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STABILITY INDICATING HPTLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF AMLODIPINE BESYLATE AND LOSARTAN POTASSIUM AND CHARACTERISATION OF ACID DEGRADANT PRODUCT OF LOSARTAN

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ABSTRACT: An accurate, precise, sensitive and selective stability indicating High-Performance Thin Layer Chromatographic method has been developed and validated for simultaneous estimation of Amlodipine besylate (AML) and Losartan potassium (LOS) in bulk and pharmaceutical dosage formulation. Chromatographic separation of both the drugs was achieved by using TLC aluminum plates pre-coated with silica gel 60 F_{254} as the stationary phase and chloroform: methanol: toluene: acetic acid (5.5: 2.5: 2: 0.05 v/v/v/v) as mobile phase. Densitometric analysis was carried out in the absorbance mode at 237 nm. The R_f values found to be 0.23 ± 0.02 and 0.74 ± 0.02 for AML and LOS respectively. The method was validated in compliance with ICH Guideline for linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, specificity, accuracy, repeatability, and robustness. Linear regression analysis data for the calibration plots showed good linear relationship over the concentration range of 100-400 ng/spot ($r^2 = 0.9993$) and 500-1750 ng/spot ($r^2 = 0.9991$) for AML and LOS respectively. The LOD and LOQ were 24.46 and 74.14 ng/spot for AML and 10.82 and 32.80 ng/spot for LOS. The recoveries of AML and LOS were found to be 99.48-99.56% and 99.51-99.89% respectively. The drugs were subjected to acidic, alkaline, oxidative, neutral and thermal degradation conditions. The degradation product of Losartan in acidic condition was carried out, and its degradation product is successfully separated and isolated by HPTLC method. Degradation product was identified by using the MS technique.

INTRODUCTION: Amlodipine besylate (AML, **Fig. 1**), chemically, (RS)-3-ethyl-5-methy-l -2 -(2-amino ethoxymethyl) -4 -(2-chlorophenyl) -1, 4-dihydro -6 -methyl -3, 5- pyridinedi carboxylate benzene sulphonate [1].

It is a long-acting calcium channel blocker, which is used as an antihypertensive agent. The dose is 5-10 mg once a daily orally [2].

Amlodipine is a dihydropyridine calcium antagonist (calcium ion antagonist or slow-channel blocker) that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle, which results in a dilatation of the main coronary and systemic arteries, decreased myocardial contractility, increased blood flow and oxygen delivery to the myocardial tissue, and decreased total peripheral resistance [3, 4].

Losartan potassium chemical name is 2-butyl-4-chloro-1-[[2'-(1H-tetrazol-5-yl) [1, 1'- biphenyl]-4-yl] methyl]- 1H-imidazole-5 -methanol, mono potassium salt **Fig. 2**. Losartan is an angiotensin-receptor blocker that may be used alone or with other agents to treat hypertension. It may also be used as an alternative agent for the treatment of systolic dysfunction, myocardial infarction, coronary artery disease, and heart failure [2, 5].
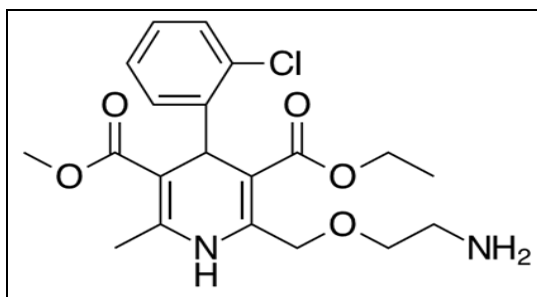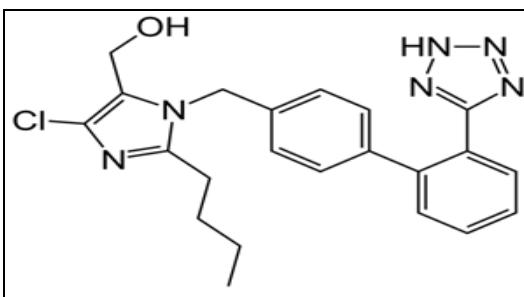

