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UV AND RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF PIRFENIDONE IN MARKETED FORMULATION

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ABSTRACT

The UV Spectrophotometric method, with water was proved to be simple, precise accurate and sensitive from the results of the validation and it is suitable method for the estimation of pirfenidone in its pharmaceutical dosage forms.

A RP-HPLC method was developed using the mobile phase of 50:48.5:1.5 %v/v methanol and water and tri etylamine. The run time of the developed method was six minutes, which reduces the solvent usage. The chromatographic conditions use ambient temperature which can yield accurate, precise results in the range of 25-35 ºC. The results of the validation parameters showed that the method is accurate and precise. Finally, it can be concluded that the methods for quantitation of pirfenidone by RP-HPLC Method in its pharmaceutical dosage forms can be applied for the routine analysis because of simplicity, accuracy, and preciseness.

Keywords: UV Spectrophotometric method, RP-HPLC, Pirfenidone

INTRODUCTION:

Pirfenidone is a novelantifibrotic drug approved for mild to moderatye idiopathic pulmonary fibrosis as orphan drug in japan and Europe. Pirfenidone is only drug which has been approved for the treatment of IPF. 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 **[1-22]**.

MATERIALS AND METHODS

Drug Profile

Pirfenidone

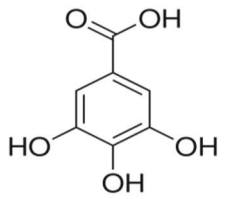

Figure 1: Structure of Pirfenidone

Molecular Formula: $C_{12}H_{11}NO$.

Molecular Weight: 185.2 Daltons.

IUPAC name: 5-methyl-1-phenyl-1,2-dihydropyridin-2-one.

Color: White solid.

Melting point: 96-97ºC.

Boiling point: 329.1±15ºC.

Storage: Stored in room temperature.

Solubility: Soluble in water, methanol and acetonitrile.

Uses: Pirfenidone is a novel compound with demonstrated Anti - inflammatory, Anti - fibrotic & Antioxidant activities that makes it a suitable candidate molecule for managing Idiopathic pulmonary fibrosis.

Determination of wavelength of maximum absorbance (λmax) of pirfenidone:

The dilution was obtained to the concentration of 10 µg / ml for pirfenidone solutions. The solution was scanned in UV range (200-400nm) in 10 mm Quartz cell against Double distilled water blank. The study of spectrum revealed that pirfenidone show a well-defined λmax at 312 nm.

Preparation of standard solutions:

Standard stock solution contain 1000 µg/ml was prepared, 10mg of pirfenidone was accurately weighed and transferred into 10 ml volumetric flask and made up to the mark with Double distilled water, standard solutions were prepared in the concentration range of 1 – 10 µg / ml by further dilution with water.

Preparation of standard calibration curve:

The absorbance's of the standard solutions in water at 1-10 µg / ml range was measured at 312 nm. Standard calibration curve was prepared by plotting maximum absorbance (λmax) versus concentration.

Linearity was studied using a regression equation.

Estimation of pirfenidone in markated formulation:

Preparation of pirfenidone test solution

A stock solution (1000 µg / ml) was prepared by taking 20 tablets, crushed, powdered; weight equivalent to 1000 µg / ml of standard pirfenidone solution was taken and dissolved in a 10 ml volumetric flask using Double distilled water. The solution sonicated for 20 min and placed undisturbed overnight, again sonicated for 15 minutes and filtered through Whattman filter paper. From the clear test stock solution, pipette out 1 ml and make up to 10 ml with Double distilled water (100 µg / ml), From that pipette out 1 ml and make up to 10 ml with Double distilled water to obtain a concentration of 10 µg/ml and serial dilitions were prepared (1 µg / ml, 3 µg / ml, 5 µg / ml, 7 µg / ml, 9 µg / ml)

Method Validation

Linearity

Prepared in 6 spikes of 1-10 µg / ml then analyze by spectrophotometrically. A graph of Concentration Vs Absorbance was plotted and correlation coefficient was calculated.

Accuracy (% Recovery)

The accuracy of the analytical method was assessed by determination of recovery for three concentrations corresponding to 50, 100 and 150 % of test solution concentration. For each concentration, three sets were prepared. The mean recovery of pirfenidone was reported.

Precision

Preparation of standard stock solution: About 10 mg of pirfenidone was taken in to 10 ml volumetric flask, and diluted up to 10 ml with diluent

A. Repeatability- Repeatability expresses the precision under the same operating conditions over a short interval of time. It means the study was performed morning and evening for all concentrations (1-10 µg / ml solution) 6 times and % RSD was calculated.

B. Intermediate precision- Intermediate precision expresses within - laboratories variations: different days, different analysts, different equipment, etc. It means the study was performed different analyst (analyst -1 and analyst -2) for all concentrations (1 -10 µg / ml solution) 6 times and % RSD was calculated.

C. Reproducibility- Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to the standardization of

methodology). It means the study was performed today and tomorrow for all concentrations (1-10 µg / ml solution) 6 times and % RSD was calculated.

Robustness

The estimation of pirfenidone was performed at different environmental conditions (room temp at 29˚C and elevated temp 35˚C).

Ruggedness

The ruggedness is to expresses within-laboratories variations: different days, different analysts, different equipment, etc

Detection Limit & Quantitation Limit

LOD and LOQ were based on the third approach and were calculated according to the 3.3σ/S and 10σ/S respectively; where σ is the standard deviation of y-intercepts of regression lines and s is the slope of the calibration curve.

RP-HPLC Method

Preparation of Standard Stock Solution:

An accurately weighed quantity of (10 mg) pirfenidone was transferred to a 10mL volumetric flask, dissolved and diluted to the mark with Methanol : Water : Triethylamine (50:48.5:1.5 %v/v) to obtain standard stock solution of 1000µg/ml.

Preparation of mobile phase:

Take 50 ml of methanol was transferred to 100 ml of volumetric flask then add 48.5 ml of double distilled water and add 1.5 ml of tri ethylamine then sonicate it for 1hr and filter the mobile phase (50:48.5:1.5 v/v).

