https://doi.org/10.33472/AFJBS.6.6.2024.1922-1936

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Development and Validation of a High-Performance Thin Layer Chromatography Method for Estimation of Polmacoxib in Pharmaceutical Bulk Form

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Article Info

Volume 6, Issue 6, June 2024

Received: 6 April 2024

Accepted: 11 May 2024

Published: 03 June 2024

doi: 10.33472/AFJBS.6.6.2024.1922-1936

ABSTRACT:

The present research outlines the development and validation of a method for the quantitative and qualitative analysis of Polmacoxib in bulk using High-Performance Thin Layer Chromatography (HPTLC). The study focuses on establishing a reliable and accurate analytical procedure to determine the concentration of Polmacoxib, ensuring its quality and adherence to regulatory standards. The method was successfully developed by employing TLC aluminium plates pre-coated with silica gel 60 F_{254} and CAMAG twin through chamber, the study optimized Toluene: Ethyl Acetate: Methanol (8:2:1 v/v/v) as mobile phase for efficient plate development at room temperature (25 ± 2°C). Utilizing CAMAG TLC scanner 4 with visionCATS user software, scanning and densitometric analysis were carried out at 322 nm (λ max), revealing sharp peaks and dense bands with an Rf value of 0.44. The developed method undergoes rigorous validation in adherence to ICH guidelines Q2 (R1), encompassing specificity, linearity, precision, LOD and LOQ, accuracy and robustness. The Linearity studies demonstrated a strong correlation (R^2=0.9919) across the concentration range of 5-30 ng/spot. The precision results met the acceptance criteria, and LOD & LOQ were determined at 700.03 ng/spot and 2100.30 ng/spot, respectively. The recovery studies showed accurate results with RSD of 99.97%. Robustness testing involved deliberate changes confirming the method's suitability for routine analysis. This validated HPTLC method establishes itself as a reliable analytical tool for the routine quantification of Polmacoxib, offering pharmaceutical researchers and technicians a practical and accurate approach. The results of this study contribute significantly to the qualitative and quantitative analysis of Polmacoxib in both bulk and pharmaceutical formulations, providing a robust foundation for quality control processes in the pharmaceutical industry.

Keywords: Polmacoxib, HPTLC, optimization, ICH, validation.

1. Introduction

Osteoarthritis is described as a chronic and painful joint disease, in which the patient experiences acute joint pain, inflammation, bone remodelling and even disability which are caused due to breakage of cartilage present between the bone joints[1]. Osteoarthritis cases has been growing more immensely in recent years due to increase in certain factors like obesity, unhealthy lifestyle, inactivity, poor posture, etc[2,3]. There are various first line medications for treatment of arthritis and inflammation which involves paracetamol, and especially for Osteoarthritis and Rheumatoid arthritis treatment there are various Cyclooxygenase-2 inhibitors, also known as COXIBs, which help in reduction of inflammation and pain like- Valdecoxib, Rofecoxib, Celecoxib, Polmacoxib. But most of the COXIBs like Valdecoxib, Rofecoxib, etc. has been removed from the market due to the increasing side effects in patients including cardiovascular toxicity and gastrointestinal toxicity[4]. Polmacoxib is one of the recently introduced COX-II inhibitor which is a Non-Steroidal Anti-inflammatory Drug (NSAID) class of drug. Polmacoxib was produced by Crystal Genomics, Korea and was approved by Korean Ministry of Food and Drug Safety (MFDS) in the year 2015[5]. Polmacoxib known as CG100649 is a 4-{3-(3-fluorophenyl)-5,5-dimethyl-4-oxo-4,5-dihydrofuran-2-yl}-benzenesulfonamide as shown in figure 1, is used to treat Osteoarthritis. It is an NSAID with selective dual inhibitory action which can inhibit both Carbonic Anhydrase (CA) enzyme and Cyclooxygenase II (COX-II) enzyme by reducing inflammation and redness in Osteoarthritis patients. It is considered better than Celecoxib as it is administered in lower dosage (2mg/day) with comparatively low side effects than Celecoxib[6].

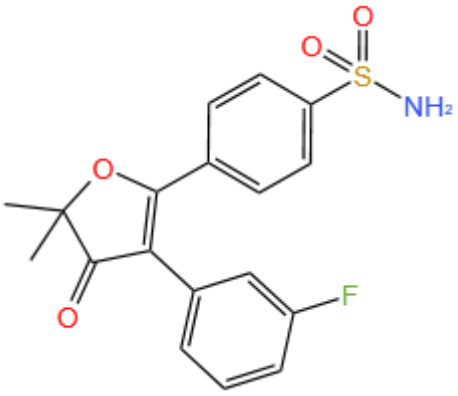

Figure 1: Polmacoxib structure

Very few analytical methods like RP-HPLC, DCS, PXRD were observed for analysis of Polmacoxib[6,14]. There were three patent information for Polmacoxib showing the development, comparison and combination with other drug products like Pregabalin [20-22]. But there was no single article which stated the High-Performance Thin Layer Chromatography (HPTLC) method for Polmacoxib. Thin Layer Chromatography (TLC) also known as planar chromatography is a world-wide analytical method for separation and quantification of any drug substance or formulation. HPTLC is the most advanced form of TLC which helps in sample separation, identification, quantification and qualification of compounds which would be of great help for routine analysis of any drug substance. HPTLC method is considered beneficial than other methods because of its versatility, low-cost analysis, better resolutions, quick and multiple scanning in single mobile phase run, gives more precision and accurate results, requires very less time and less sample is required[7,8]. Hence, HPTLC method is considered an important aspect for knowing the quality and other particulars of validation regarding Polmacoxib. The solubility profile of CG100649 was studied which explained about the low solubility of Polmacoxib in water, it was highly soluble in ethanol, DMSO, DMF,

