# A Validated Stability Indicating RP-HPLC Method for the Quantification of Pirfenidone and its Degradation Products

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## ABSTRACT

A reverse-phase high-performance liquid chromatographic (HPLC) method was utilized to accurately quantify Pirfenidone, an oral drug employed in the treatment of idiopathic pulmonary fibrosis. The method aimed to maintain sensitivity and stability throughout the analysis. The separation process involved using a C-18 (ODS 250 × 4.6mm, 5µm) stationary phase, with a mobile phase composed of a combination of Methanol and tert Butyl Methyl Ether. The flow rate was set at 1 ml/min, and the detection of Pirfenidone was carried out at a wavelength of 316 nm, with a fixed injection volume of 10 µL. A calibration curve was constructed to establish a reliable relationship between concentration and response, demonstrating linearity within the concentration range of 10-40 ppm, with an R2 value of 0.995. The method underwent validation following the criteria set by the International Conference on Harmonization (ICH). The stability of Pirfenidone was assessed under various stress conditions, including hydrolytic (acidic, alkaline, and neutral) and oxidative conditions. Remarkably, no significant degradation of Pirfenidone was observed when exposed to these conditions. The developed HPLC method proved successful in accurately estimating the quantity of Pirfenidone in both bulk samples and pharmaceutical formulations.

**Keywords:** Pirfenidone, RP-HPLC, Stability-indicating, Degradation products, Validation

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## INTRODUCTION

Pirfenidone is approved in the European Union to treat Idiopathic pulmonary fibrosis that ranges in severity. In 2011, the European Medicines Agency gave it its seal of approval for its usage. [1,2] It received approval for usage in Japan in October 2008, in India in 2010, and in 2011 in China (commercial release in 2014). It received US government approval for medical usage in October 2014. [3] In January 2017, a tablet version received US-FDA approval for practice. [4]

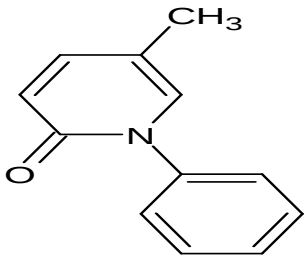


Fig 1: Structure of Pirfenidone

A small-molecule medication called pirfenidone has anti-inflammatory and anti-fibrotic effects. The precise mechanism of action of pirfenidone is not completely elucidated; however, it is believed to involve a combination of pathways. One of the proposed mechanisms suggests that pirfenidone acts by modulating inflammatory cytokines and growth factors. Research has shown that pirfenidone has the ability to reduce the levels of pro-inflammatory cytokines, specifically interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α). Moreover, pirfenidone has also demonstrated an ability to elevate the levels of anti-inflammatory cytokines, particularly interleukin-10 (IL-10). These effects on cytokines suggest that pirfenidone may exert its therapeutic effects by balancing the inflammatory response in the body. By reducing the levels of pro-inflammatory cytokines, pirfenidone may help to reduce inflammation and tissue damage.[5,6]

Additionally, pirfenidone appears to have anti-fibrotic properties, possibly preventing the synthesis of extracellular matrix proteins like collagen. Collagen is a key component of fibrotic tissue and is responsible for the scarring and stiffening of organs such as the lungs in idiopathic pulmonary fibrosis (IPF). By inhibiting collagen production, pirfenidone may help reduce fibrosis progression and preserve organ function. [7]

Pirfenidone is a phenyl-substituted pyridine with a modest molecular weight that possesses an antifibrotic effect both *in vitro* and *in vivo*. Pirfenidone has been shown in several prospective, placebo-controlled studies to slow down the progress of fibrosis and deterioration of lung idiopathic pulmonary fibrosis in patients. Chromatographic methods are broadening their application by combining themselves with analytical methods to develop new complete procedures for the evaluation. This is so that the quality of the sample, intermediates, and finished compounds can be ensured and maintained, as well as the identification of chemical entities and the evaluation of their purity[8,9]. HPLC is the best approach to have been chosen in these aspects. This research aims to create a thorough, independently validated analytical method for identifying and measuring contaminants in pharmaceutical substances[10.] Chromatography is a method based on separation that divides components according to the solvent-to-polarity ratio. When a solvent or gas interacts with a mixture, it separates the components into individual sections according to their polarity or solvency[11]. The most frequently used chromatography methods are adsorption, partition, and ion exchange. A type of liquid chromatography "method of separating components between two phases which are not miscible, and the liquid mobile phase elutes through a stationary phase on a column," is High-pressure liquid chromatography (HPLC), according to IP 2016. Based on the theory that pressure forces the mobile phase through the column at a noticeably faster rate, high-performance liquid chromatography, or HPLC, is a type of column chromatography used in biochemistry, pharmaceutical and analytical chemistry, and biomedical science to detect and separate chemicals based on their polarities and interactions with the stationary phase of the column[12,13].

