



**STABILITY INDICATING HIGH PERFORMANCE THIN LAYER LIQUID
CHROMATOGRAPHIC (HPTLC) METHOD FOR QUANTITATIVE ESTIMATION OF
AMLODIPINE AND LISINOPRIL IN PHARMACEUTICAL FORMULATIONS**

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ABSTRACT

Amlodipine (AM) and Lisinopril (LS) are used to treat hypertension. A simple, selective and stability indicating high performance thin layer chromatography (HPTLC) method has been established for analysis of AM, LS and their degradation products. Pre-coated silica gel 60F-254 TLC plates were used for the separation. n-Butanol: acetic acid: water (6:2:2 v/v/v) was optimized for the separation and determination of both drugs and the degradation products. Densitometric analysis of both drugs was carried out in absorbance mode at 560 nm. This system was found to give compact bands for AM and LS at R_f 0.69 and R_f 0.31 respectively. Linear relationships were obtained between response and amount of drug in the range 50-700 ng per band with high correlation coefficients. The method was validated for precision, robustness, and recovery. The limits of detection and quantitation were 20 and 50 ng per band (AM), 25 and 100 ng per band (LS), respectively. AM and LS were subjected degradation by acid (0.1N HCl), base (0.1N NaOH), oxidation (3% H_2O_2), dry heat, photo-light and ultraviolet. The degradation products were resolved from the pure drug with significantly different R_f values. Because the method could effectively separate the drug from its degradation products, it can be used for stability-indicating analysis.

KEYWORDS: high performance thin layer chromatography, amlodipine(AM), lisinopril (LS), pharmaceutical formulations.

INTRODUCTION

The stability-indicating assay is a method that is employed for the analysis of evaluating the stability of therapeutically important samples in pharmaceutical industry. The requirement to setup a stability indicating assay method (SIAM) has become mandatory when International Conference on Harmonization (ICH) guidelines were officially introduced, where the guide lines were clearly specified to carry out forced decomposition studies under various conditions like pH, light, oxidation, dry heat etc and the discrete separation of the drug molecule from degradation products so that the active ingredient content can be accurately measured.

Lisinopril (LS) chemically (2S)-1-[(2S)-6-amino-2-[[{(1S)-1-carboxy-3-phenylpropyl} Amino] hexanoyl] pyrrole-2-carboxylic acid, an angiotensin converting enzyme (ACE) inhibitor is used in the management of hypertension. Similarly, amlodipine (AM) chemically 3-Ethyl-5-methyl (4RS)-2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1, 4-dihydropyridine-3, 5-dicarboxylate benzene sulphonate a long-acting calcium

channel blocker used in the management of hypertension, chronic stable angina pectoris and coronary artery disease. Combined dosage form of both drugs has a marked additional effect on blood pressure as compared with their mono-therapy.

A literature survey reveals that a few analytical methods have been reported for separate determination of AM and LS of single dosage form, as well as combined dosage forms of these drugs with combination of other drugs. Methods reported include UV – visible spectrophotometry, HPLC methods. Therefore, the present studies are primarily aimed at developing a HPTLC method for the rapid quantification of the active ingredient from impurities if any and the degradation products evolved out of various stress conditions employed, thus, comprehensively making the entire study a stability indicating one. The final method is validated as per ICH guidelines.

EXPERIMENTAL

Instrumentation

Quantitative TLC analysis was performed using Linomat V automated sample applicator equipped with 100 μ l syringe and densitometric scanning performed with Camag TLC scanner III (Camag, Muttenz, Switzerland, supplied by Anchrom technologies, Mumbai) in the absorbance reflectance mode at 200 nm and operated by WINCATS Software resident in the system.

Chemicals and Reagents

All the chemicals used in the analysis were of analytical reagent grade. Standard drugs amlodipine (AM) and lisinopril (LS) were a gift samples from Mass Spectrometry Division, IICT. Combination tablets were purchased from the local pharmacies. Methanol was obtained from Merck. Water HPLC grade, acetic acid were purchased from Ranbaxy Laboratories Ltd., (S.A.S.nagar) and n-butanol was purchased from S.D.Fine Chem. (Mumbai, India).