acetone and methanol[9]. As CG100649 is hydrophobic in nature, during HPTLC it is employed as a solution dissolved in an organic solvent which allows effective separation based on their affinity for the stationary phase, which is typically a hydrophobic material like silica gel G. Various solvents were tried for solubility of Polmacoxib and various mobile phases with different ratios were experimented for the optimized selection of the HPTLC mobile phase. The sample spotted in the TLC plate which is developed in the optimized mobile phase solvent system facilitated the separation based on the hydrophobic interaction of CG100649 and the selected stationary phase. In HPTLC various detection methods can be used like UV and fluorescence detector to visualize and analyse separated compounds on the TLC plate. Hence, the aim of the present study was to develop and validate an accurate, precise, specific and robust HPTLC method for the quantitative and qualitative analysis of Polmacoxib in pharmaceutical bulk form.

Experimental Work

Materials and Methods

Polmacoxib was procured as a gift sample from Hetero Labs, Hyderabad, Telangana. Polmacoxib ^{2mg} capsules with brand name Hisaka marketed by Eris Lifesciences Limited, was purchased from the local medical market in Vadodara, Gujarat. The chemicals and solvents used for HPTLC method development and validation were all of analytical grades.

Instrumentation and Equipment

In the method development experiment, HPTLC (CAMAG, Munich, Germany) Linomat 5 auto-sampler with CAMAG micro-syringe 100.0µL. Scanner used is TLC scanner 4, HPTLC plate used is HPTLC silica gel 60 F_{254} (20 x 10 cm) plates. The twin through chamber for (20 x 10 cm) plate was used. Detection wavelength containing deuterium lamp and tungsten lamp with both UV and Fluorescence detectors was scanned at 322nm.

Identification Tests

After the procurement of the Polmacoxib API, the drug was tested and experiments were conducted for the identification of the sample and its purity. This identification tests which are the preliminary tests were conducted using all calibrated glass wares and instruments**.** The preliminary tests include – organoleptic or physical tests like appearance, odour, taste, etc., identification tests including melting point test, solubility test, and for quantification of Polmacoxib various tests conducted were – FTIR and UV spectrophotometry. During the literature survey, there were articles, papers as well as patent information which enlisted the details of Polmacoxib regarding its physical and chemical properties and from there the references were taken for conducting the identification tests [5,9,17] (Table 1)

Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) was carried out for selection of stock and sample preparation and also for optimization of mobile phase first using TLC method.

Preparation of Stock Solution: the stock solution was prepared by adding 1mg of Polmacoxib API was to 1ml of ethanol in a volumetric flask making it a 1000ppm solution. From this 1000ppm solution 1ml was pipette out and added to 10ml of ethanol (100ppm). From this again 1ml was pipette out and added to 10ml of ethanol (10ppm).

Preparation of Working Solution: working solution was prepared from the standard stock solution by withdrawing 1ml of stock solution to 10ml volumetric flask and adding 10ml ethanol (100ppm), similarly 10ppm solution was prepared.

Selection of Working Solution And Mobile Phase Ratios

Mobile phase selection was conducted on trial-and-error basis. Both 100ppm as well as 10ppm working solution were used for the TLC method. Various mobile phases were tried with very different ratios from the selected from the literature search. It was observed that the 100ppm solution of Polmacoxib was showing significant spotting on the TLC plate with some mobile phase ratios whereas the 10ppm solution did not show any spots in any mobile phase ratio. Hence, 100ppm solution was considered as the standard working solution for the HPTLC method development and its validation. (Table 3)

HPTLC Method Development

Preparation of Standard Stock Solution:

The standard stock solution is prepared similar to the stock solution prepared for TLC method. 1mg of API Polmacoxib dissolved in 1ml of ethanol (1000ppm) in a 10ml volumetric flask. This was the standard stock solution used for the HPTLC method development.

Preparation of Working Solution

From the above 1000ppm solution, 1ml of solution is pipette out and added in a 10ml volumetric flask and to this 1ml of solution 10ml of ethanol was added to prepare 100ppm solution. This 100ppm solution was used as working solution for the HPTLC method development.

Optimization of Mobile Phase and Chromatographic Conditions

Optimization of mobile phase was conducted first on TLC and then on HPTLC for final selection of appropriate mobile phase and the ratio. Various mobile phases were tried (shown in table 2) for the selection using 100ppm solution as the standard working solution. [7,10, 11]

After all the trials conducted for the mobile phase, the optimized mobile phase for HPTLC method development selected was the last mobile phase ratio [10] i.e**.** toluene: ethyl acetate: methanol (8:2:1 v/v/v) with Rf value 0.4403 and the solution of 100ppm to be used for sample spotting of Polmacoxib API. (Table 3)

The chromatographic separation has been achieved on HPTLC silica gel 60 F_{254} plates (100 x 100 mm). Samples were applied by using CAMAG Linomat 5 auto-sampler with CAMAG microliter syringe (100.0µL). samples were spotted in form of bands position Y: 8.0 mm, length: 8.0 mm, width: 0 mm. solvent front position was 80 mm, the TLC plates were developed using CAMAG twin through chamber, the migration distance was 80 mm. The chamber saturation consisting the mobile phase of toluene: ethyl acetate: methanol (8:2:1 v/v/v) at room temperature for 20 mins. The plates were then dried for 5 mins in room temperature, A CAMAG TLC scanner 4 with visionCATS user software program was used for scanning and densitometric analysis of the development plates. The slit dimension was (6 x 0.45 mm) micro and scanning speed at 100 mm/s. detectors used were one in absorbance mode 254 nm deuterium lamp filter K320, and the other one was in fluorescence mode 366 nm mercury lamp filter K320. The maximum wavelength (λ max) of the drug Polmacoxib using the mobile phase toluene: ethyl acetate: methanol (8:2:1 v/v/v) was found at 322 nm. Hence, the method of HPTLC was developed spotting Polmacoxib API solution of 100ppm (100 µg/ml) on the silica gal 60 F_{254} and run along the mobile phase – toluene: ethyl acetate: methanol (8:2:1 v/v/v) with a good resolution and optimum Rf value of 0.4403.

