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DEVELOPMENT AND VALIDATION OF A STABILITY-INDICATING RP-HPLC METHOD FOR THE ESTIMATION OF PIRFENIDONE: APPLICATION TO DEGRADATION KINETICS

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ABSTRACT

A simple, precise, and validated stability-indicating reverse phase high-performance liquid chromatography (RP-HPLC) method was developed for the estimation of pirfenidone. The primary objective was to create a robust method for routine analysis and to study the drug's degradation behavior. On a Thermo Fisher C18 column (250 × 4.6 mm, 5 µm), chromatographic separation was carried out with success using an isocratic mobile phase consisting of acetonitrile: water (40:60 v/v). The wavelength at which the eluent was detected was 317 nm. With a correlation coefficient (r^2) of 0.997, the method showed excellent linearity over a concentration range of 5–30 µg/mL. The accuracy was confirmed through recovery studies, which showed results between 98% and 102%, and the precision was established with a %RSD of less than 2%. Forced degradation studies were conducted as per ICH guidelines under acidic, alkaline, thermal, oxidative, and photolytic stress conditions. Pirfenidone showed the highest degradation under alkaline hydrolysis, following first-order kinetics with an activation energy calculated as 37.86 kJ/mol. The specificity of the method was proven, as no interference from degradation products or excipients was seen. The suggested method is robust, accurate, and reliable for quality control and stability testing of pirfenidone in bulk and pharmaceutical dosage forms.

KEYWORDS: RP-HPLC, Stability-Indicating Method, Pirfenidone, Forced Degradation, Method Validation, Kinetics.

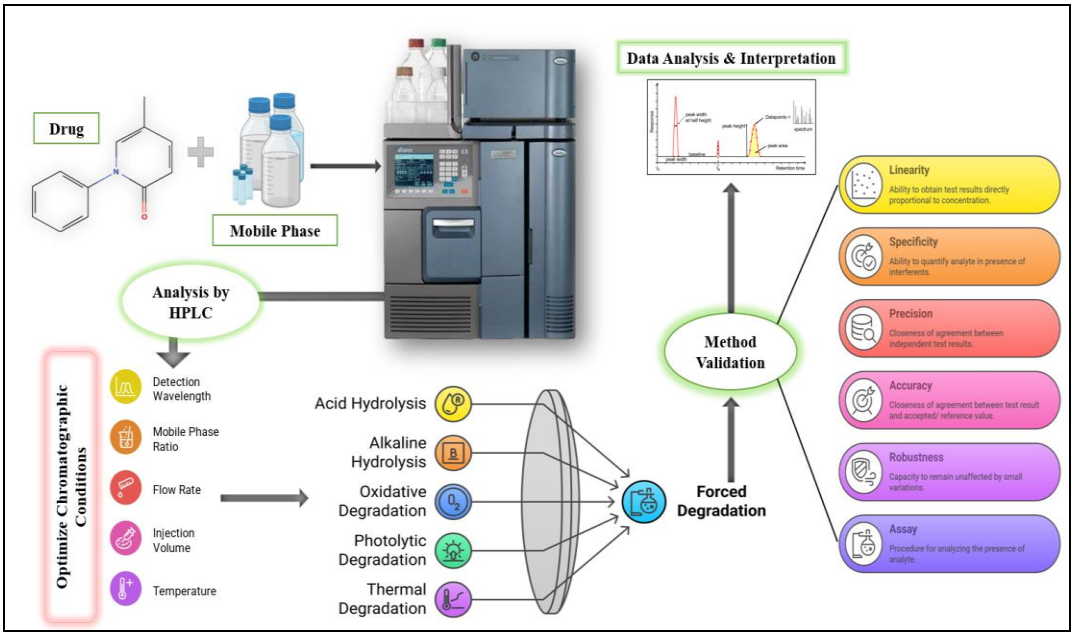

Fig 1: Graphical Abstract

INTRODUCTION

Pirfenidone (PIR) is a small molecule taken orally, known for its anti-fibrotic and anti-inflammatory actions, and is mainly prescribed for idiopathic pulmonary fibrosis (IPF). Chemically, it is 5-methyl-1-phenyl-2-(1H)-pyridone. Due to its clinical relevance, dependable analytical methods are essential for quantifying the drug in raw material and dosage forms, along with ensuring its stability. Among the various techniques, high-performance liquid chromatography (HPLC) is widely employed in pharmaceutical analysis because of its accuracy, reproducibility, and capacity to resolve complex mixtures. HPLC methods that can indicate stability are particularly important as they allow assessment of the drug even in the presence of its degraded forms, excipients, or impurities, in line with regulatory expectations [ICH Q1A(R2), Q2(R1)]. Various analytical techniques, such as HPLC and UV spectrophotometry, have previously been used to analyze PIR. Nevertheless, there remains a demand for validated methods that are simple, economical, and robust, aligning with the latest ICH requirements and incorporating comprehensive degradation profiling.

The objective of this research was to design and validate a novel RP-HPLC method that is accurate, selective, and efficient for the quantification of Pirfenidone in both bulk and its pharmaceutical dosage form. The validation process followed ICH Q2(R1) guidelines, and stress testing was performed to confirm the method's capability to monitor degradation and determine degradation kinetics under the most unstable condition.

