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R E S E A E C H A R T I C L E

Analytical Method Development and Validation for Pirfenidone (Anti-Fibrotic) in bulk drug and dosage form

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A B S T R A C T

Pirfenidone, 5-methyl-1-phenyl-2-(1H)-pyridine is a novel Anti-fibrotic agent approved for mild to moderate Idiopathic pulmonary fibrosis (IPF). Isocratic reverse phase high performance liquid chromatography (RP-HPLC) separation using a XBridge C18 column of particle size 5µm, (4.6×250mm) .The separations were achieved at the UV detection at 311nm using the mobile phase of Acetonitrile: Trifluoro acetic acid in the ratio of 50:50,with apparent pH of 2.08,the Flow rate was 0.8ml/min and the injection volume was set at 20 µl with 10mins of runtime. The retention time was observed at 4.617mins for Pirfenidone. The method was validated by using various validation parameters like accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ). The standard curve was linear over a working range of 1- 32 µg/ml and gave an average correlation factor of 0.9988 for Pirfenidone. The limit of detection and the limit of quantification were found to be 0.120µg/ml and 0.401µg/ml for Pirfenidone respectively. The method showed good recoveries and relative standard deviations of intra and inter day assay less than 2. This method can be easily and conveniently used for routine analysis of Pirfenidone in bulk Drug and tablet dosage forms.

Keywords: Pirfenidone, RP-HPLC, UV–Visible Spectrophotometer, LOD, LOQ.

A R T I C L E I N F O

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C O N T E N T S

1. Introduction

Pirfenidone is an orally available pyridinone derivative that inhibits collagen formation and is used to treat idiopathic pulmonary fibrosis. Elevations in serum enzyme levels during pirfenidone therapy are not uncommon, but it has yet to be implicated in cases of clinically apparent liver injury with jaundice. Pirfenidone is a novel Anti-fibrotic agent approved for mild to moderate Idiopathic pulmonary fibrosis. Chemically it is 5-methyl-1-phenyl-2-(1H)-pyridine[1] (Figure 1). It is a small simple heterocyclic molecule with molecular mass of 185.22 and can be administered orally. IPF- Generally a state of a progressive, fibrosing inflammatory disease of lung parenchyma of unknown cause. IPF is a rare incurable disease, often fatal, which mostly affects geriatric patients causing fibrosing interstitial pneumonia of unknown etiology[2]. Patients with IPF suffer from shortness of breath, cough and hampered daily physical activities. Treatment for IPF consists of oxygen therapy, pulmonary rehabilitation and lung transplant.[3] Pirfenidone (Esbriet) is the first drug to be licensed in Europe for treatment of IPF. It was initially approved in Japan and Europe followed by India and USA in 2010 and 2014 respectively.

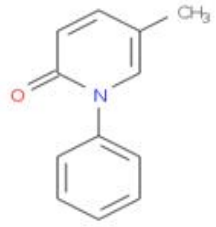

Figure 1: Chemical structure of Pirfenidone

Literature survey reveals few analytical methods were reported for the determination of pirfenidone in bulk drug and pharmaceutical preparations and in biological fluids by UV and HPLC[4], LC/MS/MS[5], High performance Liquid Chromatography[6-8], However most of the available methods have rat plasma, human plasma and also have limitations such as a long runtime, low sensitivity, uneconomical and have poor symmetry. Keeping in view of these, an attempt has been made to develop an accurate, precise and reliable RP-HPLC method for the estimation of pirfenidone in pharmaceutical dosage form. Infact no monograph of pirfenidone or its formulations available hitherto in any pharmacopoeia. Oral tablet formulation containing active pirfenidone equivalent to 200 mg is available in the territorial markets of Japan, Taiwan, Korea and India. So, the present work is taken up to development and validation of assay method for routine quality control of pirfenidone dosage form. As a matter of fact the established method was validated with respect to specificity, linearity, precision, accuracy, robustness, LOD and LOQ according to ICH Q2(R1)[9].

2. Materials and Methods

Instruments and chromatographic conditions

The Agilent Compact LC 1120 HPLC system consisting of gradient pump (LC-10AT vp pump) (4MPa or 40barr), Rheodyne injector, UV variable wavelength detector, Standard cell and Agilent syringe was used. The separations were achieved on A XBridge C18 column of particle size 5µm, (4.6×250mm) with UV detection at 311nm.

