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STABILITY INDICATING RP - HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF AMLODIPINE AND CHLORTHALIDONE IN BULK AND TABLET DOSAGE FORM

P. H. Sakpal [*1] and A. R. Chabukswar [2]

Department of Pharmaceutical Chemistry [1], Marathwada Mitra Mandal's College of Pharmacy, Off Kalewadi Phata Pimpri Road, Thergaon (Kalewadi), Pune - 411033, Maharashtra, India.
Department of Pharmaceutical Chemistry [2], School of Pharmacy, Dr. Vishwanath Karad MIT World Peace University, Kothrud, Pune - 411038, Maharashtra, India.

Keywords:

Chlorthalidone, Amlodipine, RP-HPLC, ICH Guidelines, Regression coefficient

Correspondence to Author:
Mr. Pramod H. Sakpal

Assistant Professor, Department of Pharmaceutical Chemistry, Marathwada Mitra Mandal's College of Pharmacy, Off Kalewadi Phata Pimpri Road, Thergaon (Kalewadi), Pune - 411033, Maharashtra, India.

E-mail: psakpal18@gmail.com

ABSTRACT: The RP-HPLC stability-indicating assay method has been developed and validated for the estimation of the amlodipine and chlorthalidone in bulk and combined dosage form. The method was optimized by using the mobile phase as a mixture of 0.1% formic acid: methanol: acetonitrile in the ratio of (50:5:45 v/v) at pH 3 was adjusted with orthophosphoric acid. The method was carried out on the Octadecylsilane C18 column (5 µm, 25 cm × 4.6 mm) using a flow rate of 1.0 ml per min. The method was scanned at λ_{max} 266 nm for both the drugs using a PDA detector. The retention time was found to be at 6.32 min and 5.32 min for AML and CHL respectively. The calibration curve determined at respective retention time is found to be 2.5-7.5 µg/ml and 06-18 µg/ml with a regression coefficient of 0.9990 and 0.9940 for AML and CHL respectively. The developed and validated method is reliable, simple, precise and accurate and easy to apply in the laboratories.

INTRODUCTION: Amlodipine besylate is a second-generation calcium channel blocker that is used in the therapy of hypertension and angina pectoris. Chemically, it is 3-O-ethyl 5-O-methyl 2-(2- aminoethoxymethyl)- 4- (2- chlorophenyl)- 6-methyl-1,4-dihydropyridine-3,5-dicarboxylate [1, 2] **Fig. 1**. Chlorthalidone is a long acting thiazide-like diuretic of the sulfamoylbenzamide class that is devoid of the benzothiadiazine structure.

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Chlorthalidone directly inhibits sodium and chloride reabsorption on the luminal membrane of the early segment in the distal convoluted tubule (DCT) in the kidney. This leads to an increase in sodium, chloride, bicarbonate, and potassium secretion resulting in the excretion of water. In addition, this agent, like other thiazide diuretics, decreases calcium and uric acid secretion. Chemically it is (RS) 2-chloro-5-(1-hydroxy-3-oxo-2, 3- dihydro- 1, hisoindol- 1- yl) benzene- 1-sulfonamide [3, 4] **Fig. 2**.

A literature survey for amlodipine and chlorthalidone was done. It showed that the estimation of Amlodipine individually and in combined dosage forms with other APIs like metoprolol [5], telmisartan [6], valsartan [7], hydrochlorthiazide [8], losartan [9], olmesartan [10] and

chlorthalidone [11] was performed. Similarly, the chlorthalidone was also estimated individually and in combinations with atenolol [12], clonidine [13], olmesartan [14], aniloride [15] and telmisartan [17] like drugs by using either UV-spectrophotometry or RP-HPLC. Hence, there is scope for development and validation of stability-indicating assay method using RP-HPLC for estimation of amlodipine and chlorthalidone in combined dosage. The developed method is then validated as per ICH guidelines [17].

