IJRAR.ORG E-ISSN: 2348-1269, P-ISSN: 2349-5138

INTERNATIONAL JOURNAL OF RESEARCH AND ANALYTICAL REVIEWS (IJRAR) | IJRAR.ORG

An International Open Access, Peer-reviewed, Refereed Journal

RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF CHLORTHALIDONE, CILNIDIPINE AND BISOPROLOL FUMARATE IN SYNTHETIC MIXTURE

[1*]Doshi Rutva, [2]Ronak Patel, [3]Divyakant Patel, [2]Jaymin Patel, [2]Bhumi Patel & [2]Ankita Patel

[*]Student, Pharmaceutical Quality Assurance, Sharda School of Pharmacy, Gandhinagar, Gujarat. 382610

[2]Associate Professor, Pharmaceutical Quality Assurance, Sharda School of Pharmacy, Gandhinagar, Gujarat. 382610

[3]Principal, Sharda School of Pharmacy, Gandhinagar, Gujarat. 382610

Abstract : A rapid, precise, accurate and robust RP-HPLC method for simultaneous estimation of Chlorthalidone, Cilnidipine and Bisoprolol fumarate was developed. Separation was achieved using Agilent Eclipse C18 150 x 4.6 x 5 µ column as the stationary phase and a mobile phase comprising of buffer and Methanol in gradient program setting. Analysis was conducted at a flow rate of 1.0 ml/min with detection at 231 nm using UV detector, while the column temperature was maintained at 25°C for optimal separation and resolution. Retention times for Chlorthalidone, Cilnidipine and Bisoprolol fumarate were 3.94, 4.77, and 11.50 min for Chlorthalidone, Bisoprolol fumarate and Cilnidipine respectively. The developed method was validated in accordance with ICH guidelines, demonstrating specificity, linearity and range, precision, robustness, and accuracy. The method exhibited linearity within concentration ranges of 6.25-18.75 µg/ml, 5-15 µg/ml, and 5-15 µg/ml with a correlation coefficients of 0.998, 0.999 and 0.999 for Chlorthalidone, Cilnidipine and Bisoprolol fumarate respectively. Precision and robustness showed % relative standard deviations of less than 2%. Accuracy ranged from 100.7-101.5%, 98.2-100.9%, and 98.9-100.6% for Chlorthalidone, Cilnidipine and Bisoprolol fumarate.

Keywords **:** RP-HPLC, method development, validation, Chlorthalidone, Cilnidipine, Bisoprolol fumarate, UV detector.

I. INTRODUCTION

Polypharmacy is a common issue among hypertensive patients, which can be effectively addressed through the use of fixed dose combination tablets of two or three drug molecules. By combining multiple medications into a single dosage form, FDCs promote better adherence and improved clinical outcomes.

Chlorthalidone, Cilnidipine, Bisoprolol fumarate belong to the drug class Thiazide diuretics, calcium channel blockers and beta-blockers respectively. Chlorthalidone is a removes extra water and certain electrolytes from the body by increasing the amount of urine produced.Cilnidipinerelaxes blood vessels and makes the heart more efficient at pumping blood throughout the body. Bisoprolol works specifically on the heart to slow down the heart rate. Combination therapy can be effective for management of coronary artery disease and hypertension.