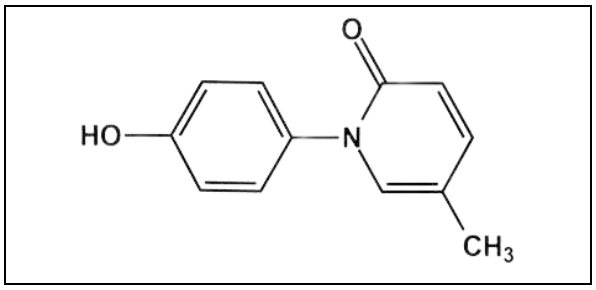

Fig. 2: Pirfenidone Structure.

MATERIALS AND METHODS

instrument and equipment

The chromatographic analysis was performed using a Waters Alliance e2695 HPLC system, integrated with a 2998 Photodiode Array (PDA) detector and managed through Empower 3 software. Separation was achieved using a Thermo Fisher C18 column (250 mm × 4.6 mm, 5 µm particle size). Other instruments included a Shimadzu UV-Visible spectrophotometer UV-1800, Shimadzu IR Affinity1 FT-IR spectrophotometer, Digital Analytical Balance (Shimadzu AUX 220), a hot air oven (MSI66, Meta Lab Scientific Industries), Ultrasonic Bath (PCI Analytics), Water Bath (BTI Pvt. Ltd.), and Vacuum Pump (INTRU Pvt. Ltd.)

Reagents & Chemicals

Pirfenidone (pharmaceutical grade, purity 99.8% w/w on anhydrous basis) was provided by Cipla, India. The solvents used included HPLC-grade acetonitrile (A.P. Enterprises) and analytical reagents such as hydrochloric acid, sodium hydroxide (flakes), and hydrogen peroxide (30% w/v), sourced from Merck and S.D. Fine-Chem Ltd. HPLC-grade water (S.D. Fine-Chem Ltd.) was used throughout the experiment. All other chemicals employed were of analytical grade.

EXPERIMENTAL

chromatographic condition

Separation of the analyte was achieved using a Thermofisher C18 column (250 × 4.6 mm, 5 µm). The mobile phase was composed of a mixture of acetonitrile and water in the ratio of 40:60 (v/v). Prior to use, the mobile phase was filtered through a 0.45 µm membrane filter and degassed. An isocratic elution was performed at a flow rate of 1.0 mL/min. The column temperature was maintained consistently (25 ± 2°C). The injection volume for each sample was set to 10 µL, and detection was carried out at a wavelength of 317 nm using the PDA detector.

Preparation Of Standard Stock Solution

A standard stock solution of Pirfenidone with a concentration of 1 mg/mL was prepared by precisely measuring 10 mg of the drug. It was dissolved in a small quantity of methanol and then diluted up to 10 mL using methanol in a volumetric flask. This primary solution was subsequently diluted with the mobile phase (Acetonitrile: Water, 40:60 v/v) to prepare working standard solutions of varying concentrations. A solution of 10 µg/mL was prepared, for routine analysis.

Preparation Of Sample Solution

The average weight of twenty marketed tablets of Pirfenidone (Label Claim: 200 mg Pirfenidone per tablet) was calculated. The tablets were crushed to a fine powder. A quantity equivalent to 10 mg of Pirfenidone was accurately weighed and placed into a 10 mL volumetric flask. It was dissolved using methanol with the help of sonication, and the final volume was made up to 10 mL with methanol to yield a 1 mg/mL solution. This solution was passed through a 0.45 µm nylon syringe filter. A 0.1 mL portion of this filtrate was diluted to 10 mL using the mobile phase to obtain a final test concentration of 10 µg/mL.

Forced degradation study

Stress testing of Pirfenidone was carried out under various conditions to assess the drug's stability and confirm the method's specificity, in line with ICH Q1A(R2) guidelines. For acidic degradation, Pirfenidone was treated with 0.1 N hydrochloric acid and heated under reflux at 70°C for 12 hours, with periodic sampling followed by neutralization and dilution. Alkaline degradation was induced by refluxing the drug in 0.1 N sodium hydroxide at 70°C for 4 hours, with

aliquots similarly processed at intervals. Oxidative degradation was conducted by treating the Pirfenidone solution with 3% hydrogen peroxide and keeping it in the dark at ambient temperature for 24 hours. Wet heat degradation involved refluxing an aqueous solution of Pirfenidone at 70°C for 12 hours, with aliquots withdrawn at 15-minute intervalsFor thermal stress, the powdered drug was exposed to dry heat in a hot air oven at 70°C for 6 hours. Photolytic degradation was carried out by placing the Pirfenidone powder under direct sunlight for 7 consecutive days, (meeting ICH specified illumination and UV energy levels). After completion of each stress condition, the resulting solutions were diluted using the mobile phase to achieve a final concentration of approximately 10 µg/mL and analyzed via HPLC to determine the extent of degradation.

