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RESEARCH ARTICLE

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DEVELOPMENT AND VALIDATION OF HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY METHOD FOR SIMULTANEOUS ESTIMATION OF AMLODIPINE BESYLATE AND INDAPAMIDE IN THEIR COMBINED DOSAGE FORM

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Abstract: A simple, precise and accurate high performance thin layer chromatographic method was developed and validated for the simultaneous estimation of amlodipine besylate and indapamide in combined tablet dosage form. Pre-coated silica gel 60F254 aluminium plate was selected as the stationary phase and Ethyl acetate: Toluene: Methanol: Ammonia 5:3.5: 1: 0.5 (v/v/v/v) was used as developing mobile phase. The detection of amlodipine and indapamide was carried out at 240nm. The method was validated for linearity, accuracy, precision, limit of detection and limit of quantitation parameters. The correlation coefficient of amlodipine and indapamide were found to be 0.998 and 0.997 respectively. The average percentage recovery of amlodipine and indapamide were 99.4-100.3 and 99.8-100.2 respectively. The proposed HPTLC method has potential applications for determination of amlodipine besylate and indapamide in combined tablet dosage form.

Keywords: HPTLC method, amlodipine, indapamide, mobile phase

INTRODUCTION

Amlodipine besylate[1-7] is chemically 3-ethyl 5-methyl 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate from the class of Calcium channel blocker and used in the treatment of hypertension, angina and cardiac arrhythmias. Indapamide is chemically 4-chloro-N-(2-methyl-2,3-dihydro-1H-indol-1-yl)-3-sulfamoylbenzamide) from non-thiazide indole derivative of chlorosulphonamide belonging to the diuretic family.

The review of literature revealed that various analytical methods involving spectrophotometry[8-12], HPLC[13-16], HPTLC[17] have been reported for Amlodipine besylate in combination with other drugs. Several analytical methods have been reported for Indapamide in single form and in combination with other drugs including spectrophotometry[18,19], HPLC[20-21], HPTLC[22].

The present work describes the development of a simple, precise, accurate and reproducible spectroscopic method for the simultaneous estimation of Amlodipine besylate and Indapamide in their combined dosage forms. The developed method was validated in accordance with ICH Guidelines[23] and successfully employed for the assay of Amlodipine besylate and Indapamide in their combined Tablet dosage form.

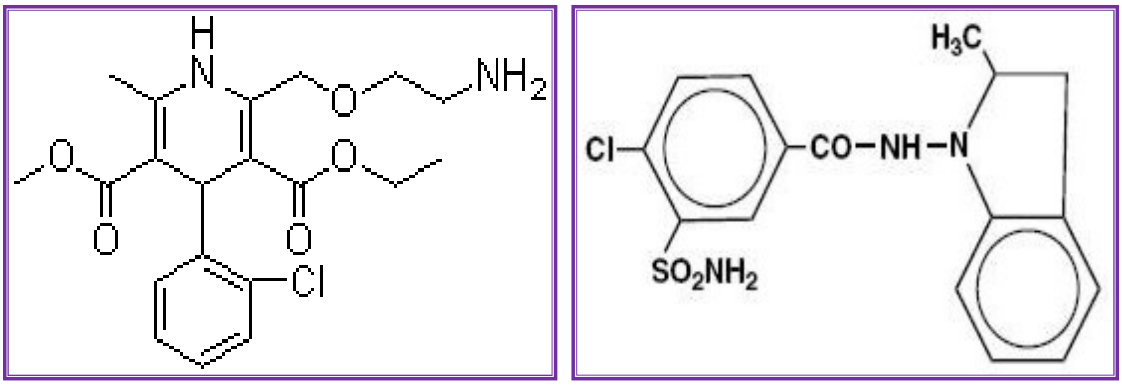

Figure 1 Chemical Structure of Amlodipine besylate and Indapamide respectively

MATERIALS AND METHODS

Reagents and chemicals

Analytically pure Amlodipine besylate and Indapamide were kindly provided by Torrent research center and Manus Aktteva pharmaceuticals, Ahmedabad. Gujarat, India respectively as gift samples. Analytical grade Ethyl acetate, Toluene, methanol and Ammonia ware purchased from RFCL limited, New Delhi, India. Tablet of Amlodipine besylate and indapamide in combine dosage form, NATRILAM, with a 5 mg Amlodipine besylate and 1.5 mg Indapamide label claim, manufactured by *Serdia Pharmaceuticals, Mumbai.*

Instruments

A Camag Muttenz High Performance Thin Layer Instrument with Linomat V Automatic sample applicator (2-500 µl) and win Cats software was used for all the spectral measurements using chamber Camag twin trough glass chamber (10 x 10cm and 20 x 10cm) having scanning speed up to 100mm/s. Calibrated analytical balance K-EA 210 (K-Roy Instrument Pvt. Ltd) was used for weighing purpose. All statistical calculations were carried out using Microsoft excel 2010 analytical tool.

