

# Pharmacokinetics and Metabolism of a Novel Antifibrotic Drug Pirfenidone, in Mice Following Intravenous Administration

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**ABSTRACT:** The present study describes the pharmacokinetics and metabolism of pirfenidone (PD), a compound which has been shown to have significant antifibrotic effects in rodent models of pulmonary and cardiac fibrosis. Despite the fact that this compound is currently in phase II clinical trials, little data are available on the metabolism and disposition of this agent in rodents or humans. Radioactive PD [benzene ring <sup>14</sup>C(U)] was administered i.v. to mice at 40 mg PD/kg body weight, and animals were killed at varying times for determination of parent compound and metabolites in various tissues. The disappearance of parent compound from the plasma followed apparent 2-compartment elimination kinetics with a terminal elimination half-life of 8.6 min. Cl (0.10 ml/min/g) and  $V_{d(ss)}$  (0.67 ml/g) indicated that PD was rapidly distributed in body water. This is consistent with the finding that peak tissue radioactivity occurred within 5 min following the i.v. administration of [<sup>14</sup>C]-PD and that well-perfused tissues, kidney>liver>lung have much higher levels of parent compound and metabolites than did fat. Two peaks isolated from plasma samples by HPLC yielded mass spectra that were consistent with initial oxidation to the alcohol followed by further metabolism to the carboxylic acid. The radioactivity recovered in the 24 h urine samples averaged 97% of the administered dose and none of that was associated with the parent compound. The short plasma half-life of parent compound in mice supports the need for additional studies in humans where the compound has been shown to have clinical benefits. Copyright © 2002 John Wiley & Sons, Ltd.

**Key words:** pirfenidone; pharmacokinetics; metabolism; antifibrotic drug

## Introduction

Idiopathic pulmonary fibrosis (IPF) is a crippling disease that leads to a decrease in lung compliance and an impairment of gas exchange due to excessive accumulation of collagen in the lung interstitium [1]. Despite recent advances in the development of new forms of therapy for IPF, it remains a highly lethal disease with a prognosis

similar to that of lung cancer. The management of IPF continues to be a challenging and perplexing clinical problem. No known effective therapies for this disease exist since most of the drugs currently in use for treatment of IPF have debilitating systemic toxicity. The most common therapy involves treatment with corticosteroids. However, less than 20% of the patients respond to this therapeutic approach, and the severe systemic side effects are well-established [2].

Pirfenidone (PD, trade name Descar), 5-methyl-1-phenyl-2-(1H) pyridone (Figure 1), is a new investigational drug. This compound has

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been demonstrated to minimize lung fibrosis in several models of drug-induced fibrosis including the bleomycin–hamster model [3] and the cyclophosphamide–mouse model [4]. In addition, we recently reported that dietary PD, starting after the second bleomycin dose in a three-dose regimen, also minimized the accumulation of collagen in the lungs [5]. Furthermore, treatment with PD and spironolactone was found to reverse the cardiac and renal fibrosis in streptozotocin-diabetic rats [6] and prevented dimethylnitrosamine-induced hepatic fibrosis in rats [7]. *In vitro* studies have demonstrated the ability of this compound to decrease proliferation of human lung fibroblasts supporting the possible uses of the compound in the treatment of obliterative bronchiolitis, a major complication of lung transplantation [8]. PD also has been shown to have beneficial effects in humans at varying stages of IPF [9], and chronic, progressive multiple sclerosis [10]. A recent comparison with other therapeutic interventions for IPF recommended further clinical trials of this drug entity [11]. However, the pharmacokinetic behavior of PD in any laboratory species is not yet known; only limited data, consisting of 0–4 h AUC values, are available in humans [12]. Therefore, the objective of this study was to determine the pharmacokinetic, metabolic disposition and tissue distribution of PD following intravenous i.v. administration in mice. The results of these studies indicate that plasma half-life of the parent compound is short and strongly support the need for further pharmacokinetic evaluation of the compound in humans.

