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

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Keywords:

Retention times, Amlodipine, Chlorthalidone, Pharmaceutical, chromatographic technique

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ABSTRACT: Background: To reduce the run and retention times of amlodipine and chlorthalidone in pharmaceutical dosage forms, a simple, affordable, time-efficient, stability-indicating, and accurate reverse-phase liquid chromatographic technique was developed. Successful chromatographic separation was achieved using Inertsil ODS-3V (250 x 4.6 mm, 5 µm particle size). A gradient consisting of water (pH 3 adjusted with formic acid) and acetonitrile was used as the mobile phase. A 20 µl injection volume was utilized, and a flow rate of 1 ml per minute was observed. The wavelength of detection was 365 nm. Method validation followed ICH norms. The medication was also found to be stable under all stress scenarios. The observed retention times for amlodipine were 1.8 minutes and chlorthalidone was 4.5 minutes. The linearity range was observed in a concentration of 50 - 150%. The percentage recovery of Chlorthalidone was 99.30% and for Amlodipine was 99.61%. The suggested technique was successfully tested and used for the estimation of amlodipine and chlorthalidone in pharmaceutical dose forms.

INTRODUCTION: Chlorthalidone is used to treat edema and hypertension, or high blood pressure. It addresses fluid excess (edema) brought on by lung, liver, kidney, or cardiac conditions. As a diuretic, chlorthalidone reduces blood pressure by flushing out excess water and specific electrolytes from the body. Additionally, it enhances blood flow and relaxes blood arteries over time. Inhibiting the reabsorption of sodium and chloride ions in the kidney's distal renal tubules is how chlorthalidone lowers blood pressure and reduces edema by increasing the excretion of water, sodium, and other electrolytes in the urine.

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Amlodipine is a drug that is often used to treat angina (chest discomfort) and hypertension (high blood pressure). It is a member of the calcium channel blocker medication class, which relaxes and widens blood vessels to facilitate the heart's blood-pumping action. This lessens the strain on the heart and lowers blood pressure. Amlodipine functions by obstructing calcium channels in the heart's and blood vessels' smooth muscle cells. Blood pressure lowers as a result of the blood vessels relaxing and reducing resistance. Additionally, it improves the heart muscle's oxygen delivery, which helps to lessen angina symptoms.

The development of a High-Performance Liquid Chromatography (HPLC) method that is rapid, reliable, affordable, reproducible, and, most importantly, very accurate and precise was the aim of this work. The demands of analysis need regular updating of analytical techniques. Our objective was to create an HPLC method that would detect

both amlodipine and chlorthalidone rapidly, reliably, selectively, sensitively, and with high accuracy. The first figure represents chlorthalidone [1] the second figure represents amlodipine [2].

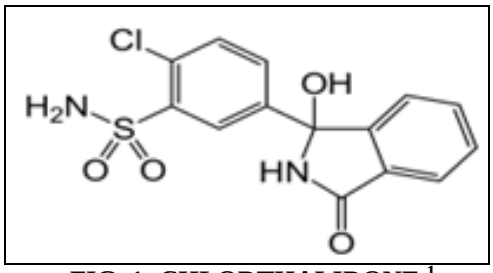

FIG. 1: CHLORTHALIDONE [1]

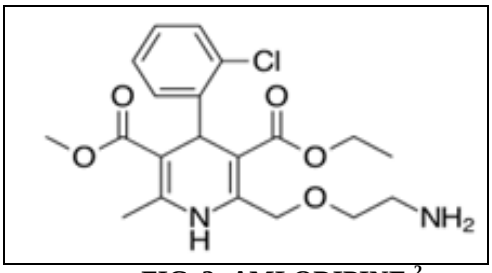

FIG. 2: AMLODIPINE [2]

MATERIAL AND METHOD: Waters 2695 with Empower 2 software and PDA detector, as well as Shimadzu LC-2010C HT HPLC system with UV detector and auto-sampler with LC solution Version 1.25 software, were used for the HPLC analysis. At 365 nm, the detection was performed using the Inertsil ODS-3V column, (250 x 4.6 mm and a particle size of 5 µm). The sample was injected at a volume of 20 µl, and the run time was 8 minutes. A combination of water and ACN was used as a gradient mobile phase. Before use, the mobile phase was degassed and filtered using a 0.45 µm nylon membrane filter.

