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STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF AMLODIPINE BESYLATE AND RAMIPRIL IN COMBINED DOSAGE FORM

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ABSTRACT

An isocratic reversed phase liquid chromatographic assay method was developed for the quantitative determination of amlodipine besylate (AML) and ramipril (RAM) in combined dosage form. A Brownlee C-18, 5 µm column with mobile phase containing 0.02 M potassium dihydrogen phosphate: methanol (40:60, v/v) adjusted to pH 3 using Orthophosphoric acid was used. The flow rate was 1.0 ml min^{-1} and effluents were monitored at 224 nm. The retention times of amlodipine besylate and ramipril were 14.6 min and 10.9 min, respectively. Amlodipine besylate and ramipril stock solutions were subjected to acid and alkali hydrolysis, chemical oxidation and dry heat degradation. The degraded product peaks were well resolved from the pure drug peak with significant difference in their retention time values. Stressed samples were assayed using developed HPLC method. The proposed method was validated with respect to linearity, accuracy, precision and robustness. The method was successfully applied to the estimation of amlodipine besylate and ramipril in combined tablet dosage forms.

INTRODUCTION

Amlodipine besylate (AML) is chemically 3-ethyl 5-methyl 2-[(2 amino ethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate. It is a long-acting calcium channel blocker (dihydropyridine class) used as an anti-hypertensive and in the treatment of angina. It is official in IP, BP. Ramipril (RAM) is chemically (2S, 3aS, 6aS)-1-[(2S)-2-{[(2S)-1-ethoxy-1-oxo-4-phenylbutan-2-yl] amino} propanol]-octahydrocyclopenta[b]pyrrole-2-carboxylic acid. It is ACE inhibitor. It lowers the production of angiotensin II and also decreases the breakdown of bradykinin, thereby relaxing arterial muscles and enlarging the arteries, allowing the heart to pump blood more easily, and increasing blood flow due to more blood being pumped into and through larger passageways. Its effect on bradykinin is also responsible for the dry cough side effect.

These drugs are now a days available in combination therapy. The rationale behind use of this drug combination is that in treatment of hypertension in patients whose blood pressure is not adequately controlled by monotherapy, oral administration of amlodipine besylate and ramipril has been found to be more effective than use of either drug alone. Combination treatment with amlodipine besylate and ramipril produces synergistic effect which reduces blood pressure [1-2]. Literature survey revealed various spectrophotometric and chromatographic methods have been reported for estimation of AML and RAM individually or in combination with other drug. For estimation of amlodipine besylate HPLC [3-6] and HPTLC [7] methods have been reported. Different methods for estimation of ramipril in single and in combination with other drugs are UV spectrophotometric [8-10], HPLC [11] and HPTLC [12]. For combination of both the drugs HPLC methods have been developed [13-16] and LC-MS/MS methods [17-18] have been developed for the same. Hence the aim of present study is to establish accurate and sensitive method and after validation in accordance with International Conference on Harmonization (ICH) guidelines [19], to use method for analysis of drug content of both in tablet dosage form.

EXPERIMENTAL CONDITIONS

Apparatus

The Liquid chromatographic system consisted of Perkin-Elmer HPLC model (VP series) containing LC-10AT (VP series) pump, variable wavelength programmable UV/VIS detector and Rheodyne injector with 20 µl fixed loop. Chromatographic analysis was performed on

Brownlee C-18 column having 250 × 4.6 mm i.d. and 5 µm particle size. All the drugs and chemicals were weighed on Shimadzu electronic balance (AX 200, Shimadzu Corp., and Japan).

Chemicals and Reagents

Analytically pure samples of AML and RAM were obtained as a gift samples from Alembic Pharmaceuticals Ltd (Baroda, India) and Century Pharmaceuticals Ltd (Baroda, India). Methanol used were of HPLC grade (SRL Chemicals, India).Tablet formulation Cardace2.5 AM (Aventis Pharmaceuticals Ltd., India) containing labeled amount of 5 mg of AML and 2.5 mg of RAM, were procured from local market.