Method Validation

The HPTLC method was validated in accordance to the ICH quality guidelines of Q2 (R1), which indicated various validation parameters like specificity, linearity, precision, accuracy, Limit of Detection (LOD) & Limit of Quantification (LOQ) and robustness [12].

Specificity

The developed HPTLC method was found to be specific for Polmacoxib API, since the blank run of solution did not show any interference of peak during scanning of Rf factor when examined the blank at 322 nm. Peak purity was also assessed during the development which should be greater than 0.990. Various identification tests were conducted for the specificity of Polmacoxib like UV, FTIR, etc. and the result showed that there was no interference of other materials or compounds and it confirm the specificity of the method.

Linearity

Linearity of the method was evaluated and calculated by selection of six concentrations of Polmacoxib bulk solution for calibration curve construction. The spotting was made each with concentration range of 500-3000 ng/spot. Plate was developed in mobile phase at 25±2°C and dried in air. Specific quantity of Polmacoxib solution was injected on the TLC plate according to the concentrations (500,1000,1500,2000,2500,3000 ng/spot). The developed plate was then scanned at wavelength of 322nm using CAMAG TLC scanner 4. Further calibration curve was created by plotting peak area vs concentrations with the help of visionCATSuser software.

Precision

Precision in validation is the parameter which explains about the degree of agreement among individual test results when the method is applied repeatedly with multiple samplings of single sample. It provides the random error percentage and is expressed in terms of %RSD. the precision is carried out in three major steps – repeatability, inter-day, intra-day studies. The acceptance criteria for the repeatability studies are ≤ 1% RSD, for intra-day and inter-day studies the acceptance criteria are ≤ 2% RSD.

Accuracy

Accuracy expresses the closeness of agreement between true value or an accepted reference value and the value found. It is also termed as trueness to the method developed. The accuracy was determined by calculating the % recovery of Polmacoxib by spiking standard solution at 50%, 100% and 150% levels to the sample solutions of Polmacoxib. The recovery value was determined and quantified to measure the trueness of the method. The acceptance criteria for accuracy study shows the % Recovery should be between 98% - 102%.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD means the minimum value at which the sample can be detected, whereas LOQ means the minimum value the sample can be quantified.

The LOD and LOQ were estimated using the calibration curve, and applying the formula for calculating the LOD and LOQ for Polmacoxib.

$LOD = 3.3 * (\sigma / S)$

$LOQ = 10 * (\sigma / S)$

Where, σ = standard deviation of the Y-intercept of calibration curve.

S = slope which is achieved from the calibration curve equation.

Robustness

Robustness is the ability of a method to remain unaffected even with slight variations are applied. The robustness studies were conducted by evaluating small variations in the

chromatographic conditions. The results were executed by implementing small changes in saturation time (±2), detection wavelength (±1) and change in the development distance/ solvent front distance (±1).
The robustness of the method was determined at the concentration of 1000 ng/spot for Polmacoxib.

2. Result and Discussion

Identification tests for Polmacoxib

Various identification tests were conducted for Polmacoxib and its quantification was also done using UV and FTIR. The physical characteristics were identified using the appearance test where the drug was found to be solid pale-yellow colour powder, sticky in nature. The melting point was also found to be around the actual range (155 - 158°C). solubility profile was matched and compared with the patent information. The drug was then tested for quantification using FTIR and UV (Figure 2 & 3).

Table 1: Preliminary tests for Polmacoxib

Sr.No.	Preliminary Tests	Experimental Result
1	Appearance	Solid powder
2	Melting point	155-158°C
3	Solubility	Soluble in ethanol, methanol, DMSO
4	FTIR	3216.74cm, 1683.01cm, 1261.14cm, 1151.96cm.
5	UV	320nm, 236.4nm, 203.7nm.