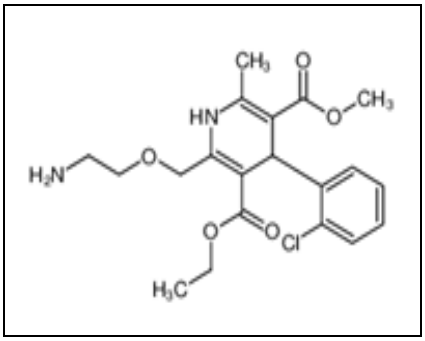

FIG. 1: STRUCTURE OF AMLODIPINE

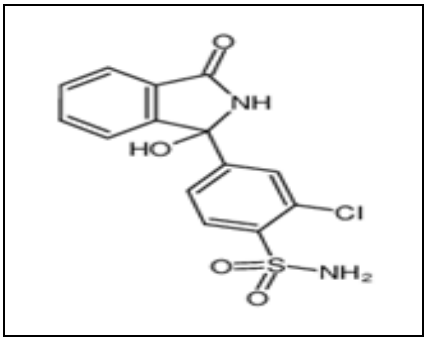

FIG. 2: STRUCTURE OF CHLORTHALIDONE

MATERIALS AND METHODS: The gift samples of amlodipine and chlorthalidone were obtained as a gift sample from IPCA Laboratories, Mumbai, India. The combined dosage form Amlodac-CH (Brand: Zydus Cadila, India, labeled claim Amlodipine 5.0 mg and Chlorthalidone 12.5 mg make Glenmark). The HPLC grade solvents used like methanol, formic acid and acetonitrile were procured from Research Lab, Pune. The Double Distilled water HPLC grade was prepared in the laboratory.

Instrumentation: The HPLC used for method development was of Agilent Technologies, India Ltd equipped with a universal loop injector (Rheodyne), PDA detector. The column used was C18 (150 mm × 4.6 mm, 5.0 µ) and the Injection volume was 20µL. The separation of both the drugs was carried out at 250 nm.

Mobile Phase: The mobile phase used was mixture of 0.1% formic acid: methanol: acetonitrile in the ratio of (50:5:45 v/v) at pH 3 was adjusted with orthophosphoric acid, the flow rate of 1.0 ml/min. The pH was adjusted with Ortho Phosphoric Acid and checked with pH meter to make Equiptronics.

Preparation of Standard Solutions: The powder equivalent to 100 mg of AML and CHL were weighed separately, taken in 100ml clean and dry volumetric flask and volume were made up with a mobile phase to give concentration 1000 µg/ml. The 10 ml of the stock solution of AML and CHL separately was pipetted out in a 100 ml volumetric flask and made-up the volume with the mobile phase to give concentration 100 µg/ml.

Linearity Study: The linearity for amlodipine was obtained by taking 0.25 ml, 0.30 ml, 0.5 ml, 0.70 ml and 0.75 ml in 10 ml volumetric flask and for chlorthalidone by taking 0.6 ml, 0.9 ml, 1.2 ml, 1.5 ml and 1.8 ml in 10 ml volumetric flask and made up the volume with mobile phase to give concentration 2.5-7.5 µg/ml and 6-18 µg/ml respectively. The injection volume was 20 µl. All the drug concentrations were repeated for three determinations and a calibration curve was constructed.

Validation of Proposed Method: The developed method is validated as per ICH guidelines.

Accuracy: The recovery study was performed at 50%, 100%, and 150% level studies. The accuracy was studied by the addition of a known amount of standard AML and CHL to a pre-analyzed sample of both the drugs and the RP-HPLC method was developed.

Precision: The Precision was studied by taking the same concentration and results were observed by taking chromatograms for calculations. The concentration found for amlodipine and chlorthalidone was 05 µg/ml and 06 µg/ml respectively. The results were obtained for 5 determinations.

Intraday and Interday Precision: The Intraday precision was obtained by analyzing, the three different concentrations 2.5µg/ml, 3.0 µg/ml and 5.0 µg/ml of AML and 06 µg/ml, 09 µg/ml, 12 µg/ml CHL for three determinations in the same

day. The Interday precision was observed by analyzing day to day variability using the above mentioned concentrations on three different days, over a period of one week.

Repeatability: It is measured by multiple injections of a homogenous sample of 05 µg/ml of AML and 06 µg/ml of CHL. It performs the instrument under HPLC chromatographic conditions.

Robustness: The robustness was observed by changing a few parameters including flow rate using 3 µg/ml of AML and 06 µg/ml of CHL.