## MATERIAL AND METHODS

The API Pirfenidone was gifted from Chitkara University and the solvent and reagents used in the study were HPLC grade methanol (Thermo Fisher Scientific India Pvt. Ltd Mumbai) and tert-Butyl Methyl Ether used in HPLC was of HPLC grade quality and was sourced from Central Drug House Pvt. Ltd. in New Delhi, India. Hydrochloric Acid and Sodium Hydroxide Pellets used for hydrolysis were also procured from the same company. The oxidizing agent utilized in the study was Hydrogen Peroxide, sourced from Thermo Fisher Scientific India Pvt. Ltd in Mumbai.

### Instrumentations

The process of separating the chromatographic components was performed using a Waters HPLC (Model-2489) system that included a manual, a column, and a UV detector. The data obtained from the experiment were analyzed using the Empower-2 software. C-18 ODS 250 ×4.6mm type of column utilized. Using s digital precision balance with 1mg sensitivity manufactured by Shimadzu Japan and a sonicator Digital Pro $^{+}$.

#### Chromatographic condition

The description outlines the method for a chromatography experiment. The separation process used a C-18 ODS 250 ×4.6mm Column. The mobile phase was an 80:20 mixture of methanol and tert-butyl methyl ether, and the flow rate was 1.0 ml/min. The eluent and run duration for 10 minutes was observed at a wavelength of 316 nm. Prior to initiating the experiment, the column was conditioned by allowing the mobile phase to flow through the system for a minimum of 30 minutes. A 10µl injection volume was employed for sample analysis. The experiment is based on the principles of liquid chromatography, where the sample components are separated more easily by pumping the mobile phase through the column. The C18 ODS column is a popular reversed-phase column that is compatible with many solvents and analytes. The use of a UV detector at 316nm suggests that the sample absorbs light in this range.

#### Mobile phase preparation

A solution was prepared using HPLC grade Methanol (80%) and tert Butyl Methyl Ether (20%). The solution was then degassed for 5 minutes using an ultrasonic water bath.

#### Standard solution preparation

To prepare a 1mg/ml solution of pirfenidone API, a 10 ml volumetric flask was utilized. The procedure involved adding a specific amount of diluent to the flask, followed by the addition of exactly 10mg of pirfenidone API. The flask was then subjected to sonication for 10 minutes to facilitate dissolution. Additional diluent was added until the solution reached the mark on the volumetric flask, ensuring a total volume of 10 ml. Then using a membrane filter to filter the solution through a 0.45 m pore size to remove any contaminants. The finished product was then accurately labelled as containing 1 mg/ml of pirfenidone API.

#### Sample solution preparation

To determine the average weight of pirfenidone tablets, a total of 20 tablets were precisely weighed, and their collective weight was divided by 20 to calculate the average weight. The tablets were subsequently crushed into a powder form using a mortar and pestle. An equivalent quantity of 10mg of the powder obtained was accurately measured and transferred into a volumetric flask with a capacity of 10 ml. To guarantee the full solubility of the medicine, diluent was added to the flask, and the mixture was sonicated for 10 minutes. To remove any impurities, the produced solution was filtered using a membrane filter with a pore size of 0.45 m. The volume of the solution was adjusted by adding more diluent until it reached the mark on the volumetric flask. The mixture was then filtered once more using a membrane filter with a pore size of 0.45 µm. For the proposed HPLC method for analyzing pirfenidone utilizing the Waters 2489 instrument, complete information on the optimal chromatographic conditions and system suitability parameters is provided in Table 1.