Optimized Chromatographic Method

Parameter	Conditions
Mobile phase	Ethyl acetate : Toluene : Methanol : Ammonia (5:3.5:1:0.5, v/v)
Stationary phase	Pre-coated silica gel G60 – F254 (100×100 mm, thickness layer 0.2 mm).
Temperature	27 ºC
Distance run (mm)	70
Chamber Saturation time (min)	25
Scanning speed (mm/sec)	20
Detection wavelength (nm)	240
Retention factor (R_f)	
AMLODIPINE	0.27 cm
INDAPAMIDE	0.61 cm
Diluent	Methanol

Preparation of Standard stock solution AML standard stock solution: (200 ng/µl)

Standard Amlodipine 20.0 mg was weighed and transferred to a 10 ml volumetric flask and dissolved in Methanol. Take 1 ml from above solution and dilute up to 10 ml with Methanol to give a solution containing 200 ng/µl Amlodipine.

IND standard stock solution: (100 ng/µl)
Standard IND 10 mg was weighed and transferred to a 10 ml volumetric flask and dissolved in Methanol. Take 1 ml from above solution and dilute up to 10 ml with Methanol to give a solution containing 100 ng/µl IND.

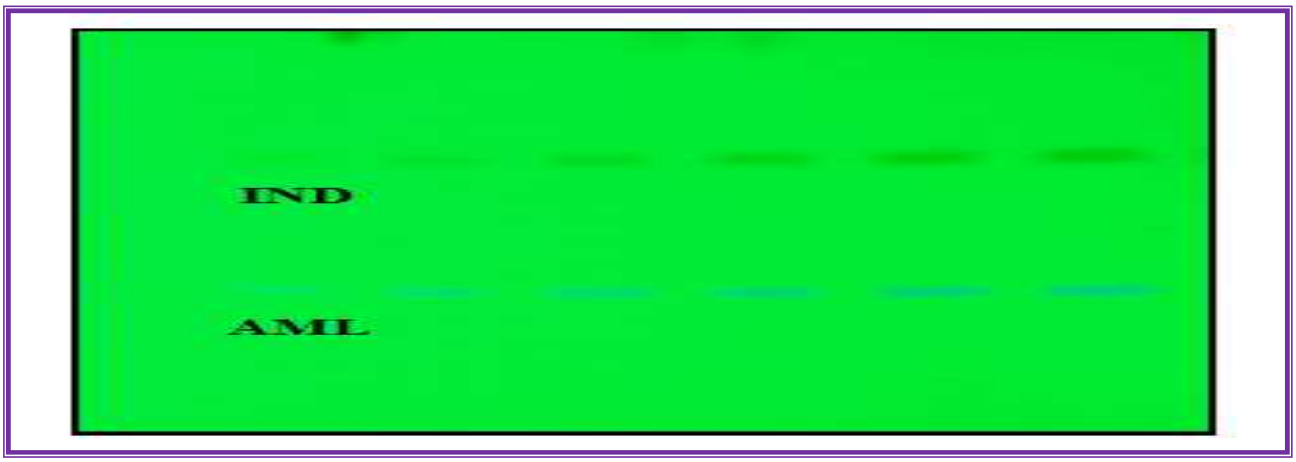

Figure 2 Photograph of developed HPTLC Plate of AML and IND

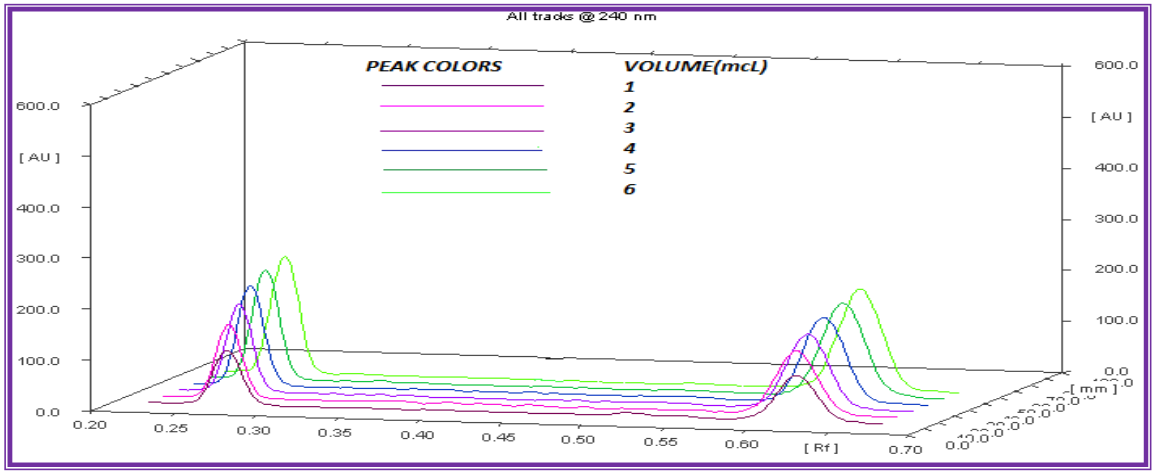

Figure 3 Overlain view of all tracks of AML and IND at 240nm

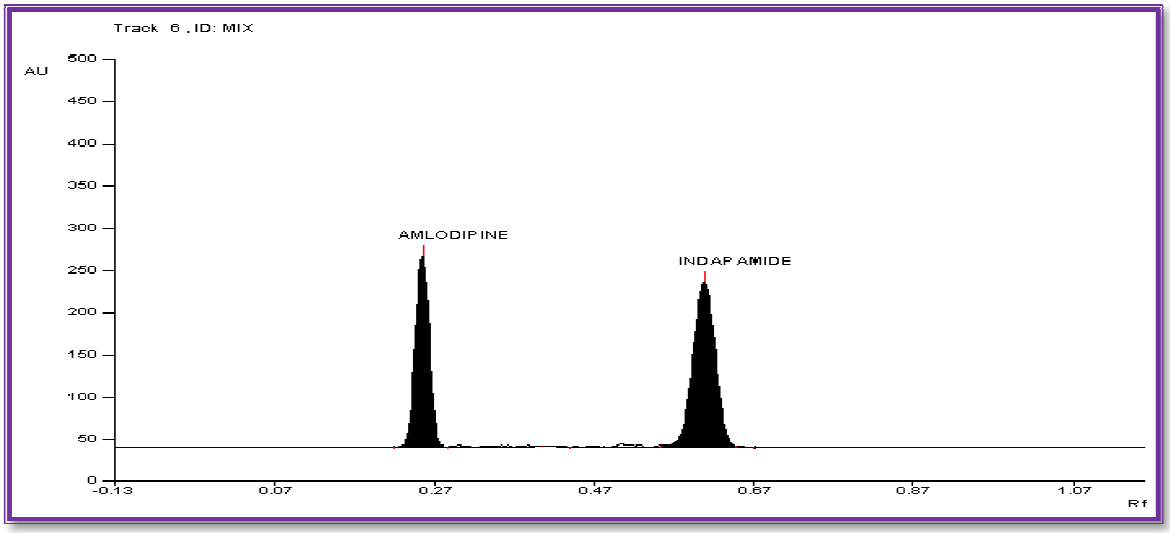

Figure 4 Densitogram of mixed standard solution containing 1200 ng/spot of AMLODIPINE and 600ng/spot of INDAPAMIDE using Mobile Phase Ethyl acetate: Toluene: Methanol: Ammonia (5:3.5:1:0.5, v/v)

Method validation

The proposed method has been extensively validated in terms of specificity, linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ), robustness and reproducibility. The accuracy was expressed in terms of percent recovery of the known amount of the standard drugs added to the known amount of the pharmaceutical dosage forms. The precision (Coefficient of Variation- C.V.) was expressed with respect to the repeatability, intra-day and inter-day variation in the expected drug concentrations. After validation, the developed methods have been applied to pharmaceutical dosage form.

Linearity

Appropriate volume of aliquot from AML and IND standard stock solution was transferred to give solutions containing 200-1200 ng/µl AML and 100-600 ng/µl IND. Each concentration was applied six times to the HPTLC plate. The plate was then developed using the previously described mobile phase and the peak areas were plotted against the corresponding concentrations to obtain the calibration curves Figure 5, 6.