Analytical weighing balance (Shimadzu AUX 220) was used for weighing, sonicator (EQUITRON230VAC, 50Hz), vaccum pump (SUPER FIT), filtration kit (TARSONS) and Nylon membrane filter (Merck Millipore) for solvents and sample filtration were used throughout the experiment. Double beam UV Visible spectrophotometer (SHIMADZU-UV 1700) was used for wavelength detection. The EZ Chrome Elite software-single channel was used for acquisition, evaluation and storage of chromatographic data.

Chemicals and Reagents:

An analytically pure sample of pirfenidone standard was procured as gift sample from Hetero Labs Ltd., Hyderabad, India. All chemicals were analytical grade. HPLC grade acetonitrile were procured from Merck Life Science Private Limited., Mumbai, India. And HPLC grade trifluoroacetic acid was procured from Merck Specialities Private Limited., Mumbai, India. Water used was of HPLC grade from Karnataka College of Pharmacy, Banglore, and Karnataka. Commercial tablets of pirfenidone formulation were produced from Apollo pharmacy. PIRFENIX tablets containing Pirfenidone with labeled amount of 200 mg per tablet are manufactured by CIPLA LTD., India.

Preparation of Reagents and Standards

Preparation of buffer

The mobile was prepared by 0.6756ml of TFA was accurately transferred in to a 1000ml volumetric flask and dissolved in HPLC water and pH was 2.08.The solution was filtered through and degassed by ultra sonicator.

Preparation of standard stock solution:

To prepare standard solution, accurately weighed 100 mg of pirfenidone was transferred in to 100 ml of volumetric flask, dissolved and diluted up to the mark with methanol to obtain stock solution containing 1 mg/ml of the drug.

Tablet sample preparation

Weigh accurately not less than 20 tablets of pirfenidone and determine average weight .Grind the tablets of pirfenidone (PIRFENIX) into fine powder. Weigh accurately an amount of tablet powder equivalent to 50 mg of pirfenidone and transfer into 50 ml volumetric flask. Add 40 ml of mobile phase and place in an ultra-sonication bath until dissolution is complete. Add mobile phase to bring up the volume to 50 ml Pipette out 1 ml of the sample solution into 10 ml volumetric flask and dilute with mobile phase up to the mark and mix well. The resulting solution was filtered using 0.2µm filter and degassed by sonication. The resulting solution is further diluted to get a concentration of approximately 10µg/ml.

Selection of detection wave length

The UV spectrum of diluted solutions for various concentration of pirfenidone in mobile phase was recorded using Double beam UV Visible spectrophotometer (SHIMADZU-UV 1700). The wave length of maximum absorbance was observed at 311 nm. This wave length was used for detection of pirfenidone.

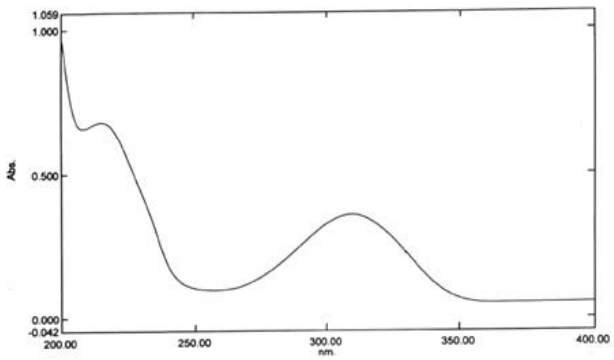

Figure 2: UV spectrum of pirfenidone (10µg/ml)

Calibration curve for pirfenidone

20 µl of each calibration sample solution (1, 2, 4, 8, 16, 32 µg/ml) were injected into the HPLC system to get the chromatograms. The average peak area and retention time were recorded. Linearity cure constructed by plotting concentration of pirfenidone on X-axis and peak area of standard pirfenidone on Y-axis. The linearity range was found to be 1-32µg/ml. The results are presented in the table 1. The standard chromatogram of pirfenidone in figure 3.RP-HPLC overlaying chromatogram of pirfenidone (1-32µg/ml) figure 4. The calibration graph of pirfenidone is presented figure 5. The results are presented in the table 1. The standard chromatogram of pirfenidone in figure 3.RP-HPLC overlaying chromatogram of pirfenidone (1-32µg /ml) figure 4. The calibration graph of pirfenidone is presented figure 5.