Validation of the proposed technique

The developed technology has been modified in accordance with the International Conference on Harmonization criteria to guarantee that it is appropriate for its intended application (ICH). The system's appropriateness, precision, specificity, linearity, accuracy, limit of detection, and limit of quantification were all improved by these changes.

Linearity

A calibration curve for pirfenidone was prepared by taking stock solutions of 10, 15, 20, 25, 30, 35, and 40 µg/ml separately in 10 ml volumetric flasks and diluting with methanol: tert butyl methyl ether (80:20) as a diluent. At each concentration level, a volume of 10 µl from each solution was injected multiple times, and the peak area obtained was noted. These recorded peak areas were then used to create a calibration curve by plotting them against the corresponding drug concentration. Within the investigated range, the findings showed a linear association between peak area and concentration. Further information and observations can be found in Table 2.

Precision

The system's precision was evaluated by determining the reproducibility and repeatability through inter-day, and intra-day precision measurements. To evaluate precision, the RP-HPLC method was employed to inject three different concentrations (10, 25, 40 ng/ml) in triplicate on two separate days. The reproducibility and repeatability of the measurements were then calculated using the standard deviation and percentage relative standard deviation.

Accuracy

In order to evaluate the accuracy of Pirfenidone, a standard working sample was created in triplicate at different concentrations (2, 4, and 6 parts per million) and analyzed through a UV spectrophotometer. The accuracy was determined by calculating the percentage of the analyte that was recovered from a known amount that was added. Table 3 displays the data from nine measurements made in three sets at three doses.

Limit of Detection and Limit of Quantification

The slope (S) and standard deviation (SD) values from the calibration curve in Table 4 were

used to determine the limit of detection (LOD) and limit of quantification (LOQ) for pirfenidone. The computation was done using the following formula:
LOD = (S x 3 x SD) (10 x SD) / S = LOQ
Here, SD stands for the response's standard deviation, and S stands for the calibration curve's slope.

System suitability

The system suitability test is a critical step in ensuring the chromatographic system's appropriateness for the intended analyses. This test involves injecting multiple samples and assessing various parameters such as peak area, theoretical plate, plate height, and tailing factor, all of which must meet the United States Pharmacopoeia's (USP) established criteria. The results of the test demonstrate that the system is appropriate for the analysis, as the RSD values observed were within the generally accepted limits (not more than 2%). This indicates that the system is precise and can deliver consistent results, as presented in Table 5.

Specificity and Selectivity

Analytical techniques need to possess specificity as a critical characteristic, which determines their ability to accurately measure the analyte's reaction amidst potential disruptive substances, such as impurities and degradation products. This factor is particularly crucial in complex samples that contain a large number of interfering components. Moreover, the selectivity of the technique can also be determined by assessing the purity of each degradation peak.

Degradation studies

Degradation studies are conducted to assess the stability of a drug under stress conditions, including acid hydrolysis, basic hydrolysis, neutral, and oxidation. Table 6 displays the conditions for force degradation.

Acid Degradation

0.1NHCl was employed to force degrade the drug sample at 60°C for 7 hours to evaluate acid degradation. The solution was then neutralized with 1N NaOH and diluted with methanol. The resulting mixture was then filtered using a membrane filter with a 0.45 µm pore size.

Base Degradation

To assess base degradation, the drug sample was subjected to 0.1N NaOH for 7 hours at $60^0$C. The mixture was then neutralized with 1N HCl and diluted with methanol. The resulting mixture was filtered using a membrane filter with 0.45 µm pore size.
Neutral Degradation

A medication is exposed to water at a temperature of 60°C for 24 hours as part of the "Neutral Degradation" procedure. The sample is then diluted with methanol before being filtered through a membrane filter with a 0.45 µm pore size.

Oxidative Degradation

The medication sample is exposed to 3 percent hydrogen peroxide at a temperature of 60°C for 6 hours as part of the "Oxidative Degradation" protocol. The sample is then combined with methanol and the resulting mixture is using 0.45 µm pore size.