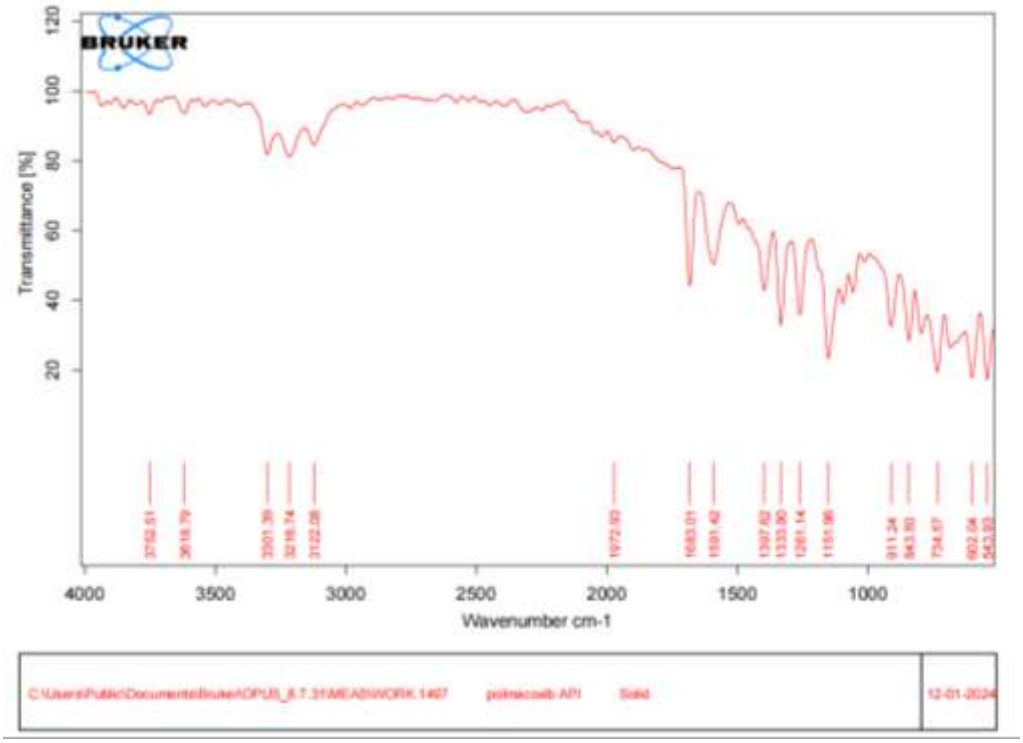

Figure 2: FTIR spectra for Identification of Polmacoxib

Table 2: Functional groups present in Polmacoxib through FTIR

Wavenumber (cm^{-1})	Functional Group
3216.74cm	N-H bond

1683.01cm	carbonyl group C=O group
1261.14cm	C-F carbon-fluorine bond
1151.96cm	presence of C-O vibration, containing ester or ether group
1151.96cm,1261.14cm, 1333.80 cm	S=O vibration

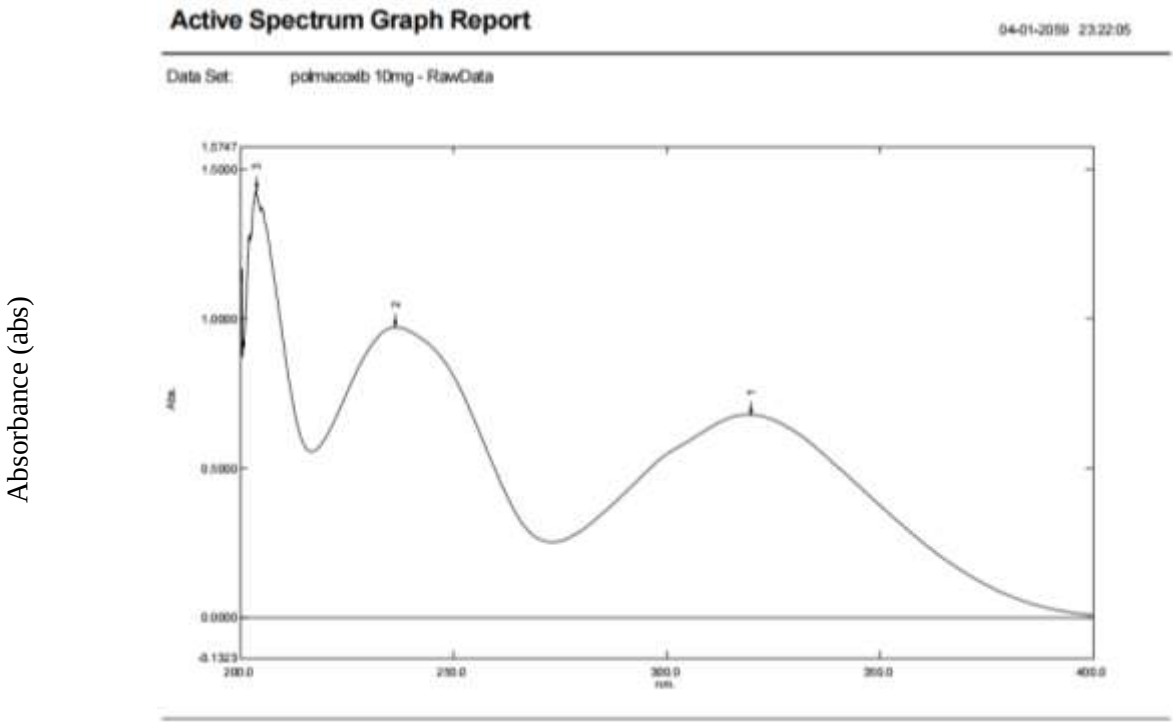

Figure 3: UV Spectra Identification for Polmacoxib

Wavelength (nm)
*UV spectrum wavelength – 320nm, 236.4nm, 203.7nm (1,2,3 wavelength peak respectively)

TLC and Mobile Phase Optimization

After various trials for selection of mobile phase, one such mobile phase was selected as the optimized mobile phase for separation and quantification of Polmacoxib by HPTLC method, i.e. toluene: ethyl acetate: methanol (8:2:1 v/v/v) as it gave sharpest peak, good resolution, dark spotting when observed under the UV light as well as fluorescence and it also gave a good Rf value to work further for HPTLC processing. Hence, this mobile phase was selected as the optimized mobile phase for Polmacoxib.