FIG. 1: CHEMICAL STRUCTURE OF AML

FIG. 2: CHEMICAL STRUCTURE OF LOS

Literature survey reveals the availability of several methods for estimation of both AML [6 - 34] and LOS [35 - 47] includes UV, HPLC, LC-MS as alone or in combination with other drugs. However, no stability indicating HPTLC method has been reported for the estimation of AML and LOS in the combined dosage form. Present work emphasizes the quantitative estimation of AML and LOS and their degradation products in the combined dosage form by HPTLC. As compared to the other analytical techniques, HPTLC is the most simple and less time-consuming technique. This technique has an advantage that many parameters can be performed at one time, which is not possible with other analytical techniques.

MATERIALS AND METHODS:

Material: AML and LOS were provided as gift samples by Apotex Pharmaceuticals Pvt. Ltd., Marketed formulation tablets (Repace-A Mfg. by Sun Pharmaceuticals) with a dose of 5 mg Amlodipine besylate and 25mg Losartan potassium were procured from the local market, Pune. Analytical grade solvents and TLC aluminum plates pre-coated with silica gel 60 F_{254} used for this study were purchased from Merck Pvt. Ltd., Mumbai.

Equipment: Chromatographic separation of the drug was performed by using TLC aluminium plates pre-coated with silica gel 60 F_{254} (10 × 10) with 250 μm thickness. Samples were applied on the plate in the form of bands of width 6 mm using Hamilton microliter syringe (100 μl). Linear ascending development was carried out in 10 × 10 cm twin trough glass chamber (CAMAG) by using as chloroform: methanol: toluene: acetic acid (5.5:2.5:2:0.05 v/v/v/v) mobile phase. The slit dimension was kept at 5 × 0.45 mm. The optimized chamber saturation time for the mobile phase was 30 min. The length of the chromatogram run was 9 cm. After development plates were air-dried and densitometric scanning was performed on a Camag TLC Scanner equipped with winCATS software version 1.4.4.6337 at 237 nm. The source of radiation utilized was a deuterium lamp.

Method Development:

Preparation of the Standard Stock Solution I: An accurately weighed 10 mg of AML, and 10 mg of LOS were transferred to 10 ml volumetric flasks, and the volume was made up to the mark with methanol, to get standard stock solution of AML A1 (1000 μg/mL) and Losartan L1 (1000 μg/mL) respectively.

Preparation of the Working Standard Solution: 1 ml of standard stock solution of A1 (1000 μg/mL) was diluted with 10 ml methanol to get working standard solution of AML (A2) 100 μg/mL. 5 ml of the standard solution of L1 (1000 μg/mL) was diluted with 10 ml methanol to get 500 μg/mL working standard solution of LOS (L2).

Preparation of Mixed Standard Solution (M): 1 ml solution of AML 1000 μg/mL and 5 ml solution of Losartan potassium 5000 μg/mL were mixed and diluted with 10 ml methanol to get mixed standard solution. This mixed standard solution (M) of containing AML 100 μg/mL and LOS 500 μg/mL respectively was used for stability indicating HPTLC analysis for simultaneous estimation of AML and LOS.

Optimization of Chromatographic Method: The major objective in developing this stability indicating HPTLC method is to achieve the resolution of AML and LOS and its degradation products.
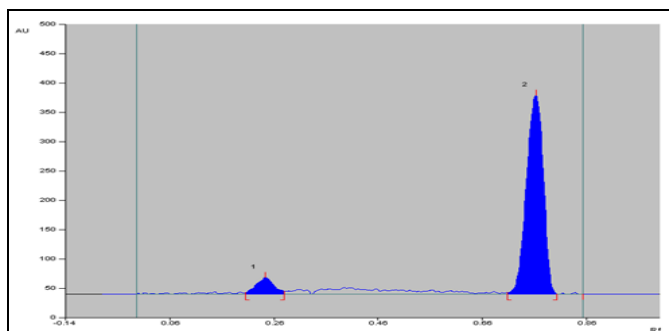