## Materials and Methods

### *Animals and chemicals*

Male mice (CD1/ICR) weighing 27–30 g, purchased from Harlan Sprague Dawley, Inc. (Houston, TX), were used in this study. The animals were maintained in animal care facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care. The mice were housed 4 per cage and had access to laboratory chow and water *ad libitum*. PD (MW 185.3, C<sub>12</sub>H<sub>11</sub>NO, Figure 1) was provided by

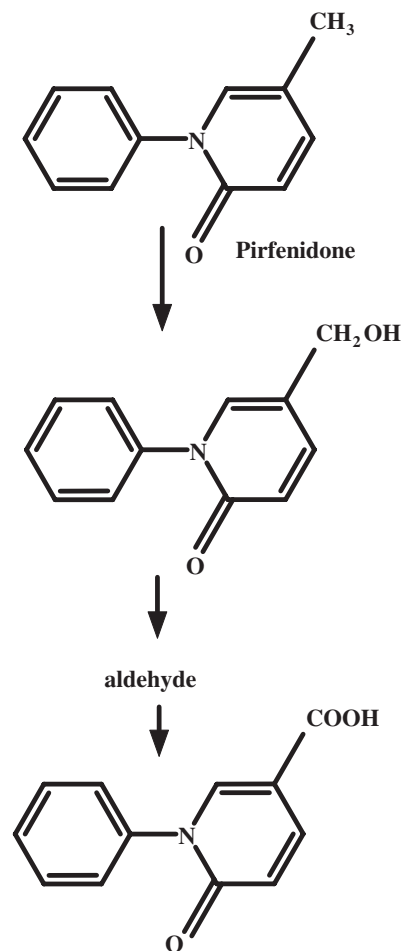


Figure 1. Structures of pirfenidone and identified metabolites

Marnac, Inc., Dallas, Tx. Radiolabeled PD [benzene ring <sup>14</sup>C(U)] was custom synthesized by Moravek Biochemicals, Inc. (Brea, CA). The specific activity of [<sup>14</sup>C]-PD was 25 mCi/mmol, and its radiochemical purity, checked by HPLC on a reverse phase column, was greater than 98%.

### *Administration of PD*

Aliquots of stock [<sup>14</sup>C]-PD in ethanol were evaporated to dryness under nitrogen and mixed with unlabeled PD dissolved in sterile isotonic saline. Each mouse received 100 μl of this solution by tail vein injection. This volume contained 2 μCi of [<sup>14</sup>C]-PD and gave a dose of approximately 40 mg PD/kg body weight. The final

specific activity of the dose solution was 610 dpm/nmol.

In a subset of experiments, mice receiving the same dose (40 mg/kg) of either [ $^{14}\text{C}$ ]-PD (2  $\mu\text{Ci}$ /mouse) or unlabeled PD were kept in glass Bioserve metabolic cages for 24 h. Urine was collected in tubes submerged in dry ice. After thawing and recording the total urine volume, an aliquot was used to determine the radioactivity, and the remainder was stored at  $-80^\circ\text{C}$  for separation and mass spectral characterization of urinary metabolites. The urine collected from mice receiving unlabeled PD was used for mass spectrometry.

#### *Measurement of radioactivity in blood and tissues*

Mice were anesthetized in a chloroform jar at different times, and blood was collected in heparinized syringes directly from the right ventricle. Blood samples were centrifuged at 600g for 15 min, and plasma was aspirated gently. Remaining erythrocytes were washed twice with isotonic saline and resuspended in saline to the original blood volume. Different organs including liver, kidneys, lung, heart, testes, spleen, pancreas, adrenal glands, skeletal muscle, intestine, epididymal fat pad, and brain were removed and rinsed 3 times in cold isotonic saline. The intestine was split open, the contents were removed, and the tissue was rinsed thoroughly in saline. A small piece of tissue (50–100 mg) was excised from each organ and transferred to a scintillation vial containing 1 ml of tissue solubilizer, Soluene 350 (Packard, Downers Grove, IL). Fifteen ml of scintillation fluid (Value made, Beckman Instruments, Fullerton, CA) was added to each vial containing either solubilized tissue or plasma (0.1 ml) and kept at room temperature for 24 h for equilibration. The samples were counted in a Beckman Scintillation Counter (Model LS 5801) with counting efficiencies ranging from 65 to 75%.