Preparation of Pirfenidone test solution:

A stock solution (1000 µg/ml) was prepared by taking 20 tablets , weight equivalent to (1000 µg/ml) of standard Gallic acid solution was taken and dissolved in a 10 ml volumetric flask using Methanol : Water : Triethylamine (50 : 48.5:1.5 %v/v). The solution sonicated for 20 min and placed undisturbed overnight, again sonicated for 15 minutes and filtered through Whattman filter paper. From the clear test stock solution, pipette out 1 ml and make up to 10 ml with solvent (100 µg/mL), From that pipette out 0.5, 1, 1.5, 2 & 2.5 ml and make up to 10 ml with solvent to obtain a concentration of 5-25µg/ml.

Method validation

Specificity

Specificity include impurities, degradants, matrix, etc.

Linearity

Accurately weighed 10 mg of pirfenidone transfer to 10 ml of volumetric

flask. Admade up the volume with Methanol : Water : Triethylamine and sonicate it for 10min (1000 µg /ml). These are prepared in 6 spikes of 5-25 µg / ml then analyze by Chromatography. A graph of concentration Vs peak area was plotted and correlation coefficient was calculated.

Accuracy (% Recovery)

The accuracy of the method was determined by calculating the recovery of Pirfenidone by the standard addition method. The accuracy of the analytical method was assessed by determination of recovery for three concentrations corresponding to 50,100 and 150 % of test solution concentration. For each concentration, three sets were prepared. The mean recovery of Pirfenidone was reported.

Preparation of standard stock solution:

About 10 mg of Pirfenidone was taken in to 10 ml volumetric flask, and diluted up to 10 ml with diluent (1000 µg / ml) from these stock solution prepared 5-25 µg /ml solution.

Preparation of 50% (30 µg /ml) solution:

Taken 2 ml solution from 20 µg / ml sample solution and 1ml solution from 1 µg/ml standard solution into 10 ml volumetric flask, made up to the volume with diluent and Peak area of these solutions was determined.

Preparation of 100% (40 µg/ml) solution:

Taken 2 ml solution from 20 µg/ml sample solution and 20 ml solution from 20 µg/ml standard solution into 10 ml volumetric flask.

Preparation of 150% (50 µg/ml) solution:

Taken 2ml solution from 20 µg/ml sample solution and 3ml solution from 30 µg/ml standard solution into 10 ml volumetric flask, made up to the volume with diluents.

Precision

The degree of reproducible results produced by a sample at different conditions **Preparation of standard stock solution:**

About 10 mg of Pirfenidone was taken in to 10 ml volumetric flask, and diluted up to 10 ml with diluent (1000 µg /ml) from these stock solution prepare 5-25 µg /ml solution.

A. Repeatability - Repeatability expresses the precision under the same operating conditions over a short interval of time. It means the study was performed morning and evening for all

concentrations (5-25 µg /ml solution) 6 times and % RSD was calculated.

B. Intermediate precision - Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

C. Reproducibility - Reproducibility expresses the precision between laboratories 6 times and % RSD was calculated.

Robustness

The estimation of Pirfenidone was performed at different environmental conditions (room temp at 29˚C and elevated temp 35˚C).

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Detection Limit & Quantitation Limit

The LOD and LOQ were based on the third approach and were calculated according to the 3.3σ/S and 10σ/S respectively; where σ is the standard deviation of y-intercepts of regression lines and s is the slope of the calibration curve.

RESULTS AND DISCUSSION

The λmax of pirfenidone was found to be 312 nm **(Figure 2)**.

Figure 2: Spectrum of pirfenidone in water at 312 nm

Selection of suitable wavelength

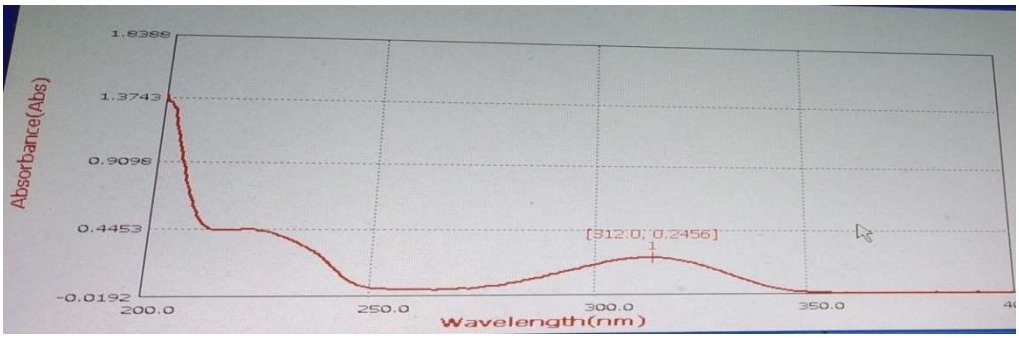

Figure 3: λmax of pirfenidone

Table 1: Standard Graph Values

S.no	Concentration(µg/ml)	Absorbance(nm)
1	1	0.0333
2	3	0.1156
3	5	0.1998
4	7	0.2880
5	9	0.3776
6	10	0.4113

