Determination and Pharmacokinetic Study of Pirfenidone in Rat Serum by High-Performance Thin-Layer Chromatography

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Abstract

A rapid, sensitive and selective high-performance thin-layer chromatography (HPTLC) method was developed and validated for the determination and pharmacokinetics of pirfenidone in rat serum. One-step protein precipitation by methanol is reported, and serum samples were separated by HPTLC using a simple mobile phase of toluene–methanol in the ratio of 8:2. The retardation factor of pirfenidone in the serum sample was 0.45 with the detection performed at 315 nm. The calibration curve was linear over the range of 100–1,200 ng/spot with a lower limit of quantitation of 40 ng/spot. The mean recovery of pirfenidone in serum was in the range of 70.6–75.8%, and intra-day and inter-day precision were both <14.1%. This method was successfully applied to the pharmacokinetic study of pirfenidone in rats on oral administration of the drug at a dose of 15.0 mg/kg.

Introduction

Pirfenidone is a small non-peptide molecule of low molecular weight, with the chemical name 5-methyl-1-phenyl-2-(1H)-pyridone, used as a novel antifibrotic agent approved for mild-to-moderate idiopathic pulmonary fibrosis (IPF) (1). It was initially reported as analgesic, antipyretic and anti-inflammatory agent by Gadekar et al. (2, 3). IPF is the most common type of idiopathic interstitial lung disease, incurable and often fatal, which mostly affects geriatric patients causing fibrosing interstitial pneumonia of unknown etiology (4–6). Pirfenidone is the only drug that has been approved for the treatment of IPF in Japan and Europe; it is also effective in cardiac, renal and hepatic fibrosis (7–11).

Liquid chromatography (LC), liquid chromatography–mass spectrometry (LC–MS) (12) and ultra-pressure liquid chromatography coupled tandem mass spectrometry (UPLC–MS–MS) (13, 14) were developed for the analysis of pirfenidone in biological samples and matrices (15). Recently, we have also reported a stability-indicating method for pirfenidone in biological samples and matrices (16). However, so far no HPTLC method for the determination of pirfenidone in biological matrix is available in the literature. Previously reported methods require high-cost equipment and more sophistication; to overcome this lacunae, we report development and optimization of a simple, sensitive and specific HPTLC method for the determination of pirfenidone in rat serum. This method is validated and successfully applied to pharmacokinetic studies of pirfenidone using single oral administration.

Experimental

Chemicals and reagents

Pirfenidone was received as a gift sample from Cipla Pharmaceutical Ltd., Mumbai, and Phenacetin (IS) was purchased from Sigma-Aldrich (St. Louis, USA). Methanol, toluene (AR) grade was purchased from Merck Specialties Private Limited, India.

HPTLC conditions

Chromatographic separation of the drugs was performed on Merck HPTLC plates pre-coated with silica gel 60 F254 [10 × 10 cm² with a layer thickness of 150–200 μm with a surface thickness deviation of ±30 μm, Catalogue no. 1055480001, E. Merck (Merck Millipore), Germany]. The sample was applied on the plates as a band with
phenacetin is shown in Figure 1.

and pirfenidone (Peak 2).

Figure 1. The densitogram and chemical structures of phenacetin (IS) (Peak 1) and pirfenidone (Peak 2).

5 mm width using a CAMAG 100 µL sample syringe (Hamilton, Switzerland) with a Linomat-IV TLC applicator. Linear ascending development was carried out in a twin-trough glass chamber (10 × 10 cm²). Densitometric scanning was performed using a CAMAG TLC-Scanner-III (version 4.0.1) supported with CAMAG Win cats® software (version 4.0.1), an electronic balance (ACCUCLAB Model ALC-210.4 Huntington valley, PA) and a sonicator (EN 30 US, Enertech Fast-clean, Mumbai, India).

A toluene–methanol mixture in the ratio of 8:2 v/v was optimized for thin-layer chromatography plate development. The chamber was saturated with the mobile phase at room temperature for 20 min. The run distance was kept at ~70 mm, and 10 mL of the mobile phase was used for a single development. The dosing speed of nitrogen applicator was kept 150 nL/s with a pre-dosage volume of 5 mL. Samples were applied as bands of 5 mm width with gaps of 6 mm in between. The developed plates were dried at room temperature for 5 min, and detection was done at 315 nm using a deuterium lamp in absorption/reflectance mode. The slide dimension of the detector was kept at 4 × 0.45 mm². The optimized densitogram of pirfenidone and phenacetin is shown in Figure 1.

