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DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING HPTLC METHOD FOR ESTIMATION OF CELECOXIB AND AMLODIPINE BESYLATE FROM SYNTHETIC MIXTURE

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Keywords:

Celecoxib, Amlodipine Besylate, HPTLC method, Forced degradation studies, Analytical method validation

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ABSTRACT: The current research paper describes a highly specific, reproducible, and efficient stability-indicating HPTLC method for estimation of Celecoxib and Amlodipine Besylate from its synthetic mixture. Chromatographic separation and quantification carried out on Merck HPTLC aluminum sheets of silica gel 60 F254 using Chloroform: Acetone: Toluene: Methanol in the ratio of 5:3:2:0.5 v/v/v/v as solvent system. This system was found to give compact and dense spots for CXB and AML with the R_f value of 0.80, 0.23 respectively. Densitometric analysis of CXB and AML was done at 238 nm and 366 nm. Regression analysis for the calibration plots was indicative of good linearity between response and concentration over the range of 0.2-1.2 µg/spot for AML and 4-24 µg/spot for CXB. Forced degradation studies were performed under different conditions. Both drugs were degraded in acidic, basic, oxidative, thermal, and photolytic conditions. In the present research, a stability-indicating HPTLC method has been developed for Celecoxib and Amlodipine Besylate. The developed method was validated as per ICHQ2R1 guidelines and was successfully applied for quantitative analysis of synthetic mixture of Celecoxib and Amlodipine Besylate.

INTRODUCTION: Celecoxib (CXB) is chemically designated as 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzene-1-sulfonamide. The empirical formula of CXB $C_{17}H_{14}F_3N_3O_2S$ and has a molecular weight of 381.373 g/mole[1]. **Fig. 1** Celecoxib is a COX-2 (cyclooxygenase-2) inhibitor, belonging to the class of non-steroidal anti-inflammatory drugs (NSAIDs) has a sulfonamide side chain that binds to a hydrophilic region near the active binding site of COX-2 & inhibits the prostaglandin synthesis by inhibition of cox-2 and gets relieving the pain due to Inflammation.

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CXB is indicated for symptomatic treatment of adult osteoarthritis (OA) and adult rheumatoid arthritis (RA), acute pain in adults, ankylosing spondylitis, painful menstruation, and juvenile rheumatoid arthritis [2]. Amlodipine besylate (AML) is chemically designated as 3-ethyl 5-methyl 2-[(2-aminoethoxy) methyl]- 4- (2- chlorophenyl)- 6-methyl-1,4-dihydropyridine-3,5-dicarboxylate. The empirical formula of AML $C_{26}H_{31}ClN_2O_8S$ [3] and has a molecular weight of 567.05 g/mole. **Fig. 2** Amlodipine belongs to the group of dihydro-pyridine Calcium channel blockers, which block the inward movement of calcium by binding to the L-type "long-acting" voltage-gated calcium channels in the heart, the vascular smooth muscle, which decreases arterial smooth muscle contraction [4].

The overall decrease in blood pressure is due to vasodilatory effects [5-7]. According to the literature survey, no analytical method was developed and

validated on AML and CXB in combination. Therefore, this particular combination is selected for the development of a suitable analytical method. This survey mentioned that the use of spectroscopic methods [8-11], high-performance liquid chromatography (HPLC) [12-27] and high-performance thin-layer chromatography (HPTLC) [28-42] for separate and combination of other substances. Therefore, this established simple, rapid, and accurate HPTLC method procedure for estimation of celecoxib and amlodipine besylate from a synthetic mixture. Forced degradation studies have an important role in the development of pharmaceuticals. ICH guidelines require that the stability of samples should be analyzed by stability-indicating assay method, which is to be developed by stress testing in conditions like hydrolytic, oxidative, thermal and photolytic and validated. HPTLC method has several advantages over HPLC methods. It is economical; samples can be analyzed with shorter run time, low mobile phase consumption per sample. It facilitates automatic sample application and scanning to the plate, can handle a large no. of samples at a time, and is sensitive. By considering all the points, the present work emphasizes on development and validation of stability-indicating the HPTLC method for CXB and AML from the synthetic mixture.