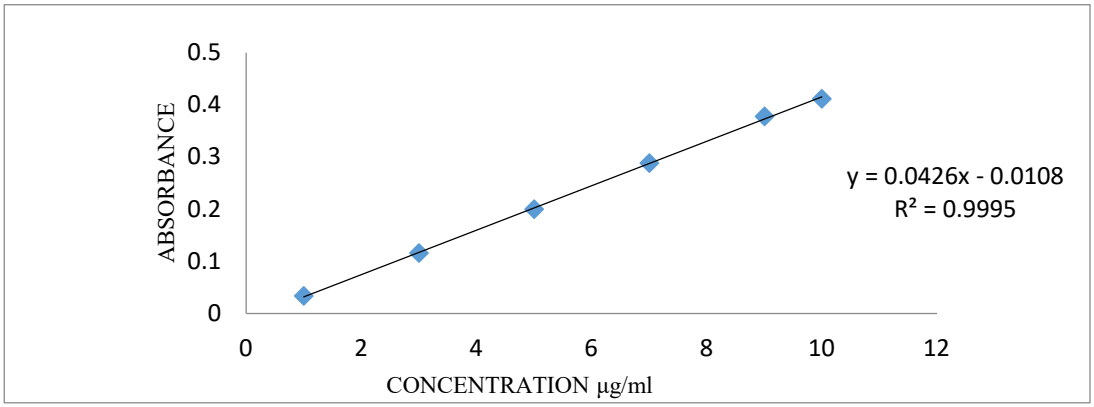

Figure 4: Calibration curve of Pirfenidone

Assay

Table 2: Assay Results

S. No.	Conc. (µg/ml)	Standard Absorbance(nm)	Test Absorbance(nm)	Amount Found	%Purity
1	5µg/ml	0.1998	0.199	262.9mg	98%

Spectroscophic Method Validation of Pirfenidone

Table 3: Linearity values

S. No.	Conc. ((µg/ml)	Absorbance (Mean, N=6)	Standard Deviation	%RSD
1	1	0.0333	0.0005	1.9
2	3	0.1156	0.0016	1.3
3	5	0.1998	0.0032	1.8
4	7	0.2880	0.0050	1.8
5	9	0.3776	0.0049	1.3
6	10	0.4113	0.0079	1.7

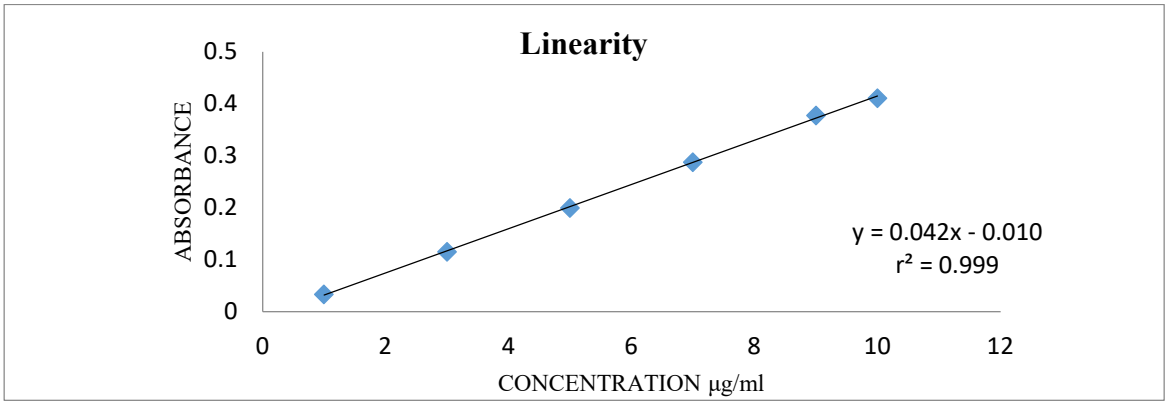

Figure 5: linearity graph (1-10µg/ml)

Table 4: Precision results

Precision		Repeatability		Reproducibility		Intermediate Precision	
		Morning	Evening	Day 1	Day 2	Analyst 1	Analyst 2
Absorbance at 3µg/ml	S1	0.1162	0.1150	0.1162	0.1153	0.1162	0.1153
	S2	0.1179	0.1175	0.1179	0.1177	0.1179	0.1195
	S3	0.1141	0.1141	0.1141	0.1141	0.1141	0.1168
	S4	0.1161	0.1165	0.1161	0.1168	0.1161	0.1171
	S5	0.1165	0.1163	0.1165	0.1161	0.1165	0.1162
	S6	0.1169	0.1170	0.1169	0.1174	0.1169	0.1188
Mean		0.1162	0.1160	0.1162	0.1174	0.1162	0.11728333
SD		0.0012528	0.00127854	0.0012528	0.00136186	0.0012528	0.00158672
RSD		0.0107742	0.01101557	0.0107742	0.01171662	0.0107742	0.01352891
%RSD		1.07%	1.101557296%	1.07%	1.17%	1.07%	1.35%

Table 5: Accuracy results

Recovery Level	Test (initial amount)	Standard (amount added)	Absorbance			Mean	%Recovery
			S1	S2	S3		
50%	5ml	2.5ml	0.297	0.295	0.296	0.296	102%
100%	5ml	5ml	0.396	0.398	0.397	0.397	99%
150%	5ml	7.5ml	0.498	0.501	0.499	0.499	98%

Table 6: Robustness results

Robustness		At Room Temperature (25°C)	Elevated Temperature (29°C)
Absorbance at 3µg/ml	S1	0.3572	0.3612
	S2	0.3658	0.3701
	S3	0.3589	0.3611
	S4	0.3661	0.3689
	S5	0.3655	0.3722
	S6	0.3589	0.3665
Mean		0.36206667	0.36666667
SD		0.00414085	0.00465389
RSD		0.01143672	0.01269242
%RSD		1.14%	1.26%

Table 7: Ruggedness results

Ruggedness		Analyst 1	Analyst 2
Absorbance at 3µg/ml	S1	0.1163	0.1153
	S2	0.1189	0.1195
	S3	0.1175	0.1168
	S4	0.1181	0.1171
	S5	0.1172	0.1162
	S6	0.1182	0.1188
Mean		0.1177	0.1172833
SD		0.00090554	0.00158672
RSD		0.0076	0.0135
%RSD		0.76%	1.35%

Table 8: LOD and LOQ

Limit of Detection	Limit of Quantification
0.242µg/ml	0.733µg/ml

Chromatographic conditions:

Table 9: Optimized chromatographic conditions:

Column	Flowrosil C_{18} column with 5 µm (Dimension)
Mobile phase	Methanol:water:triethylamine(50:48.5:1.5)%v/v
Flow rate	1ml/min
Temperature	Ambient
Run time	7min
Detection wavelength	312nm
Injection volume	10µl
Mode of operation	Isocratic elution