FIG. 3: REPRESENTATIVE DENSITOGRAM OF AMLODIPINE BESYLATE AND LOSARTAN POTASSIUM BY HPTLC METHOD

The separation was achieved by linear ascending development in 10 cm × 10 cm twin trough glass chamber using chloroform: methanol: toluene: acetic acid (5.5: 2.5: 2: 0.05 v/v/v/v) as mobile phase and detection was carried out at 237 nm. The retention factors were found to be 0.24 ± 0.02 and 0.73 ± 0.02 for AML and LOS respectively.
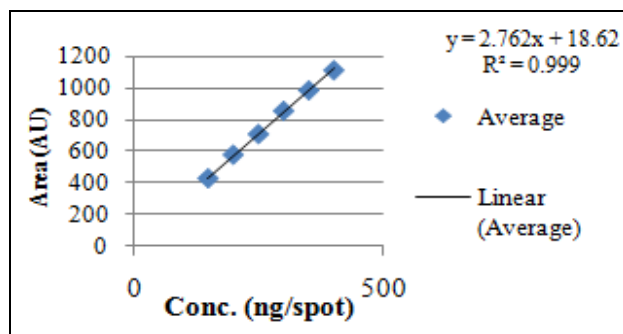
Representative densitograms of a mixed standard solution of AML and LOS is shown in **Fig. 3**.

Validation of Analytical Methods: The method was validated as per the ICH guideline [48, 49, 50, 51] for linearity, precision, accuracy, the limit of detection (LOD), the limit of quantification (LOQ), robustness and specificity.

Linearity: The Mixed standard solution (M) containing Amlodipine besylate (100 μg/mL) and Losartan potassium 500 μg/mL was applied on TLC plate in a range of 1.5, 2, 2.5, 3, 3.5 and 4 μl. The linear regression data for calibration curves (n = 3) showed a good linear relationship over a concentration range of 150-400 ng/spot for AML and 750-2000 ng/spot for LOS. The calibration curves were developed by plotting peak areas *vs.* concentrations shown in **Table 1** and **Fig 4** and **5**.

TABLE 1: REGRESSION DATA OF AML AND LOS

Parameters	Amlodipine besylate	Losartan
Linearity range (ng/spot)	150-400	750-2000
Correlation coefficient (r^2)	0.9993	0.9991
Slope	2.7628	4.3634
Intercept	18.625	3137.6

(n = 3)

FIG. 4: LINEARITY GRAPH OF AMLODIPINE BEYLATE
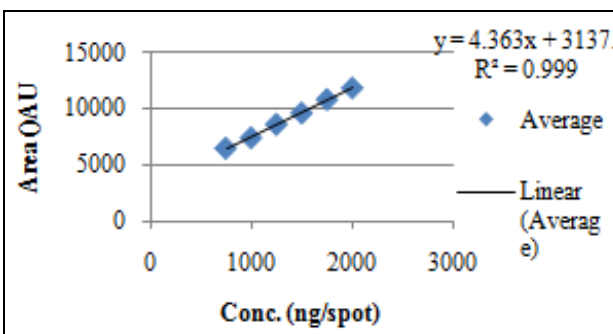
FIG. 5: LINEARITY GRAPH OF LOSARTAN POTASSIUM

Precision: The precision of the method was demonstrated by intraday precision and inter-day precision studies. Intra-day precision was determined by applying 3 spots of 2.5, 3 and 3.5 μl of mixed standard solution (M) on TLC plate for three times with different time intervals on the same day.

Inter-day precision was determined by applying 3 spots of 2.5, 3 and 3.5 μl of mixed standard solution (M) on the TLC plate within three consecutive days. The percentage of RSD was calculated. The results obtained are shown in **Table 2** and **3**.

TABLE 3: INTER-DAY PRECISION

Drug	Conc. (ng/spot)	Avg. Area	% RSD
AML	250	749.43	1.18
	300	879.6	1.31
	350	1095.5	1.24
LOS	1450	8054.63	0.33
	1500	8882.3	0.25
	1550	9771.2	1.02

% RSD = Relative Standard deviation (n=3)

TABLE 2: INTRA-DAY PRECISION

Drug	Conc. (ng/spot)	Avg. Area	% RSD
AML	250	796.7	0.74
	300	896.26	0.90
	350	1038.38	1.18
LOS	1450	8090.86	0.18
	1500	8935.63	0.34
	1550	9714.7	0.58

% RSD = Relative Standard deviation (n=3)

Recovery Study: Accuracy of the method was determined by applying the method to the drug sample AML 5 mg and LOS 25 mg combination tablet (REPACE-A) to which a known amount of Amlodipine besylate and Losartan potassium standard drug corresponding to 80, 100 and 120% of label claim have been added. The recovery was carried out by standard addition method. Percentage recoveries were calculated given in **Table 4**.