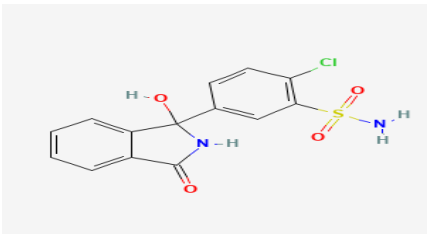

Fig.1 Structure of Chlorthalidone

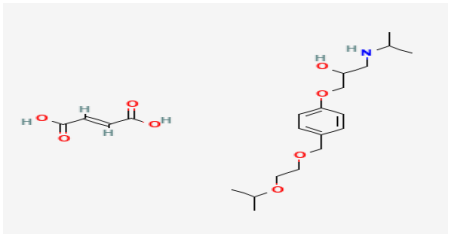

Fig.2 Structure of Bisoprolol Fumarate

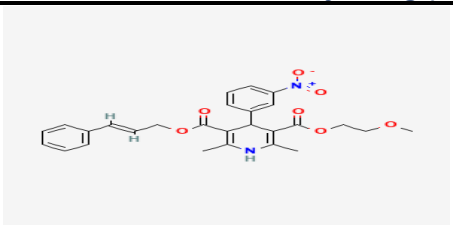

Fig.3 Structure of Cilnidipine

Chlorthalidone

2-chloro-5-(1-hydroxy-3-oxo-2,3-dihydro-1H-isoindol-1-yl)benzene-1-sulfonamide as Thiazide diuretic category its molecular weight 338.766 gram/mol Chlorthalidone prevents reabsorption of sodium and chloride through inhibition of the Na+/Cl- symporter in the cortical diluting segment of the ascending limb of the loop of Henley. Reduction of sodium reabsorption subsequently reduces extracellular fluid and plasma volume via an osmotic, sodium-driven diuresis. By increasing the delivery of sodium to the distal renal tubule, Chlorthalidone indirectly increases potassium excretion via the sodium-potassium exchange mechanism.

Cilnidipine

3-(2-methoxyethyl)5-(2E)-3-phenylprop-2-en-1-yl2,6-dimethyl-4-(3-nitrophenyl)-1,4dihydropyridine -3,5-dicarboxylate it is calcium channel blocker Cilnidipine acts on the L-type calcium channels of blood vessels by blocking the incoming calcium and suppressing the contraction of blood vessels, thereby reducing blood pressure. Cilnidipine also works on the N-type calcium channel located at the end of the sympathetic nerve, inhibiting the neither emission of nor epinephrine and suppressing the increase in stress blood pressure

Bisoprolol Fumarate

{1-[(propan-2-yl)amino]-3-(4-{[2-(propan-2-yloxy)ethoxy]methyl}phenoxy)propan-fromBeta blocker By blocking these receptors, Bisoprolol reduces the release of renin, thereby blocking the activation of the renin-angiotensin system. This dual action on the heart and kidneys makes Bisoprolol effective in managing hypertension and related conditions

II. MATERIAL & METHOD

Bisoprolol Fumarate, Chlorthalidone & Cilnidipine were procured from Cipla Limited, were procured from Torrent Pharmaceutical Limited.

HPLC grade reagent methanol, acetonitrile & milli-Q Water were used for study. AR grade reagent ortho-phosphoric acid were used for study.

HPLC method development and validation was done on a HPLC instrument (Make: Shimadzu, Model: LC 2010 CHT, Injector: 100µL fixed loop, Detector: UV Detector

Software: LC Solution), UV Detector (Shimadzu UV-1800 double-beam UV-VIS spectrophotometer, Software: UV Probe), Stationary Phase (Cosmosil C18 (250 x 4.6) 5 µm ID).

III. Experimental Work

Preparation of Buffer (pH5.5):

Buffer Preparation Weigh approximately 8.16 grams of dibasic ammonium phosphate and dissolved in 1000 mL HPLC grade water in a suitable beaker. Ensure complete dissolution through sonication and stirring. Adjust pH of the solution 5.5 with orthophosphoric acid.

Preparation of Mobile Phase

Mobile phase preparation: A mobile phase was used is a mixture of buffer and Methanol in gradient program setting.

Diluent Preparation:

Diluent: Accurately measured 750 ml (75%) of HPLC-grade Methanol and 250 ml of Phosphate Buffer (25%).Mixed and degassed the components in a digital ultrasonic bath for 15 minutes. Filtered the mixture through a 0.45 µm filter under vacuum

- **PREPARATION OF STANDARD SOLUTION:**

Chlorthalidone stock solution: To prepared stock solution, accurately weighed and transferred 12.5 mg of Chlorthalidone working standard into a 100 ml volumetric flask. Added about 50 ml of diluent and sonicated to dissolve, removing air completely. Adjusted the volume to the mark with the diluent.

Cilnidipine stock solution: Accurately weighed and transferred 10 mg of Cilnidipine working standard into a 100 ml volumetric flask. Added about 50 ml of diluent and sonicated to dissolve, removing air completely. Adjusted the volume to the mark with the diluent

Bisoprolol stock solution: Accurately weighed and transferred 24 mg of Bisoprolol fumarate working standard (equivalent to bisoprolol 10 mg, molecular weight correction factor is 0.424) into a 100 ml volumetric flask. Added about 50 ml of diluent and sonicated to dissolve, removing air completely. Adjusted the volume to the mark with the diluent.