Sensitivity: The sensitivity was determined by determining the limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ values are determined by using the SD and slope of the regression equation for the respective compound.

Specificity and Selectivity: The specificity is estimated to detect the quantity of the analyte in the presence of component that may be expected to be present in the sample matrix,

Ruggedness: The sample solutions 3 µg/ml of AML and 06 µg/ml of CHL were prepared and analyzed by two different analysts using similar operational and environmental conditions. Peak area was measured for the same concentration solutions, three times.

System Suitability Test: The system suitability testing parameters were observed for the chromatographic system and chromatographic conditions for developed methods.

Analysis of Pharmaceutical Formulation: To determine the contents of drugs in conventional tablets (Brand: Amlodac-CH, India of Zydus Cadila labeled claim amlodipine hydrochloride 5 mg and chlorthalidone 12.5 mg per tablet). The 20 tablets were weighed, their average weight was determined and they were finely powdered. Powder equivalent to 100 mg AML and CHL separately was weighed and transferred into a 100 ml volumetric flask and volume was made up with mobile phase.

The resulting solution was sonicated for 30 min and then filtered, using a 0.45 µm filter. Excipients were separated by filtration. The solution was further diluted with an optimized mobile phase to get a concentration 3 µg/ml of AML and 06 µg/ml of CHL which were subjected to proposed method and amount of AML and CHL were determined.

RESULTS AND DISCUSSION:

Optimization of Chromatographic Conditions: In the developed method optimum resolution is obtained by using the column Octadecylsilane -C18 column (5 µm, 25 cm × 4.6 mm, i.d.). The mobile phase used was a mixture of 0.1% formic acid: methanol: acetonitrile in the ratio of (50:5:45 v/v) at pH 3. The pH was adjusted with Ortho Phosphoric Acid. The separation of Amlodipine was obtained at retention Time 6.32 and 5.32 for AML and CHL respectively at a flow rate of 1.0 ml/min. The chromatogram was obtained at a maximum wavelength of 266 nm. The injection volume of 20 µL and ambient temperature was maintained during the entire separation of AML and CHL. The calibration curves were obtained at their respective retention time with good resolution. This developed method was found to be specific and validated as per ICH guidelines.

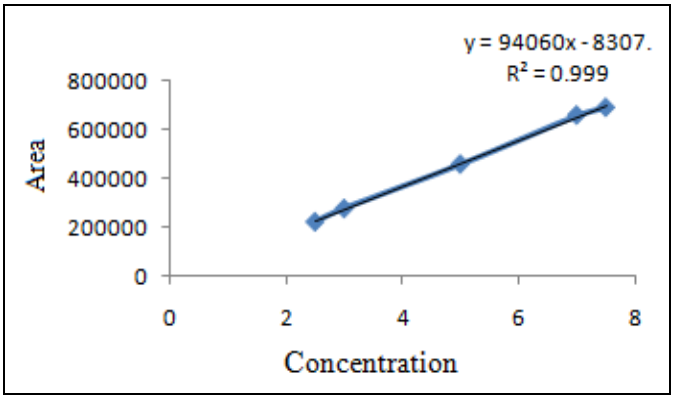

FIG. 3: CALIBRATION CURVE OF AMLODIPINE

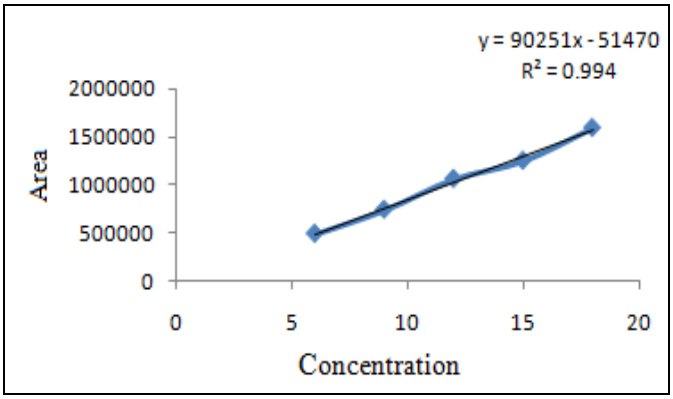

FIG. 4: CALIBRATION CURVE OF TENELIGLIPTIN

Linearity Study: Linearity was studied by preparing standard solutions at different concentration levels. The linearity range for amlodipine and chlorthalidone were found to be 2.5-7.5 µg/ml and 06-18 µg/ml respectively **Table 1**. y = 94060x - 8307 and y = 90251x – 51470 with correlation coefficient (R2) 0.9990 and 0.9940 respectively as shown in the **Fig. 3** and **4**.