Table 3: Mobile Phase Optimization

Sr.No.	Mobile Phase Ratio	Rf Value	Comment
1	Chloroform: acetone: toluene (6:2.5:1 v/v/v)	0.7365	Light spot visible, Rf value is little high, impurity was found in some peaks
2	Chloroform: ethyl acetate: methanol: ammonia (4:3:4:0.1 v/v/v/v)	0.9226	Very high Rf value, not applicable
3	Toluene: methanol: acetonitrile: ammonia (6:2:3:0.2 v/v/v/v)	0.6693	Rf was good but the peaks were not particular and merging was seen.

4	Toluene: ethyl acetate: methanol (8:2:1 v/v/v)	0.4403	Rf value is acceptable, sharp peak, dark spot visibility, good resolution.

Analytical Method Development

The HPTLC procedure was optimized for quantification of Polmacoxib. The optimized mobile phase selected for HPTLC method after various trials was toluene: ethyl acetate: methanol with mobile phase composition ratio 8:2:1 v/v/v which gave good resolution, peak purity, sharp identifiable peak, defined separation of spots, with the Rf value of 0.44. The drug gave the sharp peak with the highest peak point at λ max 322nm. Other chromatographic conditions like the saturation time, track distance, slit dimensions, spotting, detection under UV as well as fluorescence also gave good and optimized and reproducible results.

Method Validation

Specificity

There were no interfering spots or peaks of other impurities or mobile phase composition at the optimized Rf value of Polmacoxib. The peak purity was also observed to be 100% as the correlation coefficient of track in starting and ending i.e. r (s, m) and r (e, m) was found to be 0.9996 and 0.9970 respectively. (Figure 5,6).

Linearity

A good and linear correlation coefficient ($R^2 = 0.9919$) was obtained and calibration curve was created with Peak Area vs Concentration having linearity range of 500, 1000, 1500, 2000, 2500, 3000 ng/spot respectively (5-30 µg/ml). The linear calibration curve gave the correlation equation (y = 0.0008x + 0.002) where slope is 0.0008 and the y-intercept is 0.002. The linearity table and figure given below (Table 4) (Figure 4,8)

Table 4: Linearity Study of Polmacoxib.

Sr.no.	Concentration (ng/spot)	Peak Area (AU)
1	500	0.00485
2	1000	0.01024
3	1500	0.01398
4	2000	0.01729
5	2500	0.02048
6	3000	0.02436
7	Y = 0.0008x + 0.002 (Regression Equation)	
8	$R^2 = 0.9919$ (Correlation Coefficient)	

*(5-30 µg/ml**)** in Calibration Curve of Polmacoxib by HPTLC.

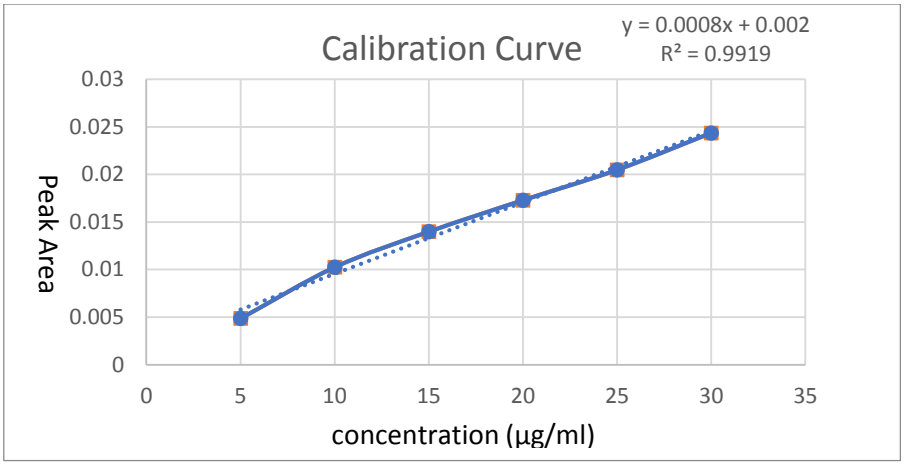

Figure 4: Calibration Curve of Polmacoxib by HPTLC

Limit of Detection (LOD) and Limit of Quantification (LOQ) (n=3)

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated using the linearity equation and the correlation coefficient and the LOD and LOQ was found to be 700.03 ng/spot and 2100.30 ng/spot respectively. (Table 5)

Table 5: LOD & LOQ studies of the method

Drug	LOD*	LOQ*
Polmacoxib	700.03 ng/spot	2100.30 ng/spot

*LOD = Limit of Detection; *LOQ = Limit of Quantification

Precision

Precision studies were divided into three compartments – repeatability, intra-day, and inter-day studies. % RSD of repeatability studies of sample application of 1000 ng/spot repeatedly for 6 times was scanned and measurements of peak areas was 0.5780%. During intra-day studies the measurements of three different concentrations in one particular day at an interval of 2 hrs with 3 observations each concentration resulted in % RSD (≤ 2%) and inter-day was performed at three different concentrations in continuous 3 days with 3 observations each resulted in % RSD (≤ 2%). The results were found to be within the acceptable criteria and hence the method was proven to be precise (table 6) (Figure 9).

Table 6: Precision Studies of The Method

Amount of concentration (ng/spot)	Intra-day studies (n=3)			Inter-day studies(n=3)		
	Mean Area	SD*	%RSD*	Mean Area	SD*	%RSD*
500	0.002260	4.1766	1.848%	0.009556	0.000178	1.866%
1500	0.005572	7.1828	1.289%	0.019153	0.000315	1.644%
3000	0.009336	0.00013	1.414%	0.027059	0.000410	1.515%
Repeatability studies (n=6)						
Amount of concentration (ng/spot)	**Mean Peak Area**		**SD***		**% RSD***	
1000	0.002771		1.6020		0.578%	

*SD = Standard Deviation; RSD = Relative Standard Deviation

Accuracy

The accuracy was carried out for quantification of Polmacoxib and acquire the recovery studies by calculating the % recovery of the drug Polmacoxib after spiking it with the standard concentration at different levels – 50%, 100% and 150%, where the average recovery was found to be 99.97% (Table 7).