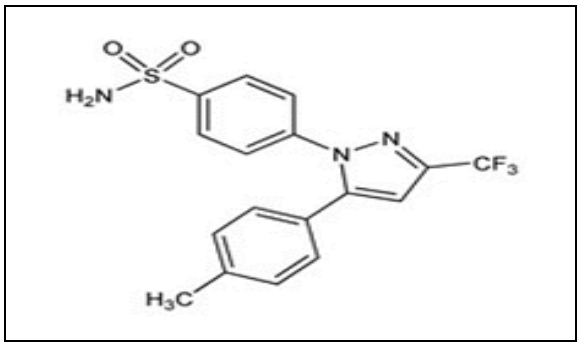

FIG. 1: CHEMICAL STRUCTURES OF CELECOXIB

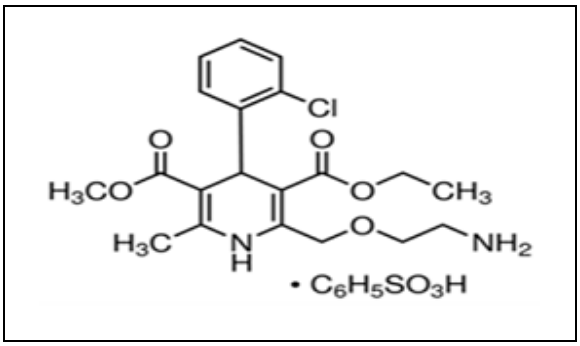

FIG. 2: CHEMICAL STRUCTURES OF AMLODIPINE BESYLATE

MATERIALS AND METHODS:

Materials and Reagents: Amlodipine besylate (AML) and Celecoxib (CXB) were obtained as gift samples from Alembic Pharmaceuticals Ltd. Toluene, Methanol (HPLC grade), Chloroform; Acetone was used as a solvent were available at research institutions.

High-Performance Thin-Layer Chromatographic Instrument: For experimental purposes, CAMAG HPTLC (Muttenz, Switzerland) was used equipped with a Linomat V sample applicator with a 100-µL applicator syringe (Hamilton, Bonaduz, Switzerland), Reprostar 3 digital camera, CAMAG TLC scanner III, WinCATS 2.43. Chromatographic separation was performed on 10 × 10 cm pre-coated with silica gel G F_{254} plates. (E. Merck, Darmstadt, Germany; supplied by Anchrom Technologists, Mumbai, India). All the active components and chemicals were weighed on Mettler Toledo's electronic balance.

Development of the HPTLC Method:

Sample Application: Standards and samples of CXB and AML were applied on the HPTLC plates in the form of narrow bands of 6 mm in length. The bands were applied 10 mm above from the bottom and 10 mm away from the left edge of the plate. Samples were applied under a continuous drying stream of nitrogen gas.

Mobile Phase and Development: The plates were developed using a mobile phase consisting of Chloroform: Acetone: Toluene: Methanol (5:3:2: 0.5 v/v/v/v). Linear ascending development was carried out in a twin-trough glass chamber equilibrated with the mobile phase vapors for 30 min. An amount of 10 ml of the mobile phase (5 mL in the trough containing the plate and 5 mL in the other trough) was used for each development and was allowed to migrate a distance of 80 mm. After development, the HPTLC plates were immediately kept on a preheated hot plate (60˚C) for instant removal of mobile phase components.

Densitometric Analysis: Densitometric scanning was performed in the absorbance mode and fluorescence mode by winCATS planar chromatography software (CAMAG). The bands were scanned at 238 nm (Absorbance mode) and 366nm (Fluorescence mode) **Fig. 3** and **4.**

The slit dimensions were kept at 5 mm length and 0.45 mm width, with a scanning rate of 20 mm s^{-1}. The concentrations of the compound were determined from the intensity of diffusely reflected light and evaluated as peak areas against concentrations using a linear regression equation.