Chemical kinetics study

The degradation kinetics of Pirfenidone were investigated under alkaline hydrolysis conditions (0.1 N NaOH) at two different temperatures, 50°C and 70°C. Samples were collected at specific time intervals, neutralized, diluted, and subsequently analyzed using HPLC. The remaining drug concentration was plotted against time to assess the degradation order (zero, first, or second) by evaluating the correlation coefficient (R^2) of the respective plots. The degradation rate constant (k) was calculated accordingly. To determine the activation energy (Ea), the Arrhenius equation was applied:

$\ln k = \ln A - Ea/RT$

where T represents temperature in Kelvin and R is the universal gas constant. A plot of ln k versus 1/T was used to derive the activation energy.

Method Validation

The RP-HPLC method developed for this study was validated as per ICH Q2(R1) guidelines, covering parameters such as linearity, accuracy, precision, specificity, limit of detection (DL), limit of quantitation (QL), and robustness.

Linearity and Calibration Curve

Working standards of pirfenidone in the range of 5–30 µg/mL by serial dilution of a 0.1 mg /mL stock solution using the mobile phase as diluent. Each solution (10 µL injection) was analysed in triplicate under isocratic HPLC conditions. (flow rate = 1.0 mL min^{-1}; detection wavelength = 317 nm). A calibration graph of mean peak area versus concentration was constructed, and linearity was evaluated by least-squares regression. The resulting equation, $y = 19731 x - 6108.3$, with a R^2 value of 0.9975, demonstrated excellent linearity across the selected concentration range.

Detection Limit And Quantitation Limit

Detection Limit (LOD) and Quantitation Limit (LOQ) were calculated based on the standard deviation of the response (σ) and the slope (S) of the calibration curve using the formulae:

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

$LOQ = 10 \times (\sigma/S)$.

The standard deviation of the y-intercepts of regression lines was used as σ.

Accuracy & Precision

To determine the accuracy of the method, a previously analyzed sample solution of Pirfenidone (PIR) with a concentration of 10 µg/mL was spiked with standard Pirfenidone solutions. This process created final concentrations equivalent to 80%, 100%, and 120% of the nominal assay concentration. The necessary volumes of the standard solutions were added to the sample solution, and the resulting mixtures were then diluted to a final volume of 10 mL using the mobile phase. To evaluate intermediate precision, each of these prepared dilutions was analyzed in triplicate over three consecutive days. The accuracy of the method was confirmed by calculating the percentage recovery of the added drug. The precision of the method, encompassing both repeatability and intermediate precision, was assessed by calculating the relative standard deviation (% RSD) from the results obtained. The outcomes of the accuracy and precision investigation are detailed in Table 5.

Specificity

The method's specificity was conducted by injecting blank (mobile phase), placebo (a mixture of common tablet excipients: lactose, sodium starch glycolate, talc, magnesium stearate), a standard solution of Pirfenidone (10 µg/mL prepared from a stock solution), and the sample solution (prepared from the pharmaceutical formulation to a concentration of 10 µg/mL PIR) into the HPLC system. Observations revealed no interfering peaks from the blank or placebo at the characteristic retention time of Pirfenidone, which was approximately 4.7 minutes. The Pirfenidone peak was observed and well-resolved in both the standard and sample solutions. Furthermore, peak purity analysis of Pirfenidone in the stressed samples and the formulation, performed using the PDA detector by comparing spectra across the peak, confirmed spectral homogeneity and the absence of co-eluting impurities, thereby establishing the specificity and stability-indicating capability of the method.

Robustness

To assess the robustness of the method, minor and intentional changes were made to the optimized chromatographic parameters. The specific parameters that were altered included the flow rate (adjusted to 0.8 mL/min and 1.2 mL/min from the original ± 0.2 mL/min) and the proportion of acetonitrile (the organic modifier) in the mobile phase. The acetonitrile percentage was varied by ± 10% v/v, resulting in concentrations of 30% and 50% ACN. The impact of these changes on the retention time, peak area, theoretical plates, and tailing factor was then observed.

RESULT AND DISCUSSION

Optimization Of Chromatographic Conditions

The creation of a reliable Stability-Indicating Method (SIM) requires the meticulous optimization of chromatographic conditions. This is essential to guarantee the effective separation, detection, and quantification of the active pharmaceutical ingredient from any potential degradation products, while adhering to system suitability criteria. Key factors in achieving selectivity and specificity include the choice of stationary phase, the composition of the mobile phase, and the detector wavelength. Preliminary characterization of Pirfenidone involved UV spectroscopic analysis, which determined the λmax to be 317 nm; this wavelength was subsequently used for PDA detection. The temperature of the column was kept at a consistent ambient level (25 ± 2°C).

Several mobile phase compositions were evaluated to achieve optimal separation and peak characteristics for Pirfenidone on a Thermofisher C18 column. Initial trials with methanol and water mixtures (in 80:20 and 70:30 v/v ratios) showed low plate counts. Switching to acetonitrile: water mixtures, early elution, and poor separation were observed at 80:20 and 70:30 ratios. A ratio of 60:40 (v/v) also failed to meet suitability criteria. Optimal performance was achieved with ACN: Water (40:60 v/v), yielding a symmetric peak at ~4.7 min, plate count >14000, tailing factor ~1.3, and clear baseline. This composition was used for all subsequent analyses.