Chromatographic Conditions

Chromatography was performed on 10 cm x 10 cm HPTLC plates pre-coated with 60F-254 (with 0.25 mm thickness; Merck, Germany) and the plates were washed with methanol and activated at 105-110⁰C for 15 min before use. The samples were applied as bands of 6 mm width, under continuous flow of nitrogen, by means of Linomat V applicator (Camag, Muttenz, Switzerland) equipped with 100 μ l syringe. A constant application rate of 4 μ l/sec was employed and the space between two bands was 10 mm. The slit dimension was kept at 5x0.45 mm and a scanning speed of 20 mm/sec was employed. Linear ascending development was carried out in 10 cm x 10 cm twin trough glass chamber (Camag, Switzerland) previously saturated with mobile phase n-butanol: acetic acid: water, 6:2:2 (v/v/v) for 30 min at room temperature. After development, TLC plates were dried, as per their detection conditions it is treated with visualizing reagents and was scanned with Camag TLC Scanner III in absorbance reflectance mode at respective wavelengths and controlled by WINCATS Software resident in the system. Evaluation was based on linear regression of peak areas.

Preparation of Standard and Commercial Samples

A standard stock solution of AM and LS 1.0 mg/ml of each were prepared separately in methanol. An appropriate dilutions (0.1 mg/ml i.e., 100 ng/ μ l) of working standards were prepared by methanol.

Twenty tablets of combined formulation (Brand name: LIPRIL-AM, Cipla Ltd., India. Label claim: 5mg of Lisinopril and 5mg of Amlodipine) were weighed and an equivalent weight of tablet content was taken accurately and dissolved in methanol, sonicated for 30 min and the volume was made up to the mark with the same solvent. The resultant solution was centrifuged at 5000 rpm for 10 min and supernatant that was filtered with Whatmann filter paper no.41 was analyzed for drug content.

Preparation of visualization reagent

In present study of work, the drugs are visualized when the plate is sprayed with ninhydrin solution and heated at 80⁰C for 10 min, a violet spot appear which can be scanned at 560 nm. [Ninhydrin solution: 100mg ninhydrin dissolved in 5 ml ethanol and 1 ml acetic acid].

Stress degradation behaviour

All degradation studies were carried out at 1 mg/mL concentration of AM and LS in 0.1 N HCl and 0.1 N NaOH solutions (1000 ng/ μ l) separately and these mixtures were refluxed for 8 hr at 80 ⁰C. The forced degradation in acidic and basic media was performed in the dark in order to exclude the possible degradative effect of light. Oxidative degradation was carried out in 3% H₂O₂ at room temperature for 6 and 24 hr. For thermal degradation, the drugs were placed in hot air oven at 80⁰c for 2 hr. Photolytic degradation were carried out in direct sunlight for 48 hours during the daytime for six days, respectively. UV degradation was carried by drugs which are evenly spread in thin layer in a covered petridish and were kept at uv short (254 nm) and uv long (366 nm) light for 12 hr. The buffer and sample solutions were filtered using 0.45 μ m micro syringe filters prior to the application.

Drug solutions stability was evaluated under the refrigerated (4⁰c) storage conditions for a period of 1 month. The concentrations of freshly made solutions and those tested after a period of 1 month did not vary much (<1%).

RESULTS AND DISCUSSION

Optimized Chromatographic Conditions

TLC procedure was optimized with a view to develop a stability-indicating assay method and a mixture of n-butanol: acetic acid: water (6:2:2 v/v/v) improved the spot characteristics and facilitated a distinct and selective separation for active ingredient as well as the degradation products. Thus, the mobile phase consisting of n-butanol: acetic acid: water (6:2:2 v/v/v) was selected whereby a sharp and symmetrical AM and LS peaks were obtained at R_f 0.34 \pm 0.02 and 0.75 \pm 0.02. AM and LS have different visualization and detection abilities, such that as when compared to their physicochemical properties, AM is an active fluorescent compound, with strong absorption at ultraviolet range (200 to 400 nm), whereas LS is poor ultraviolet absorbing substance, it requires visualization reagent for detection. Due to the presence amine groups in AM and LS the ninhydrin reagent is selected as visualizing agent and scanned at 560 nm.

