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Stability Indicating RP-HPLC Method for Estimation of Amlodipine Besylate and Clopidogrel Bisulphate in Pharmaceutical Formulation

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ABSTRACT

A stability-indicating RP-HPLC method was developed and validated for the quantitative determination of Amlodipine besylate and Clopidogrel bisulphate in tablet dosage form. An isocratic separation was achieved using Inertsil ODS-3, C_{18} Column having 4.0 × 250 mm with 5 µm particle size columns, a flow rate 1.5 mL/min and using UV detector to monitor the eluate at 239nm. The mobile phase consists of acetonitrile:0.01 M potassium dihydrogen phosphate buffer (60:40% v/v) with pH 3.0 adjusted with phosphoric acid. The drug was subjected to oxidation, hydrolysis, photolysis and thermal degradation. Fexofenadine was found to degrade in acidic, basic and oxidation condition. Complete separation of the degraded product was achieved from parent compound. All degradation products in an overall analytical run time of approximately 60 min with the parent compound Amlodipine besylate and Clopidogrel bisulphate eluting at approximately 4.11 ±0.5 min and 11.91±0.5 min. The method was linear over the concentration range of 2.5–25 µg/mL (r^2=0.999) and 75-750 µg/mL (r^2=0.999) with regression equations y =26411x + 826.43 and y =14556x + 8592.6 for amlodipine and clopidogrel respectively. The limit of detection and quantification of amlodipine was found to be 0.20 µg/ml and 0.60 µg/ml respectively for clopidogrel was 0.66 µg/ml and 2.01 µg/ml respectively. The method has the requisite accuracy, selectivity, sensitivity, precision and robustness to assay Amlodipine besylate and Clopidogrel bisulphate in tablets. Degradation products resulting from stress studies did not interfere with the detection of Amlodipine besylate and Clopidogrel bisulphate and the assay is thus stability indicating.

INTRODUCTION

Amlodipine (AML) is a long-acting calcium channel blocker used as an antihypertensive and in the treatment of angina. Amlodipine is chemically 2-[(2-Aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester.[1] Clopidogrel (CLP) is useful for the preventative management of secondary ischemic events, including myocardial infarction, stroke, and vascular deaths and also used as platelet inhibitor.[2] CLP is chemically Thieno[3,2-c]pyridine-5(4H)-aceticacid,α-(2-chlorophenyl)-6,7-dihydro-,methyl ester,(S)-,sulfate (1:1).[3] Literature survey revealed that methods have been reported for estimation of AML in plasma using HPLC-MS.[4] A few spectrophotometric and HPLC methods have been reported for estimation of AML in pure form.[5-7] Some methods have been reported for simultaneous estimation of AML and clopidogrel using HPLC.[8] Some spectrophotometric and HPLC methods have been reported for estimation of CLP in pure form and tablet dosage form.[9-11] But the reported method was found to be more time consuming and more solvent consuming as it shows the long retention time for pure drug. The proposed validated method is more economical, precise, accurate and specific for quantitative determination of AML and CLP in pharmaceutical dosage form.

MATERIALS AND METHODS

AML and CLP pure compounds were supplied by ISP Hongkong Ltd., Hyderabad, India. Tablet formulation is Numlopar tablet, Emcure Pharmaceuticals Ltd, Pune containing labeled amount of 2.5mg AML and 75mg CLP was purchased from local market. All the chemicals used were of analytical grade. HPLC grade water and solvents were used to prepare all the solutions.

HPLC instrumentation and conditions

The HPLC system consists of a pump with injecting facility programmed at 20 µl capacity per injection was used. A stability studies HPLC method was made on Water's Alliance 2690 system. The detector consists of a WATERS PDA detector–2998 and EMPOWER 2 software package operated at a wavelength of 239nm. Chromatographic responses were measured in microvolts (µV). The chromatographic separation was performed using aInertsil ODS-3, C-18 Column having 4.0 × 250 mm with 5 µm particle size. The separation was achieved using isocratic mode of elution mobile phase consisting of Acetonitrile: 0.01 M Phosphate buffer

(60:40%v/v) with pH 3.0 adjusted with dil. o-phosphoric acid at a flow rate of 1.5 ml/min. The eluent was monitored using PDA detector–2998 at a wavelength of 239nm. The column was maintained at ambient temperature and injection volume of 20 µl was used. The mobile phase was filtered through 0.45 µm Nylon membrane filter prior and degassed using sonicator for 15 min.