Accuracy

To study the accuracy synthetic powdered mixture was prepared using common excipients in college laboratory and analysis

of the same was carried out. Recovery studies were carried out by addition of standard drug to the placebo at 3 different concentration levels 80, 100, 120 %, taking into consideration percentage purity of added bulk drug samples. Each concentration was analyzed 3 times and average recoveries were measure Table 5.

Precision

The repeatability was evaluated by assaying 6 times of sample solution prepared for assay determination. The intraday and interday precision study of AML and IND was carried out by estimating different concentrations of AML (800, 1000, 1200 ng/µl) and IND (240, 300, 360 ng/µl), 3 times on the same day and on 3 different days (first, second, fifth)and the results are reported in terms of C.V. Table 8,9.

Detection limit and Quantitation limit

ICH guideline describes several approaches to determine the detection and quantitation limits. These include visual evaluation, signal-to-noise ratio and the use of standard deviation of the response and the slope of the calibration curve. In the present study, the LOD and LOQ were based on the third approach and were calculated according to the 3.3σ/S and 10σ/S criterions, respectively; where σ is the standard deviation of y-intercepts of regression lines and s is the slope of the calibration curve.

Robustness

The sample solution was prepared and then analyzed with change in the typical analytical conditions like stability of analytical solution Table 13.

Reproducibility

The absorbance readings were measured at different laboratory for sample solution using another spectrophotometer by another analyst and the values obtained were evaluated using t- test to verify their reproducibility table 10.11.

Specificity

The specificity of the method was determined by analyzing standard drug and test samples. The spot for AMl and IND in the samples was confirmed by comparing the R_f and spectrum of the spot with that of a standard Table 12. The peak purity of AML and IND was determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M) and peak end (E).

Determination of AML and IND from Combined Dosage form - Tablet

Sample preparation

Twenty tablets were weighed and finely powdered. The powder equivalent to 50 mg Amlodipine and 15 mg Indapamide was

accurately weighed and transferred to volumetric flask of 10 ml capacity. 10 ml methanol was transferred to volumetric flask and sonicated for 10 minutes. The flask was shaken and volume was made up to the mark with methanol. The above solution was filtered through whatmann filter paper (0.45µ). 1 ml of this aliquot was added to 10 ml volumetric flask. Volume was made up to the mark with Methanol to give a solution containing 500 ng/µl Amlodipine and 150 ng/µl Indapamide. This solution was used for the estimation of Amlodipine and Indapamide.

Estimation of Amlodipine and Indapamide in combined dosage form

2 µl of the prepared sample was applied on pre-washed TLC plate, developed in the above mobile phase, dried in air and photo metrically analyzed as described above. From the peak area obtained in the chromatogram, the amounts of both the drugs were calculated.

RESULTS AND DISCUSSION

The results of validation studies on simultaneous estimation method developed for AML and IND in the current study involving Ethyl acetate: Toluene: Methanol: Ammonia (5: 3.5: 1: 0.5 v/v/v/v) as the mobile phase for HPTLC are given below. The proposed method was found to be simple, specific, accurate, and precise for the routine simultaneous estimation of two drugs. The linearity range for AML and IND were found to be 200 – 1200 ng/spot and 100-600 ng/spot respectively. Regression analysis data and summary of all validation parameters is given in Table1. Precision was calculated as repeatability (% RSD) and intra and inter day variation (% RSD) for both the drugs. Accuracy was determined by calculating the recovery and the mean was determined. The LOD and LOQ were found to be 56.20 and 170.30 ng/spot respectively for AML and 30.32 and 91.90 ng/spot respectively for IND indicates sensitivity of the proposed method. The peak purity of AML and IND was assessed by comparing their respective spectra at the peak start, apex and peak end positions of the spot. The peak purity was found to be 0.9989 and 0.9993 for AML and IND respectively. The method was successfully used to determine the amounts of AML and IND present in tablets. The results obtained are in good agreement with the corresponding labeled amount. By observing the validation parameters, the method was found to be specific, accurate and precise. Hence the method can be employed for the routine analysis of these drugs in combinations.