Standard solutions, calibration standards and quality control sample

The stock solution of pirfenidone was prepared by dissolving 150 mg in 10 mL methanol, further diluted with methanol to obtain a working solution at different concentration levels: 1–12 µg/mL. Calibration standards and QC samples in serum were prepared by diluting the corresponding working solution with blank rat serum. Each working solution of pirfenidone (10 µL) was used to spike serum (1 mL) to furnish a stock solution with blank rat serum. Each working solution of pirfenidone (10 µL) was used to spike serum (1 mL) to furnish the calibration standard ranging from 10 to 120 µg/mL. QC sample solution was prepared at concentrations of 20, 50 and 100 µg/mL. IS stock solution was prepared at an initial concentration of 100 µg/mL. The IS working solution (10 µg/mL) was prepared from the stock solution. Subsequently, stock solution, working solution, calibration standard and quality controls (QCs) were immediately stored at −20°C.

Sample preparation

The serum sample was thawed to room temperature. Serum calibration standard IS and QC samples (1 mL) were transferred to a centrifuge tube, mixed with methanol. After vortex-mixing for 1 min, the samples were centrifuged at 3,000 g. The supernatant was transferred to new tubes, and the solvent was evaporated at 37°C under the stream of nitrogen. The residues were dissolved in 100 µL methanol. 1 µL of each sample was applied to the TLC plate to furnish a final calibration range of 100–1,200 ng/spot. QC samples at final concentrations of 200, 500 and 1,000 ng/spot, and each concentration was applied six times to the TLC plate.

Method validation

The method developed was validated for its selectivity, linearity, accuracy, precision, recovery and stability in accordance with the US Food and Drug Administration’s (US FDA) validation of bioanalytical methods (17). Validation runs were conducted on three consecutive days, and each validation run consisted of one set of calibration standards and six replicates of QC serum samples. The selectivity of this method was evaluated by analyzing blank rat serum, regular rat serum, blank serum spiked with pirfenidone and IS. Calibration curves were developed on analyzing the spiked calibration samples on three separate days. The peak area ratio of pirfenidone to internal standard was plotted against analyte at various concentrations, and subsequently standard curves were fitted to the equations. This was done by linear regression with a weighting factor of the reciprocal of the concentration range of 100–1,200 ng/spot. In this study, the lower limit of quantitation (LLOQ) was defined as the lowest concentration on the calibration curves. The limit of quantitation (LOQ) of drug was calculated using the following equations as per ICH guidelines:

$$LOQ = 10(\sigma/s).$$

where $\sigma$ is the standard deviation of the response and $s$ is the slope of the calibration curve.

The accuracy and precision for the proposed method were assessed by the determination of QC samples at three different concentration levels in five replicates of 200, 500 and 1,000 ng/spot, in three validation days, and the precision was expressed by the coefficient of variation (CV). The recovery was evaluated by comparing the peak area ratios of extracted QC samples with those of reference QC solutions reconstituted in blank serum extracts ($n = 5$). The recovery of the IS was determined in a similar way.

Stability

Stock and working solution stability, freeze–thaw stability, short-term stability and long-term stability studies were performed. For all the stability studies, the solution was considered to be stable if the difference between these was not >15%, as indicated in the guidelines for bioanalytical methods validation.

Stock and working solutions

The stock solutions of pirfenidone and IS were stored at −20°C and compared with a freshly made stock solution after 21 days. The maximum duration of 21 days was selected as the maximum time to keep the frozen stock solutions. The stability of working solutions of pirfenidone and IS, prepared freshly each day, was evaluated after 8 h at room temperature.

Freeze and thaw stability

Freeze–thaw stability of pirfenidone was determined by assaying the three QCs in triplicate over three freeze–thaw cycles. Three aliquots at each concentration were stored at −40°C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples
were refrozen for 24 h under the same conditions; the freeze–thaw cycle was repeated two more times.

Short-term stability
The stability of pirfenidone in serum at room temperature was investigated in triplicate along with all the three QCs stored as freshly extracted samples. Four aliquots of each concentration were stored at room temperature for 1, 2, 4 and 8 h. A maximum duration of 8 h was selected to coincide with regular daily working time.