Chemicals and Reagents: Standards for chlorthalidone and amlodipine were obtained from commercial sources, whereas Amlodac tablets were acquired from Mumbai's local market. Acetonitrile of HPLC quality was used of Finar Ltd. The remaining chemicals were all AR grade.

Preparation of Mobile Phase: Gradient mobile Phase was used for the development.

Mobile Phase A: Water (adjusted pH 3 with formic acid).

Mobile Phase B: Acetonitrile.

Diluent

Mobile Phase A: Mobile Phase B (60: 40).

Preparation of Standard Stock Solution: Precisely weighed and transferred about 62.5 mg of working standard chlorthalidone and 35 mg of working standard amlodipine besylate into a 50 ml volumetric flask, 30 ml of diluent was added and sonicated until it was dissolved completely. The flask was diluted up to the mark with diluent & mixed well.

Preparation of Final Standard Solution: 5 ml solution from standard stock solution and transferred to 50 ml volumetric flask and diluted it with diluent up to the mark and sonicated it for 5 minutes.

Preparation of Sample Solution: Dispersed a quantity of powdered tablets containing amlodipine besylate eq. to 10mg in 200mL of VF, added 170ml of Diluent sonicated for 30mins with intermittent shaking, diluted with Diluent, filtered, and centrifuged the sample

Method Development: A stability-indicating HPLC method for simultaneous estimation of Chlorthalidone and Amlodipine was developed and validated. Different combinations of mobile phases were tested to create a novel method for amlodipine and chlorthalidone on a C18 column. Several buffer systems were also methodically tested to get appropriate run times and acceptable peak shapes. Acetonitrile and water in a gradient mobile phase (pH adjusted to 3 with formic acid) showed that the peak shape was proper and the run time was shorter. Thus, the gradient mobile phase which included acetonitrile and water was chosen as the ideal mobile phase at a flow rate of 1 ml/min. The stationary phase, Inertsil ODS-3V (250 x 4.6 mm, 5 µm particle size), was used to shorten the run time. Several wavelengths were tested for drug analysis, but 375 nm was chosen as the detecting wavelength since the drug showed the highest absorption at that wavelength. The retention time was found to be 4.5 min for Chlorthalidone whereas for amlodipine, it was found to be 1.8 minutes. The chromatograms obtained are shown in **Fig. 3** and **Fig. 4.** The system suitability parameters are shown in **Table 1** and **Table 2.**

TABLE 1: SYSTEM SUITABILITY PARAMETERS OF AMLODIPINE

Parameter	Amlodipine
Retention Time	1.87
% RSD	1.15
% Assay	99.76
USP Tailing	1.24
USP Plate Count	3736