Result and Discussion

The sample showed an absorbance maximum of 316nm. Initially, methanol was used instead of acetonitrile for the analysis, but no elution was observed. A mixture of water and tetrahydrofuran (THF) in a ratio of 60:40 was tried, but the peak shape was not satisfactory. Therefore, a change in solvent was required due to THF toxicity and poor peak shape. The methanol: tert-Butyl Methyl Ether ratio was modified to 80:20% (v/v), which led to peak broadening and tailing factor. However, the peak shape improved significantly with an appropriate tailing factorThe developed HPLC method for Pirfenidone separation was optimized using a C-18 column and a mobile phase of water: tert-Butyl Methyl Ether (80:20%, v/v) at room temperature. The method was validated according to the ICH guidelines. For the suggested HPLC technique employing the Waters 2489 instrument, the optimal chromatographic conditions and system suitability parameters were shown in Table 1. It is worth noting that HPLC is a commonly used analytical method for identifying, separating, and quantifying various compounds, including pharmaceuticals. Therefore, proper optimization of the chromatographic conditions and system suitability parameters is critical to ensure the accuracy and reliability of the analytical results.

Table 1 Optimized Chromatographic Condition

| | |
|---|---|
| Flow rate | 1.0ml/min. |
| Column | Inertsil C-18ODS, (250×4.6mm),5µm |
| Mobile phase | HPLC grade Methanol: tert Butyl Methyl Ether (80:20) |
| Detector wavelength | 316 nm |
| Column temperature | Ambient |
| Run time | 10 minutes |
| Injection volume | 10 µl |
| Diluents | Methanol |
| Mode of separation | Isocratic mode |

CALIBRATION CURVE OF HPLC: A calibration curve is a crucial tool in HPLC that establishes the relationship between a compound's concentration and its corresponding peak area. Table 2 presents the HPLC peak area results, which were used to generate the calibration curve shown in Figure 4. The curve shows a linear relationship between peak area and concentration within the range of the study, indicating the proper functioning of the instrument and the reliability of the data. Additionally, the high correlation coefficient of 0.9955 confirms a strong correlation between peak area and concentration, validating the accuracy of the calibration curve as a representation of the data.

Table 2 Linearity Data of Pirfenidone

| S.no. | Drug Concentration (µg/ml) | Peak Area |
|---|---|---|
| 1. | 10 | 491316 |
| 2. | 15 | 681004 |
| 3. | 20 | 871213 |
| 4. | 25 | 1074007 |
| 5. | 30 | 1284753 |
| 6. | 35 | 1498660 |
| 7. | 40 | 1702756 |