Chromatographic conditions

A Brownlee C-18 (250×4.6 mm i.d) chromatographic column equilibrated with mobile phase 0.02M Potassium dihydrogen phosphate-methanol (40:60,v/v) adjusted to pH 3 with O-phosphoric acid (1M) was used. Mobile phase flow rate was maintained at 1 ml min^{-1} and effluents were monitored at 224 nm and the total run time was 18 min.

Preparation of standard stock solutions

AML and RAM were weighed (25 mg each) and transferred to two separate 25 ml volumetric flasks and dissolved in methanol. Volumes were made up to the mark with methanol to yield a solution containing 1000 µg ml^{-1} of AML and RAM, respectively. Aliquot from the stock solution of AML was appropriately diluted with mobile phase to obtain working standard of 100 µg ml^{-1} of AML and same procedure is followed for RAM to obtain working standard of RAM.

Method Validation

The method was validated for accuracy, precision, linearity, specificity, detection limit, quantitation limit and robustness.

Linearity

Appropriate aliquots of AML and RAM working standard solutions were taken in different 10 ml volumetric flasks and diluted up to the mark with mobile phase to obtain final concentrations of 0.01,0.1, 0.5, 1, 4,10, 20 µg ml^{-1} of AML and 1,2,5, 8,10, 20,40 µg ml^{-1} of RAM, respectively. The solutions were injected using a 20 µl fixed loop system and chromatograms were recorded. Calibration curves were constructed by plotting average peak area versus concentrations and regression equations were computed for both the drugs.

Precision

The intra-day and inter-day precision studies were carried out by estimating the corresponding responses 3 times on the same day and on 3 different days for three different concentrations of AML (4, 10, 20 μg mL^{-1}) and RAM (2, 5, 10 μg mL^{-1}), and the results are reported in terms of relative standard deviation. The instrumental precision studies were carried out by estimating response of 3 different concentrations of AML (4, 10, 20 μg mL^{-1}) and RAM (2, 5, 10 μg mL^{-1}), six times and results are reported in terms of relative standard deviation.

Accuracy

The accuracy of the method was determined by calculating recoveries of AML and RAM by method of standard additions. Known amount of AML (0, 2.5, 5, 7.5 μg mL^{-1}) and RAM (0, 1.25, 2.5, 3.75 μg mL^{-1}) were added to a pre quantified sample solution, and the amount of AML and RAM were estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve.

Detection limit and Quantitation limit

The limit of detection (LOD) is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels. Limit of quantification (LOQ) of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated using following equation as per ICH guidelines. LOD = 3.3 ×σ /S; LOQ = 10 ×σ /S; Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

Solution stability

Stability of sample solutions were studied at 25 ± 2°C for 24 h.

Robustness

Robustness of the method was studied by deliberately changing the experimental conditions like flow rate and percentage of organic phase.

Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities and degradation products. Commonly used excipients (starch, microcrystalline cellulose and magnesium stearate) were spiked into a pre weighed quantity of drugs. Chromatogram was taken by appropriate dilutions and quantities of drugs were determined.

Specificity was also studied by performing forced degradation study using acid and alkali hydrolysis, chemical oxidation and dry heat degradation studies and interference of the degradation products were investigated. AML and RAM were weighed (25 mg each) and transferred to two separate 25 ml volumetric flasks, dissolved in few ml of methanol and diluted up to the mark with methanol. These stock solutions were used for forced degradation studies.

Forced degradation studies

Alkali hydrolysis

To the different 25 ml volumetric flask, 2.5 ml stock solutions of AML and RAM were taken and 5 ml of 0.5 N NaOH was added. In another volumetric flask 2.5 ml stock solution of formulation were taken and 5 ml of 0.5 N NaOH was added to perform base hydrolysis. All flasks were heated at 80ºC for 1 h and allowed to cool to room temperature. Solutions were neutralized with 0.5 N HCl and diluted up to the mark with mobile phase. Appropriate aliquots were taken from the above solutions and diluted with mobile phase to obtain final concentration of 10 μg ml^{-1} of AML and 5 μg ml^{-1} RAM separately and in the mixture.