Table 7: % Recovery Studies of the Method

Concentration of sample (ng/spot)	% spike (standard)	Amount of Standard added (ng/spot)	Mean Peak Area	% Recovery	*SD ± % RSD
1000	50%	26.324	0.02273	98.12%	0.000169 ± 0.74%
1000	100%	52.650	0.04409	99.94%	0.000403 ± 0.91%
1000	150%	78.974	0.06602	101.32%	0.001171 ± 1.77%

*SD = Standard Deviation; RSD = Relative Standard Deviation

Robustness

The %RSD was calculated by evaluating certain small variations in the method development parameters like change in saturation time (±2), detection wavelength (±1) and small change in the development distance (±1). Low values of % RSD was obtained after deliberate changes in the said parameters. (Table 8) (Figure 10)

Table 8: Robustness of the Method

Sr.No.	Parameters	Mean Area	SD*	% RSD*	Mean % RSD
1	**Saturation time**				
	22 minutes	0.004430	1.4142	0.319%	0.382%
	18 minutes	0.003967	1.7677	0.445%	
2	**Detection Wavelength**				
	323 nm	0.00462	1.4142	0.306%	0.305%
	321 nm	0.00463	1.4142	0.305%	
3	**Development Distance**				
	8 cm	0.004940	2.8280	0.570%	0.501%
	6 cm	0.004905	2.1213	0.432%	