Preparation of stock and standard solutions

A stock solution of AML (1 mg/ml) and CLP (1 mg/ml) was prepared by accurately weighing approximately 10mg of each into 10 ml volumetric flask and dissolved with small quantity of mobile phase. The mixture was sonicated for 10 min and made up the volume with mobile phase to give concentration of 1000 µg/ml. The stock solution is protected from light using aluminum foil and stored for one week and was found to be stable during this period. Aliquots of the standard stock solutions of AML and CLP were transferred using A-grade bulb pipettes into 10 ml volumetric flasks and solutions were made up to the volume with mobile phase to give the final concentrations of 1-100µg/ml for AML and 50-850µg/ml for CLP. These stock solutions were used for preparing working standards and calibration standards. Chromatogram of AML and CLP was shown in figure 1.

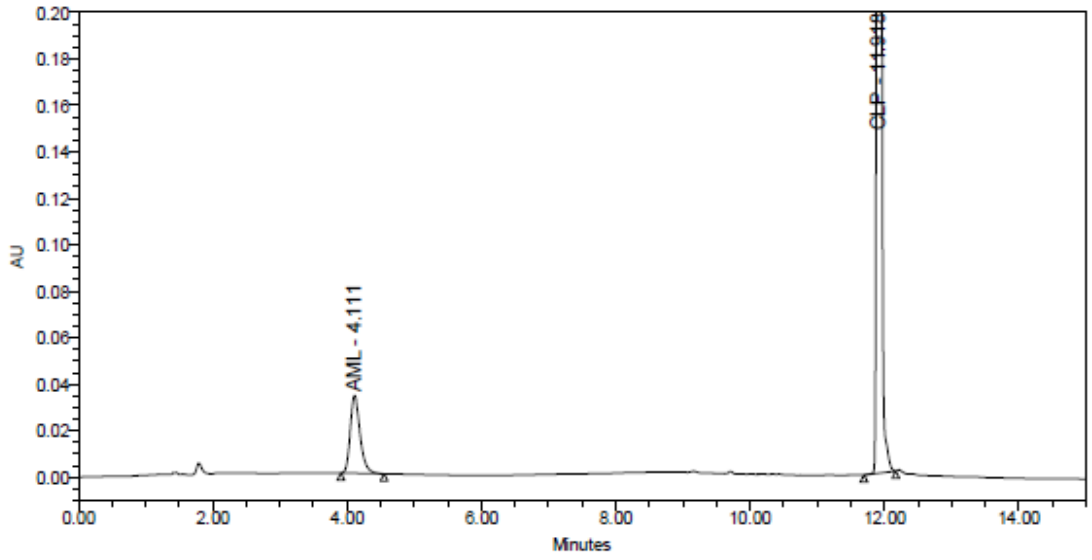

Figure 1: Chromatogram of AML and CLP. The mobile phase acetonitrile: 0.01 M potassium dihydrogen phosphate buffer (60:40; v/v) with pH 3.0 adjusted with o-

phosphoric acid and Peak of standard drug retention time TR=4.11 ±0.9 min and 11.91±0.6 min respectively, 239 nm.

Estimation of AML and CLP from Pharmaceutical dosage form

To determine the content of AML and CLP in tablets (label claim: 2.5mg AML and 75mg CLP), 20 tablets were taken and contents were weighed and mixed. An aliquot of powder equivalent to the weight of one tablet was accurately weighed and transferred to 100 ml volumetric flask and made upto volume with mobile phase. The volumetric flask was sonicated for 30 min for complete dissolution. The solutions were filtered through a 0.45 µm nylon filter. Suitable aliquots of the filtered solution was added to the volumetric flask and made up to the volume with mobile phase to yield the concentration of 1, 5, 10, 50 and 100 µg/ml. 20 µl volume of each sample solution was injected into HPLC, six times, under the conditions described above. The peak areas were measured at 239.0nm and concentrations in the samples were determined by comparing the area of sample with that of standard.