TABLE 4: DETAILS OF RECOVERY STUDIES

Drug	% Level	Initial amount (ng)	Amount added (ng)	% Recovery	% RSD
	80	300	240	99.48	0.2
AML	100	300	300	99.56	1.18
	120	300	360	99.64	1.11
	80	1500	1200	99.89	0.04
LOS	100	1500	1500	99.70	0.07
	120	1500	1800	99.51	0.2

Limit of Detection (LOD) and Limit of Quantitation (LOQ): LOD and LOQ of both drugs were calculated by using the signal-to-noise ratio following equations as per ICH guideline. Values were given in **Table 5**.

TABLE 5: LOD AND LOQ DETAILS OF AML AND LOS

Drug	LOD (ng/spot)	LOQ (ng/spot)
AML	24.46	74.14
LOS	10.82	32.80

LOD: Limit of Detection, LOQ: Limit of Quantitation

$$LOD = 3.3 \times \sigma / S \text{ and } LOQ = 10 \times \sigma / S$$

Where, σ = the standard deviation of the response. S = slope of the calibration curve.

Robustness: The robustness of the method was assessed by making small, deliberate changes in optimized condition like mobile phase composition $\pm$ 0.5 ml; wavelength $\pm$ 10 nm and saturation time $\pm$10 min. percentage RSD was calculated given in **Table 6** and **Table 7**.

TABLE 6: ROBUSTNESS DETAILS OF AML

Condition	R_f	Peak Area	%RSD
Mobile Phase Composition ($\pm$ 0.5 ml Toluene)			
Toluene: Methanol: Chloroform: Acetic Acid (1.5:2.5:5.5:0.05 v/v/v/v)	0.24	746.3	1.62
Toluene: Methanol: Chloroform: Acetic Acid (2:2.5:5.5:0.05 v/v/v/v)	0.25	742.86	
Toluene: Methanol: Chloroform: Acetic Acid (2.5:2.5:5.5:0.05 v/v/v/v)	0.26	746.3	
Wavelength ($\pm$ 10 nm)			
227 nm	0.23	751.23	0.40
237 nm	0.23	746.2	
247 nm	0.24	738.4	
Duration of Saturation ($\pm$ 10 min)			
25	0.23	740.9	1.4
30	0.24	746.2	
35	0.25	751.5	

R_f: Retention factor, % RSD: Relative Standard Deviation

TABLE 7: ROBUSTNESS DETAILS OF LOS

Condition	R_f	Peak Area	%RSD
Mobile Phase Composition ($\pm$ 0.5ml methanol)			
Toluene: Methanol: Chloroform: Acetic Acid (1.5:2.5:5.5:0.05 v/v/v/v)	0.73	9834.5	0.95
Toluene: Methanol: Chloroform: Acetic Acid (2:2.5:5.5:0.05 v/v/v/v)	0.73	9819.4	
Toluene: Methanol: Chloroform: Acetic Acid (2.5:2.5:5.5:0.05 v/v/v/v)	0.74	10007.5	
Wavelength ($\pm$ 10 nm)			
247nm	0.73	9839.7	0.70
237nm	0.73	9819.4	
257nm	0.74	9954.56	
Duration of Saturation ($\pm$ 10 min)			
20	0.72	10762.8	0.89
30	0.73	9852.73	
40	0.73	10366.9	