#### *Analysis of PD and metabolites in plasma by HPLC*

Aliquots of plasma were mixed with two volumes of 100% acetonitrile and were stored overnight at  $-20^\circ\text{C}$ . Protein was removed by

centrifugation at 16 000g for 10 min, and the supernatant was placed in a clean microcentrifuge tube and evaporated to dryness using a centrifugal vacuum concentrator. Protein pellets containing less than 1% of the total radioactivity in plasma were discarded. The residue from the dried plasma samples was redissolved in acetonitrile/water (25:75), and samples were sonicated at room temperature for 5 min. Particulate material was removed by centrifugation. An aliquot of the dissolved sample was injected onto a  $\text{C}_{18}$  reverse phase column (Phase Sep,  $250 \times 4.6 \text{ mm}^2$ ,  $5 \mu\text{m}$ ), and the parent compound and metabolites were separated under the following conditions. The mobile phase was programmed linearly from 0% solvent B to 60% solvent B over a period of 100 min at a flow rate of 1 ml/min. Solvent A consisted of 0.05% trifluoroacetic acid in water and solvent B was 0.05% trifluoroacetic acid in acetonitrile. Fractions were collected at 1 min intervals for determination of radioactivity. Total radiolabel eluting under each of the peaks was used to calculate the amount of parent compound or metabolite.

#### *Preparation and mass spectral analysis of plasma metabolites*

Pooled plasma samples from three animals treated intravenously with unlabeled PD were extracted as described above. Metabolites and parent compound were separated by semipreparative HPLC using a Ranin Dynamax column ( $250 \times 21.2 \text{ mm}^2$ ,  $8 \mu\text{m}$ ). Identical solvent compositions to those used for the analytical studies were utilized at a flow rate of 11.25 ml/min. Two metabolites, eluting at 70 and 85 min were collected, solvent was removed by lyophilization and samples were redissolved in a minimal amount of 50:50 acetonitrile/water for analysis by mass spectrometry. Mass spectral analysis of two PD metabolites and their product spectra was performed on a Finnigan LCQ ion-trap mass spectrometer (Thermo-Quest–Finnigan, San Jose, CA) using a standard API interface in positive mode with a source voltage of 4.25 kV, a capillary voltage of 38.28 V and trap DC offset of  $-10.32 \text{ V}$ . The samples were introduced into the ESI source *via* loop injection of 10  $\mu\text{l}$  sample solution with an

overall flow of 100  $\mu\text{l}/\text{min}$  of 50:50 acetonitrile/water delivered by an ABI 140B solvent delivery system.

#### *Isolation of metabolites in urine by HPLC and structural confirmation by mass spectrometry*

Urine from animals treated with labeled PD was used to examine the recovery of metabolites in the urine and to establish appropriate extraction procedures for analysis. For mass spectrometry, urine samples from mice treated with unlabeled PD were used. Frozen urine was thawed, centrifuged at 10 000g for 20 min at 4°C to remove sediment, and an aliquot was removed for determination of radioactivity. The remaining urine sample was acidified to pH 2.0 with 0.01 M  $\text{H}_2\text{SO}_4$ . Samples were extracted 3 times with two volumes of water-saturated ethyl acetate, and the ethyl acetate extracts were combined. Aliquots of the ethyl acetate and aqueous layers were counted. Ninety percent of the total radioactivity was present in the ethyl acetate extract and the remaining 10% in the aqueous phase. The remaining ethyl acetate extract was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and evaporated to dryness under vacuum. The residue was redissolved in HPLC mobile phase for separation on a Ranin Dynamax  $\text{C}_{18}$  semi-preparative HPLC column ( $21.4 \times 25 \text{ cm}^2$ , 8  $\mu\text{m}$  packing) using 0.05% trifluoroacetic acid (TFA) in  $\text{H}_2\text{O}$  (solvent A) and 0.05% TFA in acetonitrile/ $\text{H}_2\text{O}$ /40% acetonitrile (solvent B). Metabolites were separated using a linear gradient from 100% solvent A to 100% solvent B over total of 100 min. The flow rate was 11.25 ml/min. Fractions were collected directly into scintillation vials at 0.6 min increments and 500  $\mu\text{l}$  samples were counted in a liquid scintillation counter. Two metabolites, separated by HPLC and analyzed by mass spectrometry, were found to be identical to those isolated from the plasma.