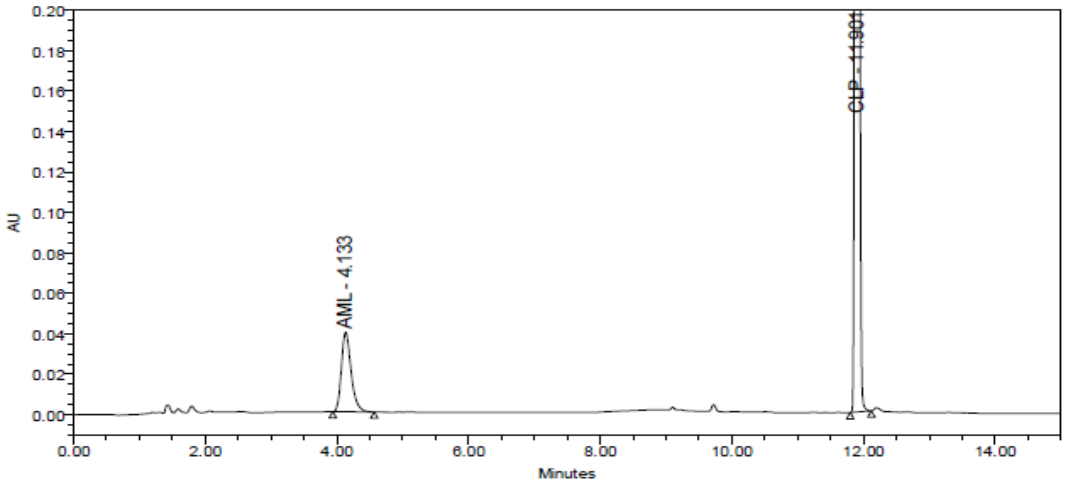

Figure 2: Chromatogram of tablet dosage form contains 12.5 µg/ml of AML and 375 µg/ml of CLP (Numlopar tablet contain 2.5mg AML and 75mg CLP

Forced degradation studies[12]

In order to determine whether the analytical method and assay were stability indicating, AML and CLP pure drugs were stressed under various conditions to conduct forced degradation studies. A stock solution of 100 µg/ml was prepared by dissolving 10mg of AML and CLP in 5 ml of mobile phase and volume was made up to 100ml with mobile phase. This solution was

used for forced degradation studies to evaluate the stability indicating property and specificity of proposed method. In all forced degradation studies, the average peak area of standard AML,CLP and degradation sample after application (20 µg/ml of HPLC) of six replicates were obtained.

Acid Degradation Studies

To 2 ml stock solution, 2 ml of 1 N hydrochloric acid was added. The solution was kept for 60 min at room temperature. The resultant solution was diluted to obtain 20 µg/ml solution and 20 µl were injected into the system and the chromatograms were recorded to assess the stability of sample. (Figure 3)

Alkali Degradation Studies

To 2 ml of stock solution, 2 ml of 1 N sodium hydroxide was added. The solution was kept for 60 min at room temperature. The resultant solution was diluted to obtain 20 µg/ml solution and 20 µl were injected into the system and the chromatograms were recorded to assess the stability of sample. (Figure 4)

Oxidation

To 2 ml stock solution, 2 ml of 3% hydrogen peroxide was added separately. The solutions were kept for 60 min at room temperature. For HPLC study, the resulting solution was diluted to obtain 20 µg/ml solution and 20 µl were injected into the system and the chromatograms were recorded to assess the stability of sample. (Figure 5)

Dry Heat Degradation Studies

The standard drug was placed in oven at 80º for 1 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 20 µg/ml solution and 20 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photostability studies

Photochemical stability of the drug was also studied by exposing the stock solution (1 mg/ml) to direct sunlight for 46 h on wooden plank and kept on terrace. For HPLC study, the resultant

solution was diluted to 20 µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample. (Figure 6)