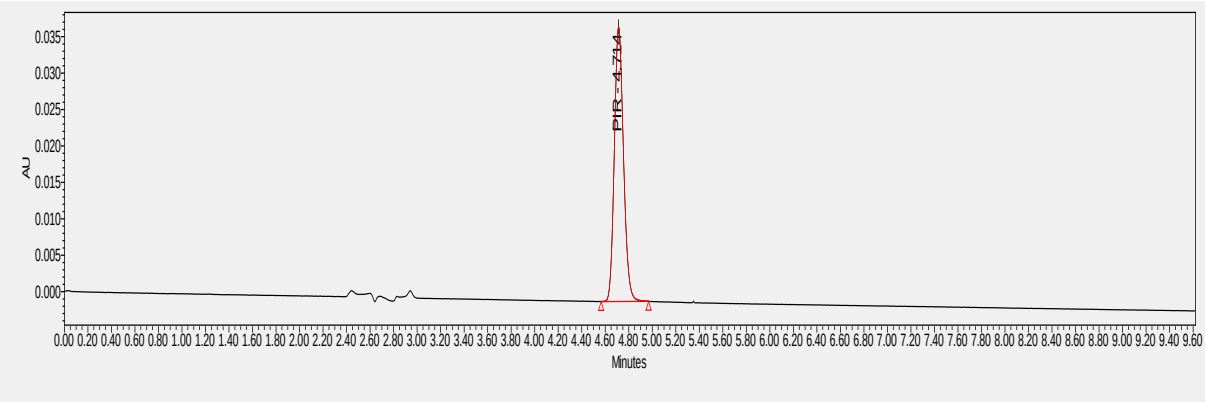

Fig. 3: Representative chromatogram of Pirfenidone in optimized chromatographic conditions.

Forced Degradation Studies

The forced degradation studies, detailed in Table 1, indicated that Pirfenidone is susceptible to a range of stress conditions, including acidic, alkaline, oxidative, thermal, and photolytic environments. Under these conditions, the degradation of Pirfenidone was observed to be between 5.60% and 9.08%. This degradation was primarily observed as a consistent and reproducible (confirmed by triplicate experiments) reduction in PIR's peak area, as no distinct degradation product peaks were separated under these conditions. Peak purity analysis confirmed the spectral homogeneity of the Pirfenidone peak in all stressed samples, establishing the method's stability-indicating capability.

Table 1: Summary of Forced Degradation Studies of PIR.

Sr. No	Degradation Parameter	Stressed conditions	% Degradation	Remark
1.	Acid Hydrolysis	0.1 N, Refluxed at 70°C for 12 hr	5.6%	No degradation products were formed
2.	Alkali Hydrolysis	0.1 N, Refluxed at 70°C for 4 hr	9.08%	No degradation products were formed
3.	Wet Hydrolysis	Refluxed at 70°C for 12 hr	6.81%	No degradation products were formed
4.	Dry heat	Kept at 70°C for 12 hr	5.8%	No degradation products were formed
5.	Oxidation	3% H_2O_2, Kept in the dark for 18 hr	6.11%	No degradation products were formed
6.	Photolytic	Exposed to sunlight for 7 days	5.63%	No degradation products were formed

Chemical Kinetics

To assess the degradation behavior of Pirfenidone specifically under alkaline conditions, a dedicated forced degradation study was performed, using a 0.1 N NaOH solution and exposing the drug to two distinct temperatures: 50°C and 70°C. The concentration of Pirfenidone was monitored over time, and the data were used to assess the order of reaction, rate constants, half-life, shelf-life, and activation energy.

Kinetics at 50°C

At 50°C, the degradation of Pirfenidone was studied over 240 minutes. The correlation coefficients (R^2) for zero, first, and second-order kinetic models were determined to be 0.9921, 0.9926, and 0.9923, respectively. The data best fit the first-order model, indicating that the degradation process adhered to first-order kinetics at this specific temperature.

- Rate constant (k): 0.00022 min^{-1}
- Half-life ($t_{1/2}$): 3143.1 min

- Shelf-life (t_{90}): 476.22 min

Kinetics at 70°C
At an elevated temperature of 70°C, a more significant degradation was observed. The R^2 values for the kinetic models were 0.9786 (zero order), 0.9811 (first order), and 0.9730 (second order). The data again showed the best fit for the first-order model.

- Rate constant (k): 0.00051 min^{-1}
- Half-life ($t_{1/2}$): 1358.82 min
- Shelf-life (t_{90}): 207.65 min

Activation Energy Determination
The activation energy (Ea) for the alkaline degradation was determined using the Arrhenius equation.

Table 2: Kinetic Data for Alkaline Degradation.