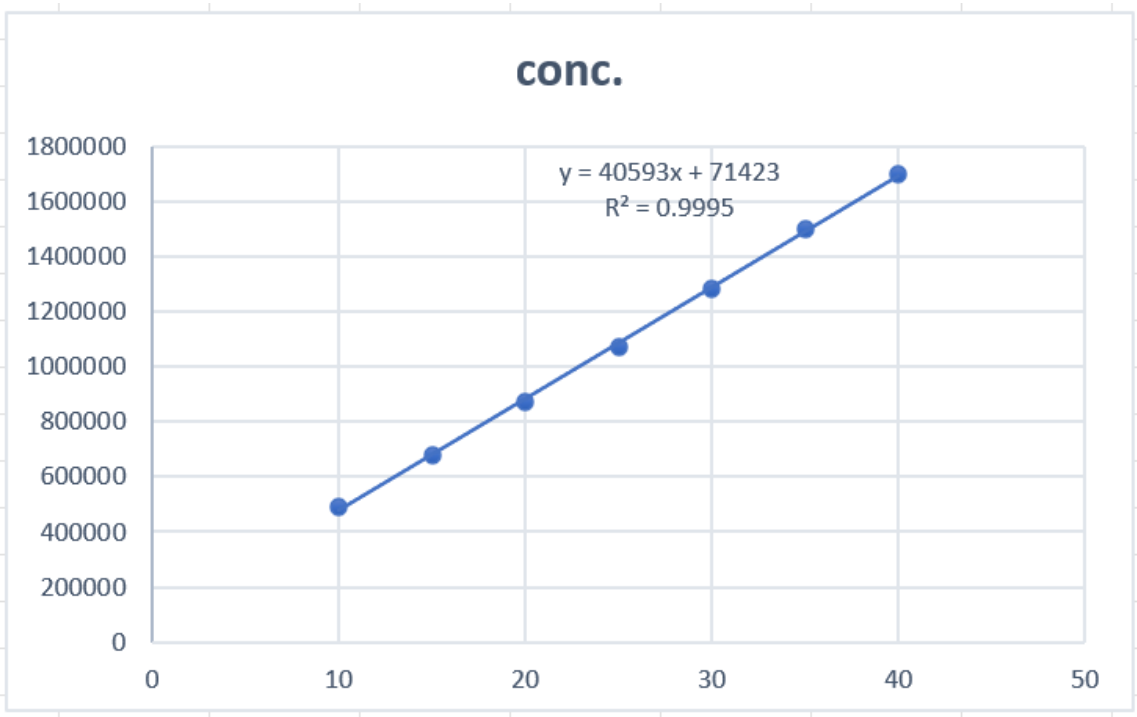


*Fig 2 Calibration curve of Pirfenidone*

Table 3 Inter-day and Intra-day Precision study of the developed method of Pirfenidone

| S.No. | Concentration ng/ml | Inter-day precision | | Intra-day precision | |
|---|---|---|---|---|---|
| | | SD | %RSD | SD | %RSD |
| 1 | 10 | 4328.62 | 0.855156 | 2552.329 | 0.503165 |
| 2 | 25 | 7281.062 | 0.617523 | 5950.219 | 0.506068 |
| 3 | 40 | 6231.446 | 0.348535 | 12230.38 | 0.681988 |

*(SD stands for Standard Deviation, (RSD) stands for Relative Standard Deviation*

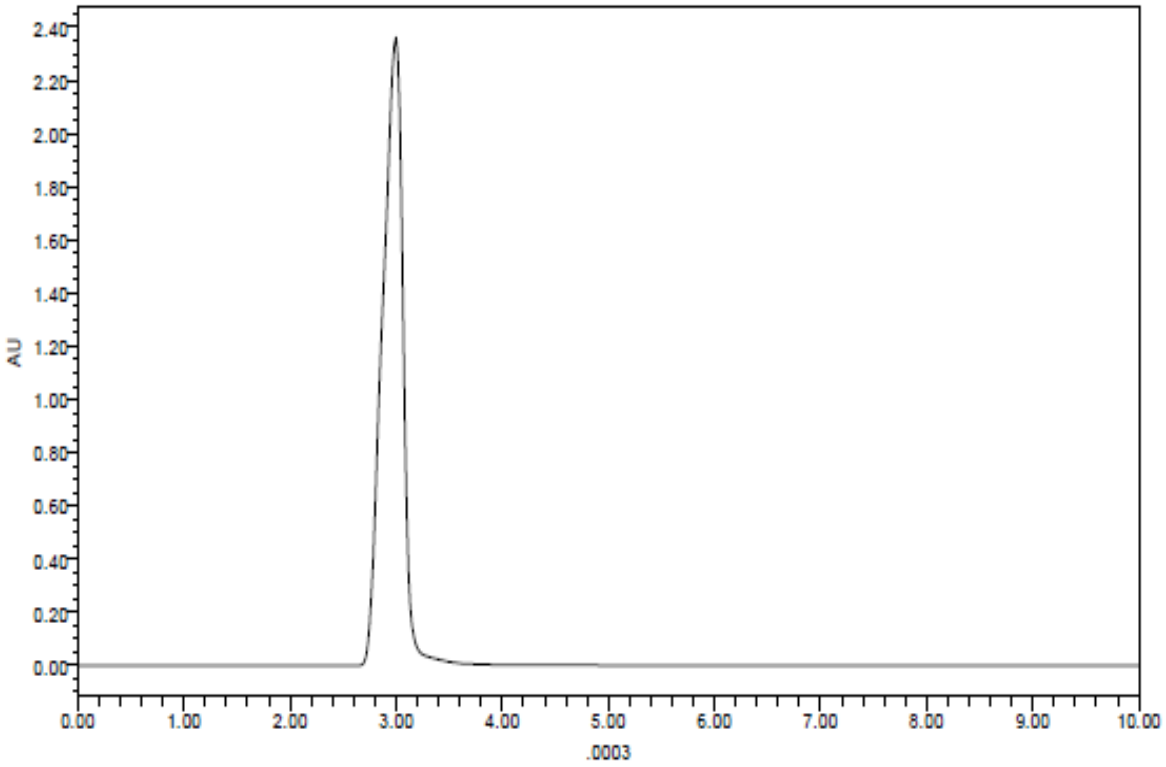


*Figure 3 Chromatogram of pure drug.*

ACCURACY: The precision of the experiment was determined using a UV spectrophotometer, and the corresponding absorbance value is represented in Table 4.