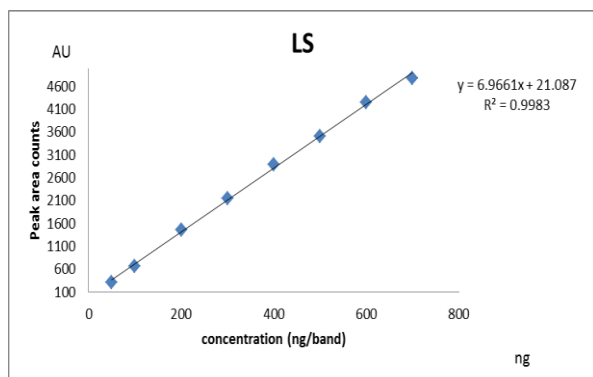
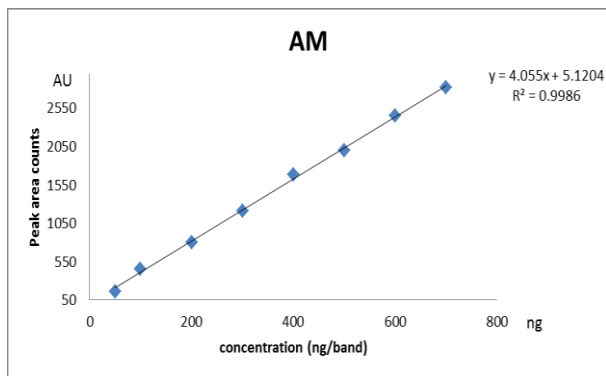
Calibration Curve

The linear regression data for the calibration curves (n=3) as shown in Table.1 offered a good linear relationship over the concentration range of 50-700 ng per band with a correlation coefficient, **r²=0.998 (AM)** and **r²=0.998 (LS)** but there was no significant difference

observed in the slopes of standard curves (ANOVA, $P > 0.05$).

Table 1: Statistical data of regression equations for AM and LS by developed HPTLC method.

Drug	AM	LS
Correlation coefficient (R ²)	0.998	0.998
Slope	5.12	21.08
Std. error of slope	0.0001	0.0001
Intercept	4.055	6.966
Std. error of intercept	0.001	0.001



Method Validation

LOD and LOQ

Limit of Detection and limit of Quantification were calculated by the method described in the Experimental section and found to be 20 and 50 ng per band (AM), 25 and 100 ng per band (LS), respectively.

Precision

Separation of the drug and different degradation products in stressed samples was found to be similar when analyses were performed using different chromatographic conditions on different days. The results of the repeatability and intermediate precision experiments are shown in Table 2.

Robustness

The low values of the % RSD, as shown in Table 3 indicated robustness of the method.

Recovery studies

Recovery studies were performed in triplicate by standard addition method at 50%, 100% and 150%. Known amounts of standard AM and LS were added to pre-analyzed samples and were subjected to the proposed HPTLC method. Results of recovery studies are presented in Table 4.

Specificity

The specificity of the method was ascertained by analyzing standard drug and the sample. The spot for AM and LS in sample were confirmed by comparing the R_f and the spectra of the spot with those of the standard sample.

Table 2: Intra- and inter day precision by HPTLC.

Concentration (ng/spot)	Repeatability		Intermediate Precision	
	Mean area(AU)± SD	% RSD	Mean area(AU)±SD	% RSD
LISINOPRIL				
200	6377.4±65.01	1.02	6374.2±89.30	1.6
300	7453.6±64.10	0.86	7429.9±86.12	1.22
400	8537.8±64.03	0.75	8519.8±92.0	1.08
AMLODIPINE				
200	2001.6±22.8	1.14	1998.9±37.7	1.89
300	3132.0±28.5	0.91	3100.6±40.3	1.3
400	4563.2±40.1	0.88	4547.8±50.9	1.12

Table 3: Robustness of the method.

Parameters	% Recovery		%RSD	
	LS	AM	LS	AM
M.P. Composition				
B: A: W (6: 2 :2 v/v/v)	100.2	99.8	0.89	1.01
B: A: W (6.5: 2.5: 2 v/v/v)	99.9	99.6	0.99	1.1
Plate run length				
8.5 cm	100.1	100.02	0.96	1.12
9.0 cm	99.92	99.81	1.01	1.1

Concentration (ng per band)				
300	100.1	99.98	0.92	1.05
400	100.05	100.01	0.99	1.03

*B: A: W – n-butanol: acetic acid: water

Table. 4: Recovery studies of AM and LS.