To make standard solution aliquot of 1 mL each stock solutions were taken into 10 mL flask. Adjusted the volume to the mark with the diluent which yields 12.5 ppm of Chlorthalidone, 10 ppm of bisoprolol and 10 ppm of Cilnidipine.

SAMPLE PREPARATION :

Synthetic mixture equivalent to one tablet labeled as containing 12.5mg of Chlorthalidone, 10 mg of Cilnidipine and 10 mg of Bisoprolol fumarate was accurately weighed and finely powdered and taken into 100 ml of clean dry volumetric flask. Sample was extracted in 50 ml of Methanol by sonication for 15 minutes. Then it was filtered through 0.45 micron syringe filter. 1 ml aliquot of clear filtrate was then diluted upto 10 ml to yield concentration of 12.5 ppm of Chlorthalidone, 10 ppm of bisoprolol and 10 ppm of Cilnidipine.

- **Synthetic Mixture Preparation**

A few patents70-71 based on formulation of antihypertensives were studied and suitable fillers, binders, distributor and anti adhesives were chosen for making a synthetic mixture. Synthetic mixture formulation was prepared using following formula:

Table 1 Synthetic Mixture Preparation

Ingredient	Amount per Tablet
Bisoprolol fumarate IP equivalent to Bisoprolol	10 mg
Cilnidipine IP	10 mg
Chlorthalidone IP	12.5 mg
Lactose	200 mg
Povidone (PVP)	10 mg
Microcrystalline Cellulose	10 mg
Talc	5 mg

- **METHOD VALIDATION :**

1. **Linearity:** Linearity assessment involved preparing five linear concentrations ranging from 6 to 18 µg/mL of Chlorthalidone, 5 to 15 µg/mL for Cilnidipine and 5 to 15 µg/mL for bisoprolol (table –25-27), followed by injection into the HPLC system and recording the detector results.

2. **Specificity**: Specificity is the validation parameter used to assess potential interference in the optimized method. The absence of interfering peaks at the retention times of the drugs. To ensure the specificity of the method, the blank, placebo, standard, sample and individual standards of Chlorthalidone, Cilnidipine and Bisoprolol fumarate were injected separately and compared with each other.

3. **Precision** : The degree of agreement between individual test results when the procedure is applied repeatedly to multiple samplings is known as precision.

4. **Accuracy**: One is to examine a sample with a known concentration and compare the measured value to the "true" value. Referencing standards are required in this situation. Spiked-placebo recovery: This method includes an addition of the reference standard to the synthetic sample matrix (placebo). After chromatographing the mixture, the measured values are compared to the true values that were predicted. Standard addition technique: This method is used in situations where a placebo is unavailable. The first sample is analyzed using this technique. Subsequently, the sample is mixed with a known quantity of the reference standard (API) and reanalyzed. Calculated differences between the two assays are compared to the anticipated outcomes. Recovery in the last two methods is expressed as a percentage of the measured value to the expected values. There are three concentration levels in the standard range at which accuracy is always measured, i. e. 50, 100 and 150 percent of the standard concentration and three replicates of each.

5. **LOD and LOQ**: Low levels of LOD and LOQ establishes that the method is sensitive enough to detect and quantify low concentrations of Chlorthalidone, Cilnidipine, and bisoprolol.

6. **Robustness** System suitability parameters were tested under each varied conditions. All the parameters were found to be within the standard range. Therefore, the method is reliable and suitable for routine analysis even under slightly varied conditions, makes it robust and suitable for practical applications