TABLE 1: LINEARITY FOR AMLODIPINE AND CHLORTHALIDONE

Amlodipine			Chlorthalidone		
Conc. (µg/ml)	**Mean peak area ± SD (n = 3)**	**% RSD**	**Conc. of (µg/ml)**	**Mean peak area ± SD (n = 3)**	**% RSD**
2.5	223658	0.030414	10	495895	0.068569
3	278105	0.108870	15	745896	0.076496
5	458965	0.071456	20	1065895	0.044401
7	658966	0.712493	25	1254789	0.139034
7.5	690258	0.046256	30	1595206	0.049631

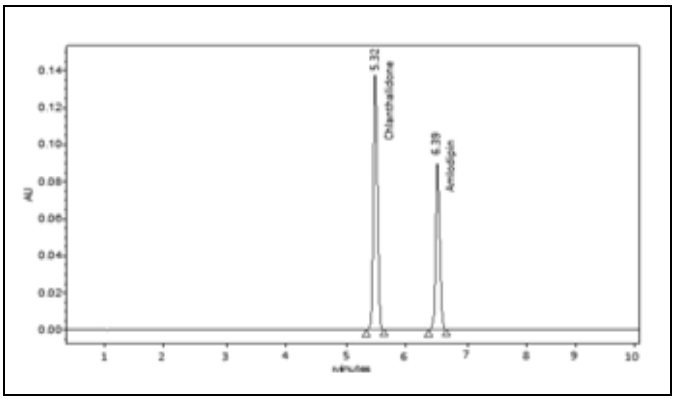

FIG. 5: CHROMATOGRAM OF STANDARD AMLODIPINE AND CHLORTHALIDONE

Method Validation:

Accuracy: To check the degree of accuracy of the method, recovery studies were performed in triplets by standard addition method at 50%, 100% and 150% concentration levels. Known amounts of standard AML and CHL were added to the pre-analyzed samples and were subjected to the proposed HPLC method. The % recovery was found to be within the limits of the acceptance criteria with an average recovery of 98.75 - 99.61% for AML and 96.58 - 103.21% for CHL. The result of recovery studies is shown in **Table 2**.

TABLE 2: RECOVERY STUDY OF AMLODIPINE AND CHLORTHALIDONE

	Level of recovery study	**Initial amount (µg/ml)**	**Amount added (µg/ml)**	**Amount recovered %**	**% Recovery**	**S.D. (n = 3)**	**% RSD**
Amlodipine	50%	3	4.5	4.48	99.61	0.077675	0.077976
	100%	3	6	5.94	99.15	0.095394	0.096212
	150%	3	7.5	7.40	98.75	0.01	0.010127
Chlorthalidone	50%	6	9	8.83	98.12	0.049329	0.050271
	100%	6	12	12.38	103.21	0.045826	0.044401
	150%	6	15	14.48	96.58	0.134288	0.139034

Precision: Precision was evaluated by carrying out six independent sample preparations of a single sample and validated as per ICH guidelines. The intra-day and inter-day precision were carried out in the same manner as described in sample preparation. Percentage relative standard deviation (% RSD) was found to be less than 2% that proves the method is precisely shown in **Tables 3** and **4**.