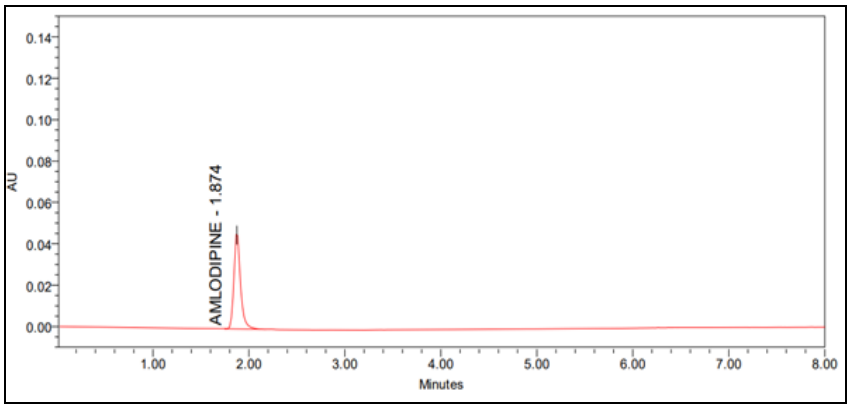

FIG. 3: REPRESENTATIVE CHROMATOGRAM OF AMLODIPINE STANDARD

TABLE 2: SYSTEM SUITABILITY PARAMETERS OF CHLORTHALIDONE

Parameter	Chlorthalidone
Retention Time	1.20
% RSD	0.63
% Assay	99.48
USP Tailing	1.29
USP Plate count	5888

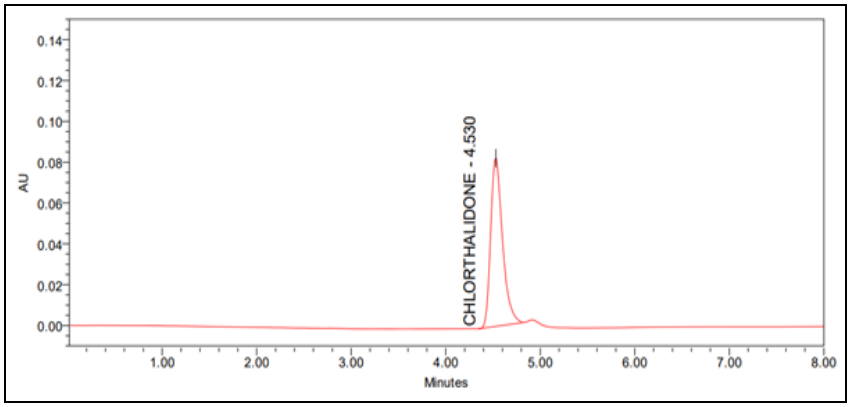

FIG. 4: REPRESENTATIVE CHROMATOGRAM OF CHLORTHALIDONE STANDARD

Material Validation: As specified in the ICH Guidelines, the goal of the method validation is to prove that the procedure is appropriate for the intended use. The above-mentioned method was validated according ICH guidelines to determine the performance characteristic of the method (expressed in terms of analytical parameters) and to meet the requirements for the method's intended use.

They were tested using the optimized chromato-graphic conditions and instruments.

Specificity: According to the methodology, the spectral purities of the peaks for amlodipine and chlorthalidone were evaluated for the interference of the tablet excipients, degradation components, or the presence of impurities. In the work, a solution containing a mixture of the tablet excipients was prepared using the sample preparations procedure to evaluate possible interfering peaks.

Linearity: The ability of an analytical method to provide test results that are directly proportional to the analyte concentration in the sample (within a

specified range) is termed linearity. Chlorthalidone and amlodipine's linearity was established by analyzing serial dilutions of a working standard stock solution. Five concentrations such as 61.30, 98.08, 122.60, 147.12, 183.90µg/ml for Chlorthalidone and 25.80, 41.28, 51.60, 61.92, 77.40µg/ml for Amlodipine were prepared as per **Table 3** and **4** and analyzed. The correlation coefficient & % Y-axis should be within the limit.

TABLE 3: LINEARITY CONCENTRATION LEVELS OF CHLORTHALIDONE

% level	Volume of stock solution	Diluted to (ml)	Final concentration in ppm
50%	2.5 ml	50	61.30
80%	4.0 ml	50	98.08
100%	5.0 ml	50	122.60
120%	6.0 ml	50	147.12
150%	7.5 ml	50	183.90

TABLE 4: LINEARITY CONCENTRATION LEVELS OF AMLODIPINE

% level	Volume of stock solution	Diluted to (ml)	Final concentration in ppm
50%	2.5 ml	50	25.80
80%	4.0 ml	50	41.28
100%	5.0 ml	50	51.60
120%	6.0 ml	50	61.92
150%	7.5 ml	50	7740

Accuracy: The recovery studies were carried out by adding a known amount of drug with a pre-analyzed sample, and the contents were reanalyzed using the proposed method, to confirm the method's accuracy and reliability. According to ICH guidelines, analytical recovery tests were carried out to check the accuracy of the developed method and evaluate the interference of formulation excipients. Three different solutions were prepared in triplicate at levels of 50%, 100%, and 150% of its predefined concentration, and the percentage mean and individual recovery were calculated. Data from the linearity was considered for accuracy.