Table 4 Accuracy Data

| Sample | Absorbance |
|---|---|
| 2ppm | 0.263 |
| 4ppm | 0.327 |
| 6ppm | 0.423 |

Table 5 LOD and LOQ value

| | |
|---|---|
| LOD | 0.262 |
| LOQ | 0.796 |

SYSTEM SUITABILITY: The evaluation of system suitability was carried out using various factors and the outcomes are presented below.

Table 6: Data on System Suitability

| S.No. | RT | AUC | N | TF | H |
|---|---|---|---|---|---|
| 1. | 2.943 | 409200 | 2183.8 | 1.09 | 44746 |
| 2. | 2.944 | 408701 | 2223.9 | 1.12 | 44255 |
| 3. | 2.949 | 406188 | 2231.2 | 1.10 | 44175 |
| 4. | 2.943 | 408735 | 2211.2 | 1.07 | 44417 |
| 5. | 2.943 | 400213 | 2199.9 | 1.09 | 44552 |
| 6. | 2.970 | 404530 | 2105.3 | 1.15 | 44316 |

*RT stands for Retention Time, AUC stands for Area Under the Curve, N stands for Number of Theoretical Plates, TF stands for Tailing Factor, H stands for Height*

SPECIFICITY AND SELECTIVITY: The chosen chromatographic conditions allowed for a retention time of 2.931 minutes for pirfenidone. In order to determine the method's specificity and selectivity, a comparison was made between a blank mobile phase chromatogram and a mobile phase sample spiked with pirfenidone. No peaks that could interfere with the analyte were detected during the retention time.

Table 7 The condition for stress degradation of pirfenidone was optimized

| Applied for degradation study | Concentration of stressor | Exposed Condition | Duration | % Degradation Product |
|---|---|---|---|---|
| Acid | 0.1NHCl | $60^0$C | 7 hours | No Degradation |
| Base | 0.1NNaOH | $60^0$C | 7hours | No Degradation |
| Neutral | $H_2O$ | RT | 24 hours | No Degradation |
| Oxidative | 3%$H_2O_2$ | $60^0$C | 6 hours | No Degradation |