TABLE 3: PRECISION OF THE AML AND CHL

	Initial amount (µg/ml)	**Amount recovered (µg/ml)**	**Mean (n=5)**	**S.D.**	**% RSD**
Amlodipine	3	3.04	101.59	0.067823	0.066762
Chlorthalidone	6	6.06	101.05	0.026458	0.026183

TABLE 4: PRECISION STUDY INTRADAY AND INTERDAY

	Intra-day				Inter-day		
	Conc.	**Amt. recovered**	**S.D. (n = 3)**	**RSD**	**Amt. recovered**	**S.D. (n = 3)**	**RSD**
AML	3	3.05	0.7014	0.4078	3.05	0.08832	0.08684
CHL	6	6.06	0.1509	0.149309	6.07	0.01527	0.015098

TABLE 5: SENSITIVITY STUDY OF AML AND CHL

Analyte	**Slope**	**S. D. (n = 3)**	**LOD**	**LOQ**
AML	94060	0.322806	1.0295×10^{-5}	3.43192×10^{-5}
CHL	94060	0.0749	1.07×10^{-5}	7.96×10^{-6}

Sensitivity: LOQ and LOD can be determined based on the visual evaluation, signal-to-noise approach and standard deviation of the response and slope. Limit of detection of AML and CHL was determined 7.0846 and 2.3173, respectively. Limit of quantitation of AML and CHL was determined 0.00021 and 7.7246, respectively **Table 5**.

Specificity and Selectivity: The method is also validated for the parameters like specificity and selectivity. There is no signal to noise ratio is observed.

System Suitability Test: System suitability parameters were observed like tailing factor, capacity factor, and theoretical plates for AML and CHL were in the acceptance criteria as per the ICH guidelines.

Analysis of Pharmaceutical Formulation: The assay was performed for both the drugs and the percentage content of AML and CHL in the tablet formulation was determined in the range as per ICH guidelines **Table 6**.

TABLE 6: ANALYSIS OF TABLET FORMULATION

	Label claim (mg)	Amount found (mg)	Mean %	S.D. (n = 3)	RSD
AML	5.0	5.04	101.28	0.1928	0.189739
CHL	12.5	12.60	100.95	0.005774	0.005719

Forced Degradation Study: The forced degradation studies were carried out for the combination and individually as per the ICH guidelines by acid degradation, alkali degradation, hydrogen peroxide degradation, photolytic degradation, and thermal degradation.

The amlodipine and chlorthalidone were dissolved separately in the mobile phase and standard stock solutions were prepared and performed for forced degradation studies under different conditions. The degradation studies for all conditions are as shown in **Fig. 6** to **15** and the results related to the peaks are tabulated in **Table 7.**

Acid Degradation Studies: Acid degradation studies were carried out by taking 25 mg of the AML and CHL in a 25 ml volumetric flask and made up the volume with distilled water to give concentration 1000µg/ml. The AML and CHL acid degradation studies were carried out by further pipetting out 5 ml of this stock solution and 5 ml 0.5 M HCl is added into it. The solution is refluxed for 01 h and 40 min. on a water bath at 70 ºC. The refluxed solution is then added in a 10 ml volumetric flask and made up the volume with methanol. The chromatogram for acid degradation studies for AML and CHL are as follows.