R_f: Retention factor, % RSD: Relative Standard Deviation

Assay: To determine the content of Amlodipine besylate and Losartan potassium in tablets (containing 5 mg Amlodipine besylate and 25 mg Losartan potassium), 20 tablets were weighed accurately, crushed and average weight was calculated. The amount equivalent to 1 tablet was taken and dissolved and diluted up to the mark in with methanol to obtain a concentration equivalent to 100 µg/mL Amlodipine besylate and 500 µg/mL Losartan potassium. 3 µl spot of mixed standard solution, M (Amlodipine besylate 100 µg/mL and Losartan potassium 500 µg/mL) and marketed formulation solution were spotted on TLC plate. The plate was dried and scanned at 237 nm using Camagwin software version 1.4.4.6337. The dendrograms recorded are shown in **Fig. 6**
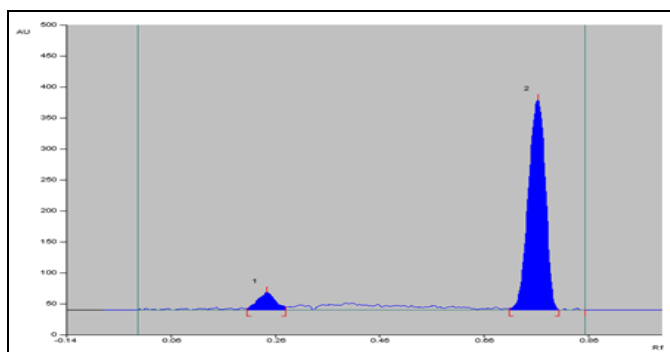

FIG. 6: HPTLC DENSITOGRAM OF AML AND LOS IN TABLET FORMULATION

Forced Degradation Studies: Forced degradation study was performed to ensure that the degradation products generated due to stress condition are not interfering with the API peak. API and drug product should be forcefully stressed by treating with acid, alkali, hydrolysis, photolysis and oxidizing agent by controlling stress agent concentration and exposure time to achieve degradation about 10-30%.

Preparation of Acid Induced Degradation Product: A 5 ml of mixed standard solution M (AML 100 µg/mL and LOS 500 µg/mL) and 5 ml of 0.1N HCl was kept at room temperature for 3 h. 3µl of the resulting solution was applied on TLC plates to get concentration equivalent to 300 ng/spot of AML and 1500 ng/spot of LOS and the plate was run with the mobile phase, dried and scanned at 237 nm The representative densitogram after Acid-induced degradation is shown in **Fig. 7**.

Preparation of Base Induced Degradation Product: A 5 ml of mixed standard solution M

(AML 100 µg/mL and LOS 500 µg/mL) and 5 ml of 0.1N NaOH was kept at room temperature for 3 h. 3 µl of the resulting solution was applied on TLC plates to get concentration equivalent to 300 ng/spot of AML and 1500 ng/spot of LOS and the plate was run with the mobile phase, dried and scanned at 237 nm The representative densitograms after Base induced degradation is shown in **Fig. 8**.

Preparation of Hydrogen Peroxide-Induced Degradation Product: A 5 ml of mixed standard solution M (AML 100 µg/mL and LOS 500 µg/mL) and 5 ml of 3% hydrogen peroxide was kept at room temperature for 3 h. 3 µl of the resulting solution was applied on TLC plates to get concentration equivalent to 300 ng/spot of AML and 1500 ng/spot of LOS and the plate was run with the mobile phase, dried and scanned at 237 nm. The representative densitograms after Base induced degradation is shown in **Fig. 9**.

Preparation of Neutral Induced Degradation Product: A 5 ml of mixed standard solution M (AML 100 µg/mL and LOS 500 µg/mL) and 5 ml distill was kept at room temperature for 3 h. 3ul of the resulting solution was applied on TLC plates to get concentration equivalent to 300 ng/spot of AML and 1500 ng/spot of LOS and the plate was run with the mobile phase, dried and scanned at 237 nm. The representative densitograms after Neutral induced degradation is shown in **Fig. 10**.

Preparation of Thermal Induced Degradation Product: 30 mg of Amlodipine besylate and 150 mg of Losartan potassium powder was mixed and put in a petri dish. Petri dish was kept in a hot air oven at 60 ºC. A powdered sample of 30 mg was withdrawn after 30 min, 1h, 2h, 3h, and 4h intervals. Spot of mixed standard solution, M (Amlodipine besylate 500 µg/mL and Losartan potassium 5 µg/mL) and the resultant degraded solution was applied on TLC plate, and the plate was run with the mobile phase. The plate was dried and scanned at 237 nm using Camagwin software version 1.4.4.6337 and densitograms were recorded in **Fig. 11**.

