accuracy50%:

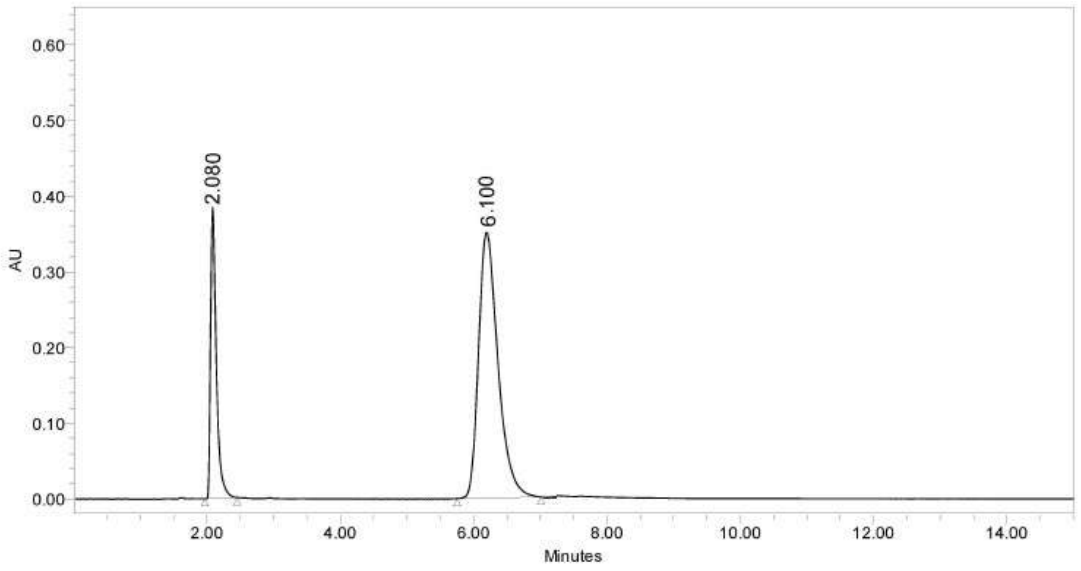


Fig. Chromatogram showing accuracy-50% injection-1

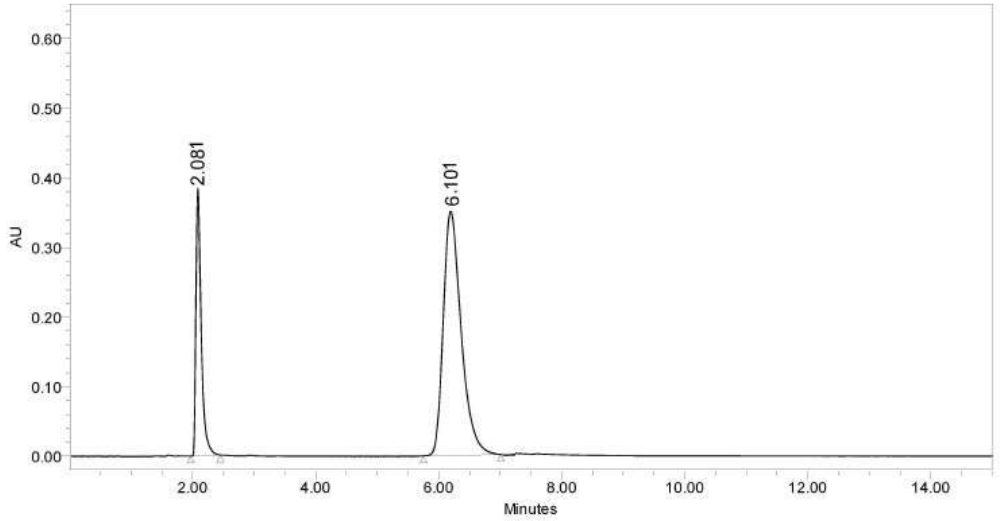


Fig. Chromatogram showing accuracy-50% injection-2

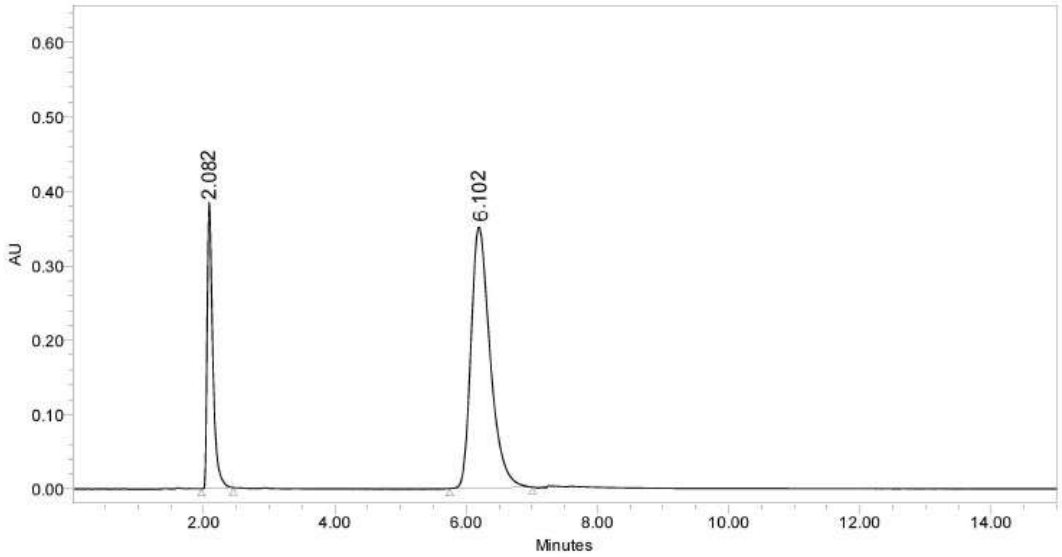


Fig. Chromatogram showing accuracy-50% injection-3

Table: Results of Accuracy for concentration-50%

S.No	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Cilnidipine	2.080	1536547	567917		1.0	5568.0	1
2	Chlorthalidone	6.100	1180807	517719	2.5	1.1	5359.2	1
3	Cilnidipine	2.081	1526986	567933		1.0	5565.5	2
4	Chlorthalidone	6.101	1046986	517733	2.5	1.1	5355.2	2
5	Cilnidipine	2.082	1567845	566924		1.0	5568.0	3
6	Chlorthalidone	6.102	1025466	519124	2.5	1.1	5359.2	3

Accuracy100%:

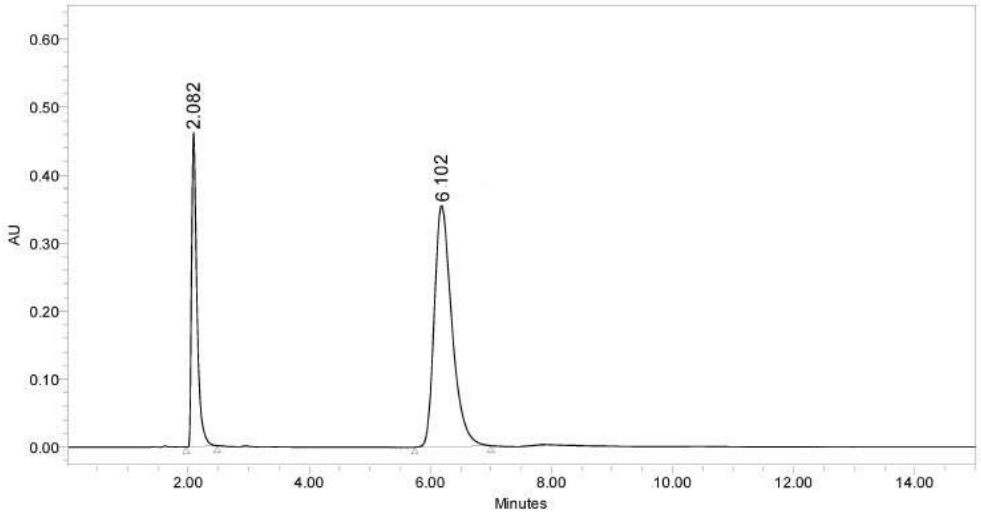


Fig Chromatogram showing accuracy-100% injection-1

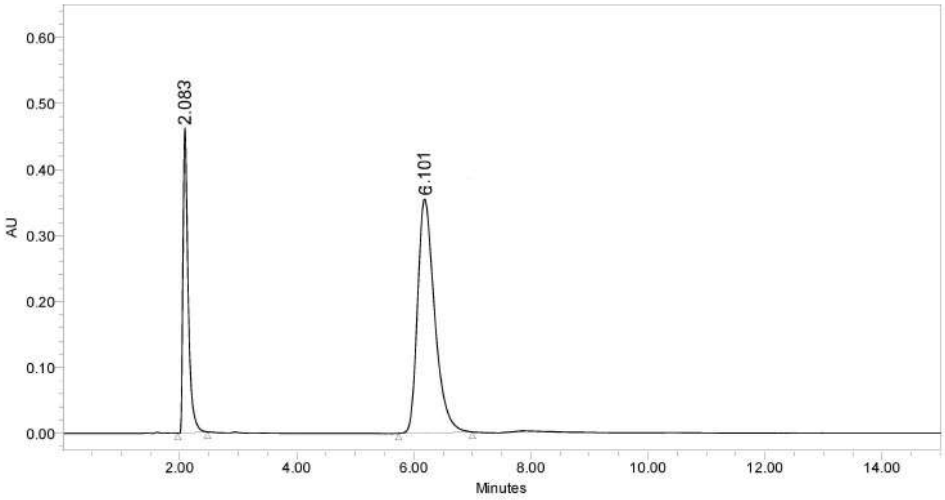


Fig: Chromatogram showing accuracy-100% injection-2

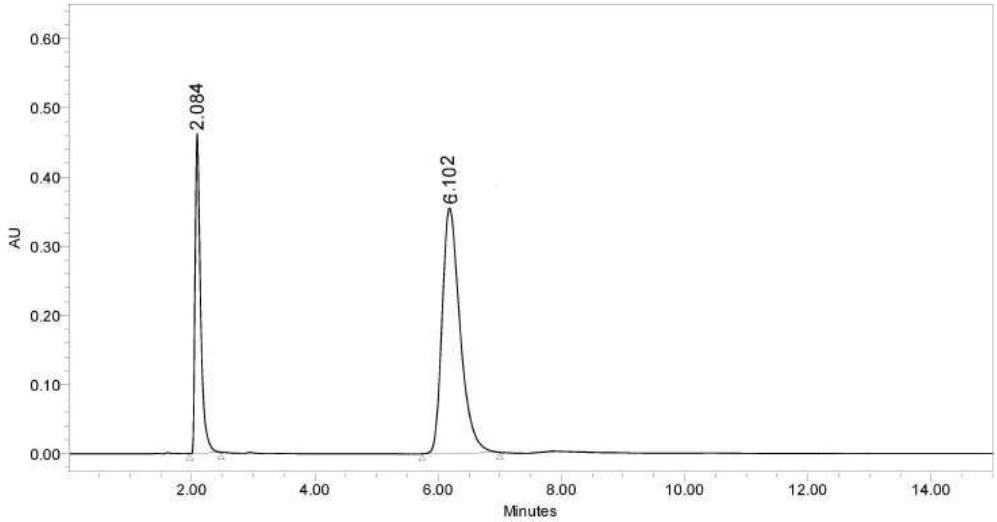


Fig: Chromatogram showing accuracy-100% injection-3

Table: Results of Accuracy for concentration-100%

S.No	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Cilnidipine	2.082	3065874	567917		1.0	5568.0	1
2	Chlorthalidone	6.102	2108433	517719	2.5	1.1	5359.2	1
3	Cilnidipine	2.083	3029381	567933		1.0	5565.5	2
4	Chlorthalidone	6.101	2084929	517733	2.5	1.1	5355.2	2
5	Cilnidipine	2.084	3012393	567917		1.0	5568.0	3
6	Chlorthalidone	6.102	2094845	517719	2.5	1.1	5359.2	3

Accuracy150%:

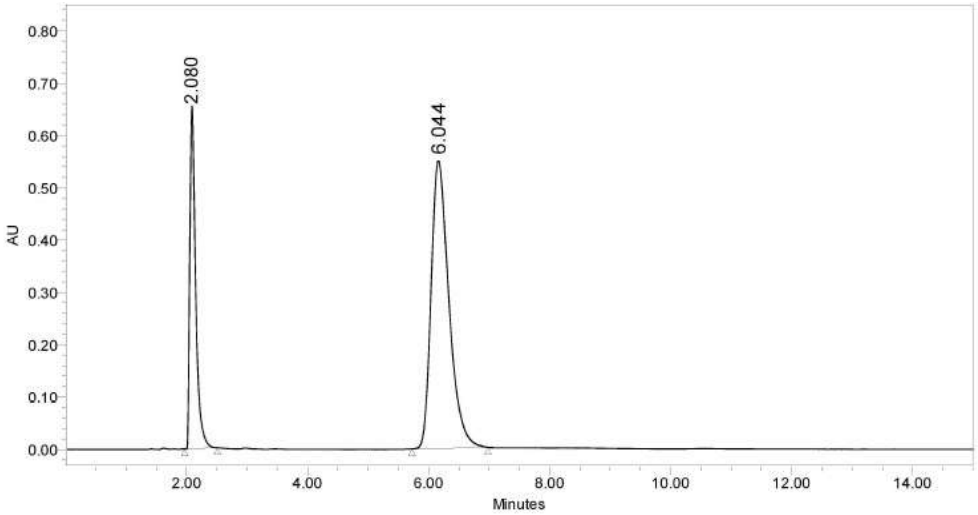


Fig Chromatogram showing accuracy-150% injection-1

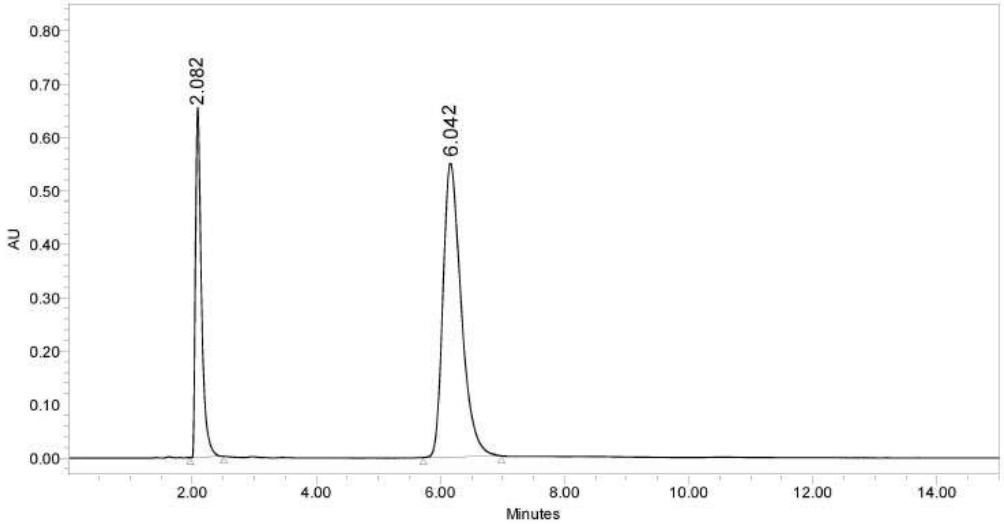


Fig. Chromatogram showing accuracy-150% injection-2

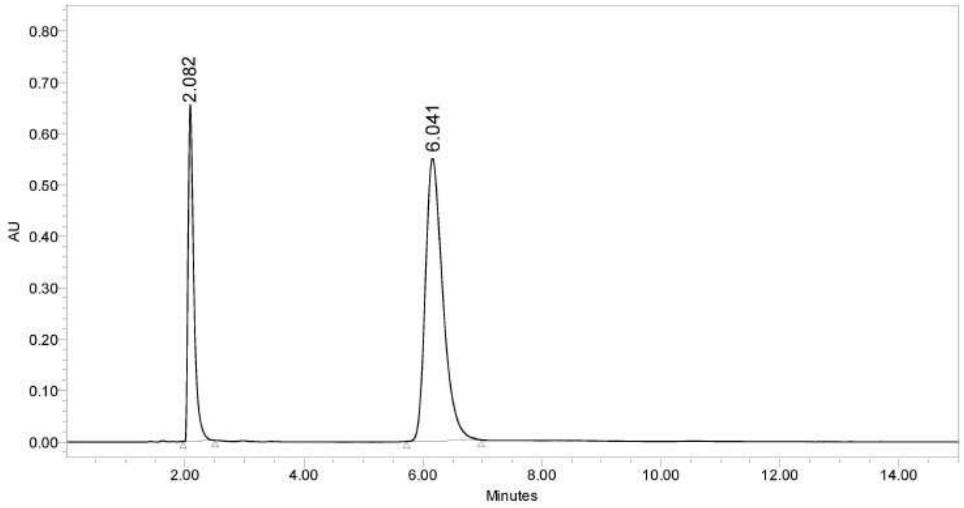


Fig. Chromatogram showing accuracy-150% injection-3

Table Results of Accuracy for concentration-150%

S.No	Name	Rt	Area	Height	USP Resolution	USP Tailing	USP plate count	Injection
1	Cilnidipine	2.080	4477263	567917		1.0	5568.0	1
2	Chlorthalidone	6.044	3094837	517719	2.5	1.1	5359.2	1
3	Cilnidipine	2.082	4482921	567933		1.0	5565.5	2
4	Chlorthalidone	6.042	3094843	517733	2.5	1.1	5355.2	2
5	Cilnidipine	2.082	4392832	568117		1.0	5568.0	3
6	Chlorthalidone	6.041	3148372	517689	2.5	1.1	5359.2	3

The accuracy results for Cilnidipine

%Concentration (at specification Level)	**Area**	**Amount Added (ppm)**	**Amount Found (ppm)**	**% Recovery**	**Mean Recovery**
50%	1543793	37.5	37.52	101.9	
100%	3035883	75	75.1	101.4	
150%	4451005	112.5	112.47	99.4	100.9%

The accuracy results for Chlorthalidone

%Concentration (at specification Level)	**Area**	**Amount Added (ppm)**	**Amount Found (ppm)**	**% Recovery**	**Mean Recovery**
50%	1084420	15	15.07	100.2	
100%	2096069	30	29.6	99.4	
150%	3112684	45	44.8	99.5	99.6%

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Method is accurate.

LIMIT OF DETECTION

The detection limit of individual analytical process is the lowest amount of analyte in a sample that can be identified but not necessarily quantitated as an exact value.

LOD= 3.3 × σ / s

Where

σ = Standard deviation of the response S = Slope of the calibration curve

Cilnidipine response standard deviation

Concentration **g/ml**	**Average Peak Area**
25	1010252
50	2049374
75	3072706
100	3921068
125	4952813

σ = 58777.45

S= 39451

Chlorthalidone response standard deviation

Concentration **g/ml**	**Average** **Peak Area**
10	8040807
20	14318417
30	21087985
40	27913928
50	34584741

σ = 176374

S= 68375

Result:

Cilnidipine:

=3.3 × 58777.45/39451

=4.9µg/ml

Chlorthalidone:

=3.3 × 176374/68375

=8.5µg/ml

LIMIT OF QUANTITATION

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

LOQ=10×σ/S

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

Cilnidipine:

=10×58777.45/39451

= 14.8µg/ml

Chlorthalidone:
=10 × 176374/68375
= 25.7µg/ml

Robustness

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Cilnidipine and Chlorthalidone. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The standard samples of Cilnidipine and Chlorthalidone were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Variation in flow

