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Development and Validation of HPTLC Method for Estimation of Amlodipine and Celecoxib in Bulk Dosage Form

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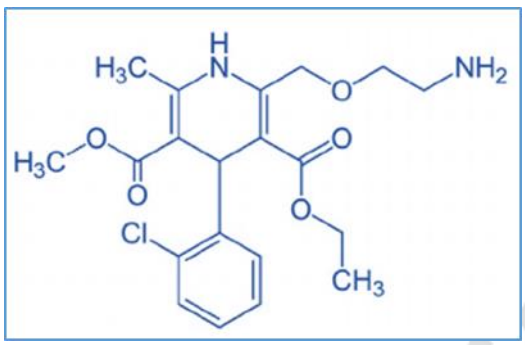
ABSTRACT

Aim: To develop a simple, precise, rapid and accurate HPTLC method for the simultaneous estimation of Amlodipine (AML) and Celecoxib (CEL) in bulk and pharmaceutical dosage forms. **Materials and Methods:** The separation of the active compounds from pharmaceutical dosage form was carried out using Chloroform: Methanol: Formic acid (5:2:0.05 v/v) as the mobile phase and no immiscibility issues were found. The densitometric scanning was carried out at 254 nm. The method was validated for linearity, accuracy, precision, LOD (Limit of Detection), LOQ (Limit of Quantification), robustness and specificity. **Results:** The Rf values (±SD) were found to be 0.74 ± 0.04 for AML and 0.97± 0.04 for CEL. Linearity was obtained in the range of 500–2500 ng/band for AML and1000–5000 ng/band for CEL with correlation coefficients r^2 = 0.995 and 0.998, respectively. The percentage recovery for both the analytes was in the range of 99.7–101.64 %. **Conclusion:** The proposed method was optimized and validated as per the ICH guidelines.

1. INTRODUCTION:

Amlodipine is a (RS)-3-ethyl 5-methyl 2-[(2-amino ethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate (Figure 1). Amlodipine is an angioselective calcium channel blocker and inhibits the movement of calcium ions into vascular smooth muscle cells and cardiac muscle cells which inhibits the contraction of cardiac muscle and vascular smooth muscle cells. Celecoxib is a 4-[5-(4-methylphenyl)-3(trifluoromethyl) pyrazol-1-yl]benzenesulfonamide (Figure 2). Celecoxib is a selective noncompetitive inhibitor of cyclooxygenase-2 (COX-2) enzyme. COX-2 is expressed heavily in inflamed tissues where it is induced by inflammatory mediators. [1, 2]



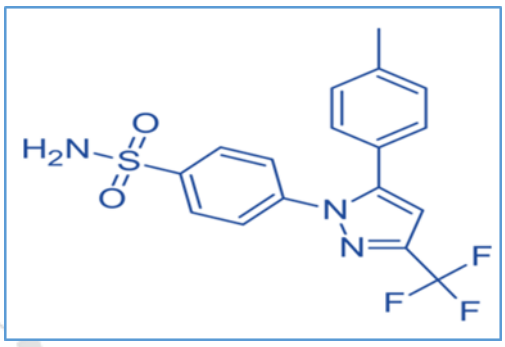


Figure No. 1: Structure of Amlodipine Figure No. 2: Structure of Celecoxib

In the literature, very few techniques for valsartan individual determination have been reported. RP-HPLC analysis [3], comparison of UV and second derivative spectrophotometric method [4], and HPLC with liquid extraction [5] are a few of the techniques used. Numerous methods have been used to determine the presence of hydrochlorothiazide, including UV [9, 10], ion pair chromatography [11], and spectrophotometry [12, 13], as well as liquid chromatography-electrospray ionisation tandem mass spectrometry [6], diffuse reflectance spectroscopy [7], and chemiluminescence analysis [8]. There is no HPTLC method for the analysis of valsartan and hydrochlorothiazide in pharmaceutical formulations, according to a literature review. The objective of this study was to develop an accurate, precise, repeatable, and specific method for analysing the drug content of tablets, which would then be used after being validated in accordance with ICH Q2 R1 guidelines [14] and the rules for good laboratory practice.

2. MATERIALS AND METHODS:

Instruments: HPTLC instrument consists of CAMAG (Muttenz, Switzerland) Linomat V sample applicator with 100 µL applicator syringe (Hamilton, Bonadauz, Switzerland). Chromatography was performed on 10 cm × 10 cm aluminum TLC plates precoated with silica gel 60-F254 (E. Merck, Darmstadt, Germany). CAMAG TLC scanner 4 was used for the densitometric scanning of the developed chromatogram.