An accurately weighed quantity of 100 mg of LOS was dissolved in 80 ml methanol. Subsequently, 1.0N HCl was added, and volume was made up to 100 ml in a graduated A-grade volumetric flask.

The resulted solution was refluxed in a round bottom flask on a temperature controlled precision water bath at 70 °C for 3.0 h. The acid degradation of the LOS was confirmed by newly developed HPTLC method, where the major degradant formed in alkaline stressed condition was isolated through preparative HPTLC technique. Working standard of LOS and isolated degradant was further subjected to Mass spectral studies. Mass spectral data provided the information regarding the molecular weight of the compound. The peak area was determined, and quantitative estimation of LOS and degradant formed in acid-stressed condition was carried out from the corresponding regression equation.
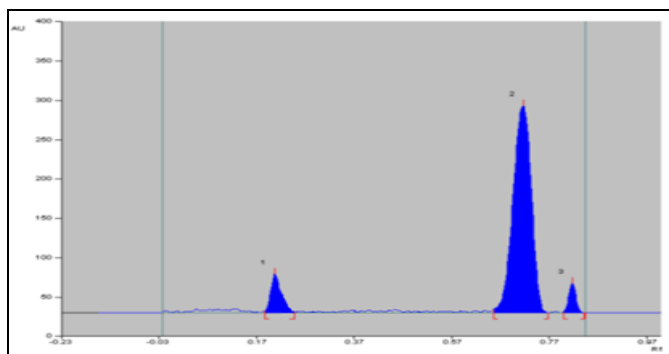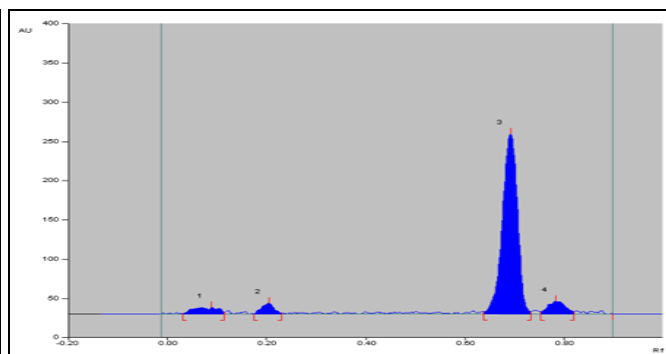

FIG. 7: ACID DEGRADATION OF AML AND LOS
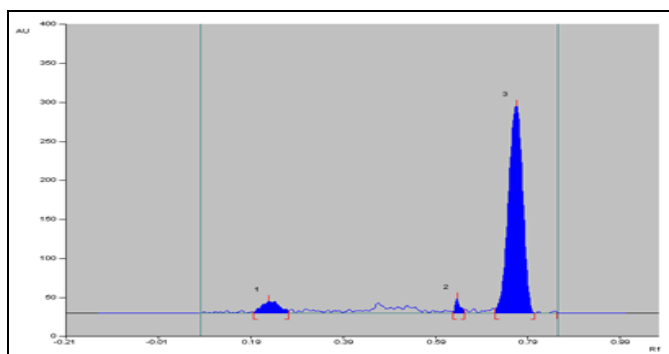

FIG. 8: BASE DEGRADATION OF AML AND LOS

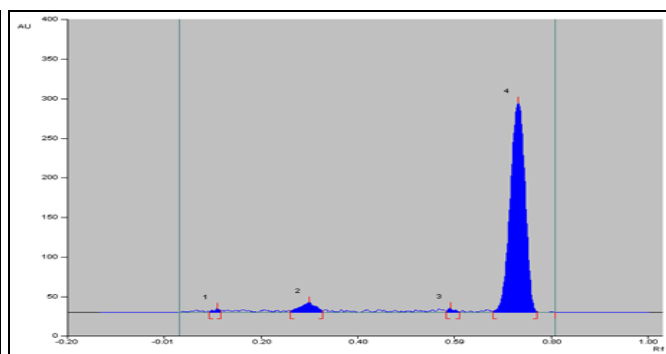**FIG. 9: HYDROGEN PEROXIDE DEGRADATION OF AML AND LOS**