Acid hydrolysis

To the different 25 ml volumetric flask, 2.5 ml stock solutions of AML and RAM were taken and 5 ml of 0.5 N HCl was added. In another volumetric flask 2.5 ml stock solution of formulations were taken and 5 ml of 0.5 N HCl was added to perform acid hydrolysis. All flasks were heated at 80ºC for 1 h and allowed to cool to room temperature. Solutions were neutralized with 0.5 N NaOH and diluted up to the mark with mobile phase. Appropriate aliquots were taken from the above solutions and diluted with mobile phase to obtain final concentration of 10 μg ml^{-1} of AML and 5 μg ml^{-1} RAM separately and in the mixture.

Oxidative stress degradation

To perform oxidative stress degradation, appropriate aliquots of stock solutions of AML and RAM were taken in two different 25 ml volumetric flasks and 5 ml of 6% hydrogen peroxide was added. Similarly, appropriate aliquots of stock solutions of AML and RAM were taken in the same 25 ml volumetric flaks and 5 ml 6% hydrogen peroxide was added. All the mixtures were heated in a water bath at 80 ^{o}C for 1 h. and allowed to cool to room temperature and diluted up to the mark with mobile phase. Appropriate aliquots were taken from above solutions and

diluted with mobile phase to obtain final concentration of 10 µg ml^{-1} of AML and 5 µg ml^{-1} RAM separately and in mixture.

Dry heat degradation

Analytically pure samples of AML and RAM were exposed in oven at 80°C for 1 h. The solids were allowed to cool and 25 mg each of AML and RAM were weighed, transferred to two separate volumetric flasks (25 ml) and dissolved in few ml of methanol. Volumes were made up to the mark with the methanol. Solutions were further diluted by mobile phase taking appropriate aliquots in 10 ml volumetric flask to obtain final concentration of 10 µg ml^{-1} of AML and 5 µg ml^{-1} of RAM. All the reaction solutions were injected in the liquid chromatographic system and chromatograms were recorded.

Analysis of marketed formulations

Twenty tablets were weighed accurately and finely powdered. Tablet powder equivalent to 5 mg AML and 2.5 mg of RAM was taken in 100 ml volumetric flask. Methanol (50 ml) was added to the above flask and the flask was sonicated for 15 minutes. The solution was filtered using whatman filter paper No.41 and volume was made up to the mark with the mobile phase. Appropriate volume of the aliquot was transferred to a 10 ml volumetric flask and the volume was made up to the mark with the mobile phase to obtain a solution containing 10 µg/ml of AML and 5 µg/ml of RAM. The solution was sonicated for 10 min. It was injected as per the above chromatographic conditions and peak areas were recorded. The quantifications were carried out by keeping these values to the straight line equation of calibration curve.

RESULTS AND DISCUSSION

Optimization of mobile phase

The objective of the method development was to resolve chromatographic peaks for active drug ingredients and degradation products produced under stressed conditions with less asymmetric factor. Various mixtures containing aqueous buffer and methanol were tried as mobile phases in the initial stage of method development. Mixture of 0.02 M KH2PO4-methanol (20+80, v/v), 0.02M KH2PO4-acetonitrile-methanol (30+40+30 V/V/V), 0.02 M KH2PO4-methanol (35+65, v/v) were tried as mobile phase but satisfactory resolution of drug and degradation peaks were not achieved. The mobile phase 0.02 M KH_2PO_4-methanol (40+60, v/v) total pH adjusted to 3 using O- phosphoric acid was found to be satisfactory and gave two symmetric and well-resolved

peaks for AML and RAM. The retention time for AML and RAM were 14.6 min and 10.9min, respectively (Fig. 3). The resolution between AML and RAM was found to be 2.4, which indicates good separation of both the compounds. The asymmetric factors for AML and RAM were 1.43 and 1.42, respectively. The mobile phase flow rate was maintained at 1 mL min^{-1}. Overlaid UV spectra of both the drugs showed that AML and RAM absorbed appreciably at 224 nm, so detection was carried out at 224 nm.