The following equations for straight line were obtained for AML and IND

Linear equation for AML**: y = 1.525x + 1119**

Linear equation for IND: **y = 4.210x + 1542**

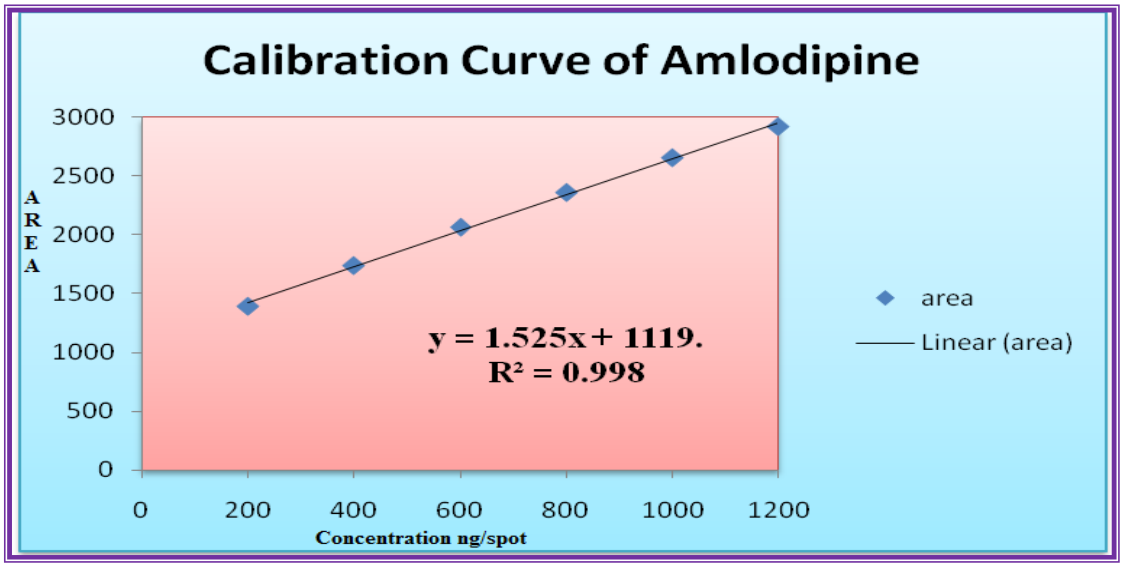

Figure 5 Calibration Curve of AML by HPTLC method

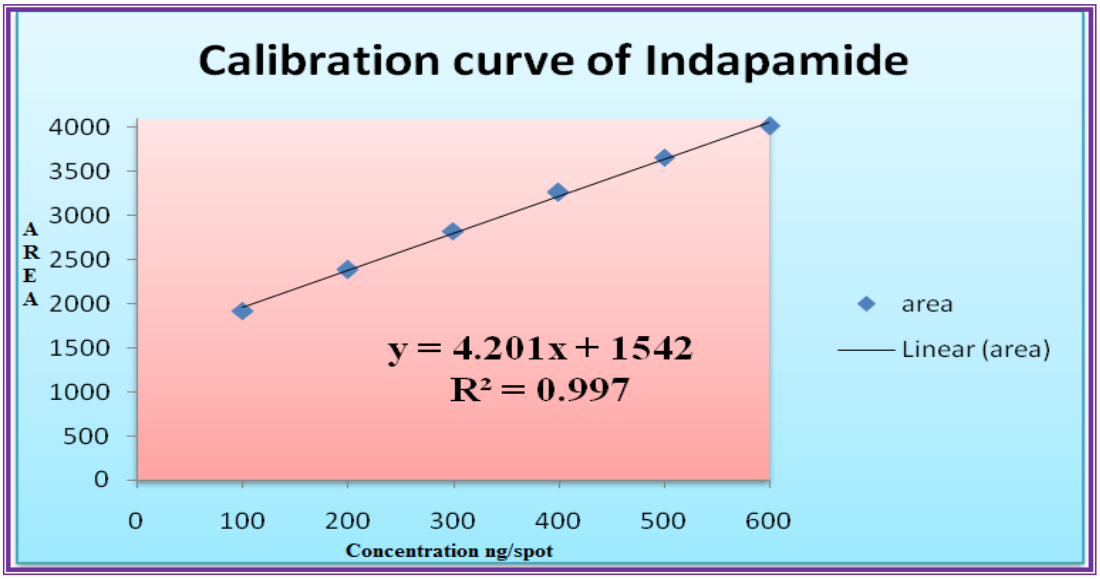

Figure 6 Calibration Curve of IND by HPTLC method

CONCLUSION

Introducing HPTLC into pharmaceutical analysis represents a major step in terms of quality assurance. Today HPTLC is rapidly becoming a routine analytical technique due to its advantages of low operating costs, high sample throughput and the need for minimum sample preparation. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase-unlike HPLC - thus reducing the analysis time and cost per analysis. The developed HPTLC technique is precise, specific and accurate. Statistical analysis proves that the method is suitable for the analysis of AML and IND in pharmaceutical formulation without any interference from the excipients. The common excipients and other additives are usually present in the tablet dosage form do not interfere in the analysis of AML and IND in method, hence it can be conveniently adopted for routine quality control.