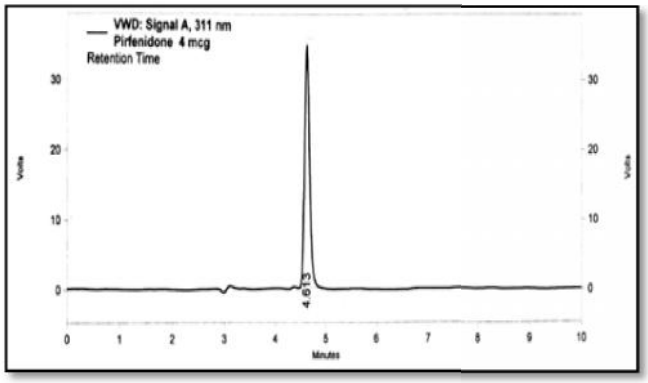

Figure 3: Chromatogram of standard pirfenidone

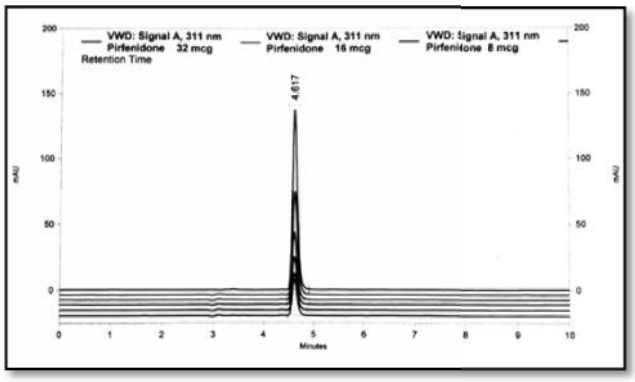

Figure 4: RP-HPLC overlaying chromatogram of pirfenidone (1-32µg/ml)

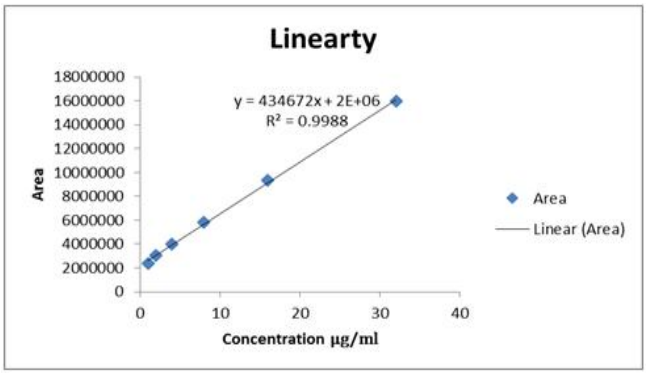

Figure 5: Calibration plot of Pirfenidone

Validation of the Proposed Method

The develop method of analysis was validated as per the ICH for the parameters like system suitability, specificity, linearity, precision, accuracy, robustness and system suitability, limit of detection (LOD) and limit of quantification (LOQ).

System suitability

The chromatographic systems used for the analysis must pass the system suitability limits before sample analysis can commence. Set up the chromatographic system; allow the HPLC system to stabilize for 40 minutes. Inject blank preparation (single injection) and standard preparation (six replicates) and record the chromatogram to evaluate the system suitability parameters like tailing factor (NMT 2), theoretical plate count (NLT 3000) and %RSD for peak area of six replicate injection of pirfenidone standard (%RSD NMT 2). The system suitability data is reported in Table 2.

Specificity

The specificity of the method was determined by observing interference of any encountered ingredients in the formulations. The test results obtained were compared with the results of those obtained for standard drug. It was shown that those ingredients were not interfering with the developed method. Further the well –shaped peaks also indicate the specificity of the method. The result of specificity is tabulated in Table 3. The chromatogram for blank indicating the specificity of developed method is presented in Figure 6.

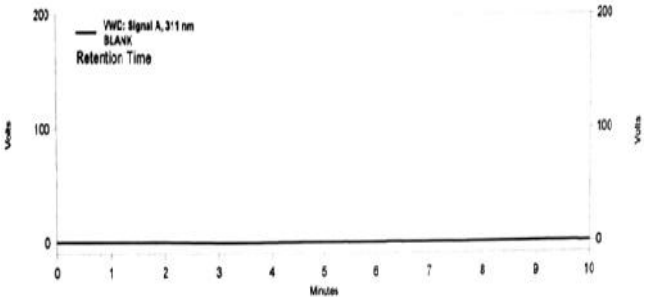

Figure 6: Chromatogram of blank

Precision: Intra-day precision was investigated by replicate application and measurements of peak area for pirfenidone for six times on the same day similar condition. Inter-day

was obtained from %RSD values obtained by repeating the six times on two different days. The percent relative standard deviation (%RSD) was calculated which is within acceptable criteria of not more than 2. The intra-day and inter day precision results were shown in the Table 4 respectively.