Reagents and chemicals: The standard drug Amlodipine and celecoxib were procured from Yarrow Chem Pvt. Ltd, Mumbai, India. The marketed formulation was procured from local market. Methanol, Chloroform, Formic acid and Ammonia analytical reagent obtained from SD fine chem, Mumbai.

Preparation of standard solution: Mixed stock standard solution containing 10mg of AML and 200 mg of CEL in 10 ml methanol. Mixed stock standard solution was further diluted with methanol to obtain working standard solutions in a concentration range of 500-2500 (ng/spot) for AML and 1000-5000 (ng/spot) for CEL. [15, 16]

Chromatographic conditions: The experiment was performed on silica gel 60 F254 aluminum sheets (10 X 10 cm) as stationary phase, using mobile phase comprised of Chloroform: Methanol: Formic acid (5:2:0.05 v/v). TLC plates were prewashed with methanol and activated in an oven at 120ºC for 15 min prior to chromatography. The solutions were applied on TLC plate in the form of bands of 8 mm width under a stream of nitrogen gas using a Camag Linomat V automatic sample applicator. The space between two bands was fixed at 14 mm. Ascending development to 70 mm was performed in 10 cm x 10 cm Camag twin trough glass chamber saturated with the mobile phase for 15 min. The developed TLC plate was air dried and then scanned between 200 to 400 nm using Camag TLC scanner 3 using Win CATS software. Both components show reasonably good response at 254nnm keeping the slit dimension of 6.00 x 0.30 mm and scanning speed of 20 mm/s. [17, 18]

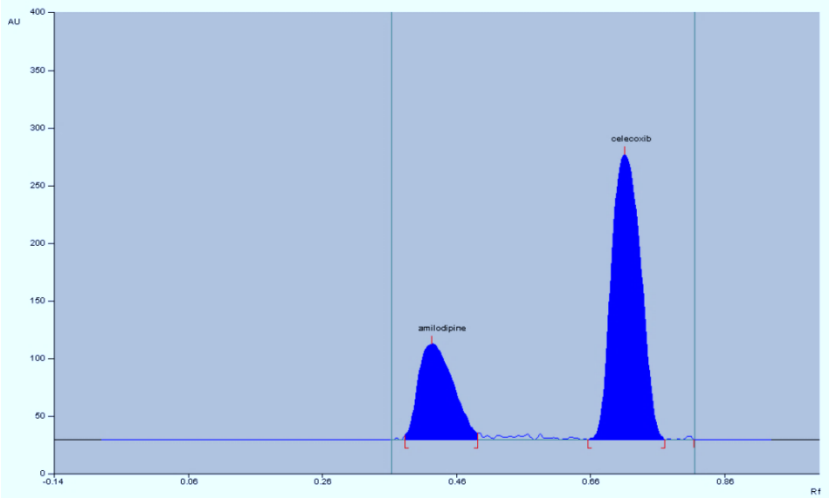


Figure No. 3: Separation of AML and CEL

Linearity (Calibration curve): Calibration curves were plotted over the concentration range of 500 – 2500 ng/spot and 1000 – 5000 ng/spot for AML and CEL, respectively. But the tablet ratio was 1:20 i.e. 10mg for AML and 200 mg for CEL. Thus, for method development the concentration range selected for AML was 500 – 2500 ng and for CEL was 1000– 5000 ng according to tablet ratio. [19]

