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Development and Substantiation of a RP-HPLC Method for Monitoring of Impurities in Pirfenidone Drug Substance

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Abstract

A simple, rapid and rugged RP-HPLC method was developed for evaluation and quantification of impurities present in Pirfenidone (PFD) drug substance. Impurities were separated and determined on a Zorbax RX-C18 column (250 mm length, 4.6 mm inner diameter and 5.0 µm particle size, octadecylsilane chemically bonded to porous silica) with 0.02 M KH_2PO_4 buffer and acetonitrile as mobile phase using a simple gradientelution program. The column flow rate of 1.0 mL per minute was used for the separation. The detection wave length was fixed at 220 nm. The method was substantiated with respect to specificity, precision, linearity, range, accuracy, ruggedness, limit of detection and quantitation. The impurities were identified as 2-hydroxy-5-methylpyridine and Iodobenzene. The linearity range obtained was 0.017 to 0.380 µg/mL for 2-hydroxy-5-methylpyridine, 0.047 to 0.382 µg/mL for Pirfenidone and 0.030 to 0.99 µg/mL for Iodobenzene with the retention times of 3.248 min, 10.608 min and 24.241 min for 2-hydroxy-5-methylpyridine, Pirfenidone and Iodobenzene, respectively. The percentage recoveries of 2-hydroxy-5-methylpyridine and Iodobenzene were in the range of 94.08% - 104.12%. The LOD and LOQ values were found 0.000005 mg/mL, 0.000017 mg/mL for 2-hydroxy-5-methylpyridine and 0.009 µg/mL, 0.030 µg/mL for Iodobenzene, respectively. The method is found to be suitable for the quantitation of impurities along with Pirfenidone drug substance. The method was validated as per the International Conference on Harmonization (ICH) guidelines.

Keywords

Development, Validation, Pirfenidone, Degradation, Quantification and RP-HPLC

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1. Introduction

Pirfenidone (PFD) is a novel antifibrotic agent approved for mild to moderate Idiopathic pulmonary fibrosis (IPF) as orphan drug. Idiopathic pulmonary fibrosis (IPF) is a progressively fibrotic disease, with no effective treatment and a median survival time of 2 - 5 years. IPF inevitably causes shortness of breath and destruction of healthy lung tissue, although some people may experience periods of stability with the disease [1] [2]. IPF is a rare incurable disease, often fatal which mostly affects geriatric patients causing fibrosing interstitial pneumonia of unknown etiology [3]. Pirfenidone is a small non-peptide molecule of low molecular weight (185.2 daltons) with the chemical name of 5-methyl-1-phenyl-2-(1H)-pyridone. A survey of literature revealed that LC-MS/MS [4] and HPLC [5]-[7] methods are reported for the determination of Pirfenidone in biological fluids [8]. Hence, an attempt has been made to develop an accurate, rapid, specific and reproducible method for determination of Pirfenidone and all two impurities in bulk drug samples and in pharmaceutical dosage forms along with method validation as per ICH norms [9] [10]. The stability tests were also performed on drug substances as per ICH norms. No methods have been reported in literature and in pharmacopoeia for the estimation of Pirfenidone and its process related impurities in pharmaceutical drug substance form. Hence, the present work is taken up to develop and validate the method for the determination of PFD and its related impurities (**Figures 1(a)-(c)**) in drug substance form. In the present paper we describe an excellent reversed phase high performance liquid chromatography (RP-HPLC) method for the separation, determination and quantification of process related impurities of Pirfenidone.

2. Experimental

2.1. Instrumentation

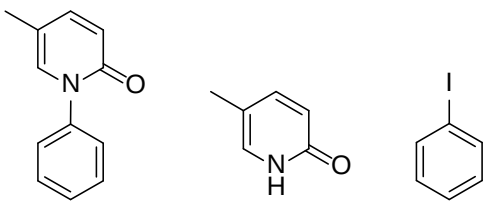
The LC system used for the method development and validation consisted of a dual piston reciprocating two Waters pumps from Waters alliance, USA (model HPLCe2695) and photo-diode array detector from Waters Crop., USA (model 2998). The HPLC system was equipped with data acquisition and processing software "Empower-2" Waters Crop., USA. Agilent Zorbax RX-C18 column (250 mm × 4.6 mm I.D., 5 µ particle size) was used.