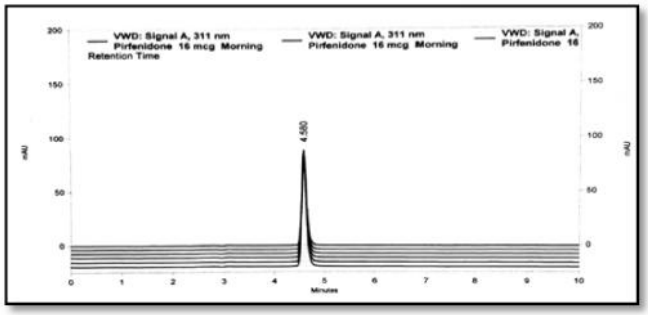

Figure 7: Chromatogram showing Intra-day precision (At morning)

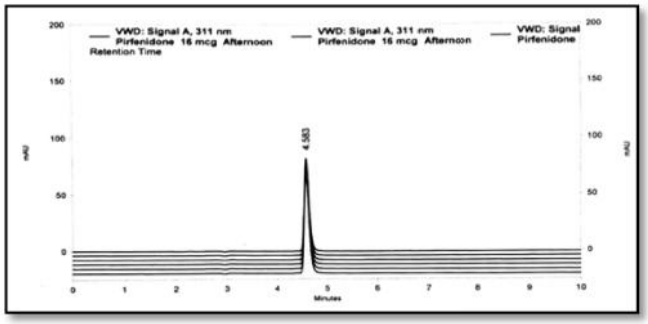

Figure 8: Chromatogram showing Intra-day precision (At afternoon)

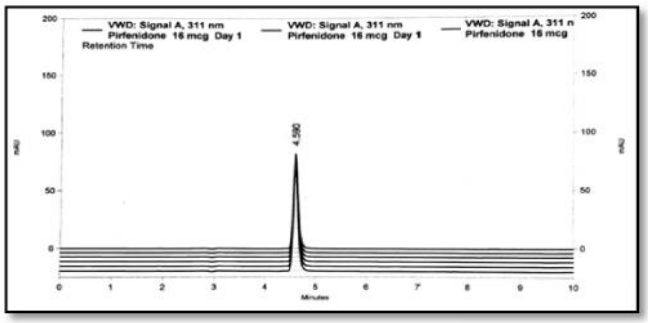

Figure 9: chromatogram showing inter-day precision (Day-1)

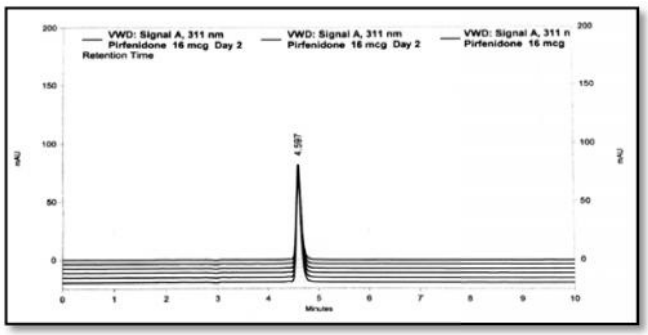

Figure 10: Chromatogram showing inter-day precision (Day-2)

Accuracy

Accuracy is degree of agreement between a measured value and the accepted reference value. The accuracy of the method was tested by triplicate at 3 different concentration equivalent to 80%, 100%, 120% of the active ingredient, by adding a known amount of pirfenidone standard to a sample with pre-determined amount of pirfenidone. The recovered amount of pirfenidone, %RSD of recovery, %recovery of each concentration is calculated to determine the accuracy. The results for accuracy study of pirfenidone are presented in Table 5.

