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DEVELOPMENT AND VALIDATION OF NOVEL HPTLC METHOD FOR THE ESTIMATION OF PIMOBENDAN IN TABLET DOSAGE FORM

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ABSTRACT:

A novel, accurate, exact, sensitive and appropriate High Performance Thin Layer Chromatography (HPTLC) method was developed and validated for the determination and estimation of Pimobendan in pharmaceutical tablet dosage form. The chromatographic separation for Pimobendan was achieved on Silica gel 60 F_{254} HPTLC plate as the stationary phase using a standardized mobile phase composed of n-Hexane: Ethyl acetate: Ethanol: Glacial acetic acid (GAA) in the following ratio of 70:15:15:1%v/v/v/v. Identification and quantification of Pimobendan were done at 330nm. The system was found to produced sharp peak and well resolved discrete band at an appropriate Rf value i.e. 0.442. The performance of proposed method was validated with respect to linearity, recovery, precision, robustness, LOD and LOQ in accordance with ICH guidelines. The linearity for molecule was found between the range of 4-40ng/band with correlation coefficient 0.9991. The LOD (limit of detection) and LOQ (limit of quantification) were found to be 1.04 ng/band and 3.16 ng/band respectively. The current method is novel since there is no reported HPTLC method for the estimation of Pimobendan in pharmaceutical tablet dosage form and method can be used successfully for quality checks and routine analysis of Pimobendan.

KEYWORDS: Pimobendan, High Performance Thin Layer Chromatography, CAMAG Linomat 5, LOD, LOQ

INTRODUCTION:

Pimobendan is an orally administered veterinary drug used as an inodilator. Pimobendan has positive inotropic and vasodilator effects. It is a calcium sensitizer and selective inhibitor of phosphodiesterase 3 (PDE3). It is used to treat canines with congestive heart failure brought on by atrioventricular valvular insufficiency. Chemically Pimobendan is 3-[2-(4-methoxyphenyl)-3*H*-benzimidazol-5-yl]-4-methyl-4,5-dihydro-1H-pyridazin-6-one having a molecular formula $C_{19}H_{18}N_4O_2$. The Pimobendan is marketed as a tablet dosage form for the treatment of congestive heart failure due to dilated cardiomyopathy. A review of the literature indicates that only few instrumental methods were available for estimating Pimobendan in tablet dosage form like UV, HPLC and no HPTLC method was reported. Hence, present study aims to develop and validate an accurate, fast, precise, selective, and cost-effective HPTLC approach for the estimation of Pimobendan in tablet dosage form.

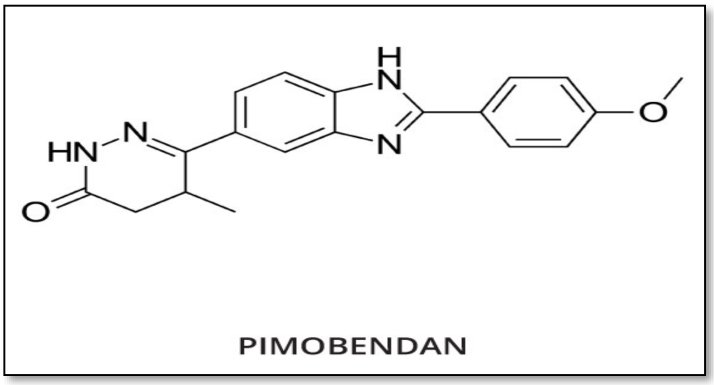

Figure no. 1: Structure of Pimobendan

INSTRUMENTATION:

Application was done by CAMAG Linomat syringe (100µl), a CAMAG Linomat 5 which sprayed samples on HPTLC plate in the form of bands with nitrogen gas generator. For the chromatographic separation of drug pre-coated HPTLC plates (silica gel 60 F254, HPTLC Plates with thickness of 250µm) were utilized and linear ascending development of the chromatographic technique was carried out in a CAMAG twin-through glass developing chamber. Chromatographic scanning was performed on CAMAG TLC scanner 4. For the visualization, imaging and documentation of chromatogram CAMAG TLC Visualizer 2 was utilized and for whole studies Vision CATS CAMAG HPTLC software version 4.0 was used.