IV. RESULTS & DISCUSSION

- **SELECION OF WAVELENGTH :**

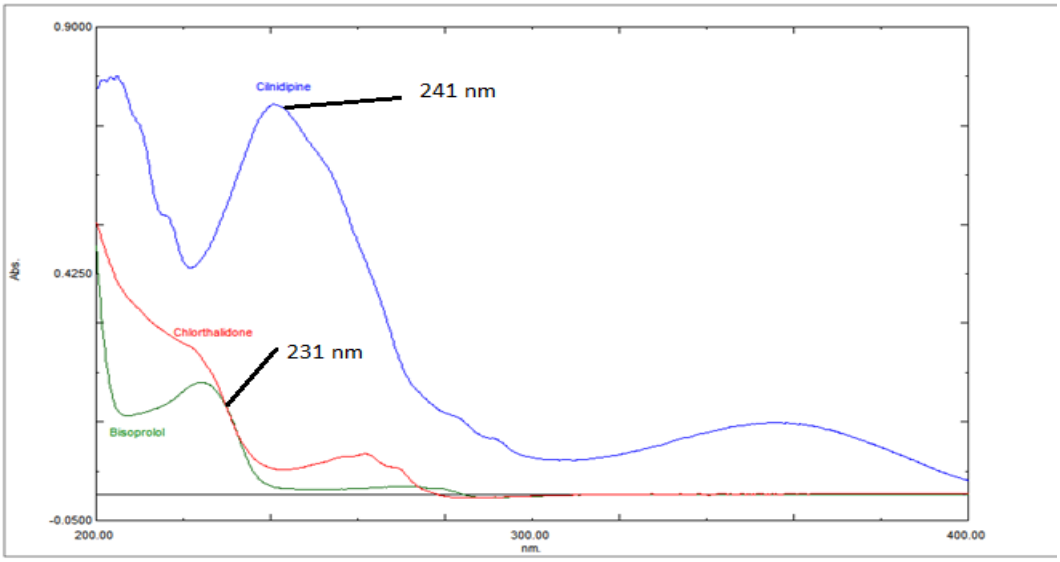

Fig.4 UV Spectra of Chlorthalidone Cilnidipine and Bisoprolol

Conclusion :
Isosbestic point for Chlorthalidone and Bisoprolol fumarate was found to be 231 nm and absorbance maxima of Cilnidipine was found to be 241 nm. Therefore, it was thought to set UV detector at around 231 nm and if the need be, we can optimize the wavelength to 241 as well.

- **CHROMATOGRAM OF FINAL TRIAL :**
  Injection: Mix standard
  Mode: Gradient
  Stationary phase: Agilent Eclipse C18 150 x 4.6 x 5 µ
  Mobile Phase :
  Mobile phase A : A mobile phase was used is a mixture of buffer and Methanol in gradient program setting..
  Mobile phase B : Methanol

Time in Minutes	A	B
0	50	50
5	15	85
10	15	85
11	50	50
16	50	50

Injection volume: 25 µL
Flow Rate: 1.0 mL/min
Column Temperature: 25 °C
Wavelength: 231nm

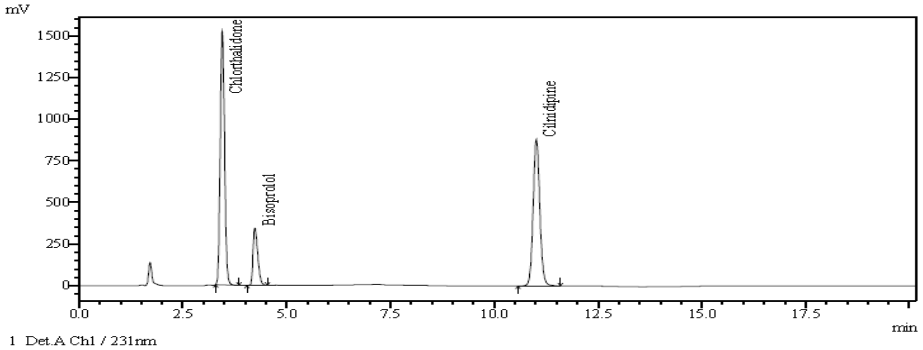

Fig.5 Chromatogram of Final

Table 2: Peak table of Final Trial

Peak#	Ret. Time	Name	Area	Area %	Resolution	T. Factor	T. Plate
1	3.444	Chlorthalidone	11137774	47.00	0.000	1.18	4599
2	4.232	Bisoprolol	2700186	11.40	3.740	1.50	6023
3	11.011	Cilnidipine	9857918	41.60	26.299	1.12	21931
Total			23695878	100.00			