Precision: The closeness of agreement between multiple measurements obtained from multiple samplings of the same homogeneous substance under specific conditions is expressed as the precision of an analytical method, repeatability of the method was checked by carrying out six independent assays of Chlorthalidone and Amlodipine. The mean area and % relative standard deviation (RSD) was calculated. % RSD should be ≤ 2%.

Intermediate Precision: Two separate repeatability trials conducted on two different days were compared in order to determine the assay method's intermediate precision. The first day's results were from the "Repeatability" analysis. Different analysts or different instruments were implemented for the second set of studies. Based on the data collected each day, the standard deviation, relative standard deviation, and mean value difference were calculated.

Robustness: An analytical procedure's resistance to small, intentional changes in method parameters is determined by its robustness, which also indicates how reliable it is under typical circumstances of operation. Variations in pH, flow, and column temperature were measured.

RESULT AND DISCUSSION: The method validation's objective was to show that the method is suitable for the intended purpose, as stated in the ICH guidelines. The method mentioned above was validated to determine the method's performance characteristics (expressed in terms of analytical parameters) and to ensure that it fulfills the requirements for the intended use.

Specificity: By comparing the chromatograms of the blank solution, placebo solution, reference solution & test solution it is observed that there is no interference of any peaks at the retention time of Chlorthalidone and Amlodipine.

The retention time of the main peaks in the chromatogram obtained with the reference solution & test solution are matching. This confirmed the specificity of the method.