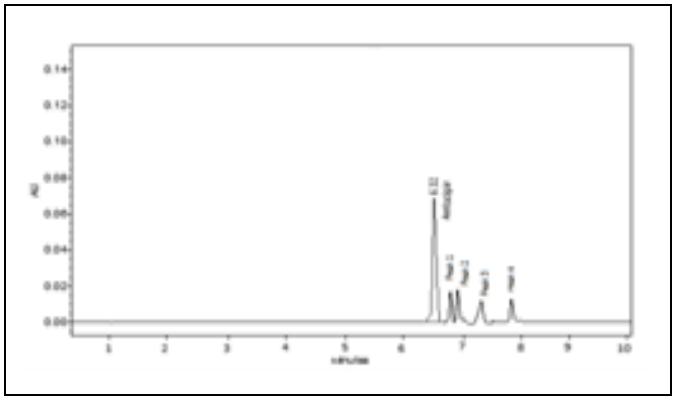

FIG. 6: ACID DEGRADATION OF AMLODIPINE

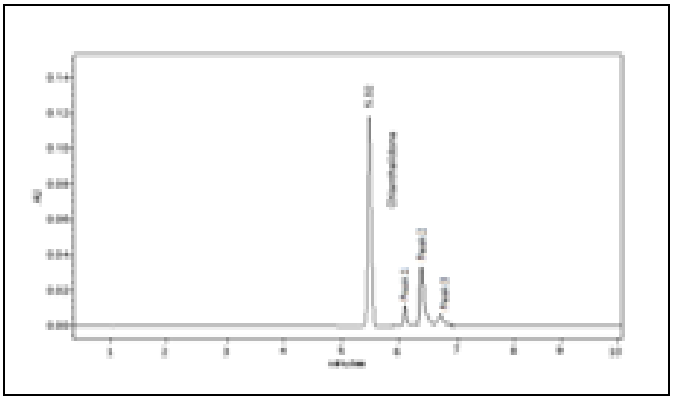

FIG. 7: ACID DEGRADATION OF CHLORTHALIDONE

Base Degradation Studies: Base degradation studies were carried out by taking 25 mg of the AML and CHL in a 25 ml volumetric flask and made up the volume with distilled water to give concentration 1000µg/ml. The AML and CHL acid degradation studies were carried out by further pipetting out 5 ml of this stock solution and 5 ml 0.5 M NaOH is added into it. The solution is refluxed for 01 h and 40 min. on a water bath at 70 ºC. The refluxed solution is then added in a 10 ml volumetric flask and made up the volume with methanol. The chromatogram for acid degradation studies for AML and CHL are as follows.

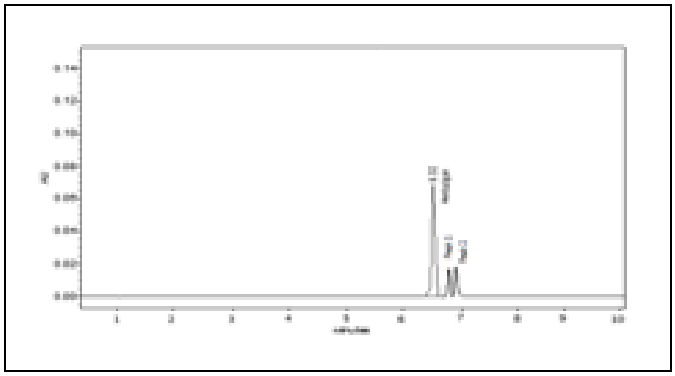

FIG. 8: BASE DEGRADATION OFAMLODIPINE

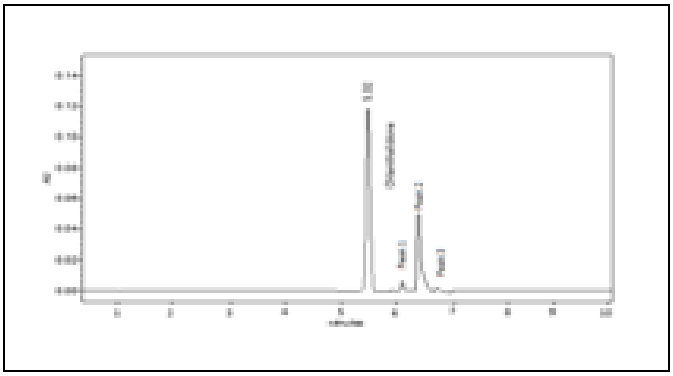

FIG. 9: BASE DEGRADATION OFCHLORTHALIDONE

Oxidative Degradation Studies: Oxidative degradation studies were carried out by taking 25 mg of the AML and CHL in a 25 ml volumetric flask and made up the volume with distilled water to give concentration 1000 µg/ml. The AML and CHL oxidative degradation studies were carried out by further pipetting out 5 ml of this stock solution and 5 ml 3% v/v hydrogen peroxide solution is added into it. The solution is refluxed for 01 h and 40 min. on a water bath at 70 ºC. The refluxed solution is then added in a 10 ml volumetric flask and made up the volume with methanol. The chromatogram for oxidative degradation studies for MET and TEN are as follows.