CHEMICAL AND REAGENTS:

Pimobendan working standard with defined potency 99.03% and Cardisure tablet containing pimobendan 1.25mg were obtained from Central Drug Testing Laboratory, Mumbai (CDTL). n-hexane of SRL grade, glacial acetic acid of E. Merck India Limited, Ethyl acetate of lab grade MS. Molychem and ethanol of Gemini associates were obtained from Central Drug Testing Laboratory, Mumbai. Precoated HPTLC silica gel 60 F_{254} aluminium sheets (20x20cm with thickness 250µm) from Sigma Aldrich, Germany were utilized for chromatographic separation of drug.

DETERMINATION OF WAVELENGTH:

A UV spectrum was obtained by scanning Pimobendan standard solution between the ranges of near UV visible region i.e. 400.0nm to 200.0nm. Pimobendan showed maximum absorbance (λ max) at 330 nm (figure no. 2). Hence, the same wavelength was selected for the analysis of Pimobendan.

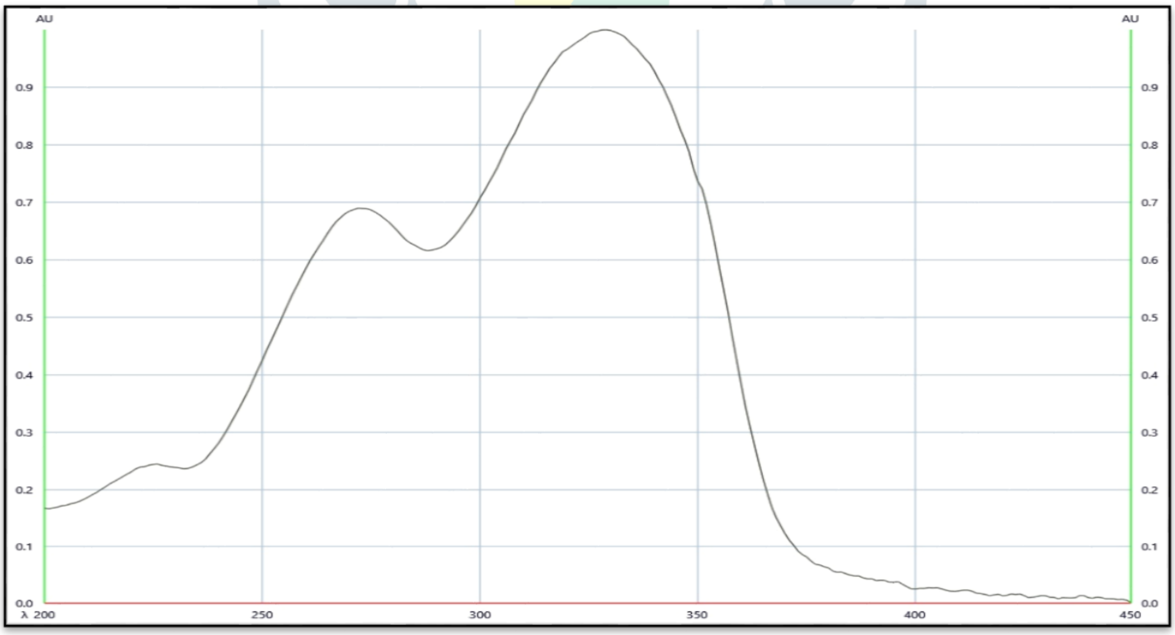

Figure no.2: UV spectra of Pimobendan

CHROMATOGRAPHIC CONDITION:

The precoated HPTLC silica gel 60 F_{254} 20 x 20 cm aluminium plate with 250 µm thickness was used as a stationary phase. Standard and sample spotting were carried out to the plate in the form of 8 mm long, narrow bands using CAMAG Linomat syringe (100 µl) and CAMAG Linomat 5 sample applicator with nitrogen gas aspirator at a constant rate of 15 nl/s. The X and Y application positions were maintained at 8 mm and 20 mm respectively to avoid edge effect. The two bands were separated by 20 mm. A CAMAG twin through glass developing chamber was saturated for 30 minutes using mobile phase containing n-Hexane: Ethyl acetate: Ethanol: Glacial acetic acid (70:15:15:1%v/v/v/v). The plate was then inserted in saturated chamber for linear ascending development of chromatogram up to run distance of 70 mm (solvent front). After development the plate was dried using dryer. The plate was later subjected to CAMAG visualizer 2 for bands visualization and imaging. A CAMAG TLC scanner 4 with a deuterium lamp was used to scan the plate at 330 nm for densitometric analysis in order to observe sharp peaks. The slit size was 6.0 mm x 3.0 mm with auto mode for keeping sensitivity and scanning speed was 100nm/s. The data processing and peak area evaluation were carried out using Vision CATS CAMAG HPTLC software version 4.0.

PREPARATION OF SOLUTION:

Selecton of Diluent:

Based on the solubility and chemical nature of Pimobendan ethanol was selected as diluent for preparation of sample and standard solutions.

Standard Preparation:

Accurately about 10 mg of Pimobendan standard was dissolved in 50 ml of ethanol in a 100 ml. volumetric flask. After sonicating, the volume was made up to 100 ml with ethanol to obtain the standard solution. This was further diluted to obtain pimobendan solution having a concentration of 12.5 µg/ml.

Sample Preparation:

Ten tablets of Cardisure containing pimobendan 1.25 mg were weighed, and the average mean weight was calculated. The tablets were then crushed to get a fine powder, and the weight equivalent of 1 tablet (233.2 mg) was transferred into a 100-ml volumetric flask, and a sufficient amount of diluent was added. The content was sonicated for 10 minutes, and the final volume was made up to the mark with diluent (12.5 µg/ml).

OPTIMIZATION OF HPTLC METHOD:

Mobile phases were chosen on the basis of chemical nature, pKa and polarity of the molecule. After working out the method on TLC the same conditions were applied with respect to the HPTLC. The precoated HPTLC silica gel 60 F_{254} aluminium plate with 250 µm thickness was used for the chromatographic separation of pimobendan. Initial trials were carried out using mobile phase containing the ratio of n-hexane: ethyl acetate: ethanol: formic acid (70:15:15:1%v/v/v/v). However, short Rf value and poor peak shape was observed. Further trials were carried out and good Rf value with symmetrical and sharp peak shape was observed by using mobile phase comprising of n-hexane: ethyl acetate: ethanol: glacial acetic acid (GAA) in the ratio of 70:15:15:1%v/v/v/v and this method was utilized throughout the analysis.

VALIDATION FOR DEVELOPED HPTLC METHOD:

In accordance with the ICH Q2 (R2) guidelines, the parameters including specificity, linearity, accuracy, precision, robustness, limit of detection, and limit of quantification were checked in order to validate the current and optimized HPTLC method.

Specificity:

Specificity of present method was assessed by comparing test sample with standard drug. The Pimobendan sample was verified through comparison with Rf and spectra of band with working standard. The peak purity of pimobendan was ascertained by comparing the peak start (S), peak apex (M), and peak end (E).

Linearity:

The linearity of an analytical method is its ability to produce test results within a given range which are directly proportional to that of the concentration of analyte in the sample. Three replicates of each concentration were used for the linearity studies on pimobendan, which were conducted within the concentration range of 4–40 ng/band. Each band spotted was of 2µl. A linear response was obtained with a co-relation coefficient of 0.9994. The plot of peak areas verses respective concentration was shown in Figure no.6 and the results of Pimobendan linearity was shown in table no.1.

Precision:

The closeness of agreement between several measurements made from repeated samplings of the same homogenous sample under specified conditions is expressed as the precision of an analytical method. Repeatability and intermediate precision studies were used to validate the precision of proposed method. % Relative standard deviation (RSD) for repeatability precision was determined by applying 25ng/band standard solution of Pimobendan six times. Each band spotted on HPTLC plate was of 2µl. The intermediate precision method was validated by inter-day and intra-day precision. Intra-day precision was carried out by the analysis of Pimobendan on the same day at three different levels i.e., low, medium and higher concentrations. And inter-day precision was carried out on three different days. The results of repeatability studies and intermediate precision studies are shown in tables 2 and 3.