Method validation

Linearity

The calibration curve for AML was found to be linear in the range of 0.01 - 20 µg mL^{-1} with a correlation coefficient of 0.9998. The calibration curve for RAM was found to be linear in the range of 1 - 40 µg mL^{-1} with a correlation coefficient of 0.9954. The standard deviation value of slope of AML and RAM were 153.4 and 3.48 which indicated strong correlation between peak area and concentration. The regression analysis of calibration curves are reported in Table 1.

Table 1: Regression Analysis of Calibration Curves

Parameters	AML	RAM
Range	0.01 – 20 µg/ml	1 – 40 µg/ml
Slope	32439.69	3970.8
SD of slope	153.4	3.48
Intercept	2206	2349.18
SD of intercept	334.6	65.2
Corr. Coefficient	0.9998	0.9954

Precision

Instrument precision was determined by performing injection repeatability test and the RSD values for AML and RAM were found to be 0.18 - 0.63% and 0.61 - 0.79%, respectively. The intra-day and inter-day precision studies were carried out and the results are reported in table III. The low RSD values indicate that the method is precise.

Accuracy

The accuracy of the method was determined by calculating recoveries of AML and RAM by method of standard addition. The recoveries found to be 99.6 – 100.1 % and 99.06 – 100.3 % for AML and RAM respectively. The high values indicate that the method is accurate.

Table 2: Accuracy study of the proposed method

Amount of sample taken (µg/ml)		Amount of standard drug added (µg/ml)		Amount of drug recovered (µg/ml)		% recovery± RSD	
AML	**RAM**	**AML**	**RAM**	**AML**	**RAM**	**AML**	**RAM**
5	8	0.0	0.0	5.03	7.96	99.90+0.21	99.49+0.45
5	8	0.5	4	7.49	12.04	100.04+0.18	100.32+1.53
5	8	1	8	9.9	15.85	100.1+0.25	99.06+0.82
5	8	1.5	12	12.38	19.85	99.6+0.27	99.26+0.77

Limit of detection and limit of quantification

The detection limits for AML and RAM were 2 ng ml^{-1} and 100 ng ml^{-1}, respectively, while quantitation limits were 10 ng ml^{-1} and 350 ng ml^{-1}, respectively. The above data shows that a nano gram quantity of both the drugs can be accurately and precisely determined.

Specificity

The specificity study was carried out to check the interference from the excipients used in the formulations by preparing synthetic mixture containing both the drugs and excipients. The chromatogram showed peaks for both the drugs without any interfering peak and the recoveries of both the drugs were above 98%.

Solution stability

The solution stability study showed that AML and RAM were stable to hydrolysis for 24 hours.

Robustness

Robustness of the method was studied by changing the flow rate of the mobile phase from 1 ml min^{-1} to 0.9 ml min^{-1} and 1.1 ml min^{-1}. Using 1.1 ml min^{-1} flow rate, retention time for AML and RAM were observed to be 9.7 and 12.8 min respectively and with 0.9 flow rate, retention time for AML and RAM were found to be 11.7 and 15.8 min respectively without affecting resolution of the drug. When a mobile phase composition was changed to 0.02 M KH_2PO_4-methanol (35+65v/v; pH 3) by increasing percentage of methanol the retention time for AML and RAM were observed to be 6.94 and 8.4 min respectively. When a mobile phase composition was changed to 0.02 M KH_2PO_4-methanol (45+55v/v; pH 3) by increasing percentage of buffer the

retention time for AML and RAM were observed to be 11.9 and 16.1 min respectively. The assay result of both the drug was found to be more than 98%.

Table 3: Summary of Validation and System Suitability Parameters

Parameters	AML	RAM
Retention time (min)	10.9	13.6
Tailing factor	1.43	1.42
Resolution	2.4	
Theoretical Plates	3287	2236
Detection limit (ng/ ml)	2	100
Quantitation limit (ng/ ml)	10	350
Accuracy(%)	99.60 - 100.1	99.06 - 100.32
Precision (% RSD)		
Intra-day (n=3)	0.53 - 1.74	0.57 – 1.40
Inter-day (n=3)	0.65 - 1.88	0.14 – 0.32
Instrument precision (% RSD)	0.18 - 0.63	0.61 – 0.79