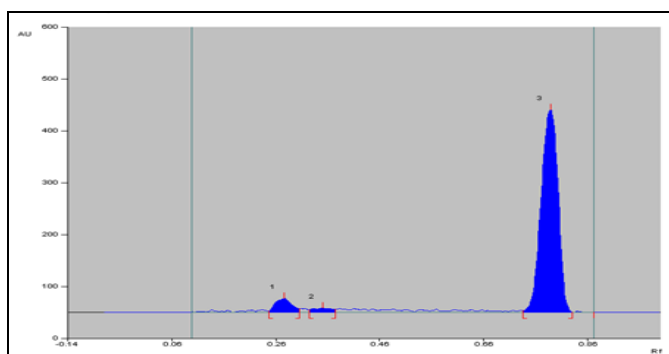**FIG 10: NEUTRAL DEGRADATION OF AML AND LOS**

FIG. 11: THERMAL DEGRADATION OF AML AND LOS ISOLATION OF ACID DEGRADATION PRODUCT OF LOSARTAN

RESULTS AND DISCUSSION: The developed HPTLC method was validated as per ICH guidelines. The developed method found to be linear within the range of 100-400 ng/spot (r^2 = 0.9993) and 500-1750 ng/spot (r^2 = 0.9991) for AML and LOS respectively. The accuracy of the method was determined at 80%, 100%, 120% level.

The % recoveries of AML and LOS were found to be 99.48-99.56% and 99.51-99.89% respectively. The LOD and LOQ of AML were found to be 24.46, and 74.14 ng/spot and the LOD and LOQ of LOS was found to be 10.82 and 32.80 ng/spot. The developed method was found to be precise as the % RSD values for intra-day and inter-day were found to be less than 2%. The method was also found to be robustness indicated by the % RSD values which are less than 2%. The summary of the validation parameters of proposed HPTLC method is shown in **Table 8**. The stress degradation studies were carried out for the drug in acid, base, oxidation, neutral and thermal conditions.

Summary of the results of stress degradation studies of AML and LOS are shown in **Table 9**.

Identification of Acid Degradant Products of Losartan: The mass spectral data of standard LOS

Fig. 12 shows the appearance of molecular ion peak at m/z 422 which indicates the molecular weight of Losartan while the spectral data of acid degradant **Fig. 13** characterized by the breaking of

the secondary amine group in imidazole ring from the structure of LOS confirms the appearance of molecular ion peak at m/z 339. The tallest peak in the spectrum appeared at m/z 395.

TABLE 8: SUMMARY OF VALIDATION PARAMETER

S. no.	Validation Parameters	Results	
		AML	Los
1	Linearity	Range:150-400 R^2: 0.9993 Slope: 2.7628 Intercept: 18.625	Range: 750-2000 R^2: 0.9991 Slope: 4.3634 Intercept: 3137.6
2	LOD	24.46	74.14
3	LOQ	10.82	32.80
4	Precision (% RSD)	Inter-day: 1.18-1.24 Intra-day: 0.2-1.18	Inter-day: 0.25-1.02 Intra-day: 0.18-.58
5	Robustness (% RSD)	0.40-1.62	0.70-.095

TABLE 9: SUMMARY OF FORCED DEGRADATION STUDY

S. no.	Degradation Condition	% Degradation		R_f of degradants	
		AML	LOS	AML	LOS
1	Acid	-	10.04	-	0.74
2	Base	8.5	13.57	0.07	0.78
3	Oxidation	0.77	4.2	-	0.63
4	Neutral	7.6	1.1	.11	0.59
5	Thermal	13.6	-	0.35	-