Long-term stability
The stability of pirfenidone in serum at −40°C was evaluated by assaying in triplicate, and all the three QCs samples were stored at this temperature for 21 days. These were not evaluated beyond 21 days as our maximal storage condition is 21 days.

Application to a pharmacokinetic study
Male Sprague–Dawley rats were used to study the pharmacokinetics of pirfenidone. The permission to use these animals for the designed protocol was obtained from the Institutional Animal Use Ethical Committee at the Rashtrasant Tukadoji Maharaj Nagpur University, India, and all the guidelines of CPCSEA were followed during animal experimentations. All animals were fasted and diet was prohibited for 12 h before the experiment, but water was provided ad libitum. Blood samples were collected from the tail vein in to centrifuge tube at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 h after oral administration of pirfenidone (15.0 mg/kg). The samples were immediately centrifuged at 3,000 g for 10 min. The serum obtained was stored at −20°C until analysis. Serum pirfenidone concentration versus time data for each rat was analyzed by Pharsight Corporation’s Phoenix WinNonlin software (Trial Version at the ACTREC, TMC, Mumbai).

Results
Method development and optimization
Liquid–liquid extraction (LLE) and solid-phase extraction methods are both time-consuming and expensive; therefore in this study, the simple protein precipitation method was used to reduce sample preparation time and make this method economical. For protein precipitation, generally methanol, acetonitrile and methanol–acetonitrile were used. Herein, we observed methanol’s higher efficiency of extraction and therefore chose it as a precipitation solvent. A number of mobile phases were evaluated for pirfenidone and IS, and the best was toluene and methanol in the ratio of 8:2. The chamber was saturated with the mobile phase at room temperature for 20 min. The run distance was kept at ~70 mm, and 10 mL of the mobile phase was used for a single
development. The dosing speed of the nitrogen applicator was kept 150 nL/s with a pre-dosage volume of 5 mL. The samples were applied as bands of 5 mm width with gaps of 6 mm in between. The developed plates were dried at room temperature for 5 min. Detection was done at 315 nm with a deuterium lamp in absorption/reflectance mode. The slit dimension of the detector was kept at 4.00 × 0.45 mm². Figure 2 shows the typical densitogram of blank serum sample, normal serum sample, sample spiked with pirfenidone and IS. Interference due to the presence of internal standard was not observed in the \( R_f \) value.

Calibration curve and sensitivity
The linear regression analysis showed that there was a good linear relationship \((r^2 > 0.997)\) between the peak area ratio and the concentration in the range of 100–1,200 ng/spot for pirfenidone in rat serum. A typical equation of the calibration curve is

\[
y = 0.006x + 0.039,
\]

where \( y \) represents the ratios of pirfenidone peak area to that of IS and \( x \) represents the serum concentration. The LLOQ for the determination of pirfenidone in serum was 40 ng/spot.

Table I. Precision, Accuracy and Recovery for Pirfenidone of the QC Sample in Rat Serum (\( n = 6 \))

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration (ng/spot)</th>
<th>CV (%)</th>
<th>Accuracy (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>intra-day</td>
<td>inter-day</td>
<td>intra-day</td>
</tr>
<tr>
<td>Pirfenidone</td>
<td>200</td>
<td>13.5</td>
<td>14.1</td>
<td>103.4</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>10.5</td>
<td>11.0</td>
<td>96.7</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>7.9</td>
<td>8.6</td>
<td>94.0</td>
</tr>
</tbody>
</table>

Table II. Stability Study of Pirfenidone Under Various Storage Conditions (\( n = 5 \))

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration (ng/spot)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 h study</td>
<td>200</td>
<td>102.3 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>97.6 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>95.8 ± 5.3</td>
</tr>
<tr>
<td>Three freeze-thaw</td>
<td>200</td>
<td>106.4 ± 4.61</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>93.5 ± 5.54</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>97.3 ± 3.43</td>
</tr>
<tr>
<td>−40°C, 21 days</td>
<td>200</td>
<td>98.3 ± 4.73</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>106.2 ± 6.81</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>95.6 ± 7.3</td>
</tr>
</tbody>
</table>