*SD = Standard Deviation; RSD = Relative Standard Deviation

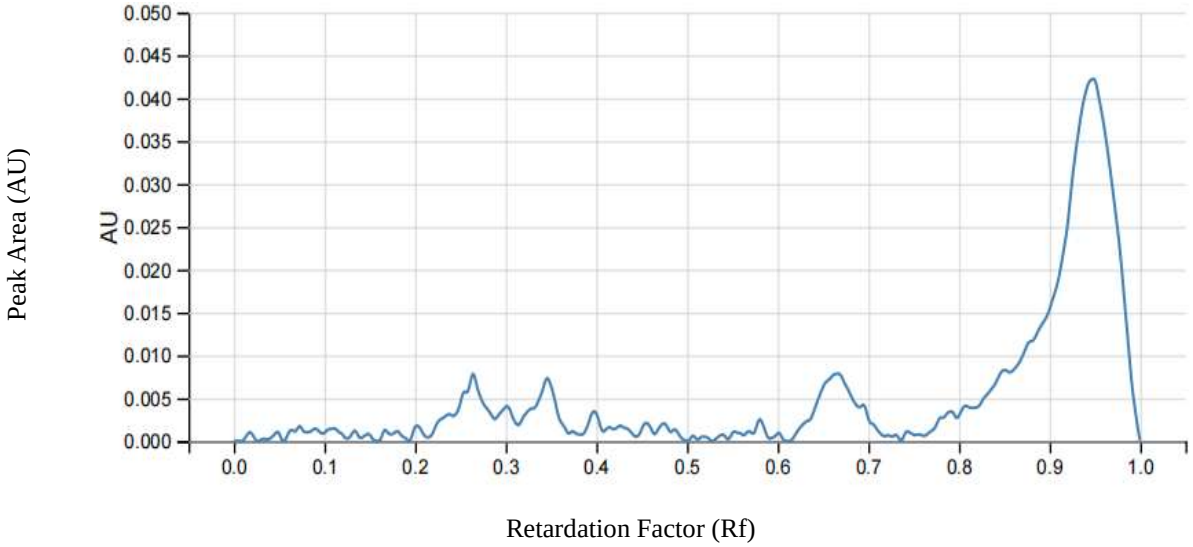

Figure 5: Chromatogram Blank of Polmacoxib

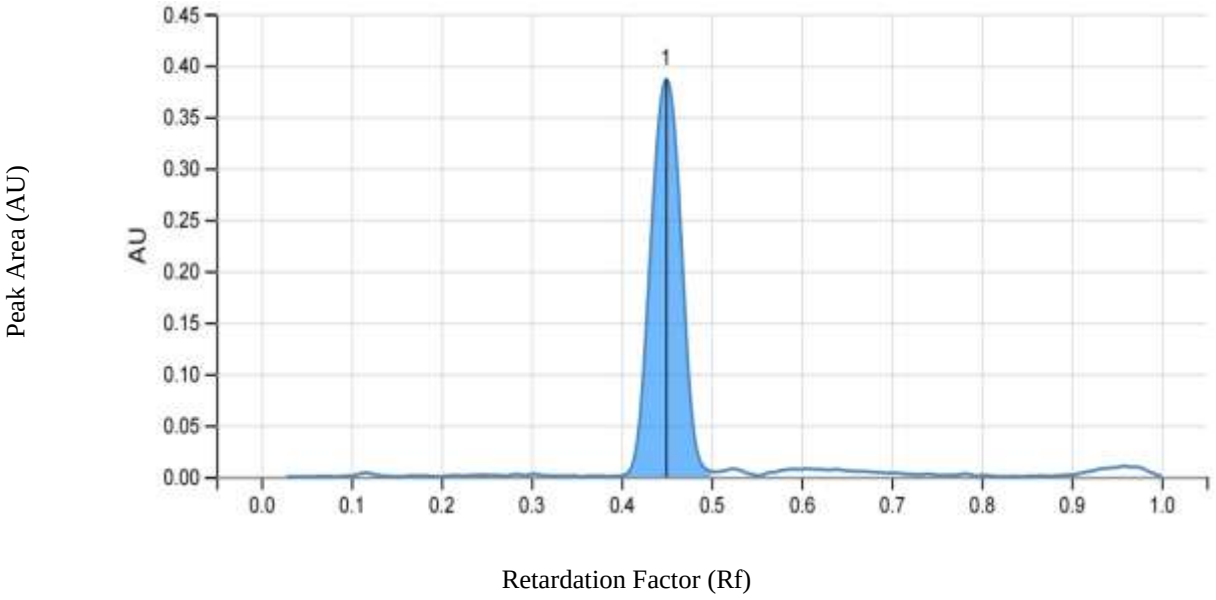

Figure 6: Chromatogram Standard of Polmacoxib (Peak no. 1)

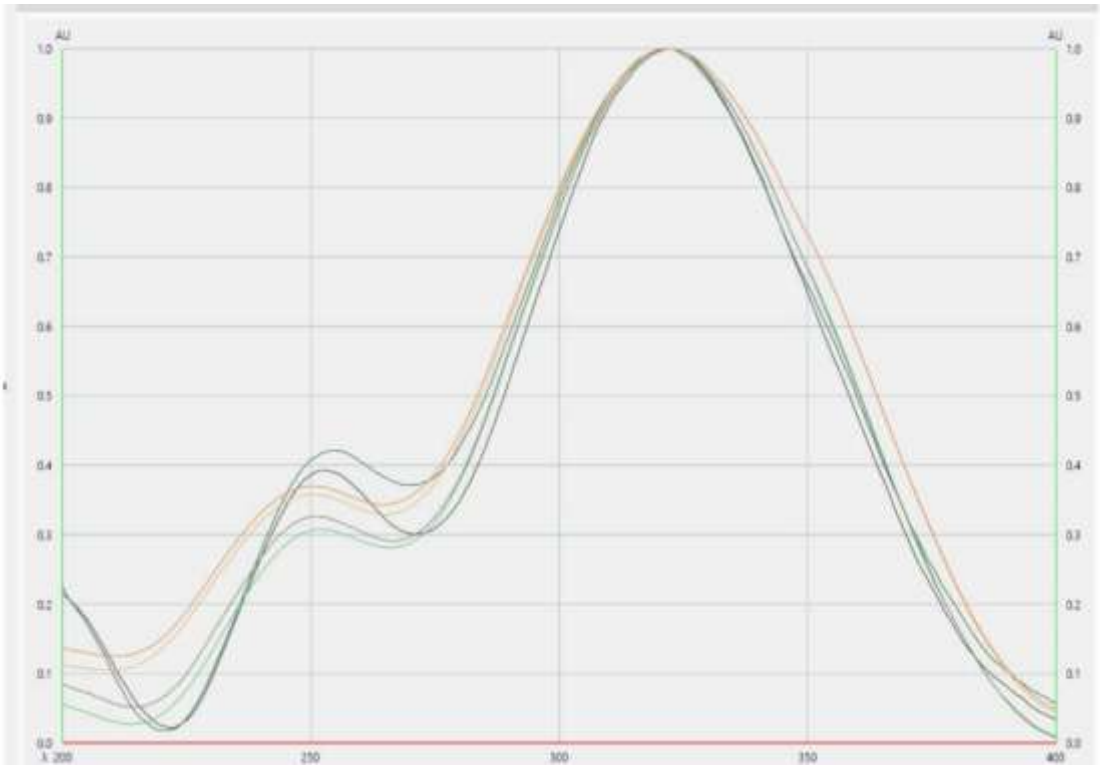

Figure 7: Overlay spectrum of Polmacoxib

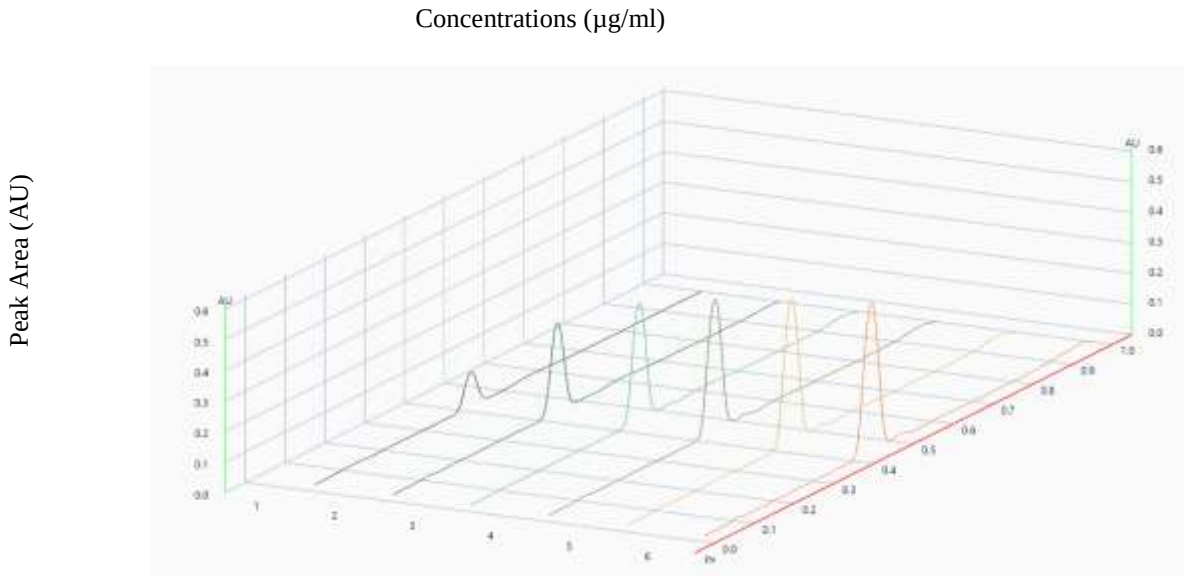

Figure 8: Series of Chromatogram showing Linear range of Polmacoxib (5 – 30 µg/ml)