2.2. Chemicals and Solvents

Samples of Pirfenidone and its two impurities [the key starting materials used for the synthesis of Pirfenidone is 2-hydroxy 5-methyl bromide and Iodobenzene. During the synthesis process of pirfedone drug substance there is every possibility that the above may be invariably carry in to API. As per ICH guidelines it should be less than 0.1%] namely 2-hydroxy-5-methylpyridine and Iodobenzene were synthesized from Dr. Konda's life sciences, Hyderabad, India. The working standards of 2-hydroxy-5-methyl pyridine 99.9%, Pirfenidone 99.7% and Iodobenzene 99.9% were provided as gift samples by Dr. Konda's Life sciences private limited, Hyderabad, India. KH_2PO_4 99.8% and HPLC grade acetonitrile 99.9%, Milli-Q water, H_3PO_4 85.0% (AR grade) were purchased from Merck Ltd, Mumbai, India.

2.3. Mobile Phase Preparation

Weigh and transfer about 2.7 g of Potassium dihydrogen phosphate in 1000 mL of Milli-Q water and sonicate to



(a) (b) (c)

Figure 1. (a) Chemical structure of Pirfenidone; (b) Chemical structure of 2-hydroxy-5-methyl pyridine; (c) Chemical structure of Iodobenzene.

dissolve. Adjust the pH to 2.5 (±0.05) using dilute Orthophosphoric acid. Filter through 0.45-micron porosity membrane and degas. Acetonitrile used as mobile phase Band diluents as blank solution.

2.4. Diluent Preparation

Buffer and acetonitrile were mixed in the ratio of 40:60 v/v to prepare diluent.

2.5. Standard Solution Preparation

The standard solutions were prepared by weighing accurately each 10 mg of 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene [working standards] and transferred into three 100 mL volumetric flask to dissolved and diluted to the mark with diluent. Further diluted 1 mL of the above solutions to 100 mL with diluent. Further diluted 2.5 mL of this solution to 10 mL with diluent to obtain the concentrations of 0.25 µg/mL of 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene.

2.6. Sample Solution Preparation

Weighed and transferred accurately 25 mg of Pirfenidone drug substance into a 50 mL volumetric flask, dissolved and diluted to the volume with diluent to produce the concentrations of 0.5 mg/mL of Pirfenidone.

2.7. Chromatographic Conditions

The chromatographic separation was achieved on Zorbax RX-C18, 25 cm × 4.6 mm, 5 µm column using a mobile phase containing mixture of KH_2PO_4 buffer (pH 2.5 with H_3PO_4) and Acetonitrile as mobile phase. The mobile phase was filtered through nylon membrane (pore size 0.45 µm). The flow rate of the mobile phase was 1.0 mL/min. The column temperature was maintained at 25˚C and the wavelength was monitored at 220 nm. The injection volume was 10 µL and the run time was 50 minutes by using with simple gradient method.

2.8. Procedure

Solutions of Pirfenidone (0.5 mg/mL) and its two impurities (0.25 µg/mL) were prepared in diluent. A 20 µL volume of each solution injected and chromatographed under the above conditions. The system suitability was determined by injecting 0.25 µg/mL solution of 2-hydroxy-5-methyl pyridine and Iodobenzene were spiked with Pirfenidone (0.5 mg/mL) and evaluated by making five replicate injections. The system was deemed to be suitable for use if the resolution was greater than 1.5 or higher and column for all the components in matrix. Standard solutions and samplesolutions were analysed the above same conditions. The content of impurity was calculated from the peak area.

3. Method Development and Method Validation

3.1. Method Development

To develop a suitable and robust RP-HPLC method for the simultaneous estimation of Pirfenidone and its related impurities in different mobile phases were employed to achieve the best separation and resolution.

The method development was made with Zorbax RX-C_{18} (250 mm × 4.6 mm I.D., 5 µ particlesize). Detection was carried out at 220 nm with the mobile phase composed 0.02 M KH_2PO_4 buffer pH 2.5 with H_3PO_4 and Acetonitrile in the ratio of 65:35 v/v at a flow rate of 1.0 mL/min was used for the separation.