Table III. The Main Pharmacokinetic Parameters After Oral Administration of 15.0 mg/kg Pirfenidone in Six Rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pirfenidone</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t_{1/2} ) (h)</td>
<td>2 ± 0.24</td>
</tr>
<tr>
<td>( C_{\text{max}} ) (ng/mL)</td>
<td>1,055 ± 120.3</td>
</tr>
<tr>
<td>( \text{AUC}_{0-\infty} ) (ng/mL h)</td>
<td>5,515 ± 432.1</td>
</tr>
<tr>
<td>( \text{MRT}_{0-\infty} ) (h)</td>
<td>6,224.32 ± 512</td>
</tr>
<tr>
<td>( MRT_{0-\infty} ) (h)</td>
<td>3.8 ± 0.04</td>
</tr>
<tr>
<td>CL/F (mL/h)</td>
<td>5.3 ± 0.07</td>
</tr>
</tbody>
</table>

Precision, accuracy and recovery
The precision of the method was determined in the form of CV for QCs at three concentration levels over three validation days. The intra-day precision was 13.5% or less, and the inter-day precision was 14.1% or less at each QC level. The accuracy of the method ranged from 94 to 103.4% at each QC level. The mean recovery of pirfenidone was 73.63%. The recovery of the IS was 80.4 ± 5.6%. The assay performance data are presented in Table I.

Stability
The stability of the pirfenidone was investigated in the rat serum, i.e., stock and working solutions, freeze and thaw stability, short-term stability and long-term stability (21 days) indicated that the analyte was stable under various storage conditions (Table II).

Application of the method in a pharmacokinetic study
The method was applied to a pharmacokinetic study in serum. The mean serum concentration–time profile after the oral administration of 15 mg/kg pirfenidone is shown in Figure 3. The main pharmacokinetic parameters are summarized in Table III.

Discussion
Pirfenidone is the only drug available for the treatment of IPF, and it also demonstrates its usefulness in cardiac, renal and hepatic fibrosis. Earlier reports for the determination of pirfenidone in biological samples had several difficulties related to time of analysis, separation, accuracy and cost-effectiveness. We have addressed these issues and developed this method on the basis of HPTLC for the determination of pirfenidone in rat serum. A simple protein precipitation method was developed using toluene–methanol mobile phase. This provided us with good and comparable results for LLE or SPE and without any interference of the IS. The developed method also exhibited good relationship between the peak area ratio and the concentration of the drug in rat serum with a lower limit of quantification of 40 ng/spot. The precision, accuracy and recovery were found to be in accordance with the guidelines provided by the US FDA, suggesting the suitability for clinical use.
of method for further use; the data are provided in Tables I and II. The stability of the drug in the serum sample was also evaluated, and it is found to be stable for long term. When this method was applied to determine the pharmacokinetics of the drug in rat serum at 15.0 mg/kg, the elimination half-life time was found to be $(t_{1/2}) = 2 \pm 0.24$, which is comparable to $1.90 \pm 0.13$ reported earlier (15). The $C_{\text{max}}$ and AUC were found to be significant at $1,055 \pm 120.3 \text{ ng/mL}$ and $5,515 \pm 432.1$, respectively. The clearance of the drug (CLz/F) was satisfactory at $642.6 \pm 0.756 \text{ mL/h}$, proving this method as satisfactory with respect to overall aspects of method development for pharmacokinetic evaluation compared with earlier reported methods of the UPLC–MS–MS method for pirfenidone.

**Conclusion**

We have successfully developed and validated a method for the determination of pirfenidone in rat serum by the application of HPTLC. To our knowledge, this is the only report for the determination of pirfenidone in rat serum by HPTLC. Earlier methods of HPLC, HPLC–MS and UPLC–MS–MS were highly expensive, time-consuming and required critical sampling. Herein, we have successfully displayed one-step protein precipitation by methanol that is fast and economical. This method was successfully applied for the pharmacokinetic study of pirfenidone in rat serum samples. The developed method is efficient and allows for parallel multiple analyses, which is a hallmark of HPTLC methods.

**Acknowledgments**

We thank Cipla Pvt., Ltd., India, for the gift sample of pirfenidone. We also thank the Pharsight Corporation for their trial version license of the Phoenix WinNonLin software (at the ACTREC, TMC, Mumbai).

**References**