Accuracy (Recovery Studies):

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value and the value found. Recovery studies were carried out using the standard addition method, which involved adding a known amount of pimobendan standard solution at three different levels—110%, 120%, and 130% of a pre-analysed sample solution. Three determinations were made at each concentration level, and the mean percentage recovery was computed and reported. The mean percentage recovery was found to be within acceptable limits and is shown in table no. 4.

Assay:

In the development and validation of HPTLC method, an assay is an investigative procedure done to quantitatively determines the amount of particular drug in a given sample. The assay results obtained shows high percentage recoveries and low Standard deviation (SD) values. The given assay concentration is with respect to 12.5 ppm of sample with spotting volume 2 µl and the results are shown in table no.5.

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

The lowest amount of analyte in the sample that can be identified but may not always be precisely measured is known as the detection limit of a particular analytical method. The lowest amount of analyte in the sample that can be quantitatively determined with appropriate precision and accuracy is the quantification limit of any unique analytical method. Using the following formulas, the LOD and LOQ were determined based on the slope of the calibration curve and the regression line:

$LOD = 3.3 \times \sigma / s$

$LOQ = 10 \times \sigma / s$

Robustness:

Robustness is the capacity of a method to remain unaffected by small, deliberate variations in the experimental conditions which have been set up for the method. For the HPTLC method, robustness was established by making changes, including a change in mobile phase composition (±0.2%), a change in chamber saturation time (±5min.), and a and a change in the distance travelled by the solvent front (±5mm), and the results were calculated and reported in table no.7.

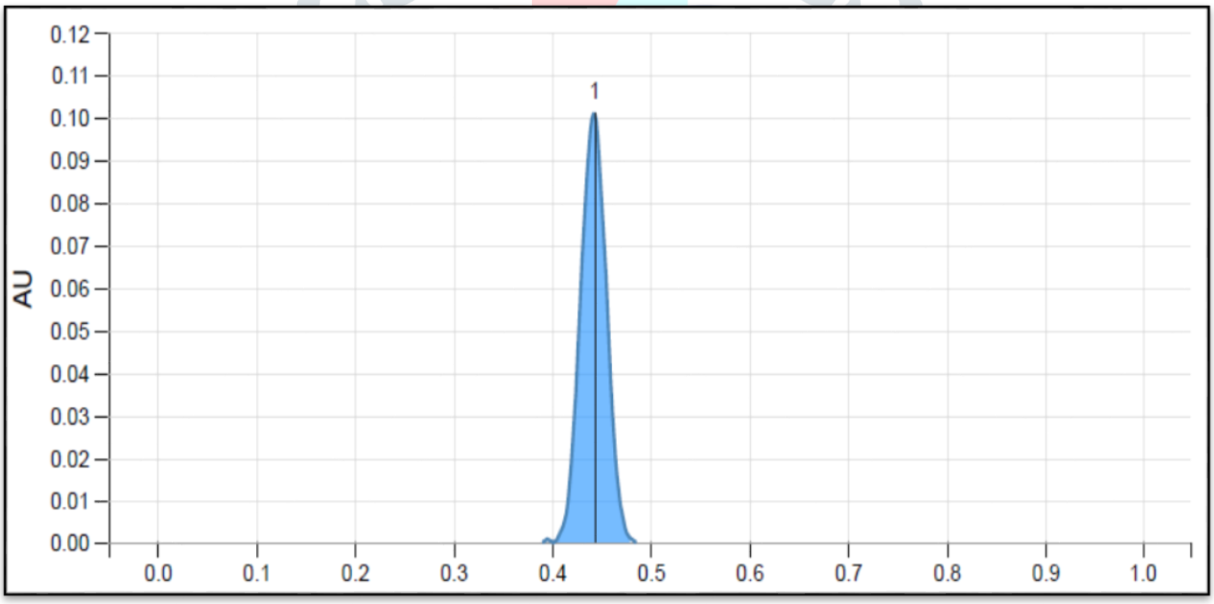

Figure no.3: Densitogram under optimized HPTLC conditions showing an Rf value of 0.44 for Pimobendan.

