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ORIGINAL RESEARCH

Metabolism and Mass Balance in Rats Following Oral Administration of the Novel Antifibrotic Drug Fluorofenidone

Wei Wu[1], Ze-neng Cheng[2]

[1]Department of Pharmacy, The First Hospital of Changsha, Changsha, Hunan, People's Republic of China; [2]Xiangya School of Pharmaceutical Sciences, Central South University, Changsha, Hunan, People's Republic of China

Correspondence: Ze-neng Cheng, Xiangya School of Pharmaceutical Sciences, Central South University, No. 172 Tongzipo Road, Yuelu District, Changsha, 410013, Hunan, People's Republic of China, Tel +86-731-82650001, Email cheng15279817797@163.com

Objective: Fluorofenidone (AKF-PD) is a novel antifibrotic small-molecule compound. The purpose of this study was to investigate the metabolic and excretory pathways of AKF-PD in rats.

Methods: High-performance liquid chromatography with mass spectrometric (HPLC-MS) detection was used to analyze the metabolites in rat urine. The metabolites were separated by chromatography and their structure was confirmed. HPLC was used to determine the contents of the parent compound and its metabolites in feces and urine after quantitative administration to study the excretion pathway.

Results: AKF-PD was mainly oxidized to the carboxyl group after methyl hydroxylation. After oral administration, the total amount of the prototype drug and its hydroxylated metabolites and carboxylated metabolites excreted from the urine and feces of rats was 87%. However, most of them are excreted in urine and feces in the form of carboxylated metabolites, and rarely excreted in the form of prototype drugs and hydroxylated metabolites. Which is that the urinary discharge of hydroxylated metabolites, fluorine ketones, and carboxylated metabolites were 0.2%, 1.1%, and 75.2%, respectively, while the fecal discharge were 0.2%, 0.3%, and 10.1%, respectively.

Conclusion: AKF-PD is mainly oxidized into 2-hydroxymethyl and 5-carboxyl AKF-PD through the Phase I metabolic reaction in rats. AKF-PD is a highly permeable compound classified by biopharmaceutics and is mainly excreted from the urine in the form of metabolites.

Keywords: fluorofenidone, metabolites, methyl hydroxylation, HPLC-MS

Introduction

Fluorofenidone (AKF-PD) is a novel antifibrotic small-molecule compound[1–3] that is the structural analog of pirfenidone.[4–6] Previous studies of the metabolic pathway of AKF-PD (see Figure 1) have found that there are two main metabolites of AKF-PD in rats:[7–12] methyl hydroxylation (M1) and carboxylated (M2) products of AKF-PD.[13] Based on the structural formula of AKF-PD, combined with the theory of drug metabolism and reference to the metabolic pathway of the homologous compound pirfenidone, a possible metabolic pathway can be predicted (see Figure 1). In the present study, after intragastric administration of AKF-PD to rats, samples of urine and feces were collected for the extraction of metabolites; the metabolite content was then determined in order to study the mass balance of AKF-PD.

Materials and Methods

Chemicals

AKF-PD (batch no.: 070701, content: 99.9%), AKF-PD hydroxylated metabolites (M1, batch no.: 070930, content: 93.0%), AKF-PD carboxylic metabolites (M2, batch no.: 070715, content: 97.8%), and carboxylated pirfenidone (batch no.: 070511, content: 99.8%) were provided by the School of Pharmacy, Central South University. Methanol (batch no.: 707108) and formic acid (batch no.: 409101) were of analytical or high-performance liquid chromatography (HPLC) grade and were

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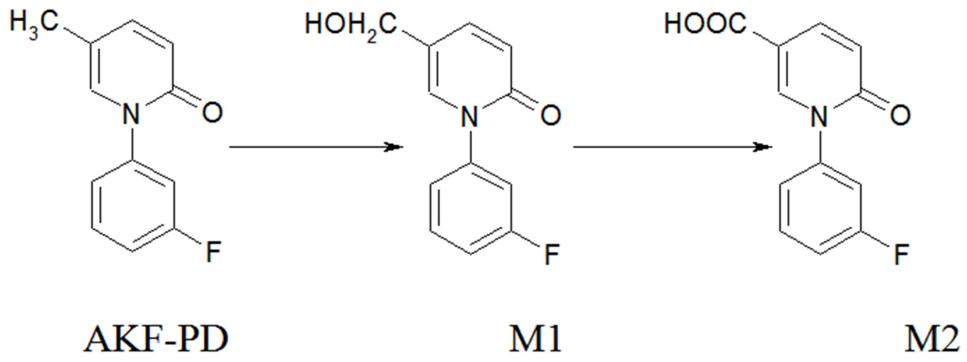

Figure 1 The metabolic pathway of AKF-PD.

supplied by Tedia, USA. Sodium carboxymethyl cellulose (CMC-Na) (batch no.: 031021) was supplied by Shantou Xilong Chemical Factory, China. Purified water was supplied by Hangzhou Wahaha Group Co., Ltd, China.