Excess of drug added to analyte (%)	Actual content (ng)	Amount of drug found (ng)		% Recovery		% RSD	
		AM	LS	AM	LS	AM	LS
0	200	199.91	200.01	99.95	100	0.95	0.85
50	250	250.23	249.96	100.09	99.98	1.09	0.99
100	300	300.01	300.1	100	100	0.8	0.71
150	350	349.96	350.08	99.98	100.02	0.98	0.98

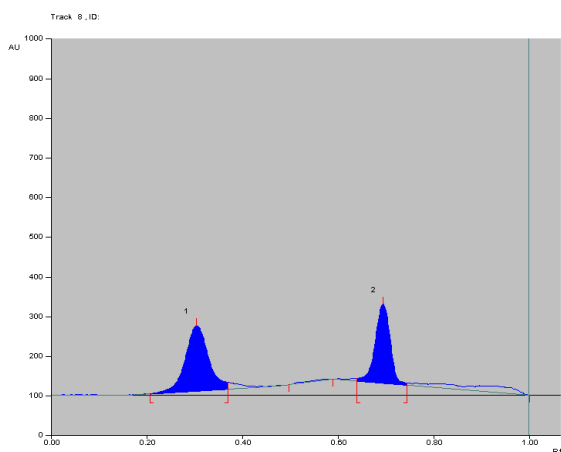


Fig: Typical densitogram of lisinopril and amlodipine besylate R_f 0.30 & 0.069 at 560 nm using n-butanol: acetic acid: water (6:2:2 v/v/v).

Stress degradation studies

Typical chromatograms presented in indicating that the active drug ingredients AM and LS under goes hydrolysis in acidic and basic as evident from the additional peak appeared at R_f 0.31 (LS) and R_f 0.69 (AM), with a proportionate reduction in the area of the main drug at R_f 0.33 (LS) and R_f 0.72 (AM). The drugs was degraded in hydrogen peroxide (3%) in dark at room temperature and shows degradation product at R_f 0.31 (LS) and R_f 0.66 (AM). The drugs also showed little amount of degradation under photo light with R_f 0.31 (LS) and R_f 0.71 (AM) and under ultraviolet light at R_f 0.32 (LS) and R_f 0.71 (AM). The active ingredients also undergo degradation under dry heat, which shows R_f 0.32 (LS) and R_f 0.63 (AM). The densitograms represented that the treated AM and LS have similar R_f that of standard active ingredients, but they showed degradation with difference in their area (AU), such that %degradation and % recovery is calculated.

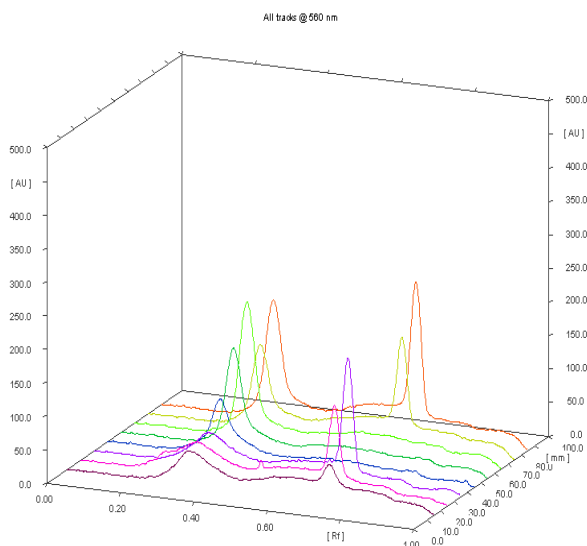


Fig: Densitogram of standard and combination tablet of AM and LS in different concentrations at 560 nm.

Stress type	Rf value		Peak area		% degradation	
	AM	LS	AM	LS	AM	LS
Normal	0.72	0.33	4563.2	8537.8	-	-
Acid degradation	0.69	0.31	1052.7	7079.9	76.93	17.1
Base degradation	0.67	0.31	1049.5	5569.4	77.01	34.8
Photo degradation	0.71	0.31	440.6	4912.3	90.34	42.5
Oxidation	0.66	0.33	1146.3	6063.0	74.49	29.0
Thermal degradation	0.65	0.32	152.3	5168.8	96.6	39.5
UV degradation	0.68	0.32	227.4	5656.5	95.02	33.8

CONCLUSION

The developed HPTLC technique is precise, specific, accurate and stability indicating. Statistical analysis proves that the method is suitable for the simultaneous analysis of AM and LS as a bulk drug and in pharmaceutical formulations without any interference from the excipients. This study is a typical example of a stability-indicating assay established following the recommendations of ICH guidelines which can also be used to determine the purity of the drug as well that is available from various sources by detecting the related impurities if any in general and for degradation studies in particular. The presented analytical data demonstrate the suitability of the method for the study of related kinetics of induced degradation of drugs AM and LS. The method may be extended to quantitative analysis of the drug in plasma and other biological fluids. As the method quantifies and resolves its degradation products from the main active drug ingredient, it can be used as a stability-indicating analytical method for quantitative estimation of amlodipine (AM) and lisinopril (LS) in pharmaceutical formulations.

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