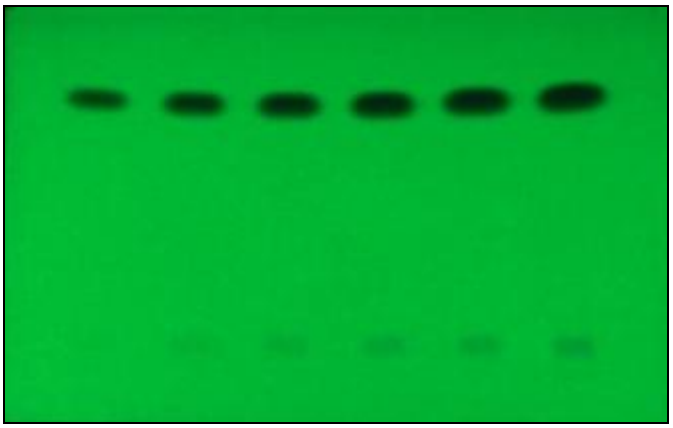

FIG. 3: DEVELOPED TLC PLATE OF CXB AND AML AT 254nm

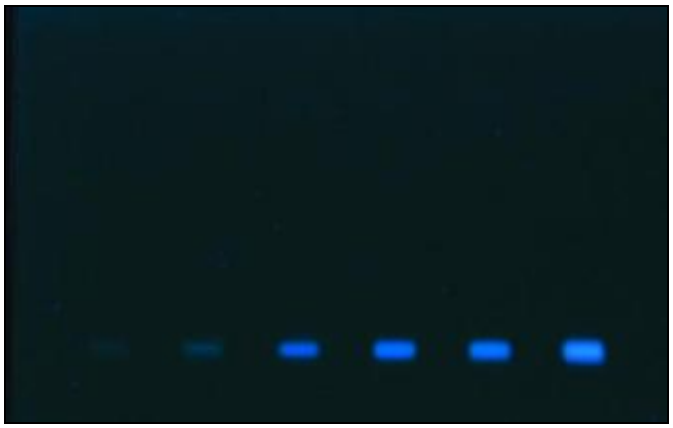

FIG. 4: DEVELOPED TLC PLATE OF CXB AND AML AT 366nm

Preparation of Standard Stock Solutions: AML (10 mg) and CXB (200mg) were weighed accurately and transferred to 10 mL volumetric flasks and dissolved in a few mL of methanol. The volumes were made up to the mark with methanol to yield a solution containing 200 µg mL^{-1}, 4000 µg mL^{-1} for AML, and CXB respectively. Aliquots from the stock solutions of CXB and AML were taken in the same volumetric flask and diluted with methanol to obtain a working standard of 200 µg mL^{-1} AML and 4000 µg mL^{-1} CXB.

Analytical Method Validation: [48] Validation of the developed HPTLC method was carried out according to the International Council on Harmonization (ICH) guidelines Q2 (R1) for linearity and range, accuracy, precision, LOD and LOQ, specificity and robustness by the following procedure.

Linearity and Range: The linearity of the method was evaluated by regression analysis at six concentration levels over a range of 0.2-1.2 ng/band, 4-24 ng/band for AML and CXB, respectively. The calibration curves were developed by plotting peak area versus concentration (n = 6).

Accuracy: The accuracy of the method was determined by calculating the % recoveries of CXB and AML. Known amounts of CXB and AML were spiked at 50, 100 and 150% to a pre-quantified physical mixture. The solutions were applied on the TLC plate and development was carried out. The peak area was measured, and amounts of CXB and AML were established using the regression equation.

Precision: Precision was evaluated in terms of intra-day and inter-day precisions. Intra-day precision was determined by analyzing sample solutions of AML (0.2,0.4,1.0 ng/band) and CXB (4, 12,20 ng/band) at three levels covering low, medium and high concentrations of the calibration curve three times on the same day (n = 3). Inter-day precision was determined by analyzing sample solutions of AML (0.2,0.4,1.0 ng/band) and CXB ((4, 12, 20 ng/band) at three levels covering low, medium, and high concentrations over three days (n = 3). The peak areas obtained were used to calculate the mean and relative standard deviation (% RSD) values.

Repeatability: Repeatability of the sample application was assessed by analyzing all concentrations 5 times on an HPTLC plate. The repeatability of the proposed method was observed by computing the percent relative standard deviation (RSD) of mean peak areas obtained from each spot of the sample.

Robustness: Deliberate minor changes were made quantitatively in the composition of mobile phase and equilibration time effects on results (R_f value and peak area) were observed. %RSD was calculated by analysis of the sample in triplicate at target concentration.

Specificity: The specificity of the proposed method was checked to quantify analyte in the presence of

matrix components, which may include impurities, degradants and excipients. The experiment was performed using excipients like cross carmellose sodium, mannitol, povidone, magnesium stearate for the preparation of the synthetic mixture. Specificity was monitored by observing peak purity at the selected wavelengths.

Method Sensitivity (LOD, LOQ): The limit of detection (LOD) was determined to detect the lowest concentration of analyte that can be detected, and the limit of quantification (LOQ) was determined to detect the lowest amount of analyte that can be quantified. LOD and LOQ were calculated using mathematical transformations as per the ICH guideline.

$$LOD = 3.3 \times \sigma / S$$

$$LOQ = 10 \times \sigma / S$$

Where σ is the standard deviation of the y-intercepts of the regression lines, S is the slope of the calibration curve

Solution Stability: The stock solution of the synthetic mixture was stored at room temperature and analyzed at the target concentration over 24 hours (at an interval of 6 hours).