Experimental Animals

Sprague–Dawley (SD) rats of both genders with a mean weight of 160–200 g were provided by the Department of Experimental Animal Science, Central South University, license no.: SCXK (Hunan) 2006-0002.

Study Design

Fourteen SD rats (seven male and seven female) were placed in metabolic cages for three days. Their feed was ground into powder, and each rat was fed 25 ± 2 g per day and given water freely. Blank urine and fecal samples of SD rats for 24h before administration were collected on the night of the third day, followed by fasting for 12h. On the morning of the fourth day, each rat was given a single gavage (120 mg), and then the urine and fecal samples of rats 24h after administration were collected.

Administration of AKF-PD and the Determination of AKF-PD and Its Hydroxylated Metabolites (M1) in Rat Urine

The AKF-PD was crushed through a 100-mesh sieve, and 0.12 g was accurately weighed and placed into a 5 mL syringe. A total of 2 mL 0.5% CMC-Na aqueous solution was added and mixed with the crushed AKF-PD. After that, the mixture was administered to the rats via gavage, and the remaining part of the syringe was washed out with methanol into a 250 mL volumetric flask (Tables 1 and 2). Water was added to the scale, which was shaken well. A total of 100 μL of this solution was added to 400 μL of internal standard carboxylated pirfenidone acetonitrile solution (5μg/mL) and mixed well. And 10ul mixing was used for the simultaneous detection of AKF-PD and its hydroxylated metabolites (M1) in rat urine samples by HPLC-UV. After that, the detection of the residual amount of AKF-PD in order to calculate an accurate dosage.

Sample Collection

After administration, urine samples at 0–3, 3–6, 6–10, 10–14, 14–24, 24–48, and 48–72 hours and feces samples at 0–24, 24–48, and 48–72 hours were collected. Before each collection, the collection funnel was rinsed with approximately 3 mL of 95% ethanol. After the rinsing solution was mixed with urine, the volume was measured and the urine samples were stored at –20°C. The feces were dried at 40°C for 12 hours and stored at room temperature, and they were weighed before measurement.

Analysis of the Urine and Feces Samples

Determination of AKF-PD and Its Carboxylated Metabolites (M2) in Rat Urine

HPLC-UV Conditions

Chromatographic conditions: HPLC ultraviolet detector (Agilent 1200 with Workstation B.03.01); chromatographic column (Synergi™ 4 μm Hydro-RP 80 Å 250×4.6 mm, Phenomenex); mobile phase: acetonitrile: 0.1% formic acid

Table 1 The Number, Basic Condition and Dosage of Each Tested Animal

Number	Gender	Weight (g)	Dosage	
			g	μmoL
1F	Female	196	0.1202	592.12
2F	Female	187	0.1202	592.12
3F	Female	193	0.1201	591.63
4F	Female	220	0.1194	588.18
5F	Female	209	0.1205	593.60
6F	Female	203	0.1192	587.19
7M	Female	213	0.1195	588.67
8M	Male	205	0.1200	591.13
9M	Male	208	0.1197	589.66
10M	Male	175	0.1201	591.63
11M	Male	274	0.1197	589.66
12M	Male	302	0.1191	586.70
13M	Male	336	0.1194	588.18
14M	Male	330	0.1201	591.63

Table 2 Dosage and Measurement of Residual Flufenidone After Administration

Number	AKF-PD Concentration (μmoL Ł-1)	Dilution Volume (mL)	Residual (μmoL)	Actual Dose (μmoL)
1F	347.98	250	87.00	505.12
2F	377.51	250	94.38	497.74
3F	255.41	500	127.71	463.92
4F	249.62	500	124.81	463.37
5F	333.38	500	166.69	426.91
6F	412.42	500	206.21	380.98
7F	265.76	500	132.88	455.79
1M	499.89	500	249.95	341.19
2M	417.81	250	104.45	485.20
3M	337.80	250	84.45	507.18
4M	458.51	250	114.63	475.03
5M	393.95	250	98.49	488.21
6M	367.45	500	183.73	404.45
7M	474.41	250	118.60	473.02

aqueous solution= 14.5:85.5(v/v) (0–22 mins), acetonitrile: 0.1% formic acid aqueous solution= 90:10 (v/v) (22.1–25 mins), acetonitrile: 0.1% formic acid aqueous solution= 14.5:85.5 (v/v) (25.1–29 mins); flow rate 1.0 mL/min; wavelength 310 nm; injection volume 10 μL.

Sample Pretreatment

A total of 100 μL of rat urine was added to 400 μL of methanol solution of carboxylated pirfenidone (25μg/mL), mixed well, and centrifuged at 15,400 × g for 10 minutes; the supernatant was extracted for injection analysis. Samples with excessive concentrations were diluted before being injected. Dilution method: 100 μL of urine was added to 900 μL of water and mixed well; 100 μL was taken for injection analysis.