3.2. Validation of Method

3.2.1. Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. The specificity of the developed LC method for Pirfenidone was carried out in the presence of its impurities namely 2-hydroxy-5-methyl pyridine and Iodobenzene. Stress studies were performed for Pirfenidone bulk drug to provide an indication of the stability indicating property and specificity of the proposed method. The following sample solutions were prepared and injected into chromatographic system to demonstrate the analytical method is selective for determination of related substances in Pirfenidone. Prepared and injected individual sample

solutions of 2-hydroxy-5-methylpyridine, Pirfenidone and Iodobenzene in diluent. Prepared a spiked sample solution by spiking of 2-hydroxy-5-methylpyridine and Iodobenzene impurity in Pirfenidone sample solution. Specificity chromatogram and results were shown in **Figure 2** and **Table 1**.

3.2.2. System Suitability

The standard solution was prepared and analysed as per the proposed method, calculated the % RSD for peak area of Pirfenidone and each specified impurity in standard solution and the % RSD for peak area of Pirfenidone and each specified impurity throughout the run injections to demonstrate system suitability for studying of each validation parameter. System suitability parameter values were shown in **Table 2**.

3.2.3. Precision

The precision of the related substance method was checked by injecting ten individual preparations of Pirfenidone (0.5 mg/mL) spiked with 0.25 µg/mL of 2-hydroxy-5-methyl pyridine and Iodobenzene with respect to Pirfenidone drug substance concentration. The % Relative Standard Deviation of area for each 2-hydroxy-5-methyl pyridine and Iodobenzene was calculated.

The intermediate precision of the method was also evaluated by using different analyst, different column and different instrument in the same laboratory. Results of system precision studies are shown in **Table 3**. To study the method precision, six replicate mixed sample solutions of 2-Hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene were injected. The percent relative standard deviation (% RSD) was calculated. Results of method precision studies were shown in **Table 4**.

3.2.4. Accuracy

The accuracy study of the impurities was carried out by standard spiking method. A known amount of standard solution was added to the permanent amount of pre-analyzed sample solution. Standard spiking method was executed at three plus one concentration levels of 50%, 100%, 120% and 150%. Recovery studies for 2-hydroxy-5-methyl pyridine and Iodobenzene results were shown in **Table 5**.

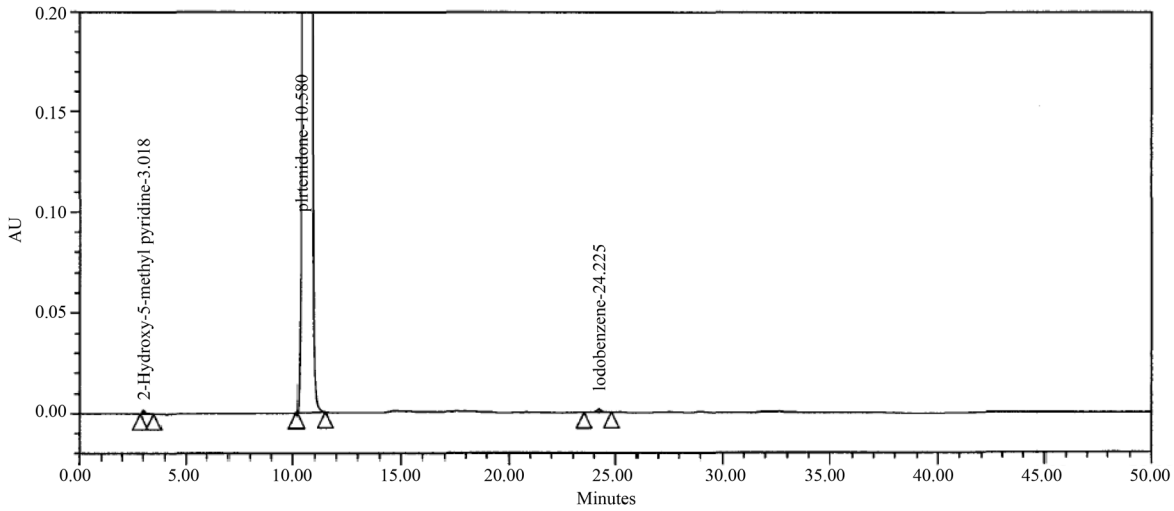


Figure 2. Typical chromatogram of specificity.

Table 1. Peak purity and resolution results.

	Name	RT	Area	% Area	Purity1 threshold	Purity1 angle	RT ratio	Resolution
1	2-hydroxy-5-methyl pyridine	3.25	3796	0.11	4.272	3.713	0.31	----
2	Pirfenidone	10.60	3,526,715	99.79	1.011	0.037	1.00	19.54
3	Iodobenzene	24.24	3725	0.11	8.054	4.249	2.29	33.31

Table 2. System suitability parameter for 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene. (a) Summary of the percentage relative standard deviation of peak area of each specified impurity and Pirfenidone in four replicate injections of standard solution in each validation parameter; (b) Summary of the percentage relative standard deviation of peak area of each specified impurity and Pirfenidone in throughout the run injections of standard solution in each validation parameter.