- **VALIDATION OF RP-HPLC METHOD :**

1. System Suitability Study :

Table 3: System Suitability study data

Sr. No.	Area of Chlorthalidone	Area of Cilnidipine	Area of Bisoprolol fumarate
1	12906268	10532587	3171226
2	12966222	10532587	3118130
3	12963427	10510762	3129514
4	12999502	10451412	3124531
5	12978835	10451412	3121667
Mean	12962851	10495752	3133014
SD	23819.2	7151.53	23823.22
% RSD	0.1	0.4	0.4
Tailing Factor	1.18	1.14	0.7
Theoretical Plates	5497	23136	1.14
Retention Time	4.045	11.62	1.48

Observation : : System suitability test results revealed tailing factors less than 2 of Chlorthalidone, Cilnidipine and Bisoprolol fumarate. The chromatographic conditions described ensured satisfactory retention and resolution for three analysts, with retention times of 4.045, 11.62 and 4.87 minutes for Chlorthalidone, Cilnidipine and Bisoprolol fumarate respectively. USP plate counts were found to be greater than 2000 for all three compounds. %RSD for repeatable injection was less than 2 for all three compounds.
The outcomes from the system suitability tests align with the USP requirements of tailing factor less than 2, USP plate counts more than 2000, less than 2 %RSD and consistent retention times.

2. Specificity :

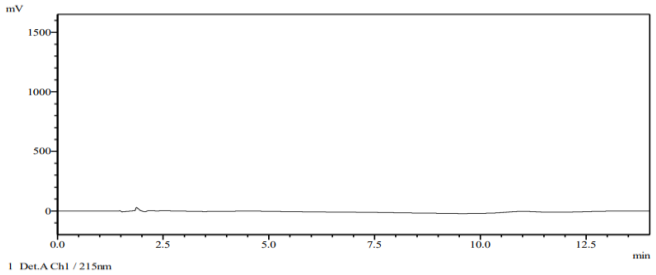

Fig.6: Chromatogram of Diluent for Specificity

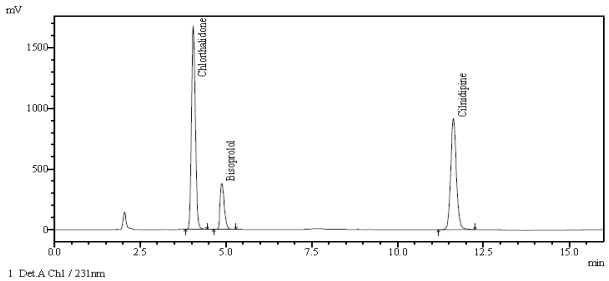

Fig.7: Chromatogram of Standard for Specificity

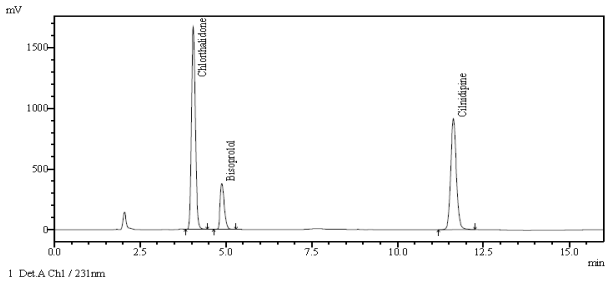

Fig. 8: Chromatogram of Sample for Specificity

Observation : When all the chromatograms were compared with each other, the target drugs peaks were observed to be without interference from other components. Therefore the developed method is specific.

3. Linearity and Range :

Table 4: Linearity data for Chlorthalidone, Cilnidipine &Bisoprolol Fumarate

	Chlorthalidone		Cilnidipine		Bisoprolol Fumarate	
Sr. No.	**Conc. (ppm)**	**Area (n = 5)**	**Conc. (ppm)**	**Area (n = 5)**	10.00	110892
1	6.25	6172419	5.00	4949269	5.00	1523563
2	9.38	8891070	7.50	7367295	7.50	2243993
3	12.50	12161196	10.00	9790873	10.00	2943093
4	15.63	14776102	12.50	11951770	12.50	3605282
5	18.75	17517502	15.00	14446597	15.00	4299577
Correlation (R)	0.99937		0.99980		0.99990	
Correlation co-efficient (R^2)	0.99874		0.99960		0.99980	
Y-Intercept	471843.01		269508.40		158452.99	
Slope	914398.87974		943165.24000		271450.20320	