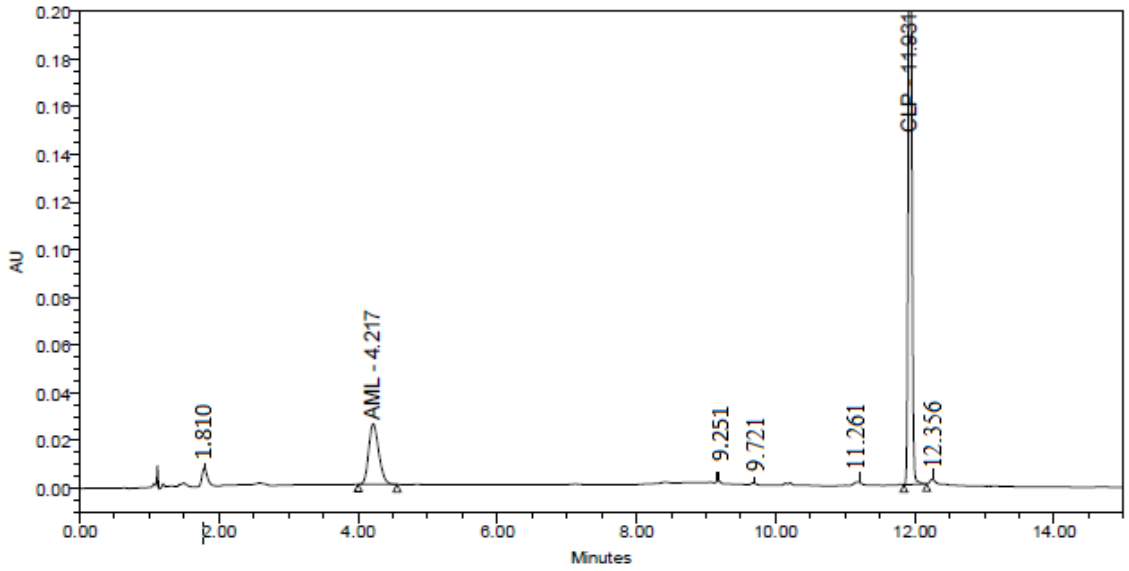

Figure 3: Chromatogram of acid-treated AML and CLP. Chromatogram of acid (1 N HCl) hydrolyzed AML and CLP showing degradation peaks at retention time (TR) 1.810 min, 9.251 min, 11.261 min, 12.256 min.

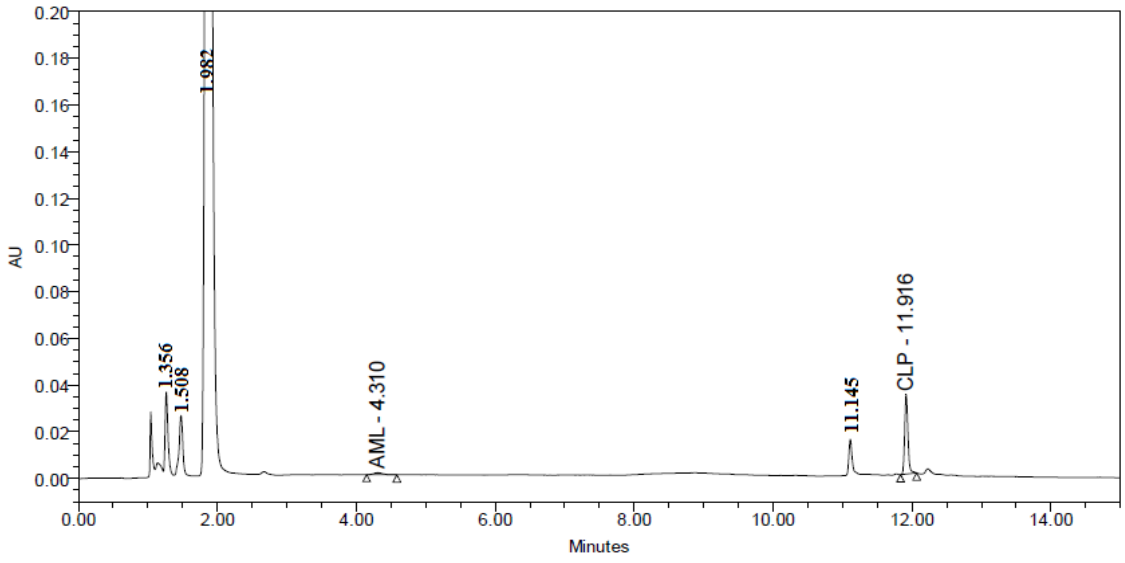

Figure 4: Chromatogram of base-treated AML and CLP. Chromatogram of base (1 N NaOH) hydrolyzed AML and CLP showing degradation peaks at retention time (TR) 1.356 min, 1.508 min, 1.982 min, 11.145 min.