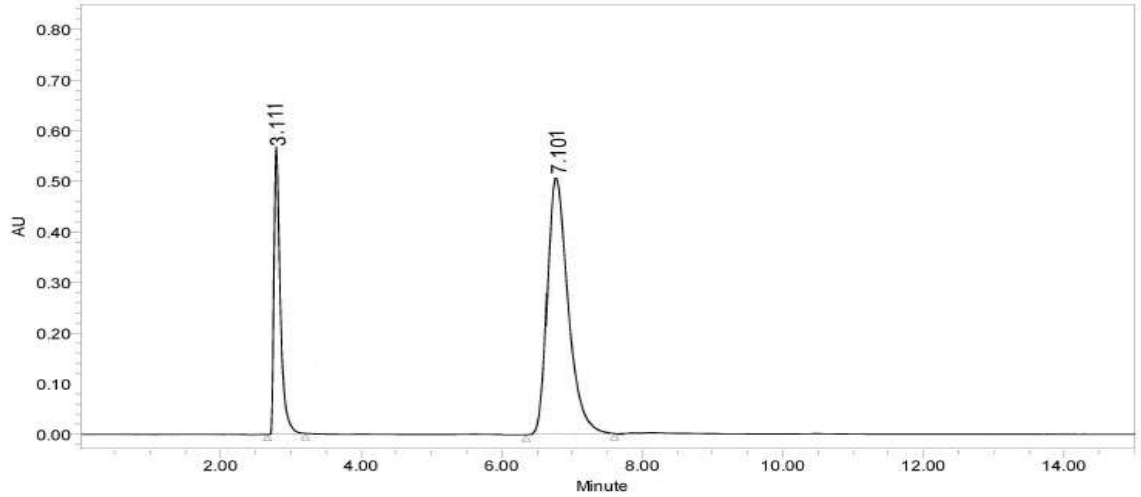


Figure: chromatogram showing less flow of 0.9ml/min

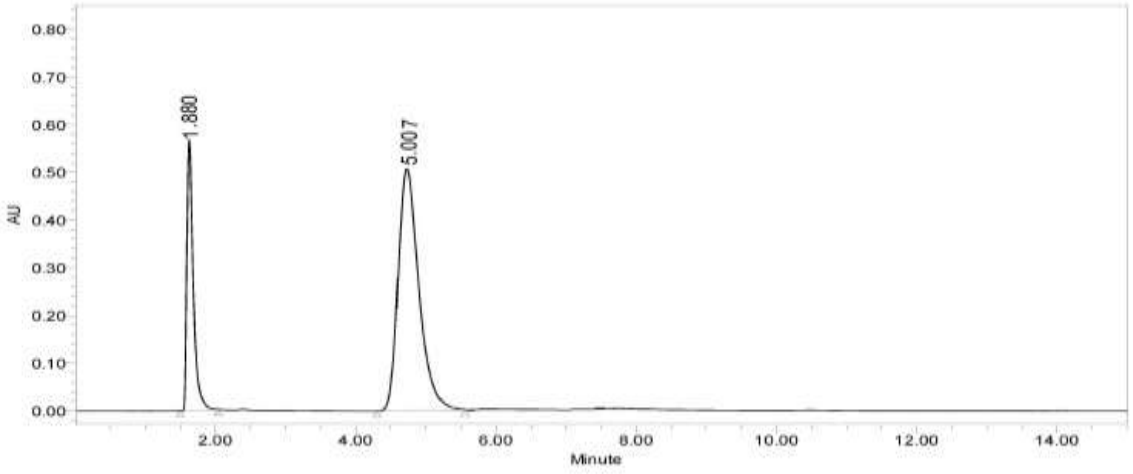


Figure: chromatogram showing more flow of 1.1 ml/min Variation of mobile phase organic composition