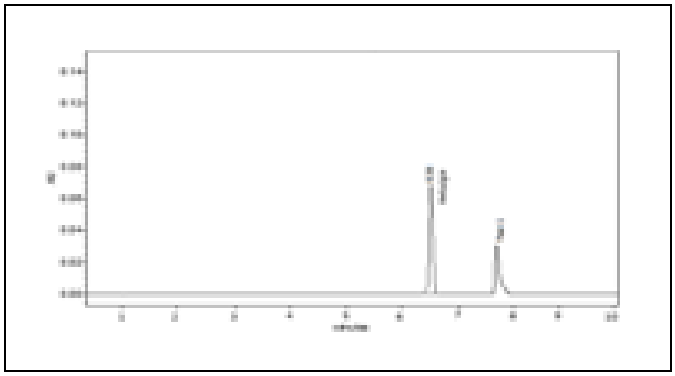

FIG. 10: OXIDATIVE DEGRADATION OF AMLODIPINE

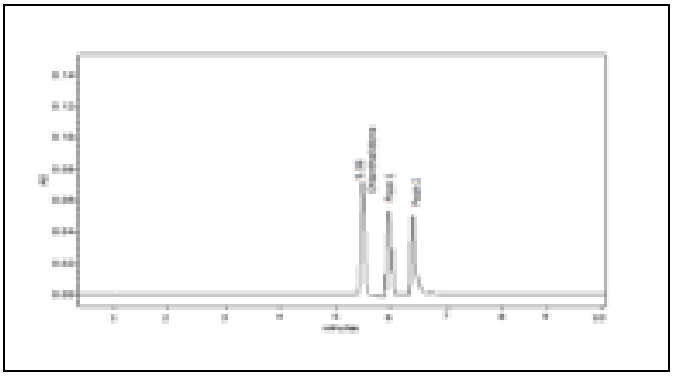

FIG. 11: OXIDATIVE DEGRADATION OF CHLORTHALIDONE

Thermal Degradation Studies: Thermal degradation studies were carried out by taking 25 mg of the TEN and MET in 25 ml volumetric flask and made up the volume with distilled water to give concentration 1000 µg/ml. The AML and CHL thermal degradation studies were carried out by further pipetting out 5 ml of this stock solution and 5 ml Distilled water is added into it. The solution is refluxed for 01 h and 40 min. on a water bath at 70 ºC. The refluxed solution is then added in a 10 ml volumetric flask and made up the volume with methanol. The chromatogram for oxidative degradation studies for AML and CHL are as follows.

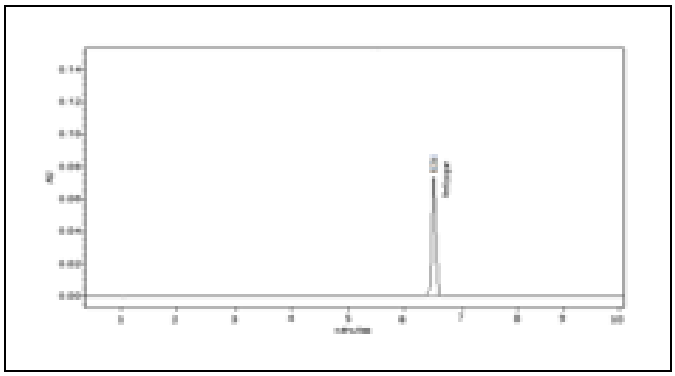

FIG. 12: THERMAL DEGRADATION OF AMLODIPINE

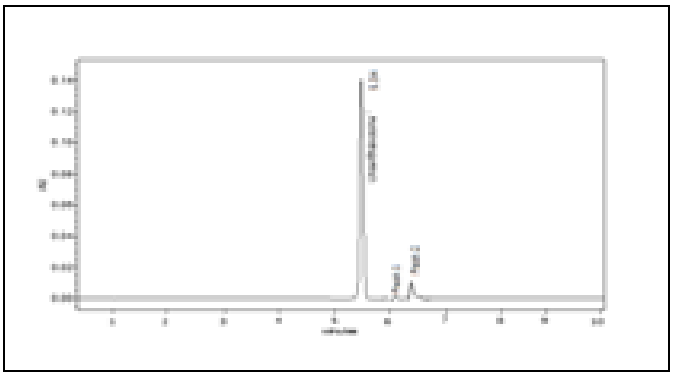

FIG. 13: THERMAL DEGRADATION OF CHLORTHALIDONE

Photolytic Degradation Studies: Photolytic degradation studies were carried out by taking 25 mg of the AML and CHL in a 25 ml volumetric flask and made up the volume with distilled water to give concentration 1000µg/ml. The AML and CHL photolytic degradation studies were carried out by further pipetting out 5 ml of this stock solution and 5 ml distilled water is added into it. The solution is kept for 24 h under UV light. The photolytic solution is then added in a 10 ml volumetric flask and made up the volume with methanol. The chromatogram for oxidative degradation studies for AML and CHL are as follows.