Acid Hydrolysis:
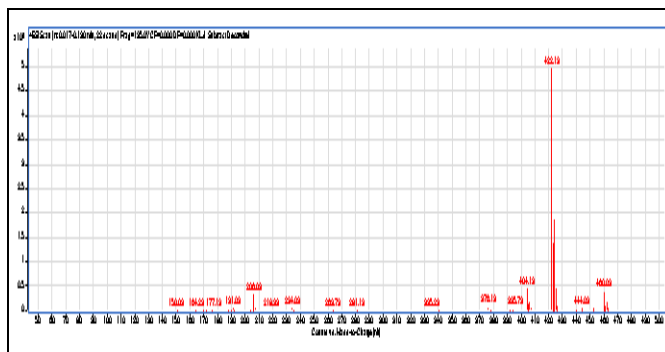
FIG. 12: MASS SPECTRA OF LOSERTAN (API)   FIG. 13: MASS SPECTRA OF 0.1 N HCl AFTER 3 h
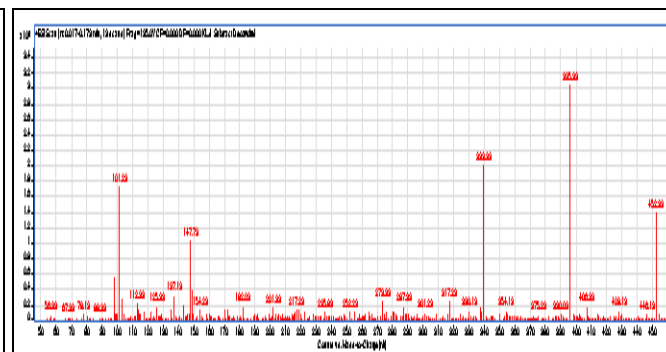
Losartan (Mass - 422 m/z)

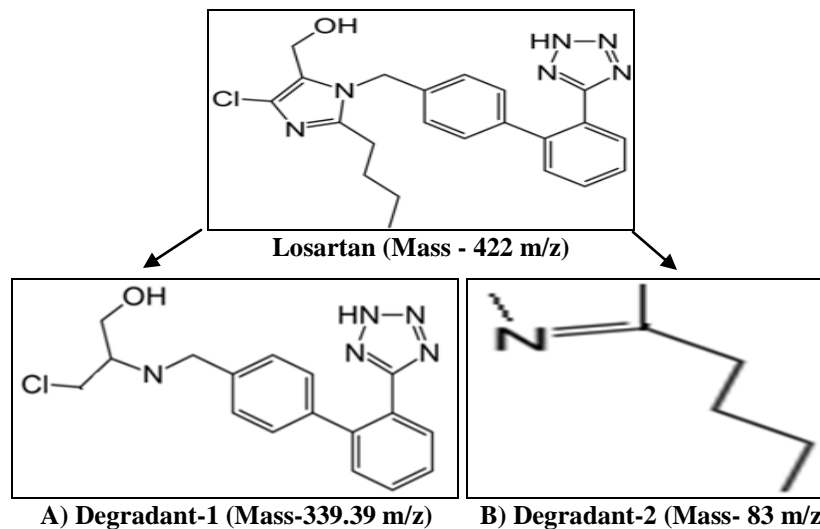A) Degradant-1 (Mass-339.39 m/z)   B) Degradant-2 (Mass- 83 m/z)
FIG. 14: FRAGMENTATION PATHWAY OF ISOLATED ACID DEGRADANT

Further, the major fragments were obtained at m/z 137, and 101 indicates the breakage of the imidazole ring. All predicted structures were satisfied with the double bonds, N-atom, and ring present in structure. The major degradation product and fragmentation of Losartan were obtained according to the suggested mechanism for the acid degradation process as indicated in **Fig 14**. From functional group and mass spectral data, Acid hydrolysis sample of Losartan was found major two degradant which 1-chloro-1-[[2'-(1H- tetrazol-5-yl) [1, 1'-biphenyl]-4-yl] methyl] -5-methanol of degradant 1 and 2-hexyleneimine of degradant 2.

CONCLUSION: A simple, specific, precise and accurate HPTLC method has been established as stability indicating a method for simultaneous estimation of AML and LOS respectively in bulk and in their pharmaceutical dosage form. ICH guidelines were followed throughout the study for method validation and stress testing. Developed HPTLC method effectively separate principle drug peak from degradation product peaks; it can be employed as stability indicating method.

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CONFLICT OF INTEREST: Nil

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