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Table 1

Result of calibration readings for AML by HPTLC method

Concentrations (µg/ml)	Area Mean ± S.D. (n=6)	Coefficient of Variation
200	1392.88±0.984	0.0706
400	1736.56±2.470	0.1422
600	2062.85±1.889	0.0916
800	2360.05±1.740	0.0737
1000	2650.55±2.068	0.0780
1200	2921.16±3.357	0.1149

Table 2

Result of calibration readings for IND by HPTLC method

Concentrations (µg/ml)	Area Mean ± S.D. (n=6)	Coefficient of Variation
100	1921.86±2.521	0.1312
200	2390.11±4.112	0.1720
300	2824.80±2.433	0.0861
400	3265.40±2.076	0.0635
500	3660.76±1.722	0.0470
600	4012.55±2.853	0.0711

Table 3

System Suitability Test Parameters

System Suitability Parameters	Proposed Method	
	AMLODIPINE	INDAPAMIDE
Peak Purity	> 0.9989	> 0.9993
Rf	0.27	0.61

Table 4

Statistical data for AML and IND by HPTLC method

Parameter	AMLODIPINE	INDAPAMIDE
Linear Range (ng/spot)	200-1200	100-600
Slope	1.525	4.201
Intercept	1119	1542
Standard deviation of slope	0.0333	0.0991
Standard deviation of intercept	25.986	38.616
Limit of Detection (ng/spot)	56.202	30.329
Limit of Quantitation (ng/spot)	170.309	91.907

Table 5

Determination of Accuracy

Spiking	Amt. of drug added		Amt. recovered		% Recovery	
	AML ng/Spot	IND ng/Spot	AML ng/Spot	IND ng/Spot	AML%	IND%
80	800	240	765.6	240.4	99.4	100.2
100	1000	300	1002.2	300.7	100.2	100.2
120	1200	360	1204.3	359.4	100.3	99.8

Table 6

Repeatability data for AML

Conc.	200 (ng/spot)	400 (ng/spot)	600 (ng/spot)	800 (ng/spot)	1000 (ng/spot)	1200 (ng/spot)
Area	1392.7	1737.9	2062.6	2360.8	2650.5	2920.4
	1391.5	1739.7	2060.3	2362.1	2652.4	2917.2
	1393.1	1734.2	2063.8	2358.4	2648.9	2922.8
	1394.3	1735.4	2065.6	2357.8	2653.6	2923.4
	1392.2	1738.5	2063.5	2361.6	2649.6	2925.6
	1393.5	1733.7	2061.3	2359.6	2648.3	2917.6
Mean	1392.8	1736.5	2062.8	2360.0	2650.5	2921.1
SD	0.984	2.470	1.889	1.740	2.069	3.357
C.V.	0.0706	0.1422	0.0916	0.0737	0.0780	0.1149

Table 7

Repeatability data for IND

Concentration	100 (ng/spot)	200 (ng/spot)	300 (ng/spot)	400 (ng/spot)	500 (ng/spot)	600 (ng/spot)
Area	1921.4	2390.6	2824.0	3265.9	3660.0	4013.2
	1923.8	2393.9	2823.8	3266.7	3661.8	4011.2
	1925.7	2388.8	2826.8	3264.2	3659.6	4017.4
	1920.4	2387.8	2824.7	3261.8	3661.2	4013.4
	1918.6	2384.2	2827.9	3267.5	3663.4	4009.5
	1921.3	2395.4	2821.1	3266.3	3658.6	4010.4
Mean	1921.8	2390.1	2824.8	3265.4	3660.7	4012.5
SD	2.521	4.112	2.433	2.076	1.722	2.853
C.V.	0.1312	0.1720	0.0861	0.0635	0.0470	0.0711