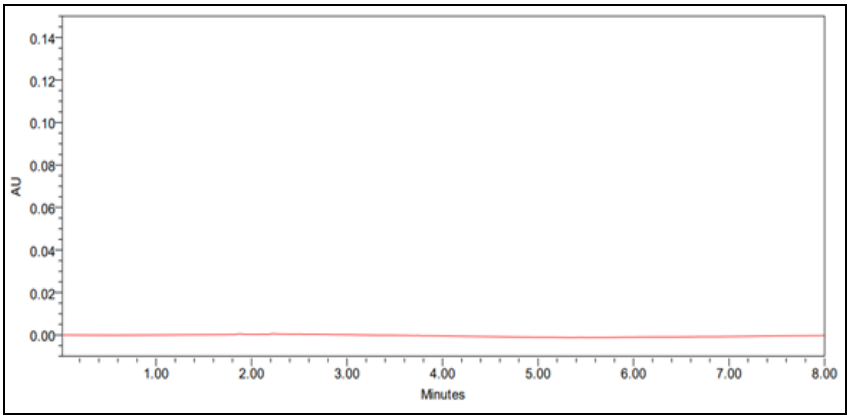

FIG. 5: CHROMATOGRAM OF BLANK SOLUTION

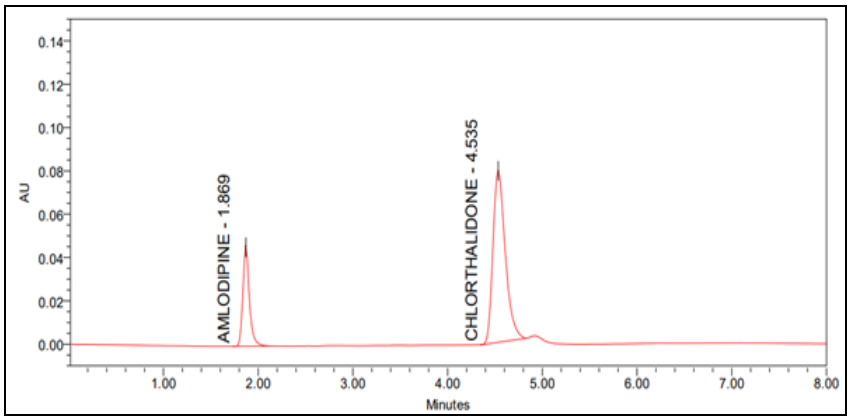

FIG. 6: CHROMATOGRAM OF STANDARD SOLUTION OF AMLODIPINE AND CHLORTHALIDONE

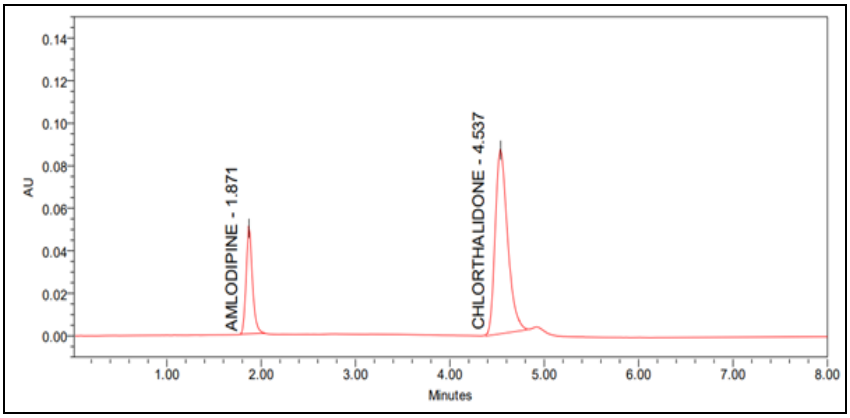

FIG. 7: CHROMATOGRAM OF SAMPLE SOLUTION OF AMLODAC

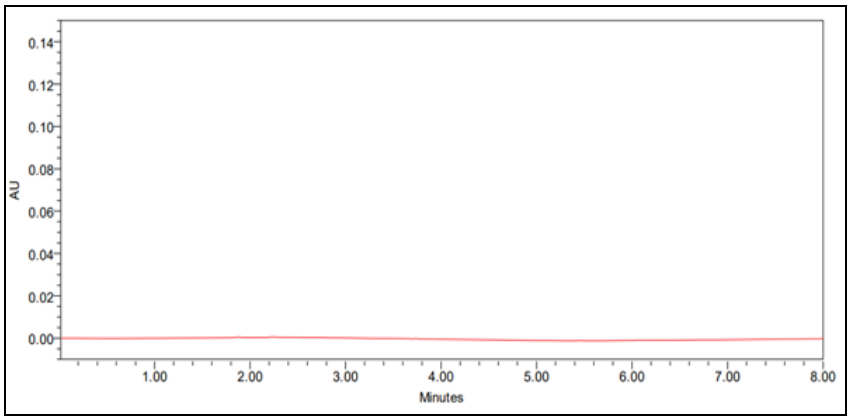

FIG. 8 : CHROMATOGRAM OF PLACEBO SOLUTION OF AMLODAC

Linearity: Five concentrations such as 61.30, 98.08, 122.60, 147.12, 183.90µg/ml for Chlorthalidone and 25.80, 41.28, 51.60, 61.92, 77.40µg/ml for Amlodipine were prepared and the linearity graph was plotted using concentration verses peak area as shown in **Fig. 9** and **Fig. 10.** Graph of Residuals against concentration was also plotted as per shown in **Fig. 11** and **Fig. 12.** A linear relationship was obtained between peak areas and quantity analyzed in the range of 50% to 150%**.** The method was considered to be linear for Chlorthalidone and Amlodipine in a range of 50 to 150% as the Correlation coefficient & %Y-axis intercept were within the limit.