#### *Data analysis*

The presence of the radiolabeled compound in the plasma and in various tissues was determined over a period of 4 h following i.v. administration of [ $^{14}\text{C}$ ]-PD. The pharmacokinetic analysis of plasma concentration–time data for parent compound was conducted using Win-

NonLin version 3 (Pharsight Corp., Cary, NC). Unweighted data were analyzed using both compartmental and non-compartmental models. For non-compartmental models, the last four time points were used to establish  $\lambda_z$ . All data are presented as mean  $\pm$  S.D. for values obtained from 4 animals at each time point and 3 animals for recovery of radioactivity in urine.

## Results

### *Pharmacokinetics*

Plasma levels of PD fell rapidly in a biexponential fashion (Figure 2A) with an apparent terminal half-life of 8.6 min and a mean residence time of 6.3 min (Table 1). Clearance values, calculated by compartmental model and statistical moment analyses (Table 1) agree, and are consistent with rapid drug disappearance. The volume of distribution at steady-state ( $V_{d(ss)}$ ) was 0.71 ml/g indicating moderate extravascular distribution of the drug (Table 1). In addition to PD, three radioactive, UV absorbing peaks were observed in the HPLC analysis of plasma samples (Figure 3). The peaks at 33 and 39 min were identified as the alcohol and the carboxylic acid derivatives of PD based on mass spectrometric analysis (see below). The metabolite eluting at 43 min was not identified. The rapid formation of both metabolites with peak levels observed at 10 min (Figure 2B) is consistent with the rapid decline in plasma levels of parent compound and with the finding that no parent compound was detected in 24 h urine samples. From the limited time points available, it appears that the half-life of metabolites in plasma is very similar to the half-life of the parent compound.

### *Mass spectral identification of metabolites in plasma*

Two metabolites, isolated from plasma samples, were identified by mass spectrometry. The parent and daughter spectra are shown in Figure 4. The mass spectrum of the peak eluting from the HPLC column at 39 min (Figure 3) had an intense signal at  $m/z$  216 Th mass. This signal represents the  $(\text{M}+\text{H})^+$  of a metabolite with a mass of

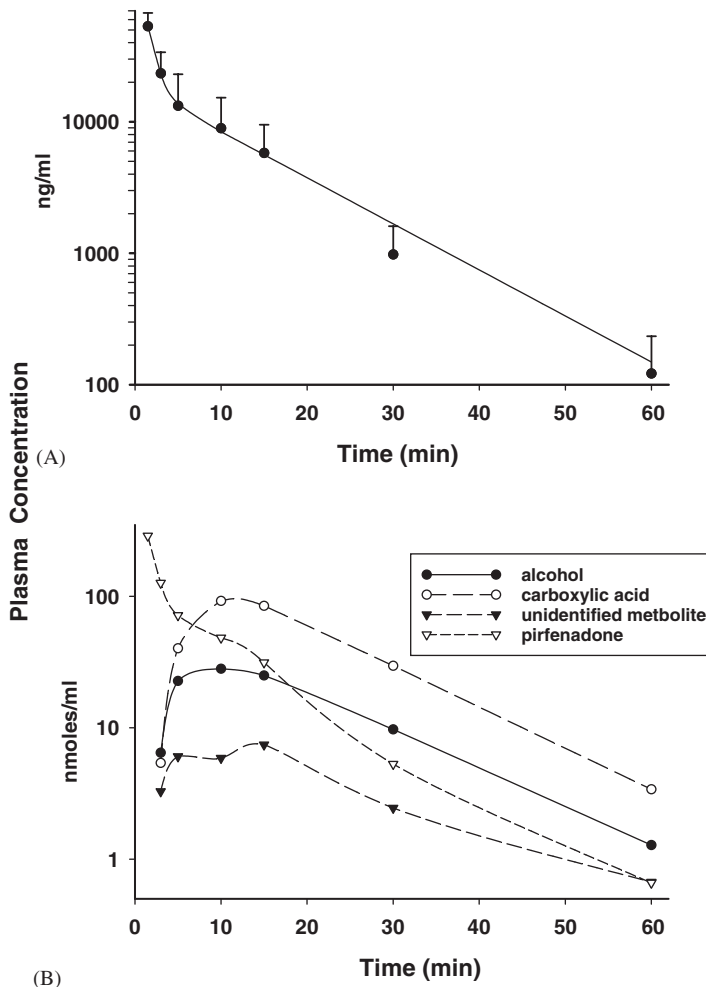


Figure 2. Time course plasma levels of pirfenidone (A) and pirfenidone and metabolites (B) after i.v. administration of <sup>14</sup>C-pirfenidone. Solid circles in A are mean plasma concentrations of parent compound ( $n = 4, \pm$  S.D.). The solid line represents the estimated plasma concentrations according to the WinNonlin best fit. In B ( $n = 4$ ), concentrations (nmoles/ml) are presented for pirfenidone, the alcohol, the carboxylic acid and an unidentified metabolite in plasma at varying times after pirfenidone administration