Table 8

Precision data for AML

Conc. µg/ml	Intraday (n=3)	C.V.	Inter day (n=3)	C.V.
600	2071.8.8±4.728	0.228	2054.5±5.774	0.281
800	2369.8±5.517	0.232	2353.1±5.056	0.214
1000	2655.9±5.267	0.198	2646.8±5.519	0.208

Table 9

Precision data for IND

Conc. µg/ml	Intraday (n=3)	C.V.	Inter day (n=3)	C.V.
300	2827.5±2.797	0.098	2825.6±4.401	0.155
400	3265.2±4.266	0.130	3261.8±4.266	0.134
500	3662.5±4.687	0.127	3660.6±4.687	0.118

Table 10

Reproducibility data for AML (50 µg/ml)

Analyst 1 Area ± S.D (n=3)	Analyst 2 Area ± S.D (n=3)	Result of t-test*	Inference
2645.06±4.465	2635.13±4.652	0.052	Not significant difference

*** At 95% confidence interval, (t-Tabulated = 4.30)**

Table 11

Reproducibility data for IND (15 µg/ml)

Analyst 1 Area ± S.D (n=3)	Analyst 2 Area ± S.D (n=3)	Result of t-test	Inference
2829.16±1.305	2824.76±3.234	0.228	Not significant difference

*** At 95% confidence interval, (t-Tabulated = 4.30)**

Table 12

Specificity and Selectivity study

Study	AML	IND
Specificity	Specific	Specific
Selectivity	Selective	Selective

Table 13

Robustness of method

Factor	Condition varied	AML 800ng/spot		IND 400ng/spot	
		Area	Mean ±S.D.	Area	S.D.
Concentration of Mobile Phase	5.1:3.4:0.9:0.5	2341.8	2350.6± 9.45	3279.2	3266.36 ±14.20
	5:3.5:1:0.5	2360.6		3268.8	
	4.9:3.6:1.1:0.5	2349.4		3251.1	
Amount of Mobile Phase	8 ml	2387.4	2366.03± 20.06	3237.4	3247.26 ±12.12
	10 ml	2363.1		3260.8	
	12 ml	2347.6		3243.6	
Plate Pretreatment	At 100^{o}C	2338.5	2350.15± 16.47	3341.6	3248.7 ±10.04
	treatment	2361.8		3255.8	

Table 14

Solvent Suitability Study

Time	Area		RESULT %	
	AML (50µg/ml)	**IND (15µg/ml)**	**AML**	**IND**
0 hr.	2642.7	2838.5	101.3	100.2
4.0 hrs.	2640.5	2835.6	100.1	100.15
8.0 hrs.	2638.7	2831.3	100.04	100
12.0 hrs.	2635.4	2830.8	99.91	99.98
24.0 hrs.	2632.1	2827.6	99.79	99.87
48.0 hrs.	2630.4	2826.4	9973	99.83

Table 15

Summary of Validation Parameters of RP-HPLC

Parameters	AML	IND
Recovery %	99.4 – 100.3	99.8 – 100.2
Repeatability(C.V., n=6)	0.0952	0.0951
Precision(C.V.)		
Intra-day (n=3)	0.198– 0.232	0.098– 0.130
Inter-day (n=3)	0.208– 0.281	0.118– 0.155
Specificity	specific	specific
Solvent suitability	Solvent suitable for 48 hrs	Solvent suitable for 48 hrs

Table 16

Assay Results of Marketed Formulation

Formulation	Actual concentration µg/ml		Amount obtained µg/ml		% AML ±SD(n=3)	% IND ±SD(n=3)
	AML	**IND**	**AML**	**IND**		
Tablet	50	15	49.70	14.95	99.33±0.305	99.68±0.823

Table 17

Optimized chromatographic conditions for AML and IND

Sr.No	Parameter	Conditions
1.	Mobile phase	Ethyl acetate : Toluene : Methanol : Ammonia (5:3.5:1:0.5, v/v)
2.	Stationary phase	Pre-coated silica gel G60 – F254 (100×100 mm, thickness layer 0.2 mm).
3.	Temperature	27 ºC
4.	Distance run (mm)	70
5.	Chamber Saturation time (min)	25
6.	Scanning speed (mm/sec)	20
7.	Detection wavelength (nm)	240
8.	Retention factor (R_f)	
	AMLODIPINE	0.27 cm
	INDAPAMIDE	0.61 cm
9.	Diluent	Methanol

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