Analysis of a Simulated Mixture: Various excipients like cross carmellose sodium, mannitol, povidone, magnesium stearate were used in appropriate quantity along with 200mg and 10mg of CXB and AML, respectively. All the components were mixed thoroughly and transfer to a 10 mL volumetric flask. All the components were dissolved by utilizing methyl alcohol and were sonicated for 15 minutes. The prepared solution was filtered by utilizing 0.22 µ Whatman filter paper. The same is diluted suitably with methyl alcohol to obtain a solution containing 4000µg.mL^{-1} CXB and 200 µg.mL^{-1} AML. 1.2 µL of the above solution was applied on an activated TLC plate and was developed by the optimized chromatographic condition. Quantitative analysis was performed by scanning the plate at a selected wavelength and the peak area was transformed to concentration by employing a developed regression equation.

Forced Degradation Study: Forced degradation study is used to identify degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule and validate the stability-indicating the power of the analytical procedures used. The nature of stress testing will depend on the individual drug substance and the type of drug product involved.

CXB and AML were subjected to various stress conditions to conduct forced degradation studies. Stress studies were carried out under the conditions of acid and base hydrolysis, oxidation, thermal, UV light as mentioned in ICH Q1A (R2).

Acid Hydrolysis: 5 ml of stock solution of CXB and AML was taken in 10 ml of the volumetric flask, 1 ml of 0.1 N HCl was added and the solution was heated in a water bath at 60 °C for 1 h. The solution was neutralized with 1 N NaOH and it was diluted up to the mark with methanol. From this solution, 3 µl was applied to the HPTLC plate, and development was carried out under optimized chromatographic conditions.

Base Hydrolysis: 5 ml of stock solution of CXB, AML was taken in 10 ml of the volumetric flask, 1 ml of 0.1 N NaOH was added and the solution was heated in a water bath at 60 °C for 1 h. The solution was neutralized with 1 N HCl and it was diluted up to the mark with methanol. From this solution, 3 µl was applied to the HPTLC plate and development was carried out under optimized chromatographic conditions.

Oxidative Degradation: 5 ml of stock solution of CXB, AML was taken in 25 ml of the volumetric flask, 1 ml of 6 % hydrogen peroxide was added. The solution was kept at room temperature for 1 h. The solution was made up to volume with methanol. From this solution, 3 µl was applied to the HPTLC plate and development was carried out under optimized chromatographic conditions.

Thermal Degradation: 10 mg of AML, 200 mg of CXB were placed in an oven at 70–80°C for 2 h. The solids were allowed to cool and transferred to volumetric flasks (10 ml) and dissolved in a few ml of methanol. Volumes were made up to the mark with the methanol. From this solution, 3 µl was applied to the HPTLC plate and development was carried out under optimized chromatographic conditions.

Photodegradation: 10 mg of AML, 200 mg of CXB were exposed to UV light for 24 h. the sample was transferred to volumetric flasks (10 ml) and dissolved in a few ml of methanol. Volumes were made up to the mark with the methanol. From this solution, 3µl was applied to the HPTLC plate, and development was carried out under optimized chromatographic conditions.

RESULTS AND DISCUSSION:

Development and Optimization: To develop the HPTLC method of analysis of CXB and AML for routine analysis, the selection of the mobile phase was carried out based on polarity and literature review. A mobile phase that would give a dense and compact band with an appropriate R_f value for CXB and AML was desired. Various mobile phases such as Ethyl acetate: methanol, methanol: n-hexane: Ethyl acetate, Chloroform: acetone: toluene were evaluated in different proportions. A mobile consisting of Chloroform: acetone: toluene: methanol (5:2:3:0.5 v/v/v/v) gave good separation of CXB and AML from its matrix. Therefore, Chloroform: acetone: toluene: methanol (5:2:3:0.5 v/v/v/v) mobile phase with a chamber saturation time of 20 min at 25 °C and solvent migration distance of 80 mm was used. These chromatographic conditions produced a well-defined, compact band of CXB and AML with optimum migration at R_f 0.80 and 0.23, respectively shown in **Fig. 5** and **Fig. 6.**