(a)

Validation parameter	% RSD of peak area in standard solution for four replicate injections (NMT 5.0%)		
	2-hydroxy-5-methylpyridine	Pirfenidone	Iodobenzene
Specificity	1.85	1.52	0.98
Determination of LOD & LOQ values	0.71	1.77	1.71
Precision at LOQ	1.44	0.98	2.14
Linearity study	1.87	1.78	1.37
Accuracy	2.08	1.22	0.65
System precision	0.91	0.48	2.18
Method precision	2.10	1.72	3.01
Intermediate precision	0.45	1.78	2.05
Robustness study-actual	1.50	1.24	1.36

(b)

Validation parameter	% RSD of peak area in standard solution for five replicate injections (NMT 5.0%)		
	2-hydroxy-5-methylpyridine	Pirfenidone	Iodobenzene
Specificity	1.84	1.54	0.85
Determination of LOD & LOQ values	0.80	1.62	1.80
Precision at LOQ	1.28	1.31	1.93
Linearity study	1.62	2.11	1.20
Accuracy	1.80	1.11	0.56
System precision	1.94	0.81	2.26
Method precision	1.91	1.54	2.99
Intermediate precision	0.52	2.92	1.83
Robustness study-actual	1.30	1.77	1.95

Table 3. System precision for 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene.

Injection No.	2-hydroxy-5-methylpyridine	Pirfenidone	Iodobenzene
1	25,874	17,170	23,610
2	26,236	17,267	22,915
3	25,895	17,230	22,410
4	26,338	17,077	22,809
5	25,932	16,759	22,934
6	24,648	17,129	21,867
7	26,081	17,079	22,387
8	26,342	17,132	22,011
9	26,346	17,155	22,421
10	25,814	17,121	22,894
Average peak area	25,951	17,112	22,626
SD	503.6315	137.7820	511.2144
% RSD	1.94	0.81	2.26

Table 4. Method precision for 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene.

Preparation No. (spiked sample)	2-hydroxy-5-methylpyridine (% w/w)	Iodobenzene (% w/w)	Single maximum impurity (% w/w)	Total impurities (% w/w)
1	0.047	0.045	0.013	0.105
2	0.046	0.046	0.013	0.105
3	0.044	0.041	0.013	0.098
4	0.042	0.044	0.014	0.100
5	0.046	0.044	0.014	0.104
6	0.043	0.046	0.011	0.100
Average (% w/w)	0.045	0.044	0.013	0.102
SD	0.0020	0.0019	0.0011	0.0030
% RSD	4.44	4.32	8.46	2.97

Table 5. Recovery studies for 2-hydroxy-5-methyl pyridine and Iodobenzene.

% level	% mean recovery of 2-hydroxy-5-methyl pyridine	% mean recovery of Iodobenzene
50	102.89	96.85
100	95.13	100.79
120	94.08	101.64
150	99.74	104.12

3.2.5. Linearity

Linearity test solutions for the related substance method were prepared by diluting stock solutions to the required concentrations. The solutions were prepared at six concentration levels of 2-Hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene from LOQ to 150% (0.25 µg/mL) of the specification level (LOQ, 25%, 50%, 75%, 100%, 125% and 150%). *i.e.*, 0.017 to 0.380 µg/mL for 2-Hydroxy-5-methyl pyridine, 0.047 to 0.382 µg/mL for Pirfenidone 0.030 to 0.379 µg/mL for Iodobenzene respectively. Twenty micro liters of each concentration was injected in duplicate into the HPLC system. From these chromatograms, the mean peak areas were calculated and linearity plots of concentration over the mean peak areas were constructed individually. The % R.S.D. value of the slope and Y-intercept of the calibration curve was calculated. The correlation coefficient should be not less than 0.99. Linearity plots are shown in **Figures 3-5**. Linearity results were shown in **Table 6**.

3.2.6. Limit of Detection and Limit of Quantification

The LOD and LOQ for 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision study was also carried out at the LOQ level by injecting six individual preparations 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene and calculating the % Relative standard deviation of the area. The LOD and LOQ values of 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene were given in **Table 7** and LOD chromatograms were shown in **Figure 6** and in **Figure 7**.