Table 4: Assay results of marketed formulation

Formulation	Labeled Amount (mg)		Amount found (mg)		% of drug found ± RSD	
	AML	RAM	AML	RAM	AML	RAM
1	5.0	2.5	4.97	2.48	99.4 ± 0.97	99.2 ± 0.83

Forced degradation study

The chromatograms of base degraded sample showed degradation product peaks at retention time (RT) 3.4 min, 4.2 min and 4.6 for AML and at 3.1 min for RAM(Figure 4). The degradation product peaks were well resolved from drug peak .The chromatogram of acid degraded samples showed degradation products peaks at 5.3 min and 6.3 for AML and at 3.2 min for RAM (Figure 5). Oxidative stress degradation sample showed no degradation peak but peaks area got decreased(Figure 6). Dry heat degradation study revealed that there is no degradation peak for both drugs but peaks area got decreased (Figure 7).

Table 5: Forced degradation study of AML and RAM for the proposed method

Conditions	Time (min)	Recovery (%)		Retention time of degradation products	
		AML	RAM	AML	RAM
Base 0.5 N NaOH	20	1.24	22.04	3.4,4.2 and 4.6	3.1
Acid 0.5 N HCl	20	2.25	76.06	5.3 and 6.3	3.2
6% hydrogen peroxide	20	90.82	54.91	-	-
Dry heat	20	71.12	86.99	-	-