Table 1. Pharmacokinetic parameters of pirfenidone in mice after i.v. administration

Pharmacokinetic term	
<i>Two-compartment model</i>	
$t_{1/2}$ (min)	8.6
Cl (ml/min/g)	0.10
<i>Statistical moment analysis</i>	
$V_{d(ss)}$ (ml/g)	0.71
Cl (ml/min/g)	0.10
MRT (min)	6.3

215 Da. The product spectrum of the molecular ion of metabolite  $m/z$  216 Th shows a strong transition to  $m/z$  198 Th and  $m/z$  188 Th ions, which occur through the loss of H<sub>2</sub>O and loss of CO from the pyridone ring. The other very intense product ion is the  $m/z$  172 Th ion, which may form via loss of 44 Da (CO<sub>2</sub>). This loss could only occur with a terminal carboxyl group on the molecule. Additional evidence for the presence of the carboxylic acid moiety is the signal at  $m/z$  170 Th which is due to a loss of 46 Da (HCOOH) (Figure 4A).

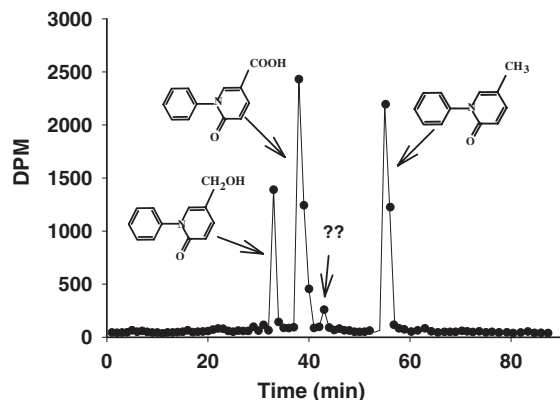


Figure 3. HPLC radioprofile of an aliquot of plasma from mice 10 min after i.v. administration of  $^{14}\text{C}$ -pirfenidone ( $2\ \mu\text{Ci}/\text{mouse}$ )

The peak eluting at 34 min (Figure 3) had an intense signal at  $m/z$  202 Th (Figure 4B) that represents the  $(\text{M}+\text{H})^+$  ion of a metabolite with mass of 201 Da. The product spectrum of molecular ion of this metabolite ( $m/z$  202 Th) shows a strong transition to  $m/z$  172 Th ion that could occur via loss of  $\text{HCHO}$  from the pyridone ring. It is highly probable that this process occurs from the terminal  $-\text{CH}_2\text{OH}$  group with the rearrangement of one H atom. The other very intense product ion is the  $m/z$  157 Th ion. This loss of 45 Da must contain the N of the pyridone ring.

#### *Tissue distribution of radioactivity*

In most tissues, the peak radioactivity occurred within 5 min, and peak levels correlated well with the degree of tissue perfusion. The levels, in descending order, ranked as: kidneys, liver, ventricle, lung, spleen, pancreas, testes, GI, brain, skeletal muscle, adrenal glands, and epididymal fat pad (Figure 5). Similar to plasma, the  $[^{14}\text{C}]$ -PD radioactivity also disappeared quickly from tissues following apparent monoexponential kinetics with half-lives ranging from 9.8 to 23.2 min (Table 2).

#### *Recovery of radioactivity in the urine and identification of major urinary metabolites*

A total of  $96.3 \pm 3.4$  (S.D.) % of the administered dose of  $[^{14}\text{C}]$ -PD was recovered in the 24 h urine samples. After acidification of the urine, 90% of

the radioactivity was extracted into ethyl acetate. Of the ethyl acetate extractable radioactivity, nearly 85% chromatographed as two, nearly equal peaks eluting from 82 to 105 min from a semi-preparative HPLC column. Parent compound was not detected in the urine. Preparative isolation of the two closely eluting peaks followed by mass spectral analysis confirmed the identity of the alcohol and the carboxylic acid metabolites of PD in urine as found in plasma. Thus, it appears that the major routes of metabolism PD involve oxidation to generate an alcohol followed by oxidation to the carboxylic acid. The lack of evidence of the intermediate aldehyde probably results from the high biological reactivity of this product.