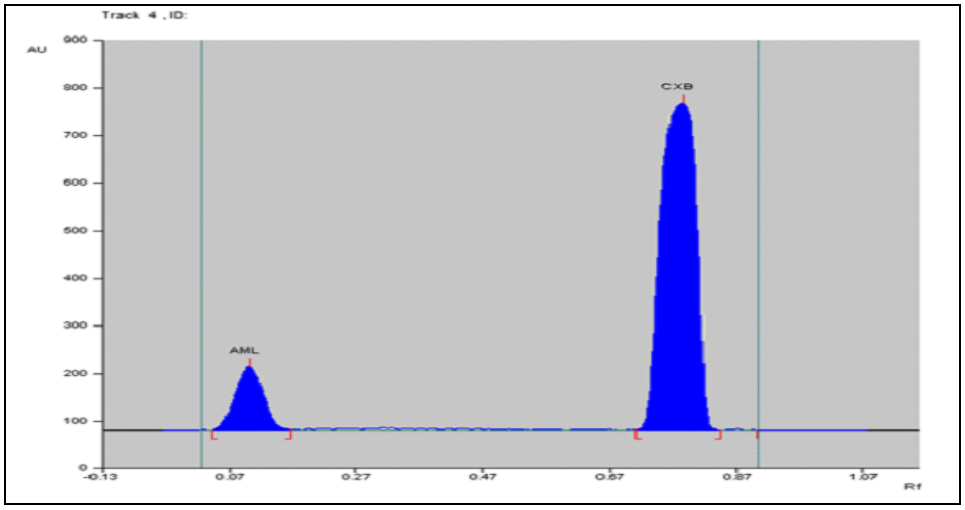

FIG. 5: DENSITOGRAM OF AML AND CXB AT 238nm IN OPTIMIZED MOBILE PHASE

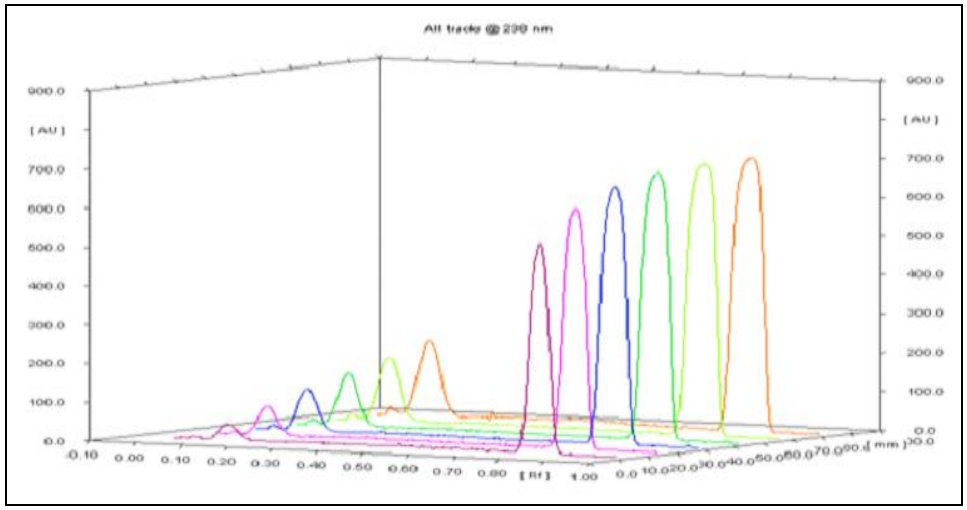

FIG. 6: THREE-DIMENSIONAL DENSITOGRAM OF CXB AND AML

Linearity and Calibration Curves: The linearity of an analytical method is its ability, within a given range, to obtain test results that are directly, or through a mathematical transformation, proportional to the concentration of the analyte. The method was found to be linear in a concentration range of 0.2–1.2 ng/band (n = 6) for AML and 4–24 ng/band (n= 6) for CXB concerning peak area.

Fig. 6 displays a three-dimensional overlay of HPTLC dendrograms of the calibration bands of CXB and AML at 238 and 366 nm. The correlation coefficient was found to be 0.996, 0.997 for CXB, and AML respectively, shown in **Table 1**. This reveals a good linear relationship over the concentration range studied, demonstrating the suitability of the method for analysis **Fig. 7, 8**.

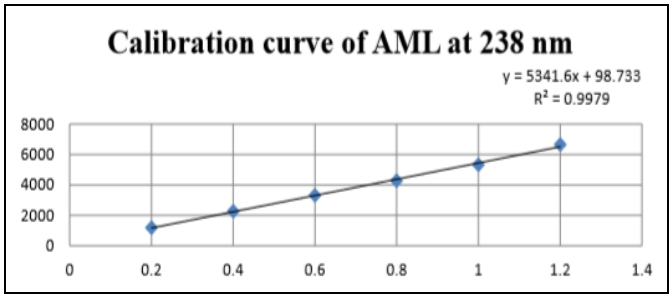

FIG. 7: REGRESSION CURVE OF AMLODIPINE BESYLATE

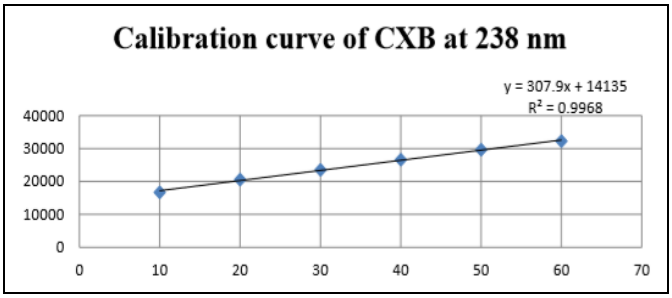

FIG. 8: REGRESSION CURVE OF CELECOXIB

TABLE 1: REGRESSION ANALYSIS OF THE CALIBRATION CURVE

Parameters	CXB	AML
Linearity [µg per band]	4-24	0.2-1.2
Correlation coefficient (r^2)	0.9968	0.9979
The slope of the regression equation	307.895	2136.733
The standard deviation of slope	2.452083	5.330916
Intercept of regression	14135	98.62667