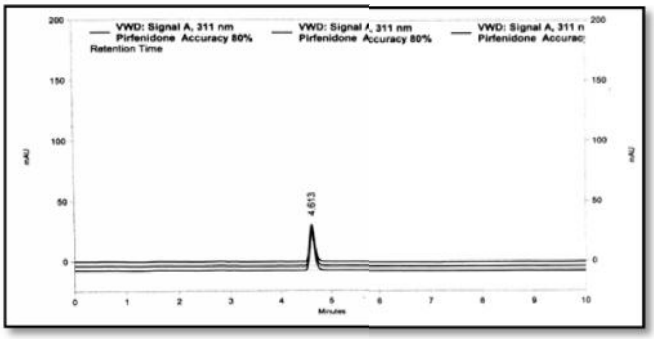

Figure-11: Chromatogram for 80% accuracy

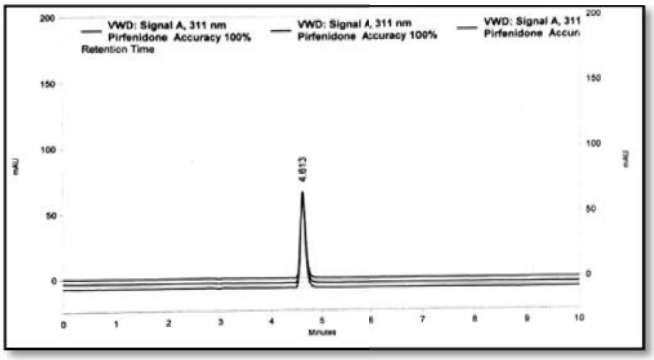

Figure-12: Chromatogram for 100% accuracy

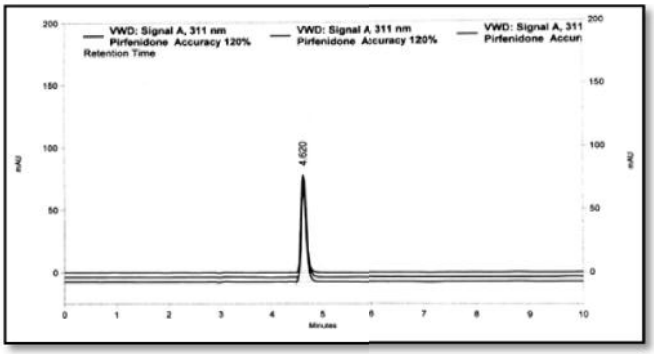

Figure-13: Chromatogram for 120% accuracy

Robustness:

Robustness is the ability to provide accurate and precise results under a variety of conditions. In order to measure the extent of method robustness, the most critical parameters were interchanged while keeping the other parameters unchanged and parallel, the chromatographic profile was observed and recorded. The studied parameters

were: the flow rate, detection of wave length, the composition of mobile phase. The results of robustness study is shown in table 6 indicated the small change in the conditions did not significantly affect the determination of Pirfenidone.

Limit of detection (LOD) and Limit of quantification (LOQ): LOD and LOQ were calculated according to ICH recommendations where the approach is based on the signal-to-noise ratio. Chromatogram signals obtained with known low concentrations of analyte was compared with the signals of blank samples. A signal to noise ratio 3:1 and 10:1 was considered for calculating LOD and LOQ respectively.

3. Results and Discussions

The present study was aimed at developing a precise, sensitive, rapid and accurate HPLC method for analysis of pirfenidone in bulk drug and dosage forms. In order to achieve phenomenal retention time and peak asymmetry, XBridge C18 column of particle size 5µm, (4.6×250mm) and mobile phase composed of acetonitrile and Trifluoroacetic acid (50:50) v/v with pH adjusted to 2.08. As column modifier at a flow rate of 0.8mL/min was selected. The retention time for pirfenidone was found to be 4.617 minutes. UV spectra of pirfenidone showed that the drug absorbance maximum at 311nm, so this wave length was selected as the detection of wave length. The correlation coefficient (0.9988) of regression was found almost equal to 1 in the range of 1-32µg/ml which states that method was linear to the concentration versus peak area responses. The precision studies were performed and the %RSD of the determination was found to 0.01 for intra-day precision and 0.01311 for inter-day precision which are within the limits which indicates that the proposed method was found to be precise. The accuracy of the method was found to be good with the overall % RSD for recovery at 80%, 100%, 120% levels were all within the limits which indicate that the proposed method was found to be accurate. On slight changes in the mobile phase ratio up to ±1%, the change in peak symmetry and retention time are within the limits which indicate the method is robust. The comparison of chromatogram of blank, standard and sample, there was no interference observed from the peaks of blank, standard, and sample it shows that the method is specific. Method validation following ICH guide lines indicated that the developed method had high sensitivity with LOD of 0.120µg/ml and LOQ of 0.401µg/ml.