## Discussion

The current work has shown that the parent compound and/or its metabolites are rapidly distributed to peripheral tissues, in many cases reaching peak concentrations within 5 min after i.v. administration. In addition, the relative concentrations of radioactivity are highest in well-perfused tissues. This is consistent with the compound's ability to reach concentrations in heart, lung, and kidneys sufficient to decrease tissue fibrosis. The high plasma clearance, short plasma half-life of parent compound (8.6 min), short tissue half-lives of parent and metabolites (9.8–23.2 min), and rapid appearance of two major oxidative metabolites in the plasma are all consistent with the view that the parent PD is rapidly and completely metabolized. These data, along with the finding that no parent compound was detected in 24 h urine samples, suggest that metabolism is the primary mechanism of drug clearance.

The fast elimination of the radiolabeled PD from plasma was consistent with the recovery of almost all administered radioactivity in urine in 24 h. The analysis of ethyl acetate extracts of acidified urine by HPLC revealed that 90% of the radioactivity was associated with two major metabolites and 10% with the aqueous phase and none with the parent compound. Two major metabolites, purified by HPLC, were later identi-

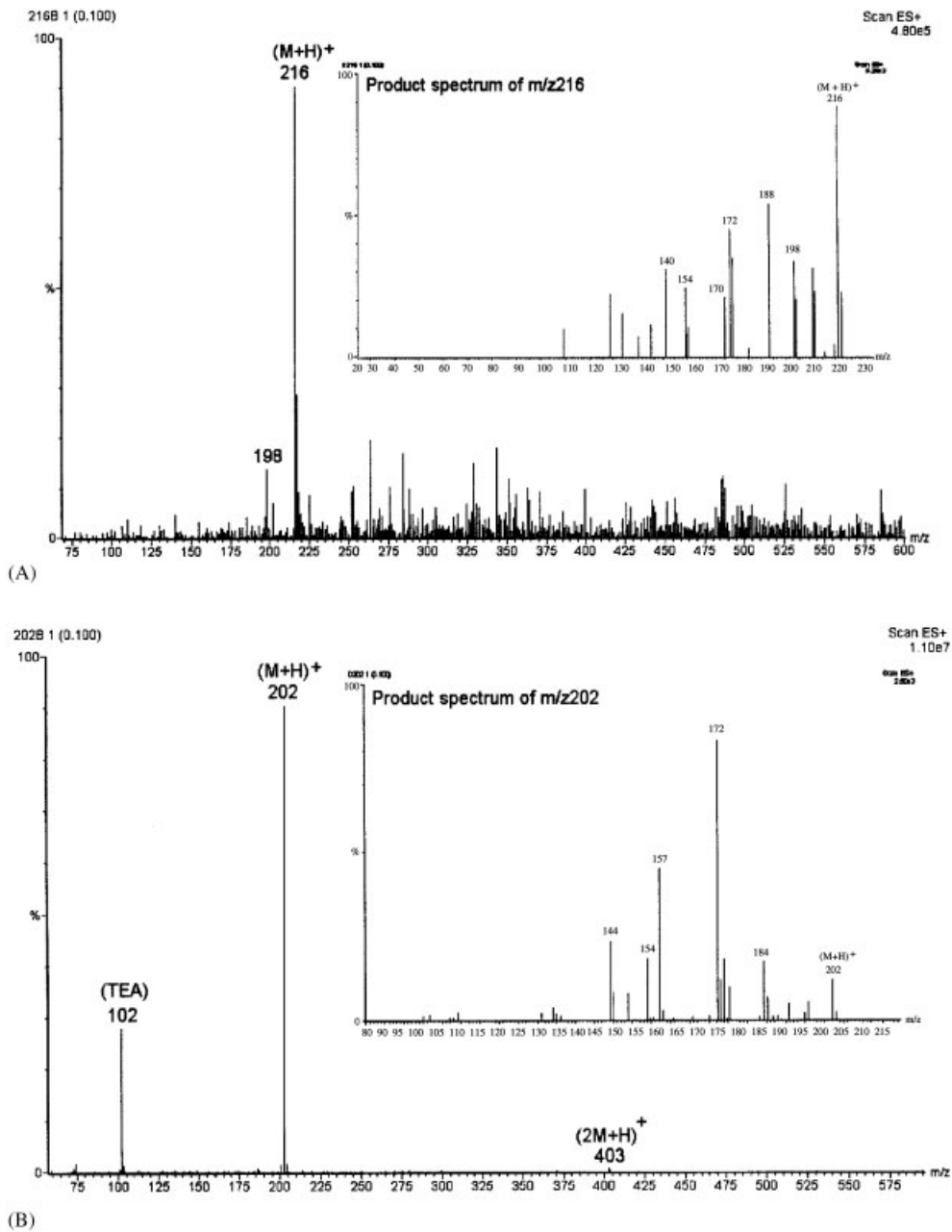


Figure 4. Representative mass spectrum of the metabolite eluting from the HPLC column at 39 min (A) or at 34 min (B). Insets: product ion spectrum of the metabolite molecular ion  $(M+H)^+$  used for identification of metabolites