Accuracy: Accuracy disclosed the amount of drug recovered from the physical mixture at three levels of standard addition. The percentage recovery of AML was found in the range of 99.33- 101.6 % w/w, and CXB was found in the range of 101.1-101.0 % w/w. Results near to the true value (100%) indicate that the method is accurate **Table 2**.

TABLE 2: SUMMARY OF THE VALIDATION PARAMETERS OF PROPOSED HPTLC METHOD

Parameters	CXB	AML
Linearity [µg.band-1]	4-24	0.2-1.2
Accuracy [%]	100.11-101.043 %	99.333-101.66 %
Repeatability	0.833-0.241	0.980-0.199
LOD	1.656µg/spot	0.02 µg/spot
LOQ	4.92 µg/spot	0.06 µg/spot
Specificity (peak purity)	0.9993	0.9985
Intra-day precision (n = 3)	0.97-0.29	0.84-0.18
Inter-day precision (n = 3)	0.43-0.32	0.5-0.4

Precision: Intra-day precision was checked by measuring the response of 3 concentrations measured 3 times a day. % RSD values for CXB and AML were found to be 0.97-0.29 % and 0.84-0.18%. Inter-day precision was checked by measuring the response of 3 concentrations measured on 3 different days. The % RSD values were found to be 0.5-0.4% for CXB and 0.43-0.32% for AML.

Repeat Ability: Repeatability study was carried out by performing scanner repeatability study and injection repeatability study by applying and analyzing CXB (4000 µg per band), TEL (200 µg per band) 6 times. The %RSD values of all drugs were found to be less than 2%.

Limit of Detection and Limit of Quantification: The LOD for CXB and AML were found to be 1.656 µg.band^{-1}, 0.02 µg. band^{-1}. The LOQ of CXB and AML were found to be 0.06µg.band^{-1}, 5.02 µg.band^{-1}. which indicates that the method is sensitive and the nanogram quantity of the drug can be estimated accurately and precisely. The validation parameters are summarized in **Table 2.**

Analysis of Simulated Mixture: The mixture was analyzed using the proposed method, which gave percentage recoveries of 99.333 ± 1.527% w/w for CXB and 100.11 ± 0.997% w/w for AML. The results of the analysis of the simulated mixture are shown in **Table 3**.

TABLE 3: RESULT OF FORCED DEGRADATION STUDIES

S. no.	Degradation Condition	% of Degradation	
		CXB	CXB
1	Acid	29.8	15.9
2	Base	27.3	19.6
3	Oxidation	22.3	18.0
4	Thermal	18.5	12.0
5	Photo	12.4	5.4

Forced Degradation Study:

Acid Hydrolysis: Chromatogram of acid hydrolysis performed at 80 °C for one hr reflux showed degradation of CXB and AML with degradation product peak at retention factor (R_f) 0.76 and 0.10 respectively in **Fig. 9.**