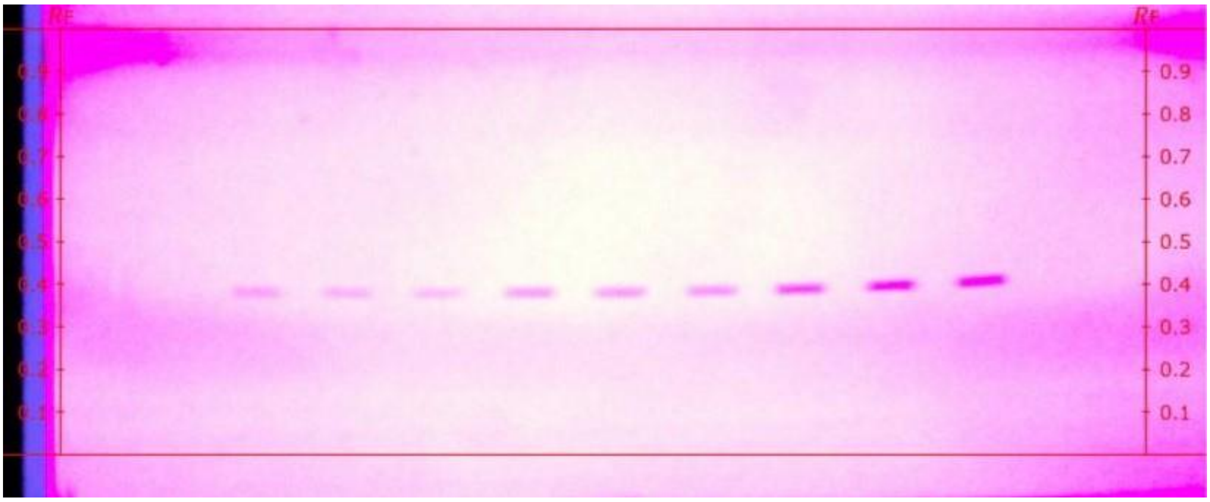

Figure no.4: Image of an HPTLC plate taken at 330nm

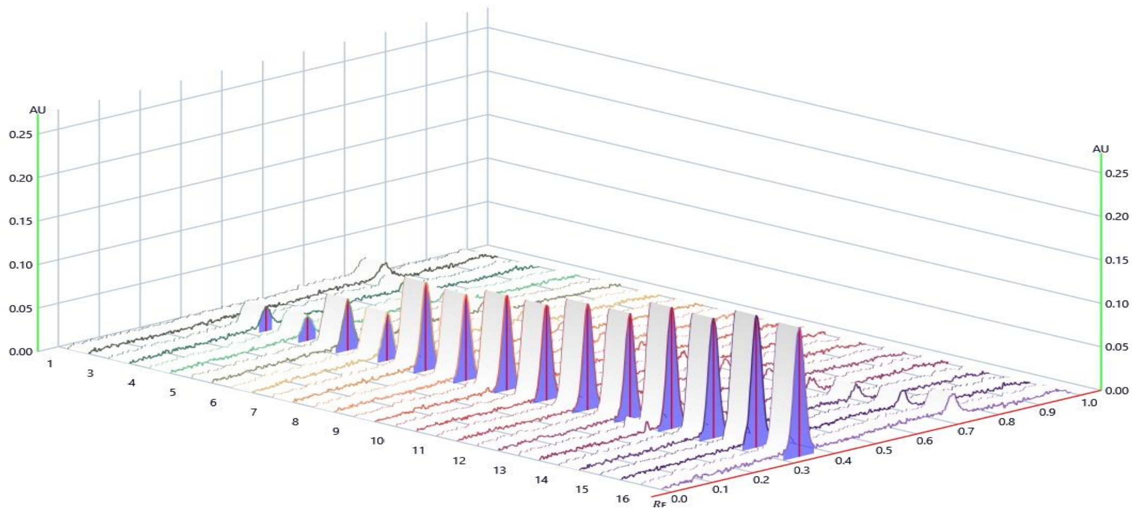

Figure no.5: 3-D densitogram for the linearity of Pimobendan

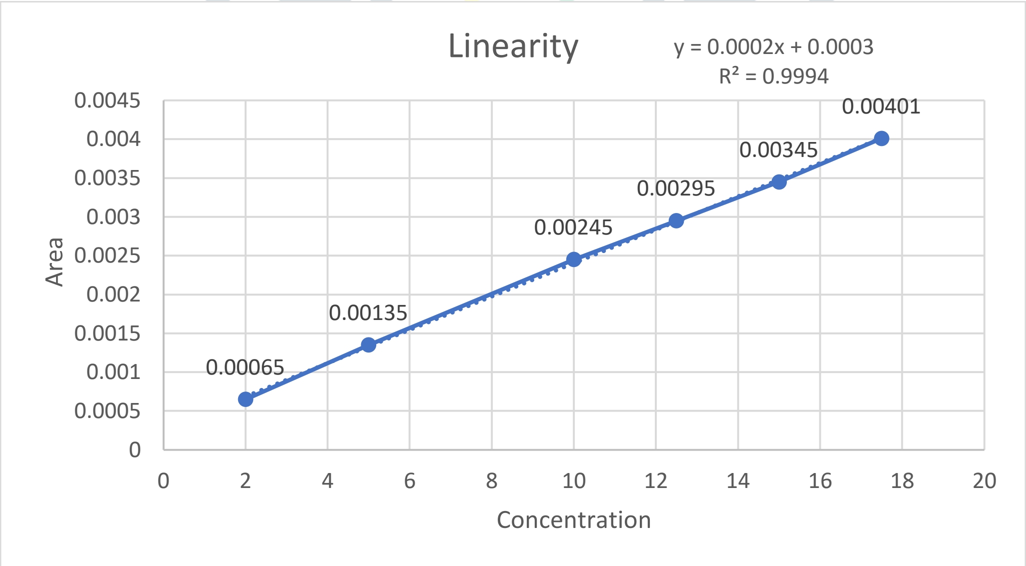

Figure no.6: Calibration curve of Pimobendan

Table no.1: Linearity concentration and area

Concentration (ng/band)	Peak area
4	0.00065
10	0.00135
20	0.00245
25	0.00295
30	0.00345
35	0.00401

Table no.2: Precision data of pimobendan as repeatability

Concentration (ng/band)	Peak area
25	0.00370
25	0.00368
25	0.00376
25	0.00374
25	0.00364
25	0.00368
Average	0.00370
SD	0.00004382
%RSD	1.184

Table no.3: Intermediate precision data of pimobendan

Concentration (ng/band)	Intra-day Precision		Inter-day Precision	
	Peak area ± SD (n=3)	%RSD	Peak area ± SD (n=3)	%RSD
12	0.00193±0.000023	1.19	0.00216±0.000015	0.70
25	0.00335±0.000036	1.07	0.00373±0.000041	1.11
36	0.00413±0.000052	1.28	0.00430±0.000049	1.14