fied by mass spectrometry. Metabolism appears to involve oxidation of the methyl group on the pyridone ring followed by subsequent formation of the carboxylic acid. The remaining 10% of the radioactivity in the aqueous phase is possibly

associated with glucuronide/sulfate conjugates generated with the functional groups present in the alcoholic and/or carboxylic acid moieties.

In summary, the rapid metabolism and clearance of PD in mice clearly underscores the need

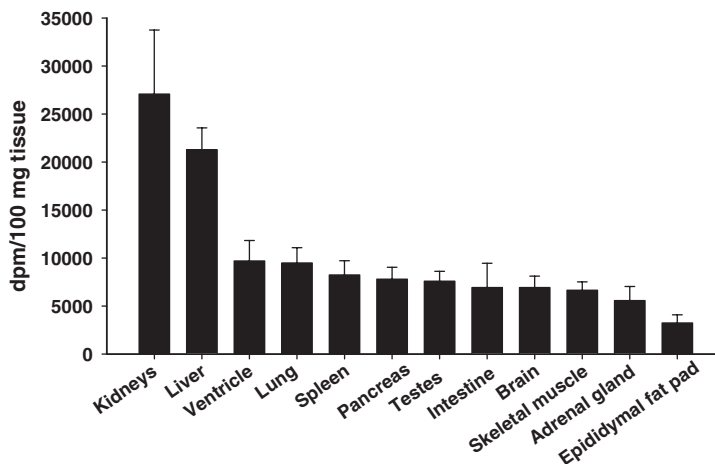


Figure 5. Tissue distribution of pirfenidone and metabolites 5 min after i.v. administration of  $^{14}\text{C}$ -pirfenidone. Values are the mean  $\pm$  S.D. obtained from 4 animals

Table 2. Tissue half-lives of radioactivity after i.v. administration of [ $^{14}\text{C}$ ]-PD<sup>a</sup>

Tissue	Half-life (min)
	Mean $\pm$ S.D. 4 mice
Kidneys	23.2 $\pm$ 2.1
Liver	17.0 $\pm$ 1.5
Ventricle	11.2 $\pm$ 1.2
Lung	12.6 $\pm$ 1.3
Spleen	13.3 $\pm$ 1.5
Pancreas	11.7 $\pm$ 1.4
Testes	14.4 $\pm$ 1.7
GI	19.8 $\pm$ 2.2
Cortex	11.3 $\pm$ 1.4
Skeletal muscle	9.8 $\pm$ 1.2
Adrenal glands	13.3 $\pm$ 1.6
Epididymal fat pad	14.1 $\pm$ 1.3
Red blood cells	13.9 $\pm$ 1.5

<sup>a</sup> Tissues were obtained 5, 10, 15, 30, 60, and 120 min after dosing.

for appropriate kinetic studies in humans. If the metabolism of pirfenadone is as rapid in humans as was observed in mice, the current 6 h oral dosing interval in humans may be suboptimal. Either modifications of the parent compound to slow oxidation of the methylene carbon [15] or preparation of a slow release formulation are approaches that may be useful in extending the time for therapeutically effective drug levels and eliminates the need for frequent dosing.

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