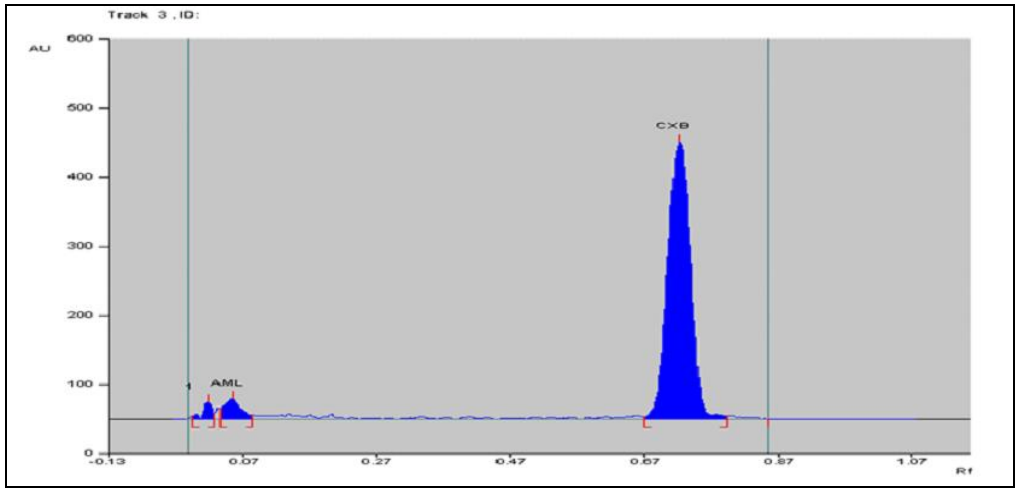

FIG. 9: DENSITOGRAM OF ACID HYDROLYSIS OF CXB AND AML

Base Hydrolysis: Chromatogram of base hydrolysis performed at 80 °C for 1 h reflux showed degradation of CXB and AML with degradation product peak at retention factor (R_f) 0.69 and 0.12 respectively shown in **Fig. 10.**

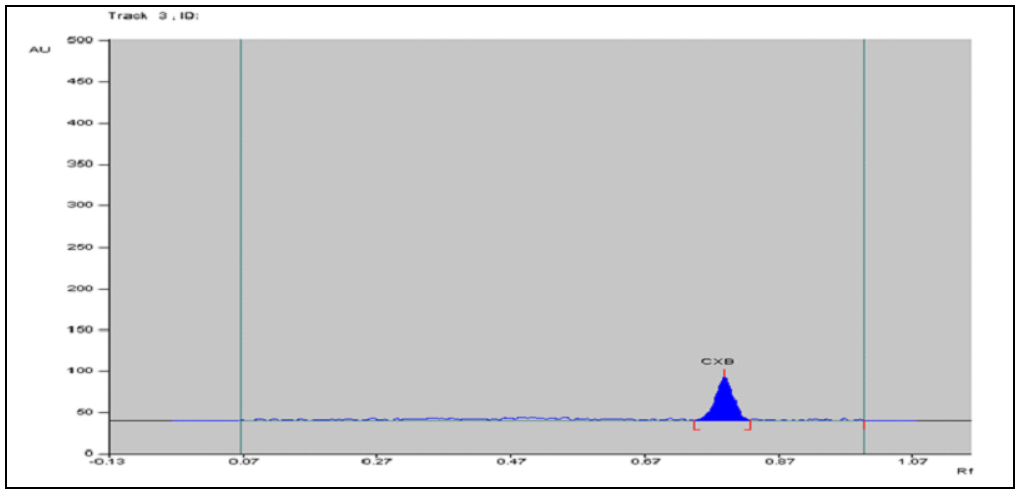

FIG. 10: DENSITOGRAM OF BASE HYDROLYSIS OF CXB AND AML

Oxidative Degradation: The chromatogram of oxidized CXB and AML with 3% hydrogen peroxide at 80 °C for 1 h reflux showed degradation of CXB and AML with degradation product peak at retention factor (R_f) 0.72 and 0.15 respectively shown in **Fig. 11.**

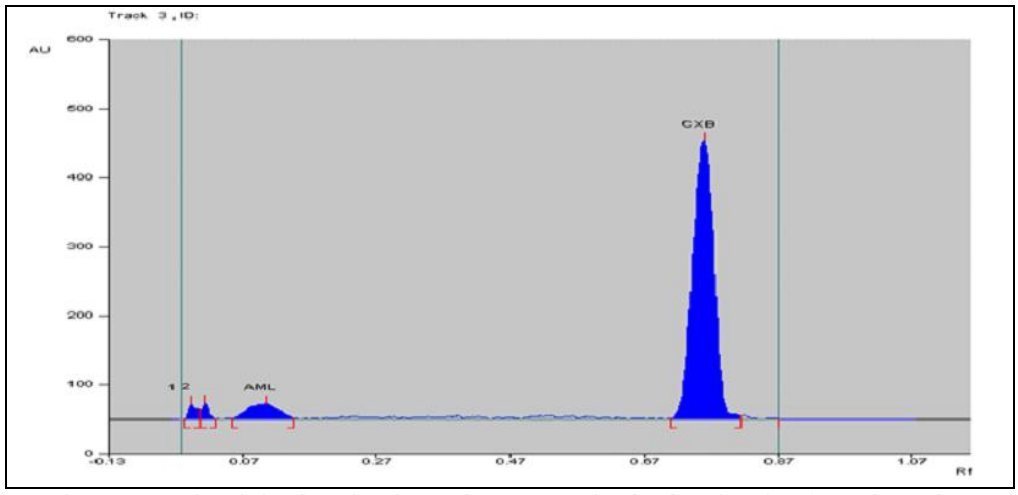

FIG. 11: DENSITOGRAM OF OXIDATIVE DEGRADATION OF CXB AND AML

Photolytic Degradation: Chromatogram of exposure to sunlight for 24 h. Showed degradation of CXB and AML with degradation product peak at retention factor (R_f) 0.80 and 0.16 respectively **Fig. 12.**