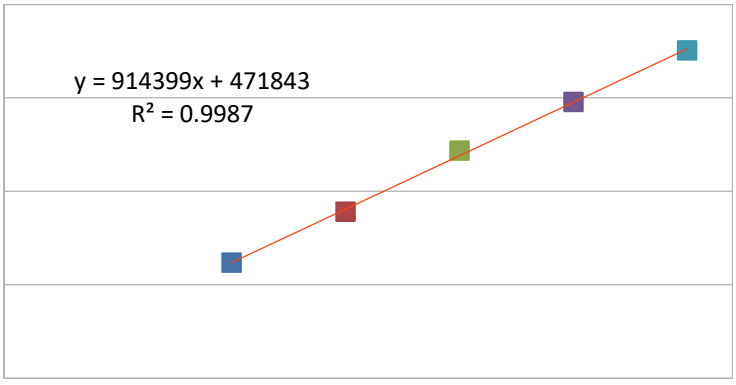

Fig. 9: Calibration curve of Chlorthalidone

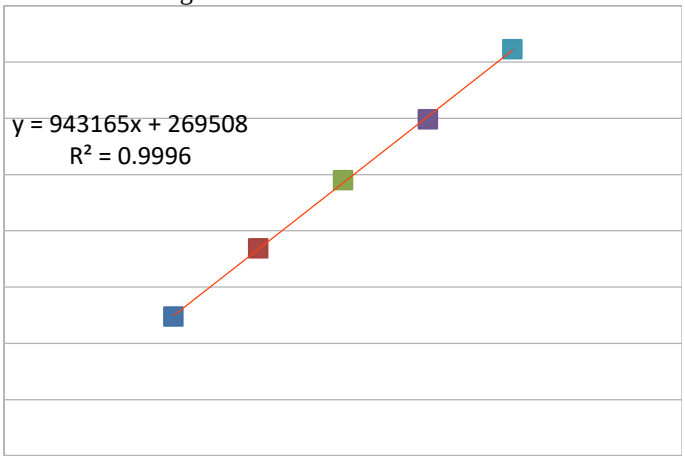

Fig. 10: Calibration curve of Cilnidipine

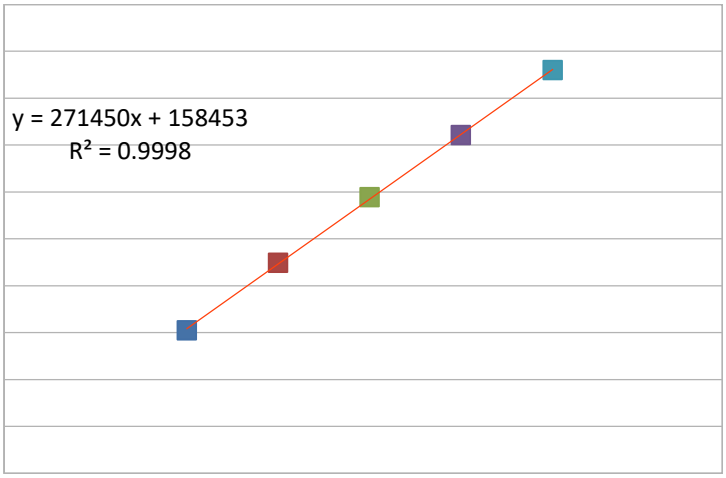

Fig. 11: Calibration curve of Bisoprolol Fumarate

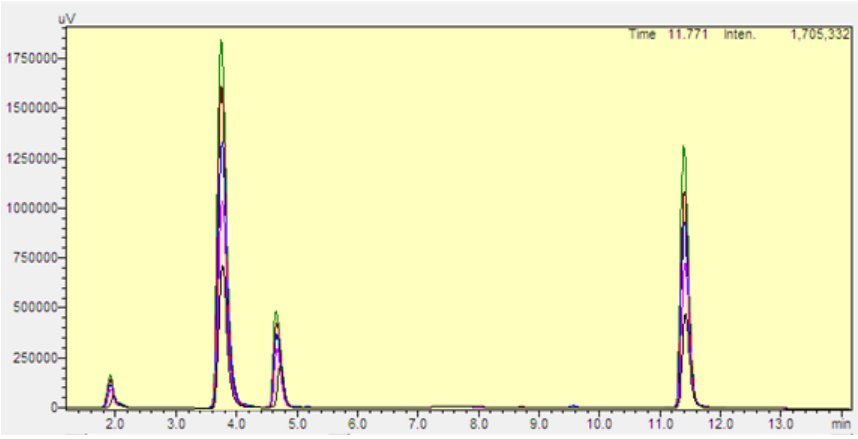

Fig. 12: Overlay Linearity chromatogram of Chlorthalidone , Cilnidipine and Bisoprolol Fumarate