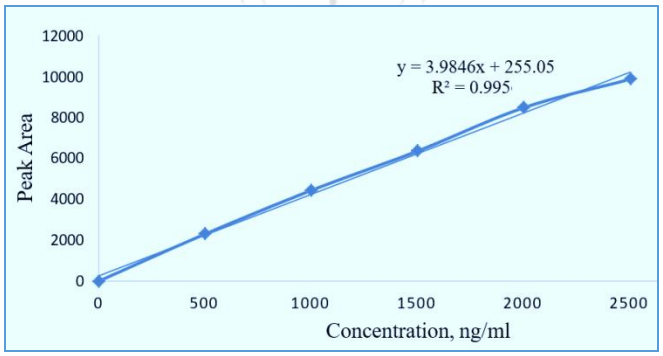


Figure No. 4: Calibration curve of Amlodipine

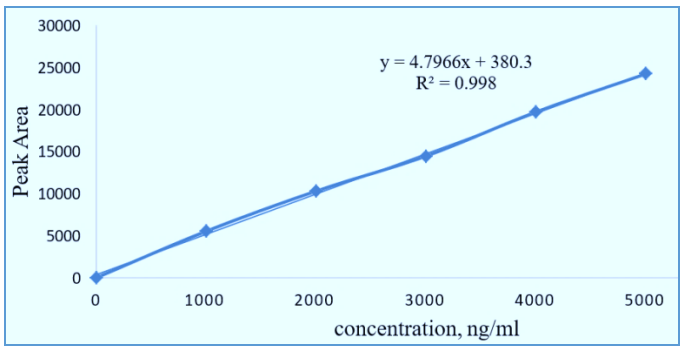


Figure No. 5: Calibration curve of Celecoxib

Standard solutions equivalent to 500, 1000, 1500, 2000 and 2500 ng spot^{-1} of AML by using standard stock solution 2500µg/ml and Standard solutions equivalent to 1000, 2000, 3000, 4000 and 5000 ng spot^{-1} of CEL by using standard stock solution 5000 µg/ml were spotted on the HPTLC plates by over spotting. Sample was applied in form of bands by using Linomat 5. Then the plate was developed in previously saturated chamber with the mobile phase Chloroform: Methanol: Formic acid (5:2:0.05 v/v). After the development, plate was scanned at 254 nm (Figure 6).

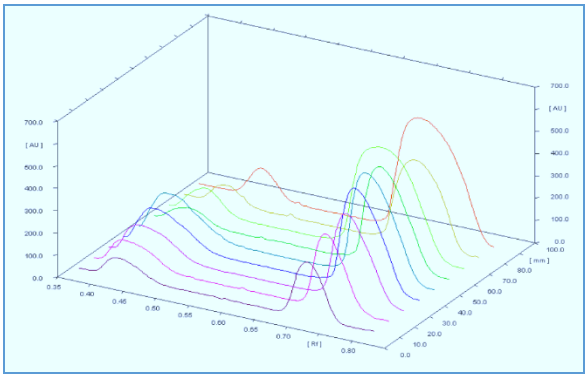


Figure No. 6: Over all 3D diagram for AML and CEL

3. VALIDATION OF HPTLC METHOD: [20, 21, 22]

Validation of the proposed method was carried out with various parameters such as precision, accuracy, specificity, LOD, LOQ, robustness and ruggedness.

Precision: Precision studies were performed by using standard solutions containing the five concentrations that are ,500, 1000, 1500, 2000 and 2500 ng/spot -1 of AML and 1000, 2000, 3000, 4000 ng/and 5000 spot -1 of CEL.

Repeatability: The precision of the method in terms of repeatability was determined by analyzing three concentrations per three replicates of AML standard solutions and CEL standard solutions. Above three concentrations of both AML and CEL were applied on plate and chromatographic development was carried out repeating each Five times. Depending on peak areas obtained for each concentration, standard deviation and percentage relative standard deviation was calculated.

Intermediate precision: Intermediate precision was assessed by analyzing standard AML solutions and CEL standard drug solutions on three consecutive days over a period of one week. The same concentrations as used in repeatability were applied to plate and it was

developed repeating each three times. Same parameters were calculated for intermediate precision. The results of the repeatability and intermediate precision were as shown in Table 1.

Table No. 1: Intra-day Studies

No. of Injection	**Conc. of AML(µg/ml)**	**Peak Area***	**% RSD***
6	1500	5674.65	0.56
No. of Injection	**Conc. of CEL(µg/ml)**	**Peak Area***	**% RSD**
6	3000	16781.67	0.69

*mean of five observations

Table No. 2: Inter-day Studies

Day	**Conc. of AML(µg/ml)**	**Peak Area***	**% RSD***
Day 1	1500	8097.65	0.69
Day 2	1500	8214.75	0.69
Day 3	1500	8345.54	0.68
Day	**Conc. of CEL(µg/ml)**	**Peak Area***	**% RSD**
Day 1	3000	18960	0.72
Day 2	3000	19086	0.71
Day 3	3000	19867	0.72

*mean of five observations

Table No. 3: Repeatability

Conc. of AML(µg/ml)	**Peak Area***	**% RSD***	**Conc. of CEL (µg/ml)**	**Peak Area***	**% RSD**
1500	8432.4	0.64	3000	18966	0.72

*mean of five observations

Accuracy: The accuracy of the method was determined by the use of standard additions at three different levels i.e. multiple level recovery studies. The reference standards of the respective drug were added to the sample solution 3000 (ng/spot) of Celecoxib and 1500 (ng/spot) of Amlodipine besylate at the level of 80%, 100% and 120%. These were further

diluted by procedure as followed in the estimation of formulation. The concentrations of the drugs present in the resulting sample solution were determined by using assay method. Each level was repeated five and the percentage recoveries were calculated as given in Table 4.