Table 1: Linearity Data for Pirfenidone

S.No	Concentration	Peak area at 311nm
1.	1 µg/ml	2349940
2.	2 µg/ml	3035576
3.	4 µg/ml	4016847
4.	8 µg/ml	5815888
5.	16 µg/ml	9335444
6.	32 µg/ml	15963853

Table 2: Optimum Chromatographic and System Suitability

S.NO	Parameter	Chromatographic condition
1	Instrument	Agilent technologies HPLC LC Compact-1120
2	Column	X Bridge C18 column of particle size 5µm, (4.6×250mm)
3	Mobile phase	Trifluoro aceticacid:Acetonitrile (50:50 v/v) (pH 2.08)
4	Flow rate	0.8 ml/min
5	Detection wave length	UV at 311 nm
6	Run time	10 minutes
7	Temperature	Ambient temperature (25±1)
8	Volume of injection loop	20µL
9	Retention time	4.613 For pirfenidone
10	Theoretical plates	10395 for 4 mcg of pirfenidone
11	Tailing factor	1.3161

Table 3: Specificity Study for Perfinidone

Name of the solution	Retention time, (t_R) min
Mobile phase	No peak
Blank	No peak
Pirfenidone, 4 mcg	4.613

Table 4: Results of precision study (Intra-day& Inter-day) for pirfenidone

S.NO	Intra-day		Inter-day	
	Peak area(Pirfenidone 16µg/ml)		Peak area (Pirfenidone 16µg/ml)	
	Morning	Afternoon	Day 1	Day 2

1	9371386	9330256	9316353	9326738
2	9369256	9329216	9312253	9326632
3	9368326	9329209	9311211	9326002
4	9367226	9328119	9310311	9325112
5	9367356	9327110	9311315	9326012
6	9366216	9327211	9314316	9326035
Average	9368294.333	9328520.167	9312626.5	9326088.5
SD	1834.419981	1251.815868	2278.856182	580.85997
%RSD	0.01	0.01	0.02	0.00622

Table 5: Accuracy for Pirfenidone

S.NO	Level of percentage recovery	Amount of drug present in sample	Amount of standard drug added	Area	Mean	Standard Deviation(SD)	%RSD	Total amount Recovered mg	% recovery
1	80%	200	160	9355508	9353136.6	40797.7	0.43	200.36	100.18
				9311205					
				9392697					
2	100%	200	200	9374625	9347680.6	30631	0.32	200.24	100.12
				9354052					
				9314365					
3	120%	200	240	9354764	9376216	19589	0.2	200.74	100.37

Table 6: Robustness results of Pirfenidone

S.No	Parameter	Optimized	Used	Retention time(t_R), min
1.	Flow rate (±0.1mL/min)	**0.8 mL/min**	0.8mL/min	4.617
			0.79mL/min	4.657
			0.81mL/min	4.540
2.	Detection of wave length (±2nm)	**311nm**	311 nm	4.617
			309 nm	4.607
			313 nm	4.597
3.	Mobile phase composition (Acetonitrile:TFA)	**50:50v/v**	50:50 ,v/v	4.617
			51:49 ,v/v	4.463
			49:51 ,v/v	4.693

Table No 7: LOD and LOQ for estimation of Pirfenidone.

S.NO	Name of the Drug	Signal/noise ratio(S/N)	LOD µg/ml	LOQ µg/ml
1.	Pirfenidone	99.7039	0.120	0.401

4. Conclusion

The developed RP-HPLC method for the quantification of Pirfenidone has various advantages like less retention time, low solvent consumption, excellent peak symmetry and phenomenal linearity, highly sensitive, precise, accurate and robust. The mobile phase can be easily prepared and diluents are economical and readily available and it does not need sample preparation with sophisticated techniques. The proposed RP-HPLC method was suitable technique for the determination of Pirfenidone. All the parameters analyzing Pirfenidone met the criteria of ICH guidelines for Method Validation. The HPLC method is more sensitive, precise and accurate compared to the spectrophotometric methods. The proposed method can be used for the routine analysis of pirfenidone in pharmaceutical dosage forms for routine application in quality control laboratories without interference of excipients.

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