Table no.4: Recovery studies of Pimobendan

% Level added	Amount spiked (ng/band)	Amount recovered (mg/tab)	%Recovery	%Mean recovery	%RSD
110 110 110	27.5 27.5 27.5	1.39 1.38 1.38	101.54 100.64 100.94	101.04	0.4535
120 120 120	30 30 30	1.52 1.51 1.50	101.44 100.84 100.54	100.94	0.4539

130 130 130	32.5 32.5 32.5	1.64 1.63 1.64	101.34 100.74 101.64	101.04	0.4535

Table no.5: Assay calculations

Sr. no.	Area of Standard at 330nm	Peak Area	% Assay
1	0.00339	0.00326	99.98
2		0.00328	100.70
3		0.00325	99.94
4		0.00329	101.17
5		0.00328	100.86
6		0.00326	100.09
Average			100.46
SD			0.522
% RSD			0.519

Table no.6: LOD & LOQ

Parameters	**Pimobendan**
Linearity Range (ng/band)	4-35
Regression Equation	0.0002x+0.0003
Slope	0.000215
Y-Intercept	0.000255
Correlation Coefficient	0.999426
Standard Error	3.41E-05
Observations	6
Limit of Detection (µg/ml)	0.5241~1.04ng/band
Limit of Quantification (µg/ml)	1.5882~3.16ng/band