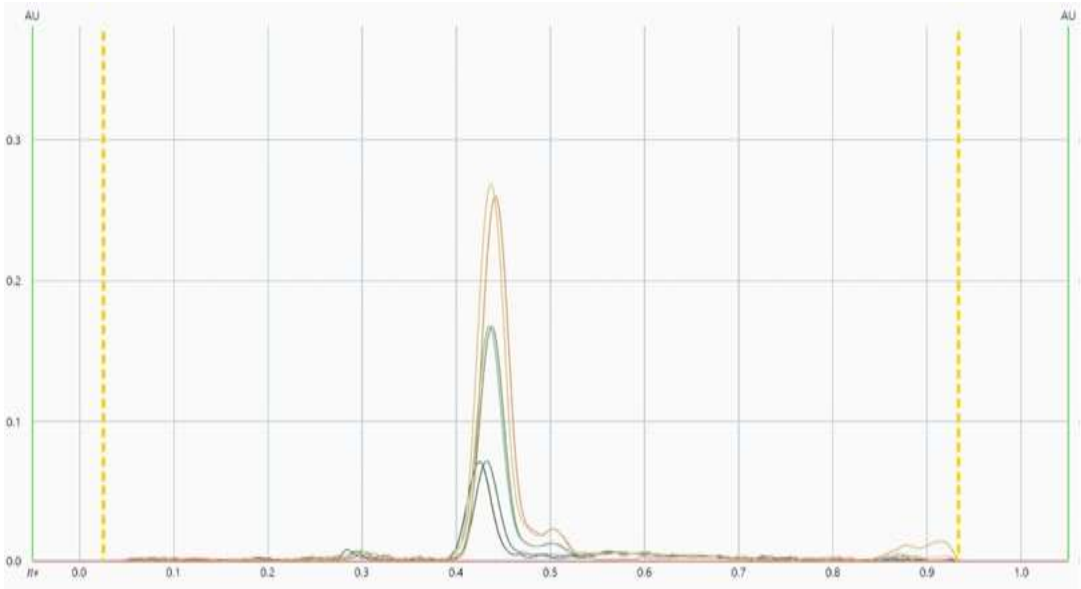

Figure 9: Precision Study Graph

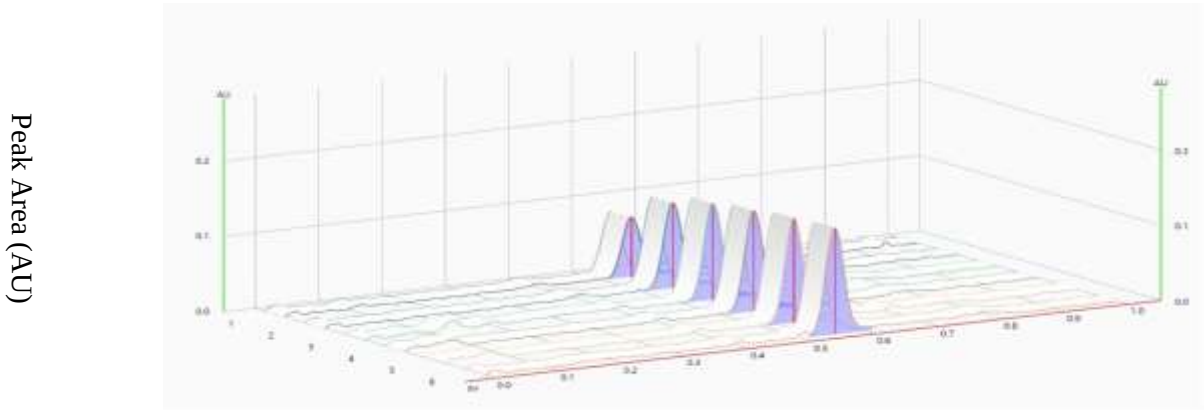

Figure 10: Robustness studies (Change in Wavelength)

3. Conclusion

The HPTLC method was successfully developed and validated for the quantification, estimation and determination of Polmacoxib in its pharmaceutical bulk form. There has not been any HPTLC method developed for the dual inhibitor NSAID – Polmacoxib, hence this present method development is needed for the determination of Polmacoxib at a single detection wavelength at 322 nm. This developed and validated HPTLC method is sufficiently precise, specific, reliable, robust and accurate with high recovery rate. The analytical method and its statistical reports prove the present method to be reproducible and selective for the routine analysis of Polmacoxib. This validated method complies with the ICH guidelines Q2 (R1) and all its validation Parameters have been reported to be within the acceptance range. This method is economical as it requires very less solvent and multiple sampling can be done using single TLC plate for analysis. This HPTLC method can be used for both qualitative and

quantitative analysis of Polmacoxib in different laboratories. This proposed method has been proved to show good resolution, sharp peak identification, linearity and RSD values less than 2% which indicates that this method is suitable for the determination of Polmacoxib.

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