Temp (°C)	Temp (K)	1/T (K^{-1})	k (min^{-1})	ln k
50	343.15	0.002914177	0.0005	-7.60090246
70	323.15	0.003094538	0.00022	-8.421883012

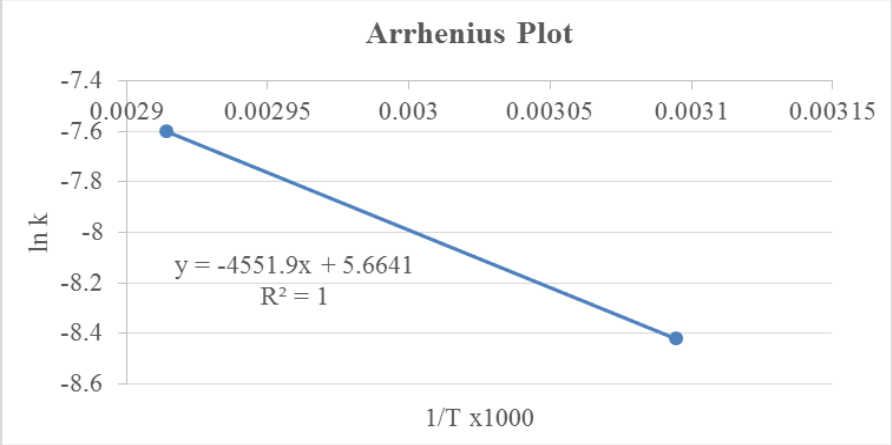

Fig 4. Arrhenius plot for alkaline (0.1 N NaOH) degradation of Pirfenidone

The rate constants obtained at 50°C and 70°C were used to plot ln k against 1/T. From the resulting Arrhenius plot, the following linear regression equation was obtained:
ln k = −4551.9 (1/T) + 5.6641
From this equation:

- Slope (m) = –4551.9
- Gas constant (R) = 8.314 J/mol·K
- Activation energy (Ea) =

$$Ea = -m \times R$$
$$= 45519 \times 8.314$$
$$= 37863.32 \text{ J/ mol}$$
$$Ea \approx 37.86 \text{kJ/mol}$$

Method Validation
Linearity:
The method demonstrated excellent inearity for Pirfenidone across a concentration range of 5-30 µg/mL. From the calibration curve, a linear regression equation of y = 19731x - 6108.3 was produced, along with a correlation coefficient (R^2) of 0.9975. This indicates a significant proportional relationship between the concentration and the corresponding peak area.

Table 3: Linearity Data of PIR.

Concentration (µg/ml)	Area				±SD	%RSD
	1	2	3	Mean		
5	108631	108687	108601	108640	43.65	0.040
10	194060	193956	194056	194024	58.92	0.030
15	310197	309902	310006	310035	149.62	0.048
20	397140	397037	396555	396911	312.29	0.079
25	508360	509394	510122	509292	885.42	0.174
30	591641	590830	591976	591482	589.25	0.100
Equation: y = 19731x – 6108.3						
Regression: 0.997						

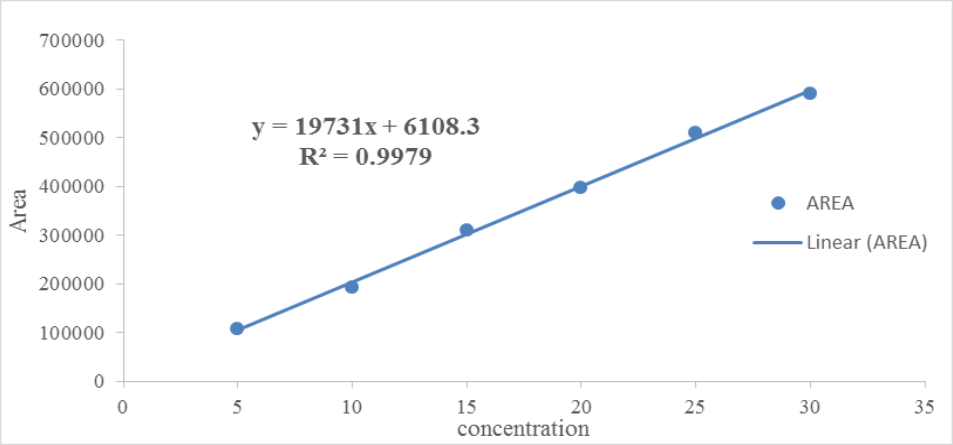

Fig. 5: Calibration curve for Pirfenidone.

Detection Limit & Quantitation Limit

An evaluation of the method's sensitivity was performed, which established a detection limit of 1.54 µg/mL and a quantitation limit of 4.66 µg/mL.

Assay

The applicability of the validated method for routine analysis was confirmed by assaying a marketed tablet formulation of Pirfenidone (label claim: 200 mg). The amount of Pirfenidone found was 199.68 mg per tablet, corresponding to 99.84% of the label claim, which falls within the typical pharmacopeial limits (e.g., 90-110% or 95-105%), demonstrating the method's suitability for quality control of pharmaceutical dosage forms.

Table 4: Assay of PIR.

Sr. No	Amount added (µg/mL)	Amount found (µg/mL)	% Assay	% Mean Assay	SD	%RSD
1.	10	9.82	99.50573	99.84	0.294	0.29%
2.	10	9.87	99.98815			
3.	10	9.87	100.0381			
Label claim: 200mg			Amount found: 199.68			

Specificity

To assess the specificity of the newly developed RP-HPLC method for PIR, a chromatographic analysis was performed using blank, placebo, standard, and formulation samples.

Blank Chromatogram

At the 4.8-minute retention time characteristic of PIR, no peak was detected, confirming the absence of interference from the diluent.