3.2.7. Robustness

The robustness of an analytical method is a gauge of its ability to stay unchanged by little, but deliberate variations in method parameter and provides a sign of its dependability throughout normal usage. The robustness was performed in different flow rate 1.0 mL/min ± 0.2 mL/min, different pH of Mobile phase 2.5 ± 0.2, Buffer strength 0.02 ± 0.05 M, Injection volume 20 µL ± 10 µL and Gradient program solvent strength 65% ± 2%. Relative standard deviation was observed for all impurities with Pirfenidone are similar in all changed condition in 0.05% impurities spiked with Pirfenidone. The consequences of robustness study are presented in **Table 8**.

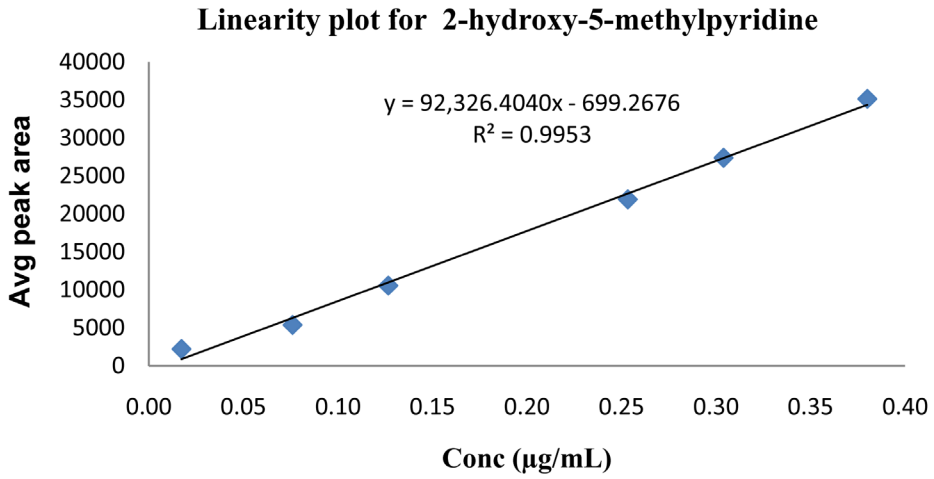


Figure 3. Linearity plot for 2-hydroxy-5-methyl pyridine.

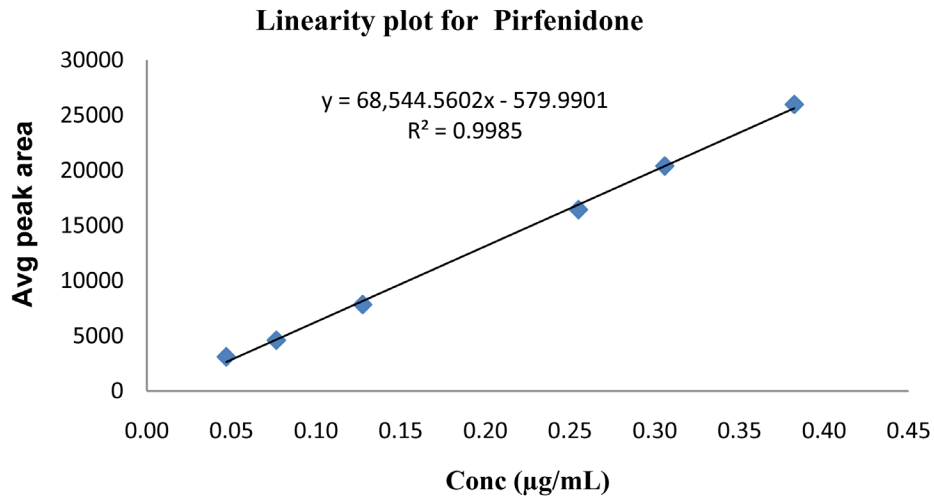


Figure 4. Linearity plot for Pirfenidone.