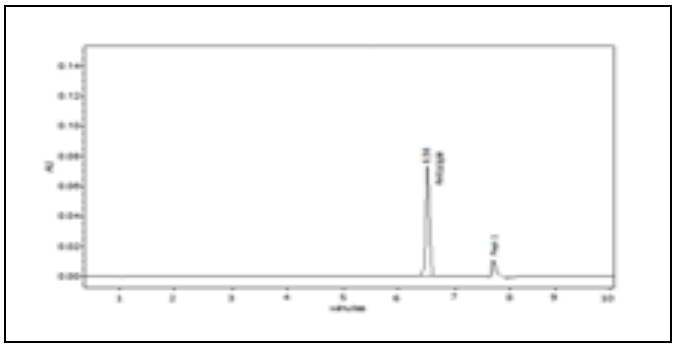

FIG. 14: PHOTOLYTIC DEGRADATION OF AMLODIPINE

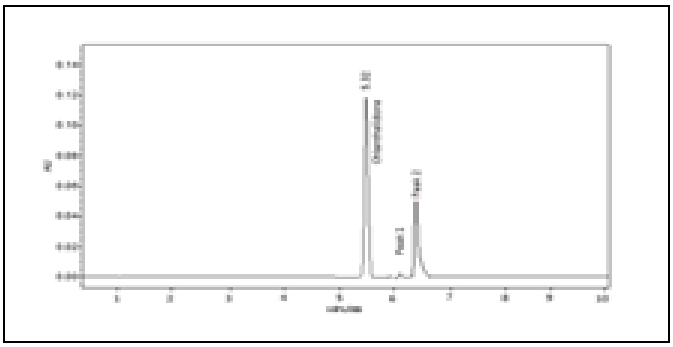

FIG. 15: PHOTOLYTIC DEGRADATION FOR CHLORTHALIDONE

TABLE 7: FORCED DEGRADATION STUDY FOR AMLODIPINE AND CHLORTHALIDONE

Analyte	API	Degradation Conditions	% Degradation	Retention time of Degradation Product
Acid	AML	01 h 40 min 70 ºC	9.47, 9.46, 7.79, 6.51	6.90, 6.95, 7.24, 7.91
Degradation	CHL	01 h 40 min 70 ºC	7.77, 12.69, 6.36	6.12, 6.59, 6.88
Base	AML	01 h 40 min 70 ºC	7.78, 7.77	6.91, 6.99
Degradation	CHL	01 h 40 min 70 ºC	3.99, 1.78, 3.99	6.13, 6.66, 6.91
Oxidative	AML	01 h 40 min 70 ºC	18.25	7.91
Degradation	CHL	01 h 40 min 70 ºC	45.70, 39.54	6.05, 6.56
Thermal	AML	01 h 40 min 70 ºC	--	--
Degradation	CHL	01 h 40 min 70 ºC	0.25, 1.20	6.14, 6.53
Photolytic	AML	24 h	7.54	7.89
Degradation	CHL	24 h	0.29, 24.01	6.15, 6.67

CONCLUSION: Here, an attempt was to develop the stability-indicating assay method for the estimation of Amlodipine and Chlorthalidone in combined dosage form by using RP-HPLC. The mobile phase was optimized for better separation of the amlodipine and chlorthalidone with good resolution at flow rate of 1.0 ml per minute. The separation was obtained by scanning at 266 nm using a PDA detector. The linearity was obtained with a good correlation coefficient at their respective retention time. The developed method was validated as per ICH guidelines by using statistical parameters.

This was followed by thermal degradation of both the drugs under different forced degradation conditions like acid, base, oxidative, thermal and photolytic study. The reliable, specific, reproducible and efficient method is developed and validated which can be easily acceptable for laboratory applications.

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CONFLICTS OF INTEREST: The authors alone are responsible for the content and writing of this article; hence there is no conflicts of interest.

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