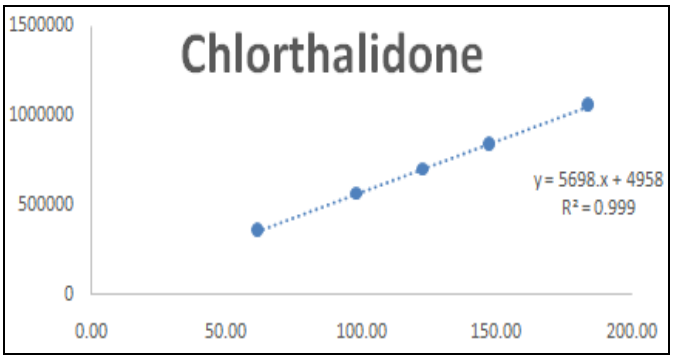

FIG. 9: LINEARITY PLOT FOR CHLORTHALIDONE

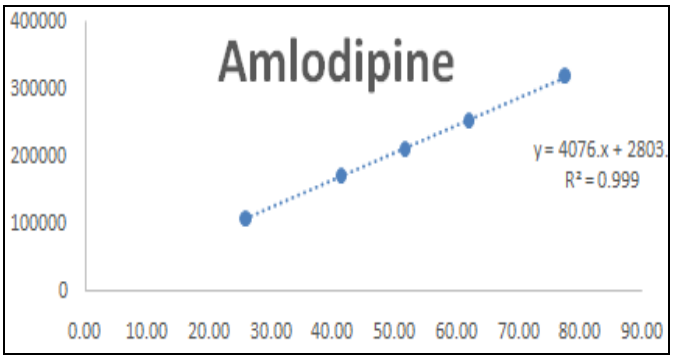

FIG. 10: LINEARITY PLOT FOR AMLODIPINE

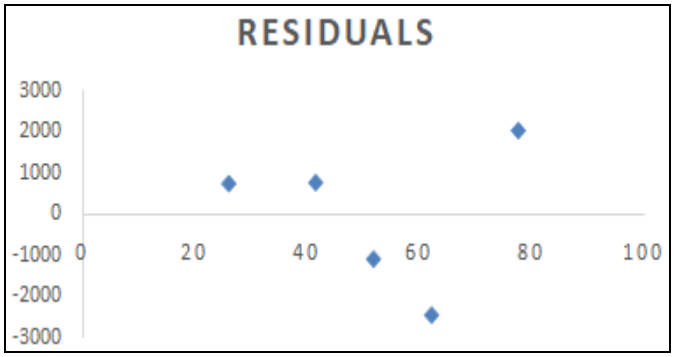

FIG. 11: PLOT OF RESIDUALS AGAINST CONCENTRATION FOR AMLODIPINE

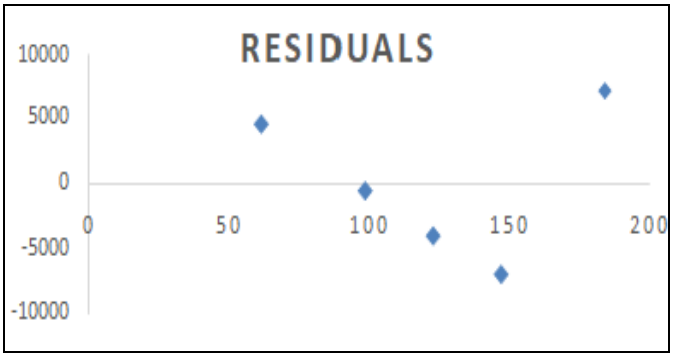

FIG. 12: PLOT OF RESIDUALS AGAINST CONCENTRATION FOR CHLORTHALIDONE

TABLE 5: OBSERVATION TABLE FOR LINEARITY OF AMLODIPINE

Parameter for Linearity	Values	Acceptance Criteria
Correlation coefficient R	0.999	≥ 0.999
%Y – axis intercept	1.63	≤ ± 5 %
The slope of the regression line	4076.88	To be reported
Residual sum of squares	12346072.53	To be reported