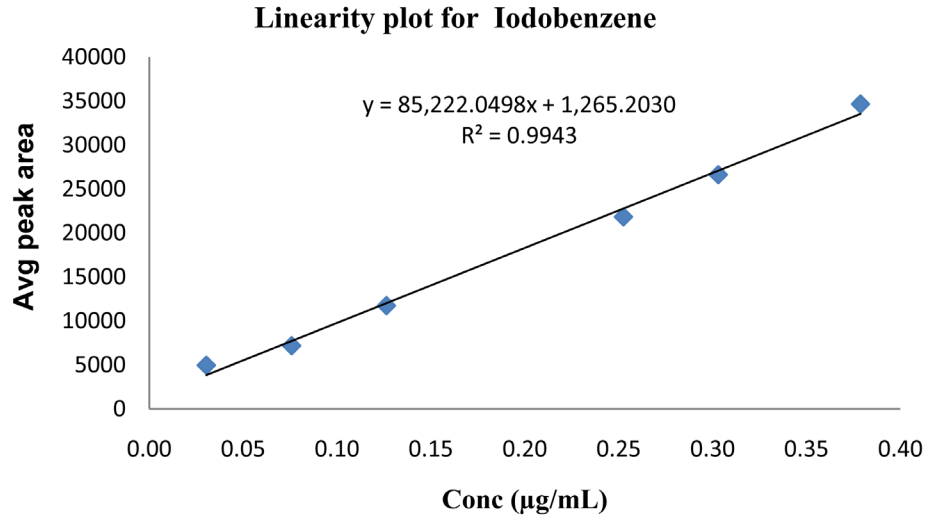


Figure 5. Linearity plot for Iodobenzene.

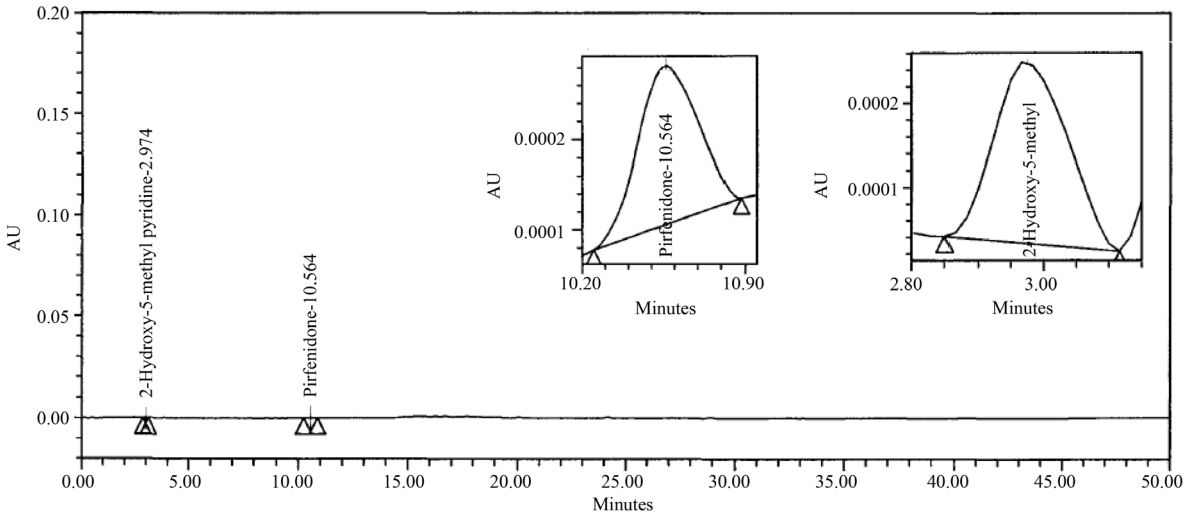


Figure 6. Typical LOQ chromatogram for 2-hydroxy-5-methyl pyridine and Pirfenidone.