Table no.7: robustness of the developed method

Change in Mobile phase ratio (70:15:15:1%v/v/v/v ± 0.2% in n-Hexane content)			
Ratio	**Rf**	**Area ± SD (ng/band)**	**%RSD**
69.8:15:15:1 v/v/v/v	0.42	0.003497±0.000011	0.330
70.2:15:15:1 v/v/v/v	0.45	0.003493±0.000025	0.720
Change in saturation time (30min. ± 5min.)			
Saturation time	**Rf**	**Area ± SD (ng/band)**	**%RSD**
25 min.	0.42	0.00344±0.000020	0.603
35 min.	0.42	0.00346±0.000025	0.725
Change in distance travelled (70mm ± 5mm)			

Distance travelled (solvent front)	Rf	Area ± SD (ng/band)	%RSD
65 mm	0.45	0.003447±0.000020	0.603
75 mm	0.43	0.003443±0.000020	0.604

RESULTS AND DISCUSSION:

A new high performance thin layer chromatography (HPTLC) method that is quick, easy to use, appropriate, and precise was developed and proven to be effective for estimating pimobendan in pharmaceutical tablet dosage form. The developed method for pimobendan provided results with a mobile phase containing n-hexane: ethyl acetate: ethanol: GAA (v/v/v/v) using HPTLC silica gel 60 F254 plates. Pimobendan's peak purity was ascertained by comparing its spectra at the peak start (S), peak apex (M), and peak end (E) positions. Pimobendan shows a linear response between the ranges 4-40 ng/band. The corresponding linear correlation coefficient (r^2) was 0.9994 respectively. The precision was performed by using repeatability precision and intermediate precision (intra-inter day precision) method. Relative standard deviation (RSD) for the repeatability studies was determined to be 1.18%. It was determined that the percentage RSD values for intra- and inter-day precision were within the acceptable limits. The developed method was found to be precise as the % RSD values found within range as per ICH guidelines. For the recovery studies the average of percent mean recovery at the level of 110%, 120% and 130% was found to be 101.04% and mean % RSD value was found to be 0.453 which is within the limit hence, the method was accurate. The results for recovery studies are shown in table no.4. The percentage of the observed assay for pimobendan in the dosage form of Cardisure tablets was determined to be 100.46% and results are shown in table no.5. Limit of Detection (LOD) was found to be 1.04ng/band and Limit of Quantification was found to be 3.16mng/band which indicates the sensitivity of method developed. Reproducible outcomes in the form of accurate Rf values and low % RSD values demonstrated the method's robustness. The results remained unaffected by small deliberate variations in the parameters.

Table no.8: Combined results of all parameters

Parameters	Pimobendan
Linearity Linearity range Correlation coefficient	 4-40ng/band 0.9994
Precision Repeatability Intra-Day Inter-Day	%RSD 1.18% 1.07-1.28% 0.70-1.14%
Accuracy (%level) 110% 120% 130%	 101.04% 100.94% 101.04%
Assay Mean recovery %RSD	 100.46% 0.519
LOD	1.04ng/band
LOQ	3.16ng/band
Robustness Change in Mobile phase Change in Saturation time Change in Distance travelled	%RSD 0.330-0.720% 0.603-0.725% 0.603-0.604%

CONCLUSION:

The present HPTLC method for quantitative and qualitative estimation of pimobendan is novel, rapid, precise, accurate, simple and robust. In accordance with ICH Q2(R2) guidelines, the suggested approach was effectively developed and validated for the parameters of linearity, precision, accuracy studies, assay, LOD & LOQ, and robustness. The results for all validation parameters were within the acceptable limit. It is important to note that there were no prior HPTLC methods available for the analysis of pimobendan in pharmaceutical tablet dosage form. Therefore, this method represents a valuable contribution. The developed method should be taken into consideration due to its convenience in term of sample preparation, less solvent consumption, the high bandwidth of HPTLC i.e. up to 15 samples per plate which saves time and cost and also the method helps to carry out content uniformity test with less time duration. Thus, routine analysis and quality control of pimobendan in pharmaceutical tablet dosage form can be accomplished using the proposed HPTLC approach.

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