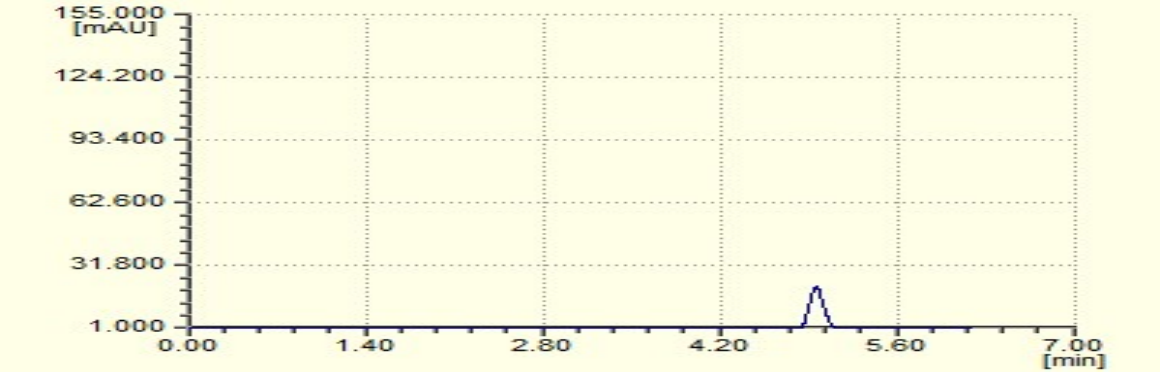

Figure 6: Optimized Chromatogram for Pirfenidone

Table 10: Chromatogram results for optimized method

Mobile phase	**Methanol:water:tri ethylamine(50:48.5:1.5)%v/v)**
Stationary phase	**Flowrosil C_{18} column with 5 µm (Dimension)**
Wavelength	**312nm**
Run time	**7min**
Flow rate	**1ml/min**
Injection volume	**10µg/ml**
Mode of operation	**Isocratic elution**
Temperature	**Ambient**

Calibration curve

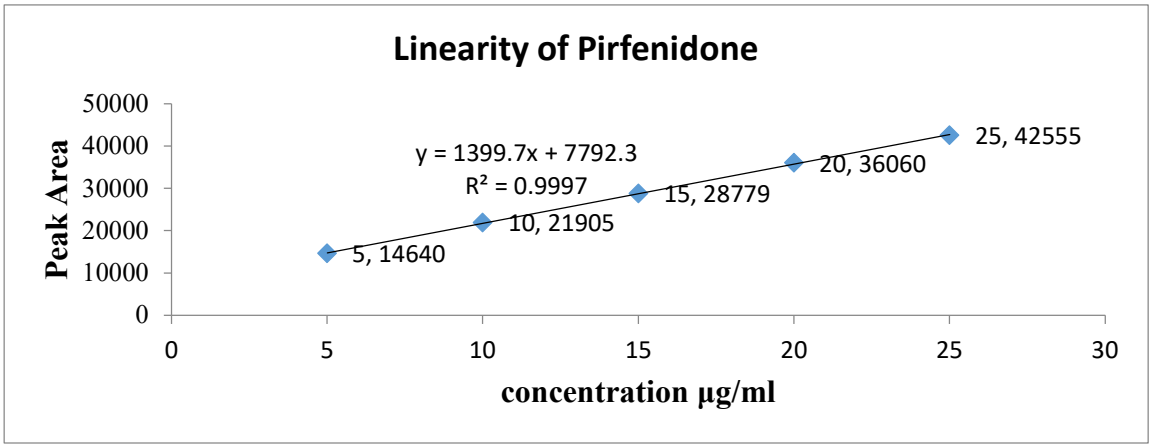

Figure 7: Calibration curve for Pirfenidone

Table 11: Assay Results

S. No.	Concentration (µg/ml)	Standard peak area (Mean±SD), n=3	Test peak area (Mean±SD), n=3	Amount of Pirfenidone found in mg(n=3)	Mean % recovery of Pirfenidone
1	**20µg/ml**	**36060**	**35560**	**19.848**	**99.24**

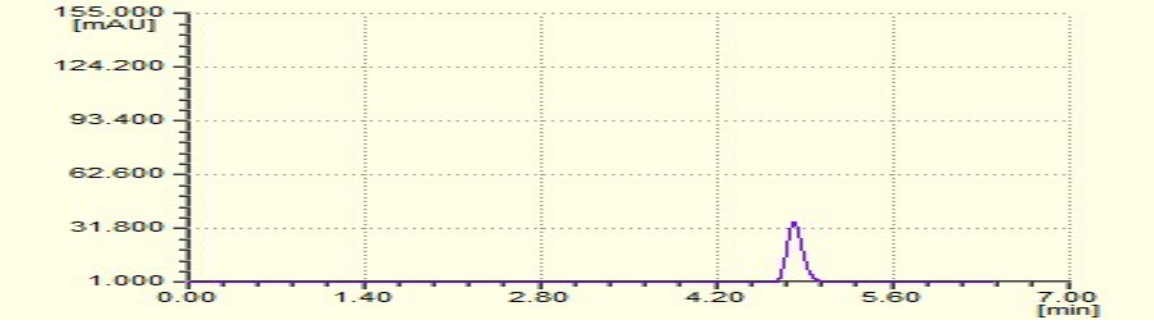

Figure 8: Chromatogram for 20 (µg/ml) Assay

Table 12: Chromatogram results for 20 (µg/ml) Assay

S. No.	Drug Name	Retention time(min)	Peak area	Theoretical plates	Tailing factor
1	pirfenidone	4.82	35560	5110.60	1.72

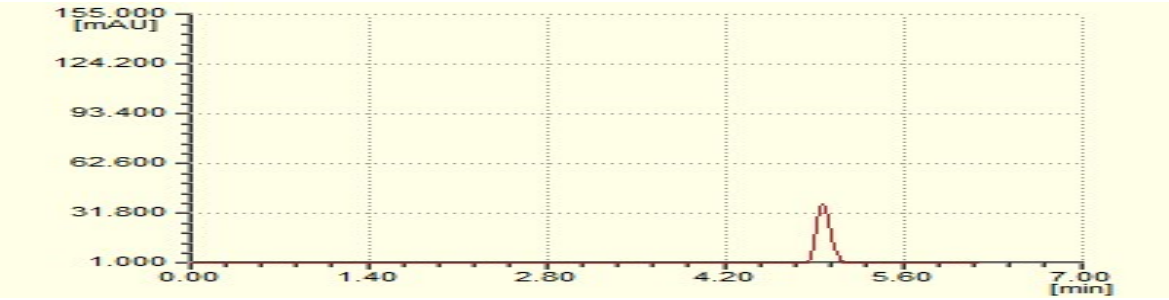

Figure 9: Chromatogram for (20 µg/ml) Standard

Table 13: Chromatogram results for (20µg/ml) standard

S. No.	Drug Name	Retention time(min)	Peak area	Theoretical plates	Tailing factor
1	pirfenidone	4.9	36060	5900.78	1.85

Validation

Specificity

Peaks of other marker were not found at retention time of 4.97 min.