Table No. 4: Accuracy (Recovery studies)

Recovery level	**Initial amount (ng)**		**Concentration of excess drug added (ng) %**		**Recovery (n = 5)**	
	AML	**CEL**	**AML**	**CEL**	**AML**	**CEL**
80%	1500	3000	999.97	1999.66	99.98	99.99
100%	1500	3000	1499.87	2998.99	100.01	101.05
120%	1500	3000	1989.09	3988.88	99.09	101.03
Mean					99.99	100.69

LOD and LOQ: The limit of detection (LOD) and limit of quantification (LOQ) were calculated by using the equations 1 and 2.

$$LOD = 3.3 \times \sigma / S \quad \ldots\ldots\ldots\ldots (1)$$

$$LOQ = 10 \times \sigma / S \quad \ldots\ldots\ldots\ldots (2)$$

Where σ is the standard deviation of intercept, S is the slope of the calibration curve.

Table No. 5: LOD and LOQ

Parameter	**AML (ng/spot)**	**CEL(ng/spot)**
LOD	0.064	0.065
LOQ	0.068	0.073

*mean of five observations

Reproducibility and Robustness: Reproducibility of the method was checked by performing the chromatographic assays with the help of two various laboratories and the variations in the results were checked. Robustness was checked by changing the chamber (20 cm x 10 cm) and migration distance (80 mm) for the mobile phase. Plates were developed according to proposed method and peak areas were recorded. To compare the data, percentage relative standard deviation was calculated for each parameter.

Table No. 6: Ruggedness

Drug	Concentration (ng/spot)	Mean Peak area*	% R.S.D*
Day I, Analyst I			
AML	1500	8432.0	0.63
CELE	3000	19459.6	0.71
Day II, Analyst II			
AML	1500	8367.6	0.72
CEL	3000	19476.6	0.80

*mean of five values.

Table No. 7: Robustness studies

		Parameter	
Modification	Percentage Recovery (%)	AML	CEL
Mobile Phase Ratio	5:2:0.5	99.76	99.65
Development Distance	9 mm	99.05	98.90
Detection Wavelength(nm)	254 nm	98.06	99.02
Slit Dimension	6.00 x .30m micro	98.96	98.99

4. RESULTS AND DISCUSSION:

For the simultaneous assessment of AML and CEL in tablet dose form, the HPTLC method was devised. The goal of this research was to develop an exact, precise, repeatable, and specific approach that could be used to determine the drug content of tablets after being validated in accordance with ICH recommendations.

The method was based on separation of the two drugs followed by densitometric measurement of their spots at 254 nm. The separation was carried out on Merck TLC aluminium sheets of silica gel 60 F254 using Chloroform: Methanol: Formic acid (5:2:0.05 v/v) as mobile phase. Linearity was assessed by visual inspection of plot of concentration versus peak area. The graphs were found to be linear in the range 500 – 2500 ng/spot with correlation coefficient value 0.995 for AML and 1000 – 5000 ng/spot with correlation coefficient value 0.998 for CEL. The sensitivity of method was assessed by determining LOD

and LOQ. For AML, LOD and LOQ was found to be 0.064 and 0.068 ng respectively. For CEL, the LOD and LOQ was found to be 0.065 and 0.073 ng, respectively.

According to ICH guidelines, assay procedure should include application of method to an analyte of known purity which was carried out by using standard mixture and purity was found to be 99.99 % for AML and 100.69 % for CEL. Next step is to apply the procedure to marketed formulation and compare the results with standard.

Accuracy was carried out in terms of recovery at 80%, 100% and 120% level including 3 concentrations per 3 replicates. The mean percentage recovery was found to be 99.99 % for AML and 100.64 % for CEL. It was observed that the calculated results of percentage recovery were expressing closeness of agreement between official limits. Hence method can be said to be as accurate according to ICH guidelines.

The method was found to be precise as observed by results obtained in repeatability and intermediate precision which expresses the closeness of agreement between a series of measurements obtained from multiple sampling of same sample under prescribed conditions.

Reproducibility was assessed by means of an inter laboratory experiments which gave similar estimations for both drugs. Robustness of the method was assessed by studying two parameters (change in chamber and change in migration distance) and effects on the results were examined. % R.S.D. values less than 2 showed the reliability of an analysis with respect to deliberate variations in method parameters.

5. CONCLUSION:

The HPTLC approach is described in the proposed study for estimating AML and CEL. The procedure was examined and determined to be straightforward, delicate, exact, and precise. With no interference from the excipients, statistical analysis demonstrated that the approach was reliable and selective for the analysis of AML and CEL. The technique worked well for figuring out the drug's composition.

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