TABLE 6: OBSERVATION TABLE FOR LINEARITY OF CHLORTHALIDONE

Parameter for Linearity	Values	Acceptance Criteria
Correlation coefficient R	0.999	≥ 0.999
%Y – axis intercept	0.88	≤ ± 5 %
The slope of the regression line	5698.48	To be reported
Residual sum of squares	138815367.9	To be reported

Accuracy: The percentage recovery of Amlodipine and Chlorthalidone was tabulated in **Table 7**. Since the individual recovery percentage was between 97% and 103% of the acceptance criteria and the mean recovery percentage was between 98% and 102% of the acceptance criteria, the method was considered accurate

TABLE 7: RECOVERY AT DIFFERENT CONCENTRATION LEVELS

Accuracy level	% Recovery of Amlodipine	% Recovery of Chlorthalidone
50%	99.93	99.47
	100.11	100.30

	98.90	99.62
100%	99.89	98.76
	99.89	98.44
	98.93	98.74
150%	99.77	99.51
	99.05	99.47
	100.04	99.66
Mean recovery	99.61	99.33
Minimum recovery	98.93	98.44
Maximum recovery	100.11	100.30

Precision: Since, the relative standard deviation from six determinations was well inside the acceptability level of ≤2%, the method's exactness, as defined by precision and method, was considered to be precise. Refer to **Table 8.**

TABLE 8: METHOD PRECISION OF AMLODAC TABLET

Sample No.	% Assay of Amlodipine	% Assay of Chlorthalidone
Sample 01	100.05	99.64
Sample 02	99.95	99.65
Sample 03	99.70	99.07
Sample 04	99.29	99.39
Sample 05	99.78	99.74
Sample 06	99.82	99.41
Mean	99.76	99.48
STD Dev	0.26	0.25
% RSD	0.26	0.25

Intermediate Precision: Two separate repeatability trials carried out over two different days were compared to determine the intermediate precision of the method. Refer to **Table 9** for the % Assay of Amlodipine and Chlorthalidone and **Table 10** and **11** for a comparison of two independent repeatability.

TABLE 9: INTERMEDIATE PRECISION OF AMLODAC TABLET

Sample No.	% Assay of Amlodipine	% Assay of Chlorthalidone
Sample 01	99.94	99.48
Sample 02	99.80	100.98
Sample 03	99.36	100.12
Sample 04	99.66	99.05
Sample 05	99.90	99.32
Sample 06	99.50	97.52
Mean	99.69	99.41
STD Dev	0.23	1.15
% RSD	0.23	1.16

TABLE 10: COMPARISON OF TWO INDEPENDENT REPEATABILITY OF AMLODIPINE FOR AMLODAC TABLET

Parameter	1st-day Repeatability	2nd-day Repeatability
Number of determinations	6	6
Mean (%) assay	99.76	99.69
RSD (%)	0.26	0.23
Mean value difference (%) Acceptance Criteria: < 2.0 % absolute	0.07	

TABLE 11: COMPARISON OF TWO INDEPENDENT REPEATABILITY OF CHLORTHALIDONE FOR AMLODAC TABLET

Parameter	1st-day Repeatability	2nd-day Repeatability
Number of determinations	6	6
Mean (%) assay	99.48	99.41
RSD (%)	0.25	1.16
Mean value difference (%) Acceptance Criteria: < 2.0 % absolute	0.07	

Robustness: Since all of the examined robustness parameters matched the system suitability requirement, the method was considered to be robust. A deliberate change of a parameter had no significant effect on the performance of the method, indicating the robustness of the HPLC method that was developed. The results are shown in **Table 12** and **Table 13.**

TABLE 12: ROBUSTNESS RESULT FOR AMLODIPINE FOR AMLODAC TABLET

Parameter	System suitability		% Assay
	% RSD	STD deviation	
Flow rate			
0.8 ml/min	0.19	502.07	99.81
1.2 ml/min	0.50	883.99	99.54
Buffer pH			
pH 2.8	0.18	510.63	100.22
pH 3.2	0.21	614.55	99.93
Column oven temperature			
28	0.16	330.56	100.14
32	0.13	275.87	99.61