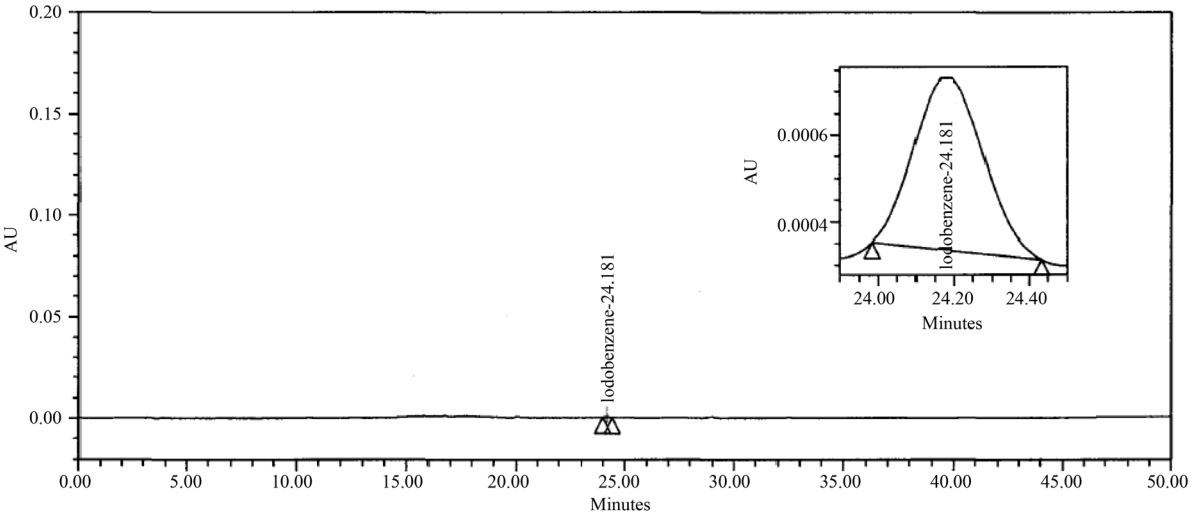


Figure 7. Typical LOQ chromatogram for Iodobenzene.

Table 6. Linearity of 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene.

S. No.	2-hydroxy-5-methyl pyridine		Pirfenidone		Iodobenzene	
	Concentration (mg/ml)	Mean peak area	Concentration (mg/ml)	Mean peak area	Concentration (mg/ml)	Mean peak area
1	0.000017	2228	0.000047	3099	0.000030	4960
2	0.000076	5392	0.000076	4605	0.000076	7191
3	0.000127	10,572	0.000127	7838	0.000126	11,738
4	0.000253	21,925	0.000255	16,447	0.000253	21,835
5	0.000304	27,385	0.000306	20,401	0.000303	26,625
6	0.000380	35,152	0.000382	25,985	0.000379	34,658

Table 7. LOD and LOQ for 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene.

S. No	Parameter	2-hydroxy-5-methyl pyridine	Pirfenidone	Iodobenzene
1	LOD (%)	0.001 (0.005 µg/mL)	0.003 (0.014 µg/mL)	0.002 (0.009 µg/mL)
2	LOQ (%)	0.005 (3.4 µg/mL)	0.01 (9.4 µg/mL)	0.01 (6.0 µg/mL)

Table 8. Robustness for 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene.

Parameter	2-hydroxy-5-methyl pyridine	Pirfenidone	Iodobenzene
0.8 ml/min	2.24	1.75	0.95
1.0 ml/min	1.50	1.24	1.36
1.2 ml/min	1.96	1.68	0.5
pH 2.3	0.67	1.51	0.82
pH 2.5	1.50	1.24	1.36
pH 2.7	3.80	1.32	2.48
Buffer strength 0.015 M	1.68	3.16	1.54
Buffer strength 0.020 M	1.50	1.24	1.36
Buffer strength 0.025 M	0.43	1.55	1.55
Injection volume 10 µL	1.00	2.18	3.92
Injection volume 20 µL	1.50	1.24	1.36
Injection volume 30 µL	2.66	1.89	0.82
Gradient program 63:37 v/v	0.50	3.38	0.93
Gradient program 65:35 v/v	1.50	1.24	1.36
Gradient program 73:33 v/v	0.84	2.07	1.55

3.2.8. Ruggedness

The related substances analysis was performed in different instrument, different column, different analyst and different dates. The results of ruggedness study are given in **Table 9**.

3.3. Stability

The stability of the drug in solution during analysis was determined by analysis of samples in the 0.1 N sodium hydroxide and acetonitrile was evaluated by analyzing solutions were spiked with impurities at specification level. The solutions were tested after 24 hours at room temperature and the results established that the samples were constant at these circumstances. Simultaneously the solution of Pirfenidone in the 0.1 N Hydrochloric acid and Acetonitrile was stored for 24 hours and analysed on the next day. No significant change was observed with the chromatogram. The total amount of impurities was found to be a maximum of 0.04% in the sample. The described method is satisfactory toobtain accurate and consistent values for the determination of related substances in Pirfenidone drugsubstance. From these results it could be seen that the developed method was simple and useful for monitoring of potential impurities of Pirfenidone drug substance. The results of stability study are given in **Table 10**.