Blank:

Figure 10: Blank injection chromatogram for specificity

Standard:

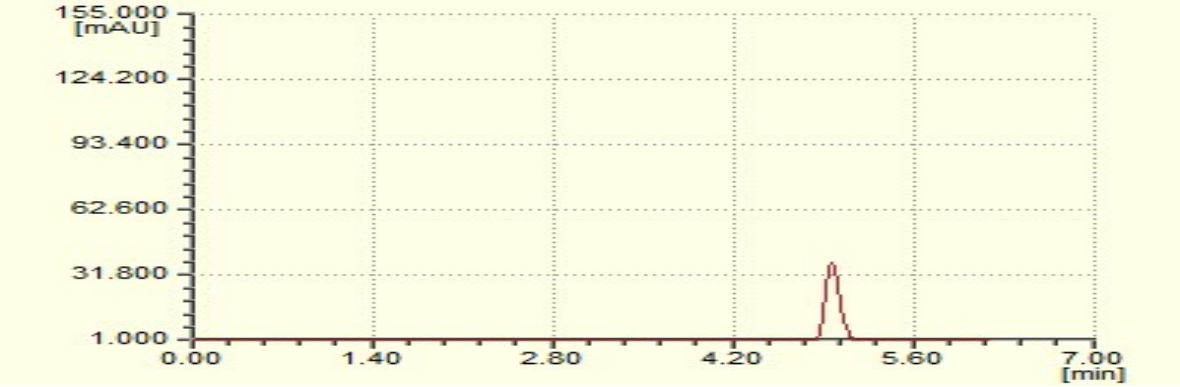

Figure 11: Chromatogram of Standard Pirfenidone

Sample:

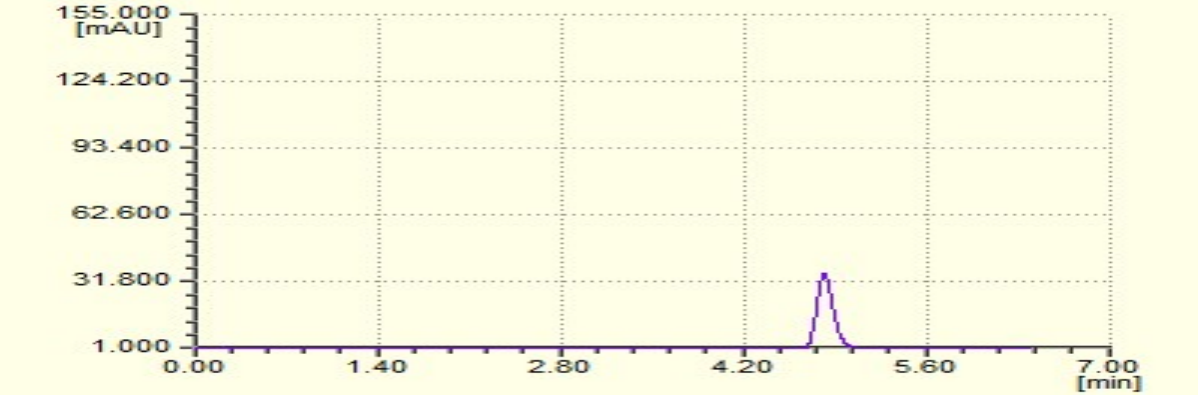

Figure 12: Chromatogram of Test Pirfenidone

Linearity 5 µg/ml

Figure 13: Linearity (5µg/ml) Chromatogram

Linearity 10 µg/ml

Figure 14: Linearity (10µg/ml) Chromatogram

Linearity 15µg/ml

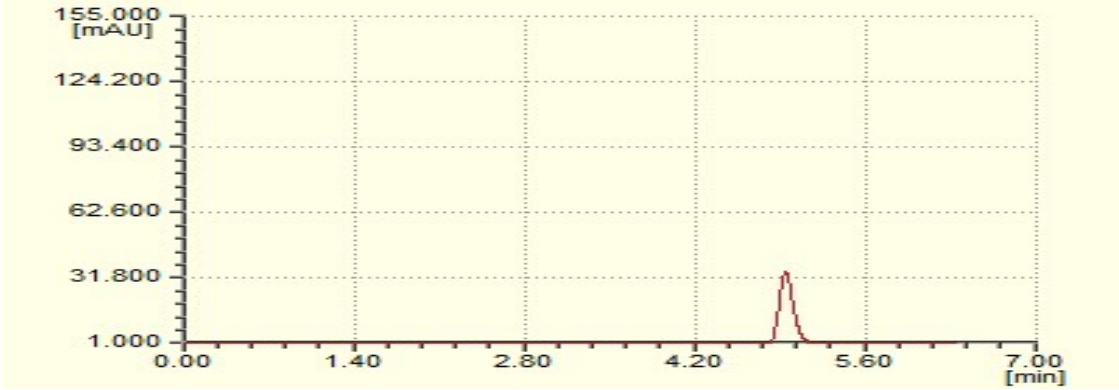

Figure 15: Linearity (15µg/ml) Chromatogram

Linearity 20 µg/ml

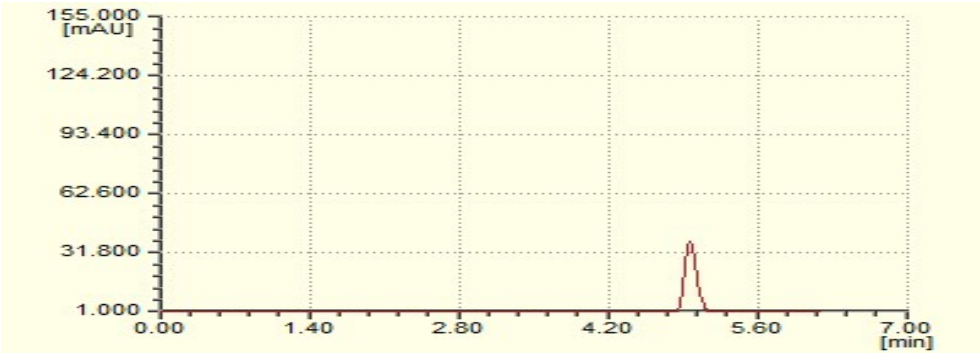

Figure 16: Linearity (20µg/ml) Chromatogram

Linearity 25 µg/ml

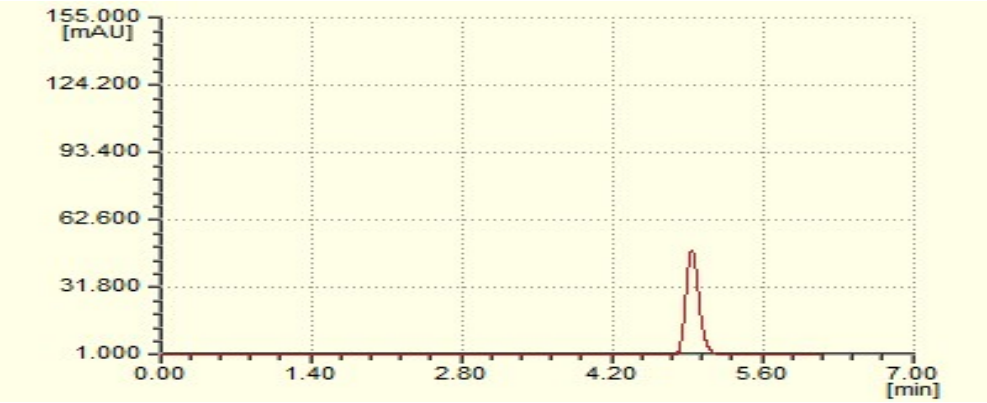

Figure 17: Linearity (25µg/ml) Chromatogram

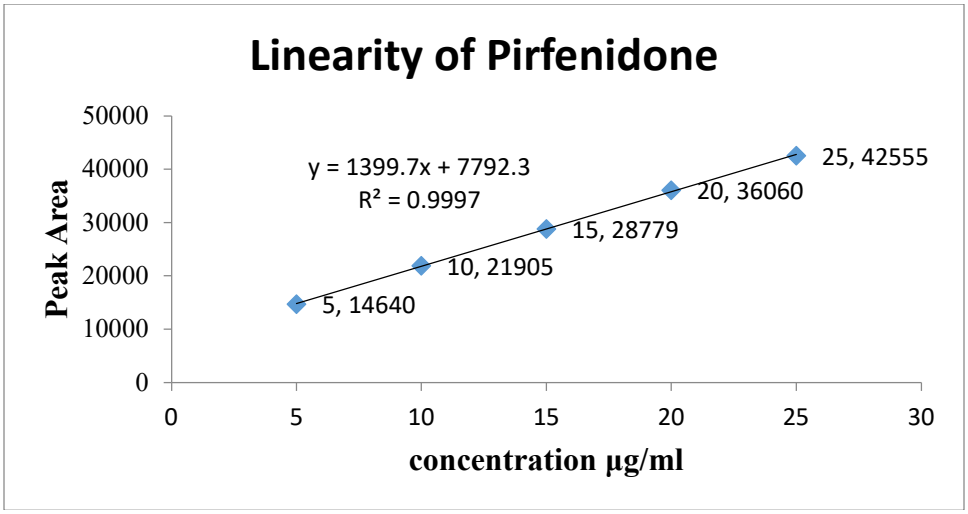

Figure 18: Linearity of Pirfenidone

Accuracy

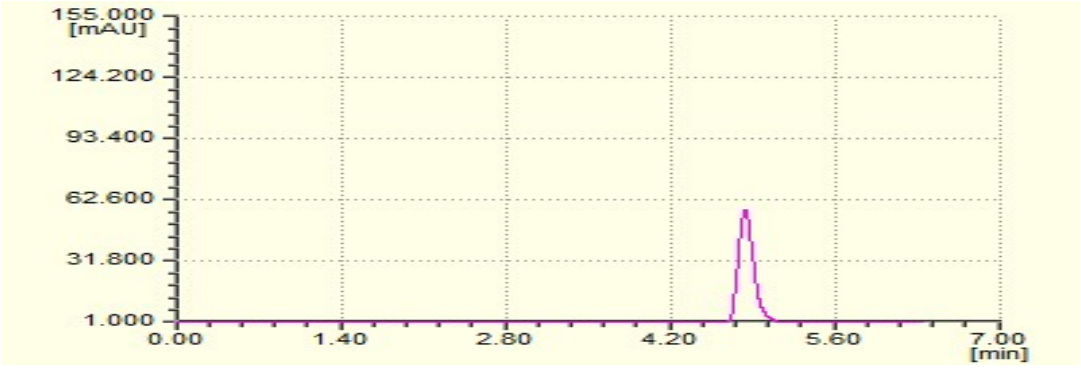

Figure 19: Chromatogram for Accuracy 50% recovery level

❖ **Accuracy 100% Recovery level**

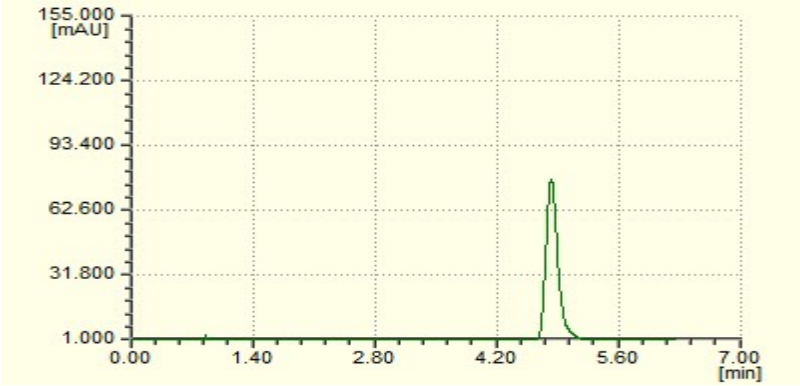

Figure 20: Chromatogram for Accuracy 100% recovery level

Accuracy 150% Recovery level

Figure 21: Chromatogram for Accuracy 150% recovery level

Table 14: Accuracy Results

Recovery level	Concentration(µg/ml)			Peak area n=3	%Mean Recovery
	Test (Initial amount)	Standard (Amount added)	Predicted Concentration		
50%	20µg/ml	10µg/ml	30µg/ml	57520	101%
100%	20µg/ml	20µg/ml	40µg/ml	71376	100%