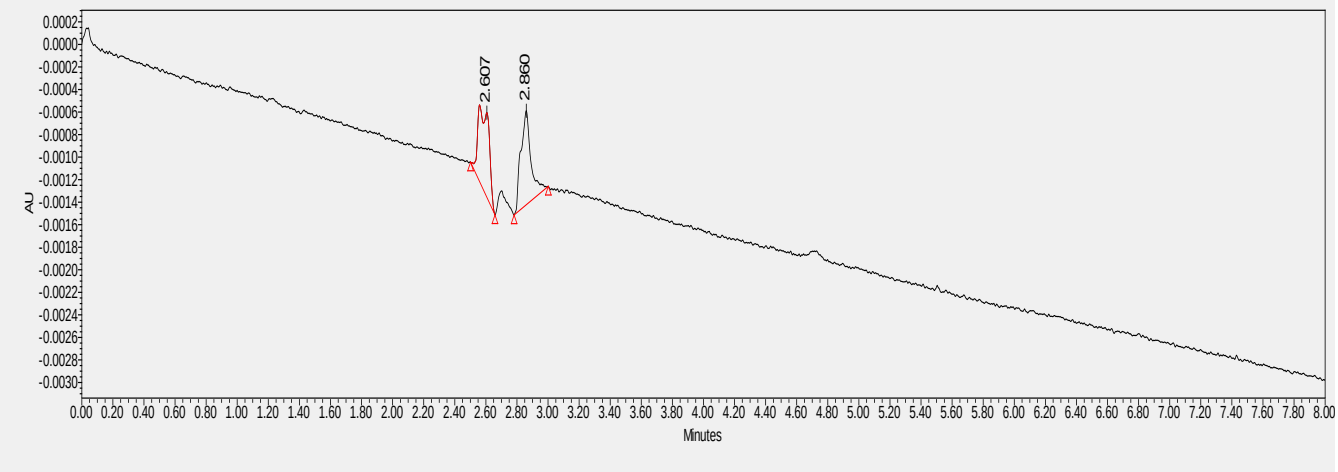

Fig. 6: Representative chromatogram for blank.

Placebo Chromatogram

Minor peaks appeared at different retention times, but none at or near 4.8 min, indicating that excipients did not interfere with Pirfenidone detection (Fig. 7).

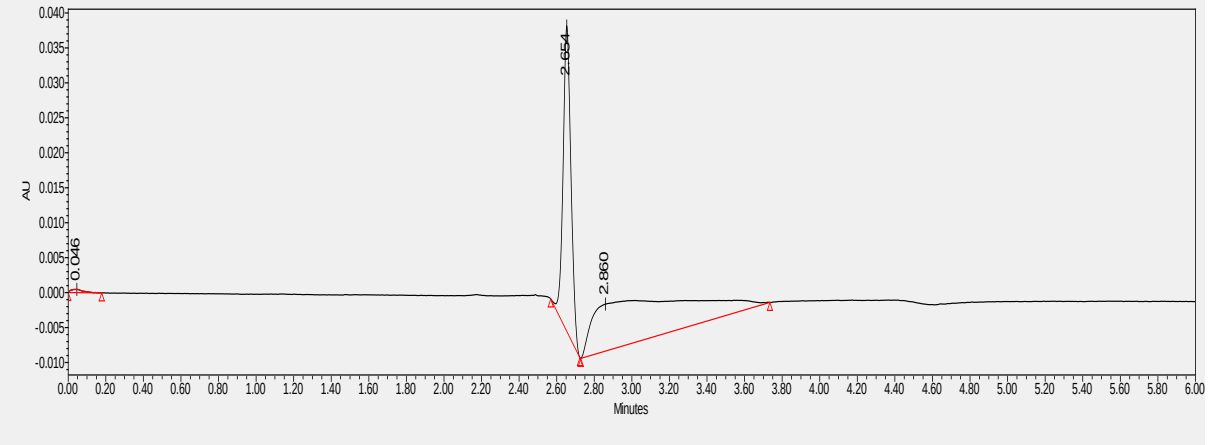

Fig. 7 Representative chromatogram for Placebo.

Standard Chromatogram

A sharp, symmetric peak was obtained at 4.7 min without adjacent or co-eluting peaks.

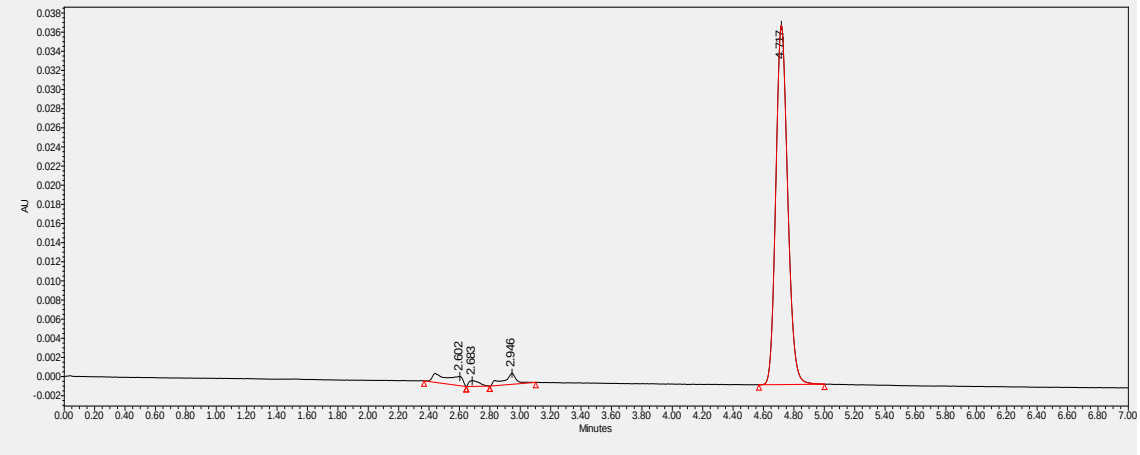

Fig. 8: Representative chromatogram for Standard.