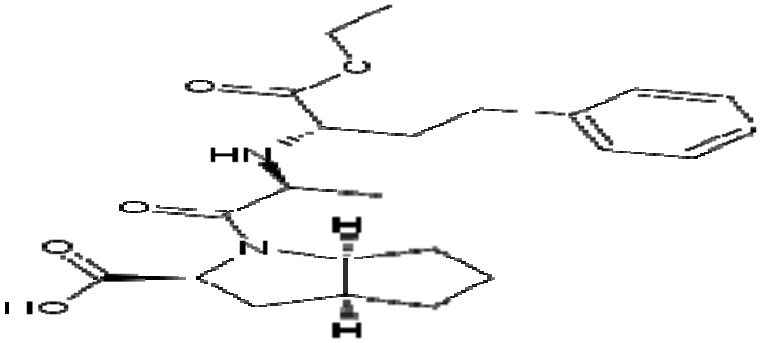

Fig. 1 Structure of ramipril

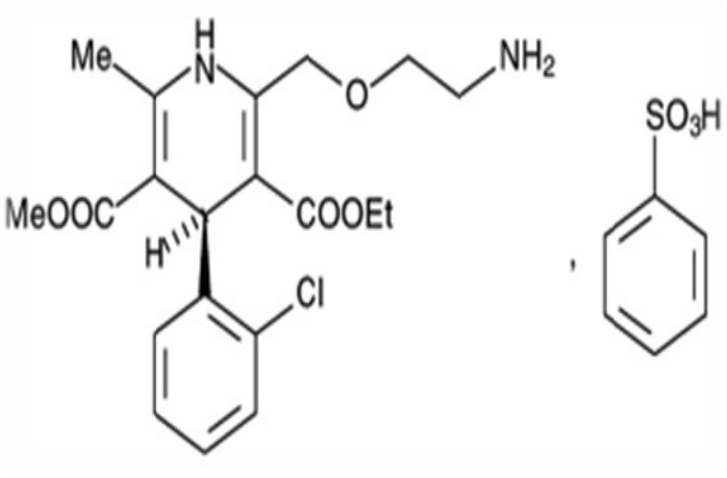

Fig. 2 Structure of amlodipine besylate

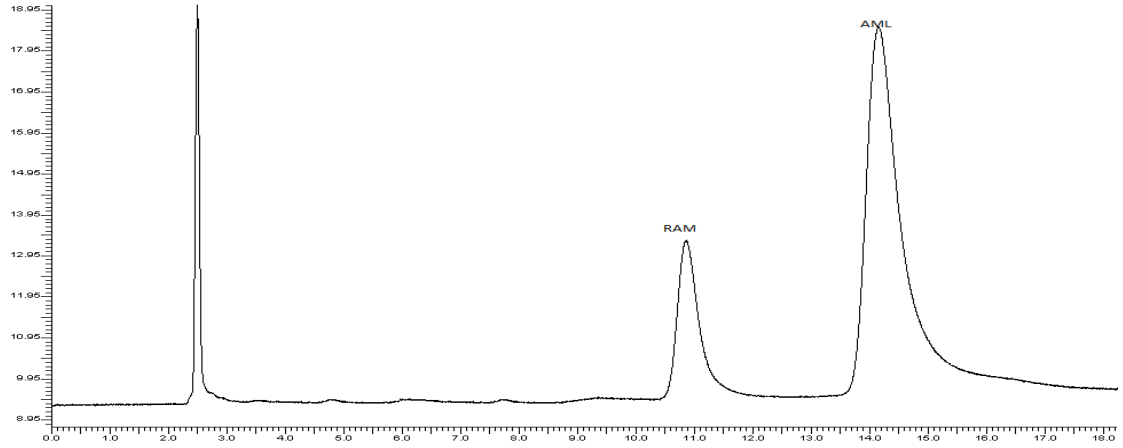

Fig. 3. Liquid chromatogram showing well resolved peaks of AML and RAM

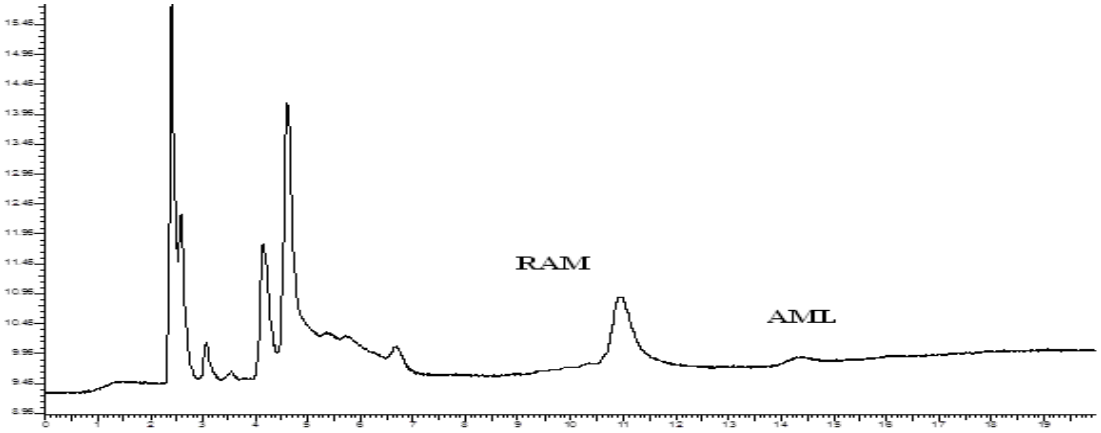

Fig. 4. Chromatogram of base (0.5 N NaOH) treated AML and RAM at 80 ^{o}C for 1 h

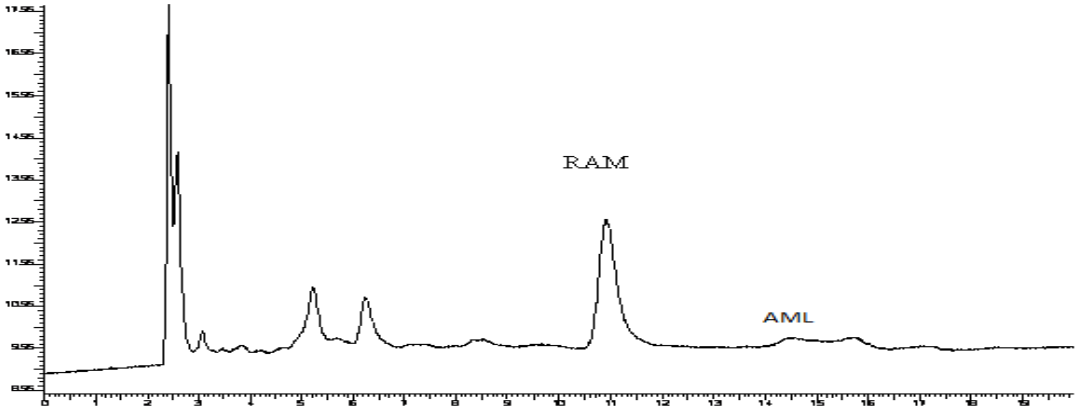

Fig. 5. Chromatogram of acid (0.5 N HCl) treated AML and RAM at 80 ^{o}C for 1 h.