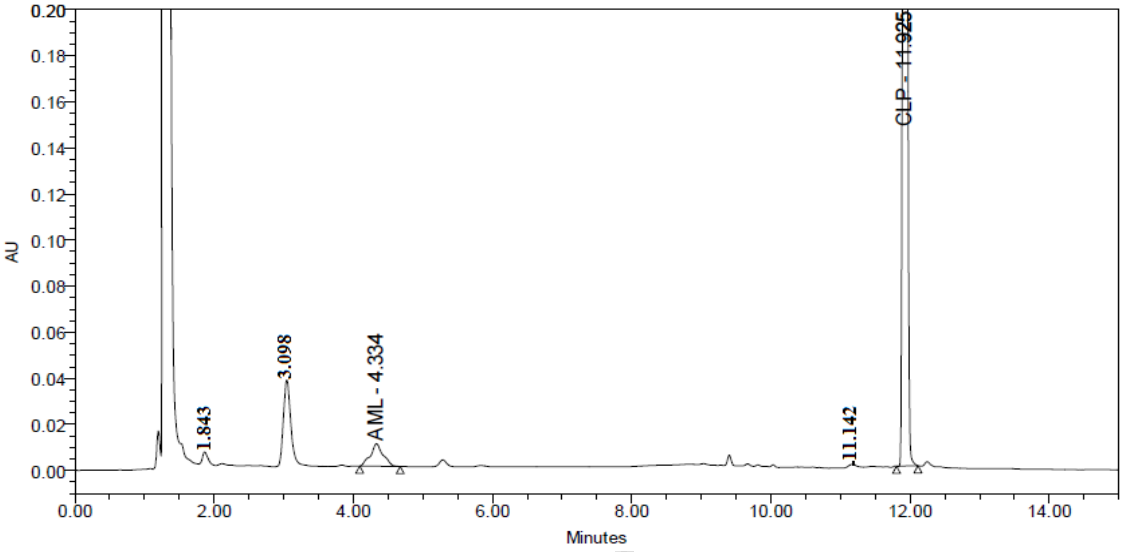

Figure 5: Chromatogram of H_2O_2 treated AML and CLP. Chromatogram of chemically oxidized AML and CLP using 3% H_2O_2 showing degradation peak at T_R 1.523 min, 1.843 min, 3.098 min, 11.142 min.

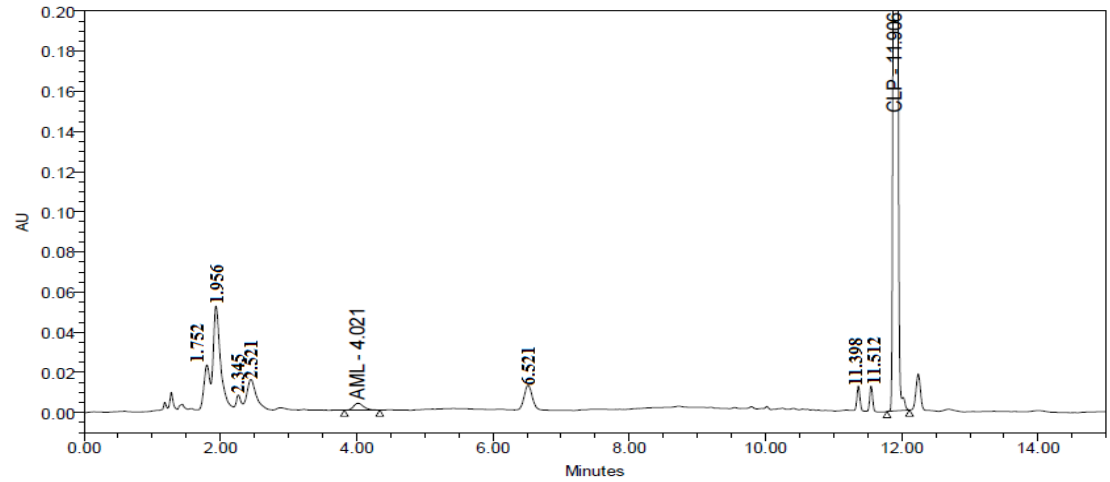

Fig. 6: Chromatogram of Photodegradation of AML and CLP. Chromatogram of AML and CLP using UV-light showing degradation peak at T_R 1.956 min, 2.521 min, 6.521 min, 11.298 min.

RESULTS AND DISCUSSION

The HPLC procedure was optimized with a view to develop a stability indicating assay method. Pure drug along with its degraded products were injected and run in different solvent systems. Initially water and methanol, acetonitrile and water in different ratios were tried. Acetonitrile:

methanol (50:50 v/v) was not able to give good peak symmetry with acceptable retention time. An attempt to improve peak symmetry was made by adding phosphate buffer to the mobile phase. The presence of phosphate buffer in mobile phase resulted in excellent overall chromatography with appropriate peak symmetry and complete baseline resolution. Finally, the mobile phase consisting of acetonitrile: 0.01 M potassium dihydrogen phosphate buffer (60:40 v/v) with pH 3.0 adjusted with o-phosphoric acid was selected for validation purpose and stability studies. The method was validated with respect to the parameters including linearity, limit detection (LOD), limit of quantitation (LOQ), recovery, precision, accuracy, robustness and selectivity and a summary of validation parameters were presented in Table 1.