Peak purity analysis showed a purity angle of 0.127, which was lower than the threshold angle of 0.287, confirming spectral purity and absence of interference (Fig. 9).

Formulation Chromatogram

The test sample showed a well-defined Pirfenidone peak at 4.707 min, along with a few minor peaks at other retention times. The peak remained resolved and consistent in retention time and shape.

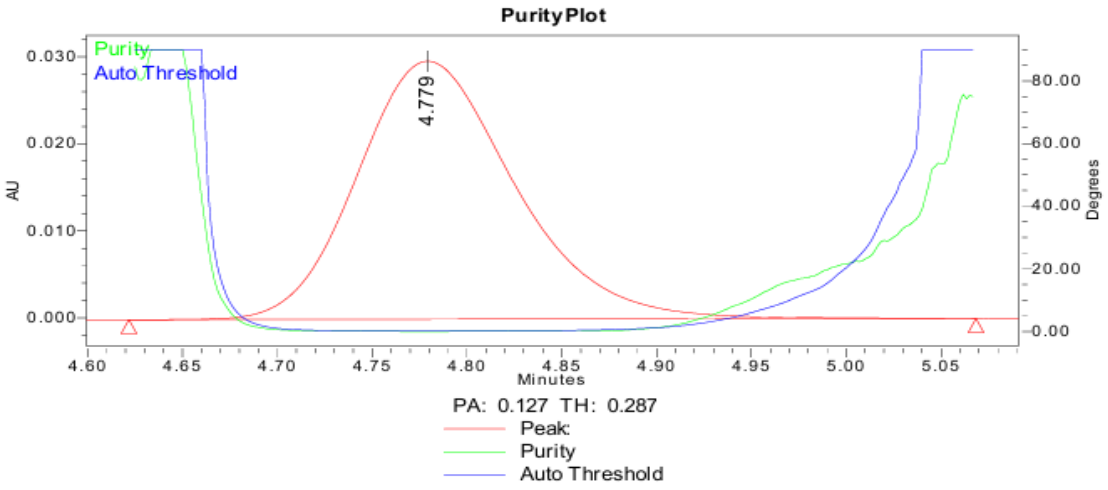

Fig. 9. Purity Plot for Standard.

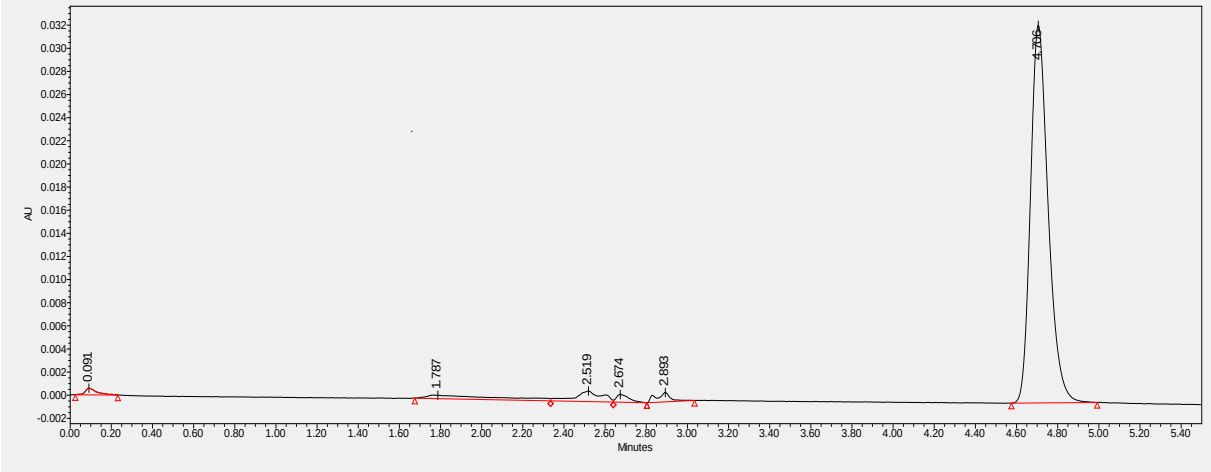

Fig. 10: Representative chromatogram for Formulation.

The purity angle (0.108) was below the threshold angle (0.302), confirming the absence of co-eluting impurities and demonstrating the method's specificity in the presence of formulation matrix (Fig. 11).

Accuracy & Precision

Accuracy was evaluated at 80%, 100%, and 120% of the label claim. Mean % recovery ranged from 99.86% to 100.0% with %RSD below 0.04, indicating high accuracy. Precision was confirmed through intra- and inter-day studies with low standard deviation and %RSD values (<0.05) across all levels.