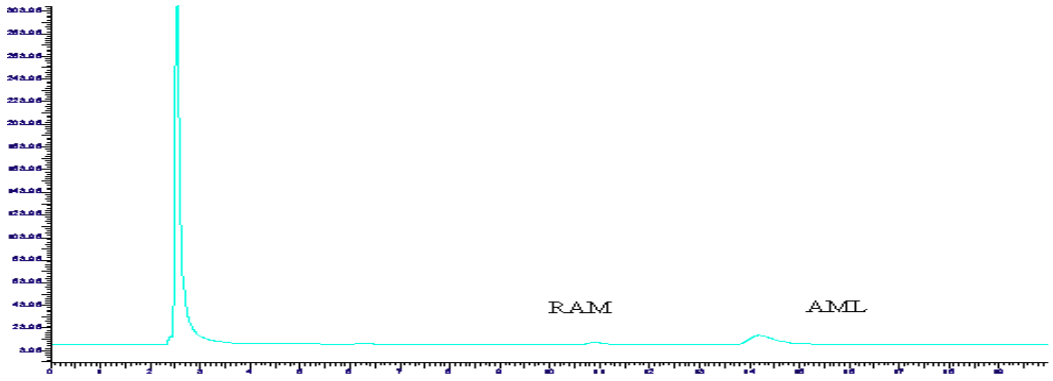

Fig. 6. Chromatogram of 6% hydrogen peroxide treated AML and RAM at 80 ^{o}C for 1 h.

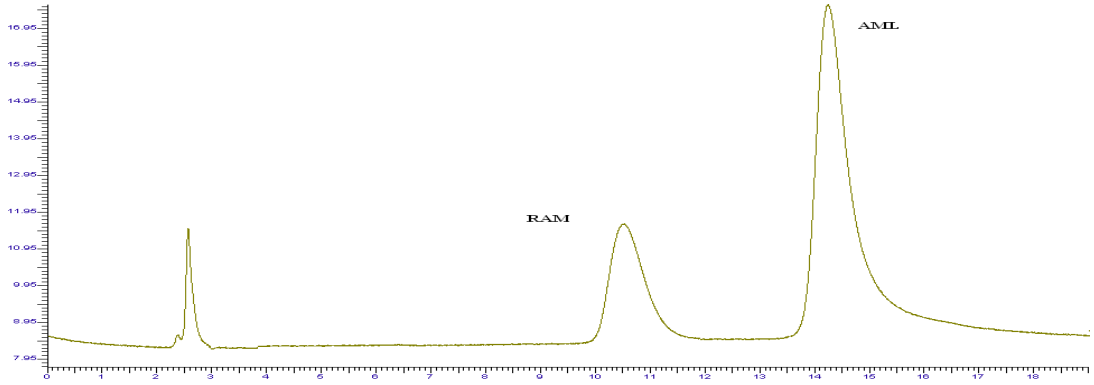

Fig. 7. Chromatogram of dry heat degradation study of AML and RAM at 80 ^{o}C for 1 h.

CONCLUSION

Proposed study describes stability indicating liquid chromatographic method for the estimation of AML and RAM in pharmaceutical dosage form. The method was validated and found to be simple, sensitive, accurate and precise. The method was successfully used for determination of drug in its pharmaceutical formulation. Also the above results indicate the suitability of the method for acid, base, dry heat and oxidative degradation study of drug. As the method separates the drugs from its degradation products, it can be used for analysis of stability samples of drug. In future, isolation and characterization of degradation product can be carried out and pharmacological effect of isolated degradation product can be evaluated.

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