AML and CLP showed linearity in the concentration range of 2.5–25 µg/ml (r^2= 0.9996) and 75-750 µg/ml (r^2= 0.9999) respectively for HPLC under PDA detector. Linearity was evaluated by determining ten standard working solutions containing various concentrations thrice in triplicate. Peak area of AML and CLP were plotted versus AML and CLP concentration and linear regression analysis performed on the resultant curve. For HPLC method, the linearity of calibration graphs and adherence of the system to Beer's law was validated by high value of correlation coefficient and standard deviation for intercept value was less than 2%.

The LOD and LOQ were determined based on signal-to-noise ratios and were based on analytical responses of 3 and 10 times the background noise, respectively. The LOD was found to be 0.2 µg/ml for AML and 0.66 for CLP. The LOQ was found to be 0.60 µg/ml for AML and 2.01 for CLP.

Proposed method when used for extraction and simultaneous estimation of AML and CLP from pharmaceutical dosage form after spiking with additional drug, afforded recovery of 98.36 to 100.99% for AML and 98.84 to 100.33% for CLP and mean recovery of AML and CLP from the marked formulation are listed in Table 2.

The precision of assay was determined with respect to both repeatability and reproducibility. An amount of the product powder equivalent to 100% of label claim of AML and CLP was accurately weighed and assayed. System repeatability was determined by six replicate applications and six times measurement of sample solution at the analytical concentration. The

repeatability of sample application and measurement of peak area for active compound were expressed in terms of % RSD (Relative standard deviation).

To evaluate HPLC method robustness, a few parameters were deliberately varied. The parameters included variation of C_{18} columns from different detection wavelength, flow rate and pH of mobile phase in different lots. Each parameter (except column from different detection wavelength and solvents of different lots) were changed at three levels (-1, 0 and 1) and examined. One factor at a time was changed to estimate the effect. Thus, replicate injections (n=6) of standard solution of three concentration levels were performed under small changes of six chromatographic parameters. Results indicate that the selected factors remained unaffected by small variations in these parameters. It was also found that variation of the detection wavelength by ± 2 nm (237 and 241nm), mobile phase (buffer) pH by 0.2 units (pH 2.8 and 3.2), and mobile phase flow rate was altered by 0.1 ml/min. (i.e 1.4 and 1.6 ml/min.). Insignificant difference in peak areas and less variability in retention time were observed. The results are given in Table 3.

Results of stress testing studies indicated a high degree of selectivity of this method of AML and CLP. Typical chromatograms obtained from the assay of pure sample and stressed samples are shown in figures 1, 2, 3, 4, 5 and 6 respectively. The retention time for AML and CLP were found to be 4.11±0.5 min and 11.91±0.5 min respectively for six replicates. The peaks obtained were sharp and have clear baseline separation.

AML and CLP were prone to hydrolysis. All the main degradation products were separated from the parent compound. AML and CLP were found to be stable under dry heat conditions and also no decomposition was seen on exposure of solid drug powder to light, which was kept in day light for 46 h. The drug was unstable under basic stress conditions when kept for 1 h under room temperature. The drug CLP was degraded approximately to 80.53%. Also it was unstable in acidic conditions when kept for 1 h at room temperature. The drug AML was degraded approximately to 63.66%. When kept under oxidative stress conditions with 3% H_2O_2 for 1 h at room temperature. The drug was degraded to around 98.00%, when kept under basic condition. Stability of stock solution was determined by quantitation of AML and CLP, comparison to

freshly prepared standard. No significant change was observed in the stock solution response, relatively to freshly prepared standard (Table 4).

The proposed method was applied for the determination of AML and CLP in tablet dosage form and results of the assays yielded 99.14% and 98.76% respectively with % RSD<2 of label claim of the tablets. The results of the assay indicate that the method is selective for the assay of AML and CLP without interference from the excipients used in these tablets (Table 5). The theoretical plates were 3562 and 319380 (more than 2000) and tailing factor was 1.34 and 1.09 (less than 2) for the AML and CLP combination peak respectively. The %RSD of the retention times was found to be <2 for both drugs (Table 6).