ANOVA analysis yielded F-values lower than the F-tabulated value at α = 0.05, indicating no significant variability between days and confirming method precision and reproducibility

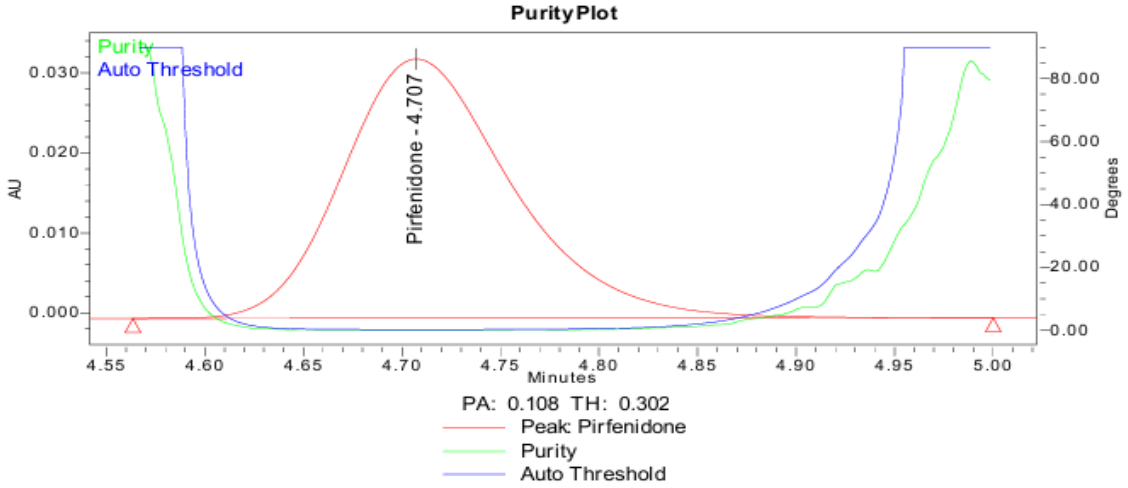

Fig. 11: Purity plot of Formulation.

Table 5. Accuracy & Precision of Pirfenidone at three concentration levels across three days

Amount Added	Amount Found mg			Within the mean square	Between mean square	F-value
	Day 1	Day 2	Day 3			
80% (160 mg) Mean	159.89	159.88	159.91	0.004122	0.000533333	0.129380054
%Recovery	99.93	99.93	99.94			
SD	0.07	0.03	0.05			
%RSD	0.04	0.02	0.03			
100% (200 mg) Mean	199.80	199.76	199.71	0.004355	0.006077778	1.395408163
%Recovery	99.90	99.88	99.86			
SD	0.05	0.04	0.07			
%RSD	0.03	0.02	0.03			
120%	239.90	239.84	239.80	0.00157	0.0076	4.85106383

(240 mg) Mean						
%Recovery	100.0	99.9	99.9			
SD	0.03	0.03	0.04			
%RSD	0.01	0.01	0.02			

Robustness

Minor variations in flow rate and organic modifier ratio showed consistent chromatographic performance. Each condition was tested in triplicate, and the mean results are tabulated below:

Table 6: Effect of Flow Rate on Chromatographic Parameters.

Flow Rate (mL/min)	RT (min)	Area	Tailing	Plate Count
0.8	5.8	236404	1.3	16141
1.0	4.7	195461	1.3	14757
1.2	3.9	157136	1.3	13832

Flow Rate Variation (0.8, 1.0, 1.2 mL/min): Retention time and peak area decreased with increased flow rate. Plate count slightly reduced, while the tailing factor remained constant at 1.3 (Table 6).

Table 7: Effect of Organic Modifier on Chromatographic Parameters.

Organic Modifier Ratio	RT (min)	Area	Tailing	Plate Count
30:70	7.1	192016	1.3	16172
40:60	4.7	195461	1.3	14757
50:50	3.7	188206	1.3	14671

Organic Modifier Ratio (30:70, 40:60, 50:50): Higher organic content resulted in shorter retention times and slightly lower plate counts. Peak shape remained unaffected, with a consistent tailing factor across all conditions.

CONCLUSION

A robust and reliable RP-HPLC method for estimating Pirfenidone was successfully developed and validated. The method exhibits excellent linearity, accuracy, precision, specificity, and robustness in line with ICH guidelines. The stability-indicating nature of the method was confirmed through forced degradation studies, with alkaline conditions causing the highest degradation following first-order kinetics. The moderate activation energy calculated for alkaline degradation further supports the drug's susceptibility to base-catalyzed hydrolysis. In summary, this validated analytical technique is highly suitable for routine quality control and for assessing the stability of Pirfenidone in pharmaceutical products.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS CONTRIBUTION

- **Harshada S. Patil:** Conceptualization of the study, method development, experimental work, data collection and analysis, and preparation of the original draft manuscript.
- **Dr. Sandeep S. Sonawane:** Supervision, methodology guidance, and manuscript review.

FIGURE CAPTIONS

Fig 1. Graphical Abstract
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Fig 3. Representative chromatogram of Pirfenidone in optimized chromatographic conditions
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