TABLE 13: ROBUSTNESS RESULT FOR CHLORTHALIDONE FOR AMLODAC TABLET

Parameter	System suitability		% Assay
	% RSD	STD deviation	
Flow rate			
0.8 ml/min	0.27	2376.48	98.94
1.2 ml/min	0.68	3875.05	98.18
Buffer pH			
pH 2.8	0.33	3215.82	99.41
pH 3.2	0.33	3209.18	99.40
Column oven temperature			
28	0.36	2537.45	98.39
32	0.47	3303.28	99.22

Stability of Analytical Solution: For 24 hrs, the sample and standard prepared were compared to a freshly prepared standard. The results were found to be within the ± 2% acceptance limit. See **Table 14.**

TABLE 14: SOLUTION STABILITY RESULTS FOR AMLODAC TABLET

	Solution stability report of Amlodipine		Solution stability report of Chlorthalidone	
Stability condition	**% Assay**	**% Absolute Difference**	**% Assay**	**% Absolute Difference**
Initial	100.05	--	99.64	--
6 hrs	99.70	0.35	99.12	0.52
12hrs	99.38	0.67	100.10	-0.46
24 hrs	99.40	0.65	99.43	0.21

Degradation Studies: Stress testing is required under the International Conference on Harmonization (ICH) guideline entitled Stability testing of new drug substances and products to determine the inherent stability characteristics of the active ingredient. This study aimed to apply the proposed method to stress degradation studies of amlodipine and chlorthalidone.

Temperature Stress Studies: Exposed crushed sample powder to 105 degrees for 8 hours. Then proceeded for preparation as per sample preparation.

Stress Degradation by Hydrolysis Under Acidic Conditions: Took sample in 100 ml flask added 5 ml of 1 N HCl and heated at 70 degrees in water bath for 1 hour.

Cooled to room temperature, neutralized with 5 ml of 1 N NaOH, and then further procedure as per sample preparation

Stress Degradation by Hydrolysis Under Akaline Conditions: Took sample in 100 ml flask added 5 ml of 1 N NaOH and heated at 70 degrees in water bath for 1 hour. Cooled to room temperature, neutralized with 5 ml of 1 N HCl, and then further procedure as per sample preparation.

Oxidative Degradation: To the sample, added 5ml of 30% hydrogen peroxide and kept aside for 1 hour, further procedure as per sample preparation.

TABLE 15: FORCE DEGRADATION FOR AMLODIPINE FOR AMLODAC TABLET

Condition	Time	%Degradation
Acidic Degradation	30 min	No Degradation
Alkaline Degradation	30 min	1.64%
Oxidative Degradation	30 min	No Degradation
Thermal Degradation	30 min	0.47 %

TABLE 16: FORCE DEGRADATION FOR CHLORTHALIDONE FOR AMLODAC TABLET

Condition	Time	%Degradation
Acidic Degradation	30 min	No Degradation
Alkaline Degradation	30 min	0.8 %
Oxidative Degradation	30 min	No Degradation
Thermal Degradation	30 min	No Degradation

CONCLUSION: This study developed and validated a reverse-phased liquid chromatographic method that is simple, economical, time-efficient, and accurate for the estimation of Chlorthalidone and Amlodipine in the pharmaceutical dosage form in accordance with the ICH guidelines. The solution stability as described previously was observed on a short-term and long-term basis. All stability study results were within acceptable limits. According to **Table 15, 16**, the drug was found to degrade significantly under acidic, basic, oxidative, and thermal conditions. The current work is worthwhile as the developed HPLC method is selective, simple, rapid, and cost-effective which can be very beneficial for the routine analysis of Amlodipine and Chlorthalidone in dosage forms.

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CONFLICTS OF INTEREST: We Declare that there are no conflicts of interest between us.

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