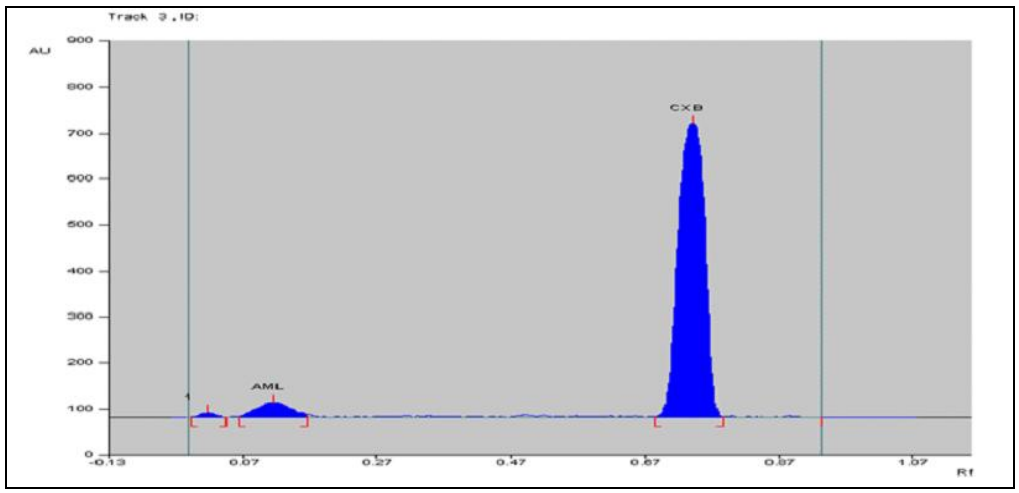

FIG. 12: DENSITOGRAM OF PHOTODEGRADATION OF CXB AND AMLTHERMAL DEGRADATION

Thermal Degradation: Chromatogram exposed to dry heat at 80 °C for 2 hrs. Showed degradation of CXB and AML with degradation product peak at retention factor (R_f) 0.79 and 0.15 respectively shown in **Fig. 13**. The results of the forced degradation study are shown in **Table 3.**

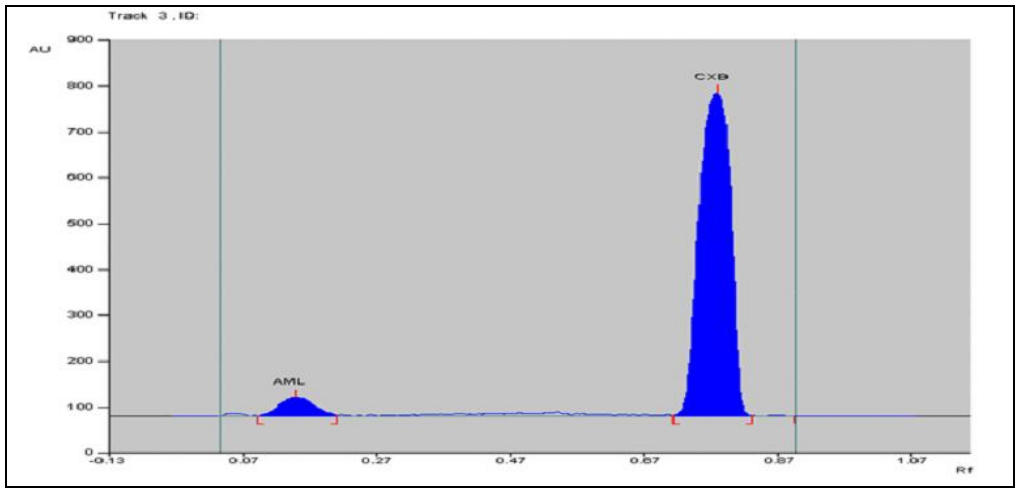

FIG. 13: DENSITOGRAM OF THERMAL DEGRADATION OF CXB AND AML

CONCLUSION: The HPTLC method was developed and validated as per ICH Q2 R1 guidelines and forced degradation for the determination of Amlodipine Besylate and Celecoxib in their combined dosage form. The present method was found to be economical in terms of cost and time. Commonly used excipient didn't interfere in the estimation of CXB and AML so the method was found to be specific. The method was also found to be repeatable and precise.

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CONFLICTS OF INTEREST: The authors declare that there is no conflict of interest.

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