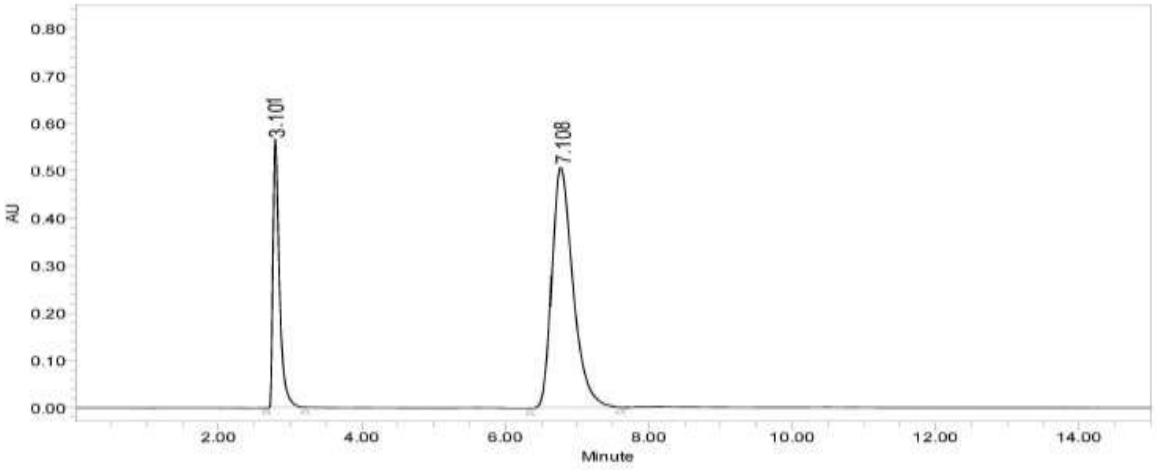


Figure: chromatogram showing less organic composition

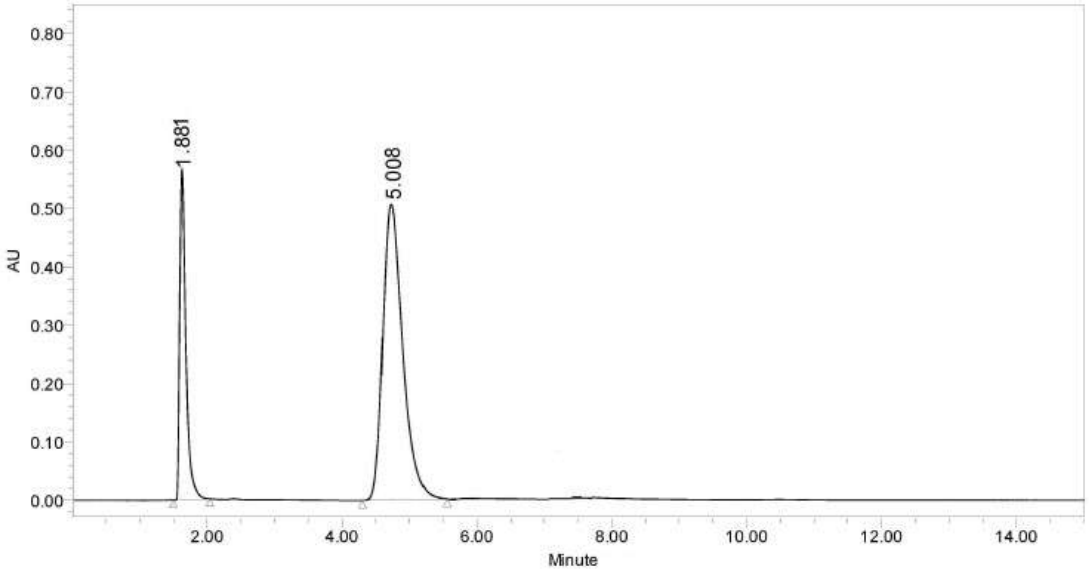


Figure: chromatogram showing more organic composition

Table: Results for Robustness CILNIDIPINE:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Flow rate of 1.0 mL/min	3425413	2.088	5568.2	1.0
Flow rate of 0.9 mL/min	3425282	3.111	5922.2	1.2
Flow rate of 1.1 mL/min	3517879	1.880	5868.8	1.2
Less aqueous phase	3175485	3.101	5836.2	1.2
More aqueous phase	3365431	1.881	5282.6	1.1

Acceptance criteria:
The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

CHLORTHALIDONE:

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Flow rate of 1.0 mL/min	2029854	6.068	5359.2	1.1
Flow rate of 0.9 mL/min	1738319	7.101	5999.1	1.2
Flow rate of 1.1 mL/min	1638304	5.007	5989.2	1.1
Less aqueous phase	1973724	7.108	5387.2	1.1
More aqueous phase	2102838	5.008	5938.1	1.1

VIII. SUMMARY

The analytical method was developed by studying different parameters. Absorbance maximum was found to be at 234nm and the peak purity was excellent.10µl Injection volume was selected which gave a good peak area. The column used for study was Altima C18 because good peak was obtained. The suitable temperature was found to be 35ºC for the nature of drug solution. The fixed flow rate was 1.0ml/min because of good peak area and satisfactory retention time.

Mobile phase is Acetonitrile, Methanol and Phosphate buffer pH4.6 (10:25:65 v/v) was fixed due to good symmetrical peak. This mobile phase was selected for the proposed study.

Run time selected was 14min because analyte gave peak around 2.088, 6.068 and also to reduce the total run time. The percentage recovery was found to be 98.0-102. It was linear and precise over the same range. System and method precision was found to be accurate and within the range.

The analytical method was found linearity over the range 25-125ppm of Cilnidipine and 10- 50ppm of Chlorthalidone of the target concentration.The analytical method passed both ruggedness and robustness tests.

IX. CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of cilnidipine and Chlorthalidone in bulk drug and pharmaceutical dosage form.Diluted samples are directly used without any preliminary chemical derivatisation process due to non complicated method.

Solubility of Cilnidipine and Chlorthalidone was in ethanol, methanol and sparingly soluble in water.
ACN, Methanol and Phosphate buffer pH4.6 (10:25:65 v/v) was chosen as the mobile phase. The solvent system which was used in this method is bearable economically.
The %RSD values was within 2 and method was found to be precise.
The results presented in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods.
This process could be used for the routine identification of Cilnidipine and Chlorthalidone in bulk drug and in Pharmaceutical dosage forms.

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