4. Results and Discussion

The main objective of the Analytical method is to separate Pirfenidone from 2-hydroxy-5-methyl pyridine and Iodobenzene. Impurities were not separated properly and peak shapes were not good by using different stationary phases such as C8, and different mobile phases. The Chromatographic separation was achieved on a Zorbax RX C18 (250 mm × 4.6 mm I.D., 5 µ particle size), flow rate of 1.0 mL/min.

Table 9. Ruggedness for 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene.

Ruggedness	2-hydroxy-5-methylpyridine (% RSD)	Iodobenzene (% RSD)	Single maximum impurity (% RSD)	Total impurities (% RSD)
Analyst-1	4.44	4.32	8.46	2.97
Analyst-2	0.00	1.00	3.33	0.35

Table 10. Stability data for Pirfenidone and its related substances.

Stability condition	Purity (area%)	2-hydroxy-5-methylpyridine (area%)	Iodobenzene (area%)	Single maximum impurity (area%)	Total impurities (area%)
Pirfenidone @ 5 N HCl-RT after 24 hours	99.91	ND	ND	0.01	0.09
Pirfenidone @ 5 N NaOH-RT 24 hours	99.90	ND	ND	0.03	0.07

In preliminary experiments all the impurities and Pirfenidone were subjected to RP-HPLC on Inertsil-C_{18} column with Ammonium acetate buffer (0.05 M) and Methanol as solvents. Peak shapes were not good when mobile phase used as diluent. In another attempt, Water:Acetonitrile:Triflouro acetic acid (50:50:0.10 v/v) have been tried and the chromatogram was recorded. Peak shapes as well as separation were not good. Finally the mobile phase was replaced with 0.02 M KH_2PO_4 buffer, pH 2.5 with H_3PO_4 and Acetonitrile in the ratio of 65:35 v/v. Good separation and quantification was achieved with simple gradient program. The sample injection volume was 10 µl. Detection was carried out at 220 nm and chromatographic run time was 50 min. A typical chromatogram of a mixture of impurities along with Pirfenidone shown in **Figure 1**. The peaks were identified by injecting and comparing with the retention times of the individual compounds. The order of elution was 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene. The method was substantiated with respect to the Specificity, precision (System precision, Method precision and Intermediate precision), accuracy, linearity, limit of detection Limit of Quantitation, Precision at Limit of Quantitation, Robustness and Solution stability.

The retention times for 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene was found to be 3.25, 10.60 and 24.24 min respectively and the peak shapes were good. The results of system suitability parameters indicate good performance and hence the method is specific. For system precision study, the % Relative Standard Deviation was found to be 1.94, 0.81 and 2.26 for 2-hydroxy-5-methyl pyridine, Pirfenidone and Iodobenzene respectively, which are well within the acceptable criteria of not more than 4.0% for ten standard injections. For method precision study, the % Relative Standard Deviation was found to be 4.44, 4.32, 8.46 and 2.97 for 2-hydroxy-5-methyl pyridine, Iodobenzene, single maximum impurity and Total impurities respectively, which are well within the acceptable criteria of not more than 10.0% for six spiked samples. This reveals that the method is quite precise.

In method precision, the percentage RSD for the each specified impurity and total impurities (Pirfenidone spiked with each specified impurity at 100.0% level) obtained was in the range of 2.97% to 8.46% at the working concentration, The percentage relative standard deviation for the each specified impurity [Pirfenidone spiked sample prepared six times (intermediate precision) at the specification level] are in the range of 0.00% to 1.00% at the working concentration.

The percentage relative standard deviation for the single maximum unspecified impurity and total impurities [Pirfenidone spiked sample prepared six times (intermediate precision) at the specification level] are in the range of 0.36% to 3.33% at the working concentration. This indicates that the analytical method was robust and rugged.

5. Conclusion

A simple, specific, linear, accurate and precise normal phase HPLC method was successfully developed, which was capable of separating the two impurities from Pirfenidone. The developed and validated method can be used for the related substances testing of Pirfenidone. The developed method is also stable and can be used for the quantitative determination of related substances in bulk materials.

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Abbreviations

ICH: international conference on harmonization
mm: milli meter
nm: nano meter
µ: micron
µL: micro litre
mL: millilitre
%: percent
RSD: relative standard deviation
v/v: volume/volume
LOD: limit of detection
LOQ: limit of quantitation
min: minute
N: normality
AR: analytical reagent
mg: milli gram