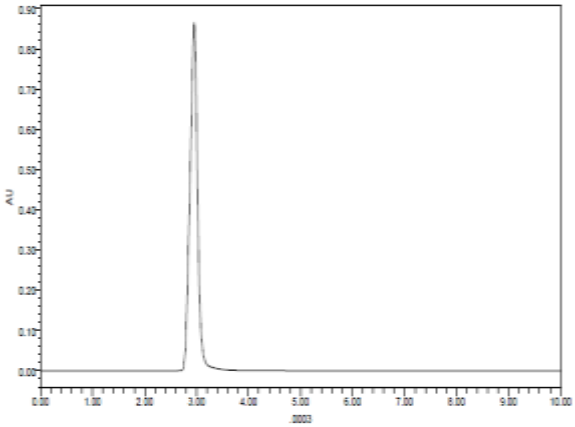

*Figure 4 Chromatogram of pirfenidone sample*

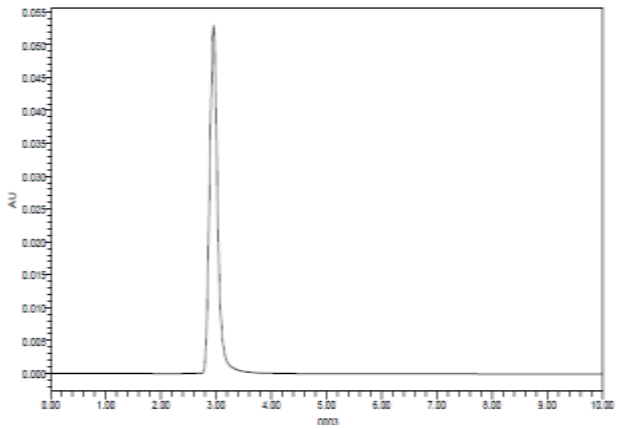

*Figure 5 Chromatogram of acid stress sample*

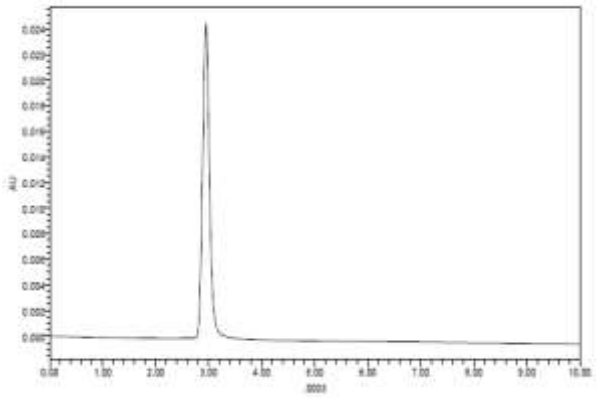

*Figure 6 Chromatogram of base stress sample*

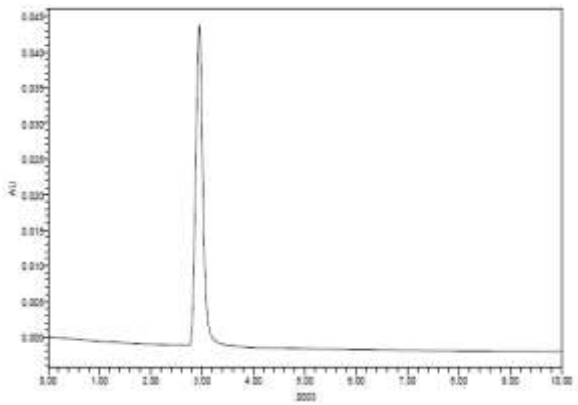

*figure 7 Chromatogram of neutral stress sample*

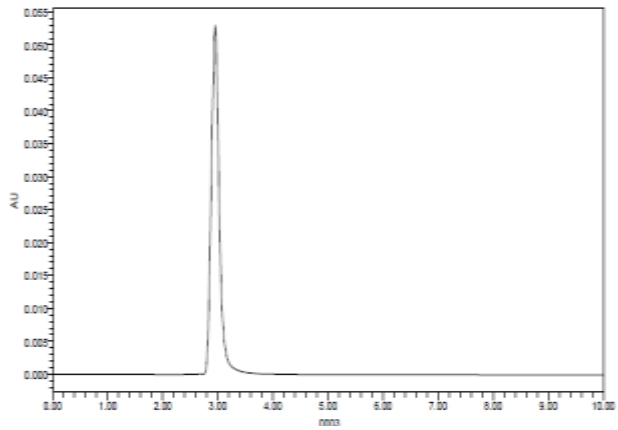

*Figure 8 Chromatogram of $H_2O_2$ stress sample*

## CONCLUSION

Chromatographic methods play a crucial role in the identification of chemical compounds, determination of their purity, and ensuring the accurate quantification of drug samples. The aim of this study was to develop a comprehensive and validated method to detect and quantify

impurities in Pirfenidone, a pharmaceutical drug utilized in the treatment of Idiopathic Pulmonary Fibrosis. A reverse-phase high-performance liquid chromatography (HPLC) technique was employed for the separation and analysis of pirfenidone. The chosen stationary phase was Inertsil C18 (250×4.6mm, 5.0mm), while the mobile phase consisted of a mixture of methanol and tert-butyl methyl ether in a ratio of 80:20% v/v. The detection of pirfenidone was achieved using a UV detector set at a wavelength of 316 nm. Chromatography was conducted at a flow rate of 1.0 ml/min. The method proved suitable for routine pirfenidone analysis, as demonstrated by recovery studies, and exhibited no interference from excipients. Various system suitability parameters, including efficiency, resolution, and tailing factor, were determined, while precision was assessed through repeated sample injections. The method underwent validation for linearity, precision, and accuracy, and exhibited (LOD), (LOQ) rendering it suitable for quality control purposes. Overall, the developed RP-HPLC method is characterized by simplicity, accuracy, and a short analysis time, making it well-suited for routine analysis of pirfenidone.

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