Observation :

Correlation coefficients for Chlorthalidone, Cilnidipine and Bisoprolol fumarate were found to be 0.998, 0.999 and 0.999 respectively. The correlation coefficients are within the specified minimum value (≤0.998), validate the precision and reliability of the calibration curves. This indicates that the method is suitable for accurately quantifying Chlorthalidone, Cilnidipine and Bisoprolol fumarate concentrations within the broad linear ranges of 50% to 150% of the target concentration.

Summary of Validation Parameter

Table 5: Summary of Validation Parameter

Parameters	Chlorthalidone	Cilnidipine	Bisoprolol fumarate
System suitability			
Theoretical Plates (N)	5497	23136	1.14
Asymmetry	1.18	1.14	0.7
Retention Time	4.045	11.62	1.48
Precision			
Intraday (% RSD)	0.90	0.90	0.66
Intraday (% RSD)	0.06-0.50	0.15-0.88	.21-0.67
Robustness	Robust	Robust	Robust

Linearity			
Range (µg/ml)	6.25-18.75 µg/mL	5-15 µg/ml	5-15 µg/ml
Correlation co-efficient (R^2)	0.998	0.999	0.999
Accuracy (%)	100.7-101.5	98.2-100.9	98.9-100.6

V. CONCLUSION

A reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous quantification of Chlorthalidone, Bisoprolol fumarate and Cilnidipine in synthetic mixture. The amounts determined by the proposed method ranged from 96.9 to 99.1 %, 97.9-100.3 %, 97.5-101.1% for Chlorthalidone, Cilnidipine and Bisoprolol fumarate respectively, falling within the acceptance criteria of 95% to 105% (as per USP). The outcomes suggest that the proposed method is simple, accurate and specific. Linearity was observed across a concentration range of 6.25-18.75 µg/ml, 5-15 µg/ml, 5-15 µg/ml for standard Chlorthalidone, Cilnidipine and Bisoprolol fumarate. This project presents a gradient HPLC method utilizing UV detection, ensuring sufficient sensitivity for analysis of all three compound.

REFERENCES

I. Laurent, S., "Antihypertensive drugs," *Pharmacol Res*., **2017**, 124, 116-125.

II. NCD Risk Factor Collaboration (NCD-RisC). Worldwide trends in hypertension prevalence and progress in treatment and control from 1990 to 2019: a pooled analysis of 1201 population-representative studies with 104 million participants" *Lancet*. **2021**, 398(10304), 957-980.

III. WHO Guidelines, Guideline for the Pharmacological Treatment of Hypertension in Adults, accessed on August 24, **2021**https://www.who.int/publications/i/item/9789240033986

IV. Anchala, R., Kannuri, N. K., Pant, H., Khan, H., Franco, O. H., Di Angelantonio, E., &Prabhakar, D. "Hypertension in India: a systematic review and meta-analysis of prevalence, awareness, and control of hypertension." *Journal of Hypertension*, **2014**, 32(6), 1170-1177.

V. Lim, S. S., Vos, T., Flaxman, A. D., Danaei, G., Shibuya, K., Adair-Rohani, H., & Lopez, A. D. "A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010." *The Lancet*, **2012**, 380(9859), 2224-2260.

VI. Belleza Marianne, Accessed on July 21, **2023**, https://nurseslabs.com/hypertension/

VII. Dr. Raghu, High Blood Pressure (Hypertension) Symptoms, Causes & Treatment, https://drraghu.com/services/conditions/high-blood-pressure

VIII. V.R. Meyer, In Encyclopedia of Analytical Science, 2nd Edn. Elsevier publications, **2005**, 99-104