This methodwas used to develop and validate a liquid chromatographic analytical method that can be used for assay of AML and CLP in a pharmaceutical dosage form. Degradation products produced as a result of stress did not interfere with detection of AML, CLP and the assay method can thus be regarded as stability indicating. This HPLC method for assay of AML and CLP in a tablet formulation was successfully developed and validated for its intended purpose. The method was shown to specific, linear, precise, accurate, and robust. Because the method separates AML, CLP and all the degradation products formed under variety of stress conditions it can be regarded as stability indicating. This method is recommended to the industry for quality control of drug content in pharmaceutical preparations. From this study we can conclude that there was no special precaution required during manufacturing and storage of formulation because there was no observed any degradation at room temperature in any of the condition.

Table 1: Summary of Validation Parameters of Amlodipine and Clopidogrel

Parameters	**AML**	**CLP**
Detection wavelength (nm)	239	239
Linear range (µg/mL)	2.5–25 µg/mL	75-750 µg/mL
Correlation coefficient (r^2)	0.9996	0.9999
Linear Regression Equation (y = mx + c)	y =26411x + 826.43	y =14556x + 8592.6
Resolution	-	42.82
LOD (µg/mL)	0.20	0.66
LOQ (µg/mL)	0.60	2.01
Recovery (%)	98.36-100.99	98.84-100.33
Intermediate Precision	0.64	0.65

*Average of six determinations.

Table 2: Recovery of Amlodipine and Clopidogrel

Sr. No.	**Level (%)**	**% recovery± S.D.***		**%RSD***	
		AML	**CLP**	**AML**	**CLP**
1	80%	98.36±0.51	100.33±0.12	0.52	0.12
2	100%	100.99±0.92	99.23±0.49	0.91	0.49
3	120%	98.42±0.61	98.84±0.30	0.62	0.30

*SD: standard deviation

Table 3: Robustness Study of Amlodipine and Clopidogrel

Parameters		AML		%RSD*	CLP		%RSD*
		RT	Peak area (AU)		RT	Peak area (AU)	
Detection wavelength (nm)	237	4.06	318760.5	1.50	11.93	5391737	0.57
	239	4.06	326476		11.94	5438025	
	241	4.07	317612		11.93	5379509	
Mobile phase pH	2.8	4.03	318370.5	1.37	11.84	5832201	0.60
	3.0	4.08	324015		11.92	5793246	
	3.2	4.09	327136		11.97	5762437	
Mobile phase flow rate (ml/min.)	1.4	4.22	320950	1.66	12.15	5438025	1.18
	1.5	4.09	331804		11.93	5318781	
	1.6	3.84	326377		11.72	5339457	

*RSD: Relative standard Deviation

Table 4: Results of Degradation Study of Amlodipine and Clopidogrel

Degradation conditions	Duration (Days)	Assay of Drug (%)		%Degradation±%RSD*		Additional peaks
		AML	CLP	AML	CLP	
Acid hydrolysis (1.0M HCl)/25°C	1	75.54	19.47	24.45±0.23	80.53±0.45	5
Base hydrolysis (1.0M NaOH)/25°C	1	2.02	2.0	97.97±1.05	98.00±0.68	4
Oxidation (5% H_2O_2)/ 25°C	1	36.33	88.73	63.66±0.77	11.27±1.12	3
UV-light	3	10.01	63.12	89.98±0.89	36.88±0.69	7
Sunlight	3	98.09	98.24	-	-	-
Thermal at 105°C	14	98.64	98.74	-	-	-

*n=3

Table 5: Assay Results ForAmlodipine and Clopidogrel

Formulation	Label claim (AML; CLP)	Amount found (mg) mean±SD (*n*=3)		Assay (%)		RSD (%)	
		AML	CLP	AML	CLP	AML	CLP
Numlopar tablet	2.5 mg, 75 mg	2.45±0.56	74.72±1.02	98.15	99.63	0.08	0.74

Table 6: System Suitability Data of Amlodipine and Clopidogrel

Parameter	AML*	CLP*
Retention Time *(R_T)*	4.10 min	11.92 min
Tailing factor *(T)*	1.34	1.09
Theoretical plates *(N)*	3562	319380
Resolution *(RS)*	-	42.82
%RSD of R_T	0.83	0.07

*n=6

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