150%	20µg/ml	30µg/ml	50µg/ml	84516	98%

PRECISION

❖ **Repeatability (Morning)**

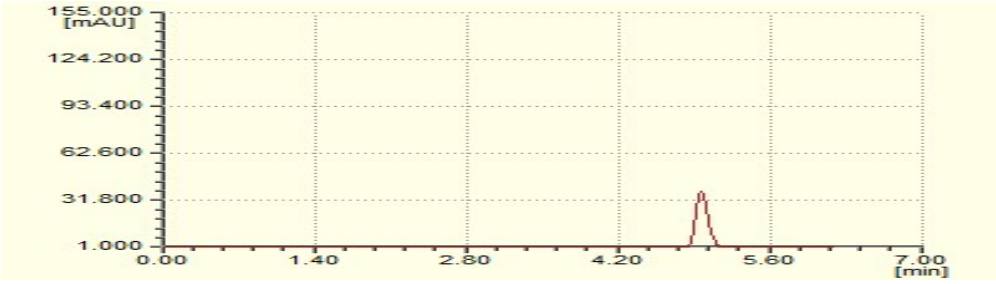

Figure 22: Precision Chromatogram of Standard Pirfenidone at 20 µg/ml

Repeatability (Evening)

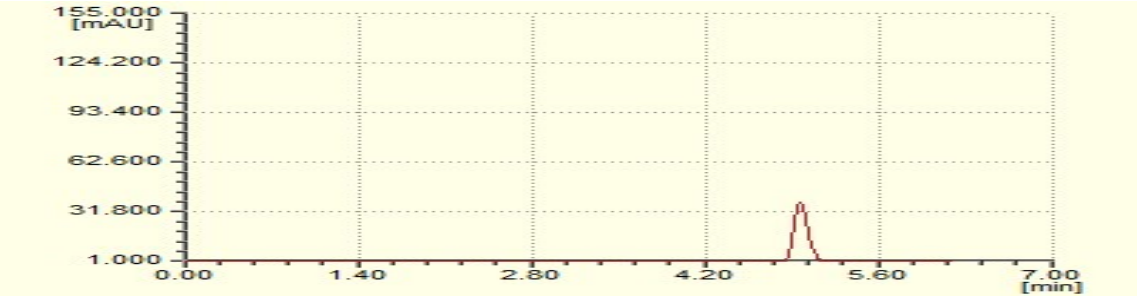

Figure 23: Precision Chromatogram of Standard Pirfenidone at 20 µg/ml

Table 15: Chromatogram results for Repeatability

Conc.20µg/ml Spikes	repeatability Evening	Retention time(min)	Theoretical plates	Tailing factor
1	36069	4.98	5900.78	1.68
2	36012	4.94	5969	1.85
3	36115	4.95	5741	1.78
4	36541	4.95	5966	1.71
5	35899	4.97	5568	1.80
6	36140	4.98	5988	1.79

- **Reproducibility (Day 1)**

Table 16: Chromatogram results for Reproducibility

Conc.20µg/ml Spikes	repeatability Day 1	Retention time(min)	Theoretical plates	Tailing factor
1	36060	4.98	5900	1.78
2	36012	4.94	5869	1.85
3	36105	4.95	5741	1.68
4	35987	4.95	5966	1.91
5	35899	4.97	5768	1.89
6	36014	4.98	5988	1.75

Reproducibility (Day 2)

Figure 24: Precision Chromatogram of Standard Pirfenidone at 20 µg/ml

Robustness:

Flow rate 0.8 ml/min

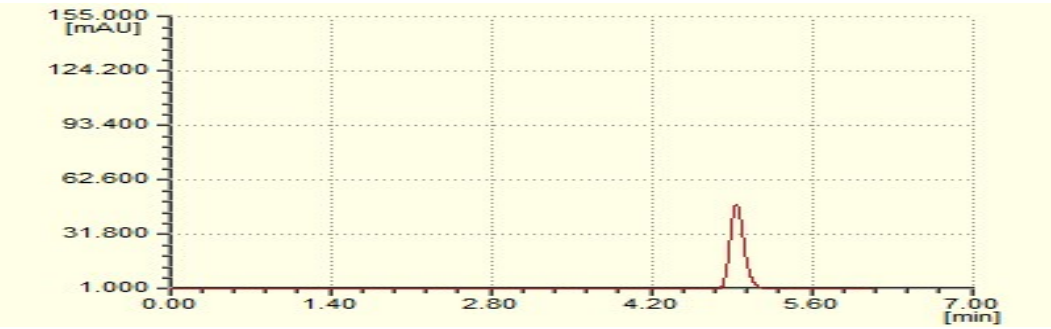

Figure 25: Chromatogram for Robustness (Flow rate 0.8 ml/min)

Flow rate 1.2 mL/min:

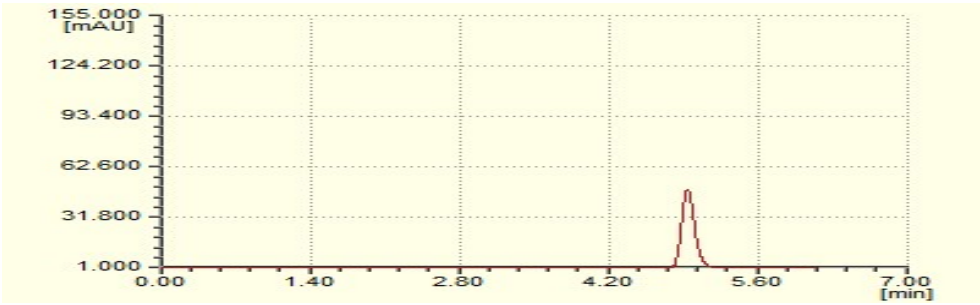

Figure 26: Chromatogram for Robustness (Flow rate 1.2 ml/min)

Table 17: Robustness Results

Robustness		Flow rate 0.8ml/min		Flow rate 1.2ml/min	
Retention time = 4.97		Retention time	Peak area	Retention time	Peak area
Absorbance at 20µg/ml	S1	4.98	36060	4.92	36561
	S2	4.96	36115	4.91	36772
	S3	4.96	36899	4.93	35754
	S4	4.98	35879	4.93	36112
	S5	4.99	36174	4.92	36453
	S6	4.98	36172	4.91	36528
Mean (n=6)		4.97	36216.5	4.975	36294.5
Standard deviation (SD)		0.01549193	351.65139	0.01378405	369.274288
RSD		0.00311709	0.0097097	0.00277066	0.01017439
%RSD		0.31%	0.97%	0.27%	1.01%

Ruggedness

- **Analyst-1**

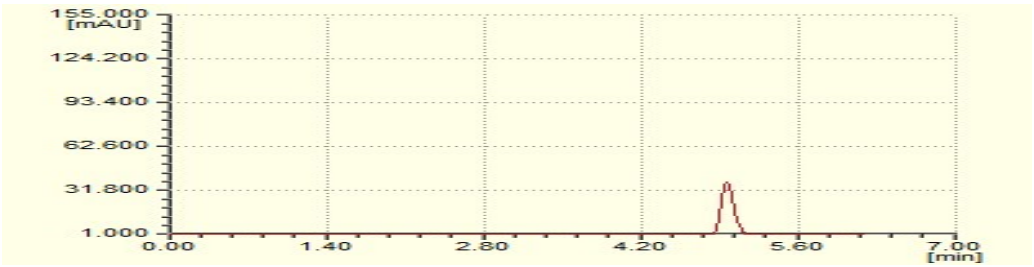

Figure 27: Chromatogram for Ruggedness

❖ **Analyst-2**

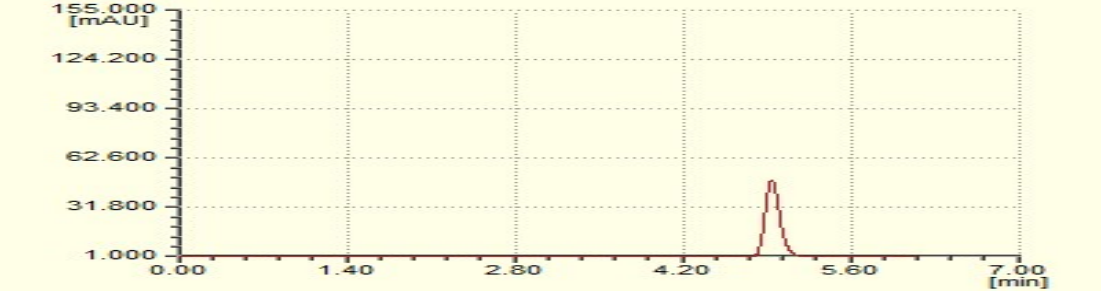

Figure 28: Chromatogram for Ruggedness

Table 18: Ruggedness Results

Ruggdness		Analyst 1		Analyst 2	
		Retention time	Peak area	Retention time	Peak area
Absorbance at 20µg/ml	S1	4.95	36060	4.92	36151
	S2	4.94	36012	4.94	37015
	S3	4.94	36105	4.93	36001
	S4	4.97	36211	4.92	36060
	S5	4.96	35899	4.93	36200
	S6	4.95	36014	4.92	36001
Mean(n=6)		4.951666667	36050.17	4.92666667	36238
Standard deviation		0.01169045	157.7857	0.00816497	389.040872
RSD		0.00236091	0.00437	0.0016573	0.01073572
%RSD		0.23%	0.43%	0.16%	1.07%

Limit of detection and Limit of quantification

Table 19: LOD & LOQ

Limit of Detection	Limit of Quantification
0.05	0.15

- The λmax of Pirfenidone in water was found to be 312nm. Water was used as a diluent. The drug exhibited the linearity in the concentration range of 1-10µg/ml with correlation coefficient of 0.999. The precision studies of the method revealed results of % R.S.D values less than 2% indicating that the developed method is precise. And the % recovery of the drug was found to be 98-102% by UV method.
- The RP-HPLC method show best results in terms of linearity, accuracy, precision, LOD, LOQ. Elution carried out using Methanol : water : Triethylamine (50:48.5:1.5%v/v).The linearity range was found to be 5-25µg/ml with correlation coefficient of 0.999 the percentage recovery was found to be 92%.

CONCLUSION

Literature survey indicates that the methods for the determination of

pirfenidone by RP-HPLC method were time consuming and costlier. So the present work aimed for the development of sensitive, economical and simpler methods for the estimation of pirfenidone in its pharmaceutical dosage forms.

A RP-HPLC method was developed using the mobile phase of 50:48.5:1.5 % v/v methanol and water and tri etylamine. The run time of the developed method was six minutes, which reduces the solvent usage. The chromatographic conditions use ambient temperature which can yield accurate, precise results in the range of 25-35 ºC. The results of the validation parameters showed that the method is accurate and precise.

Finally, it can be concluded that the methods for quantitation of pirfenidone by RP-HPLC Method in its pharmaceutical dosage forms can be applied for the routine analysis because of simplicity, accuracy, and preciseness.

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