Simultaneous Determination of AKF-PD and Its Hydroxylated Metabolites (M1) in Rat Urine

HPLC-UV Conditions

Chromatographic conditions: HPLC ultraviolet detector (Shimadzu LC-2010AHT with LC Solution workstation); chromatographic column (Synergi™ 4 μm Hydro-RP 80 Å 250×4.6 mm, Phenomenex); mobile phase: acetonitrile: 0.1% formic acid aqueous solution = 14.5: 85.5 (v/v) (0–15 mins), acetonitrile: 0.1% formic acid aqueous solution = 40: 60 (v/v) (15.1–22 mins), acetonitrile: 0.1% formic acid aqueous solution = 14.5: 85.5 (v/v) (22.1–26.2 min); flow rate 1.0 mL/min; wavelength 310nm; injection volume 10μL.

Treatment of Samples

A total of 100 μL of rat urine was added to 400 μL of acetonitrile solution (5ug/mL) of carboxylated pirfenidone as an internal standard, mixed well, and centrifuged at 15,400 ×g for 10 minutes. The supernatant was extracted for injection analysis.

Simultaneous Determination of AKF-PD and Its Metabolites (M1 and M2) in Rat Feces

HPLC-UV Conditions

Chromatographic conditions: HPLC ultraviolet detector (Shimadzu LC-2010AHT with LC Solution workstation); chromatographic column (Synergi™ 4 μm Hydro-RP 80 Å 250x4.6 mm, Phenomenex); mobile phase: acetonitrile 0.1% formic acid aqueous solution = 14.5: 85.5(v/v) (0–21 mins), acetonitrile : 0.1% formic acid aqueous solution = 35: 65 (v/v) (21.1–28 mins), acetonitrile : 0.1% formic acid aqueous solution = 14.5: 85.5 (v/v) (28.1–34 mins); flow rate 1.0 mL/min; wavelength 310 nm; injection volume 10 μL.

Treatment of Fecal Samples

The feces samples of rat taken at 24 hours were stored and weighed at room temperature after drying. They were then soaked in 10 mL of methanol for 10 hours and ground in a mortar, before being extracted with a small amount of methanol several times. The extract was transferred to a 25mL volumetric flask, methanol was added, and the flask was shaken well, before the solution was filtered through a 0.45μm microporous membrane. A total of 100μL of internal standard carboxylated pirfenidone acetonitrile (25μg/mL) was added to 1mL of the filtrate and mixed. The residue was dried under nitrogen flow, dissolved in 100μL of 50% methanol aqueous solution, and centrifuged at 15,400 ×g for 10 minutes. The supernatant was taken for sample analysis.

Results

Dosage

Table 3 shows the basic information and administration information of each experimental animal. The actual dose was equal to the dose given with the remaining dose subtracted. After gavage of the rats, the remaining part of the syringe was washed out with methanol into a 250mL or 500mL volume flask, to which water was added. The flask was shaken well, and 100μL of the solution was added to 400ul of internal standard carboxylated pirfenidone acetonitrile solution (5μg/mL) and mixed well. AKF-PD and its hydroxylated metabolites (M1) were simultaneously detected with 10ul of the mixture through the HPLC-UV. After that, the detection of the residual amount of AKF-PD would be detected.

Determination of AKF-PD and Its Metabolites in the Urine and Feces of Rats

The content detection results for M1, M2, and AKF-PD in the rat urine and feces samples are shown in Table 3. Flufenidone was mainly excreted in the form of carboxylated metabolites (M2) in rats urin and feces, mainly through kidneys. And the recovery rate of the drug was about 87.07%. There was no statistical difference in total excretion and carboxylated metabolites (M2) between male and female rats (Figure 2).

Table 3 The Content and Total Recovery Rate of AKF-PD and Its Metabolites M1 and M2

Animal Number	The Amount of Excretion in Rat Urine (μmoL)			The Amount of Excretion in Rat Feces (μmoL)			Total Excretion (μmoL)	Dosage (μmoL)	Recovery Rate %
	M2	AKF-PD	M1	M2	AKF-PD	M1			
1F	364.15	0.37	2.02	67.00	0.74	0.95	435.24	505.12	86.17
2F	386.42	1.02	3.83	49.33	1.54	1.32	443.45	497.74	89.09
3F	340.19	0.36	2.86	29.38	0.60	1.05	374.44	463.92	80.71
4F	351.31	0.88	4.21	10.77	0.95	0.83	368.94	463.37	79.62
5F	342.83	2.22	5.81	10.83	1.16	0.73	363.58	426.91	85.17
6F	308.95	0.34	3.11	31.85	1.20	0.98	346.43	380.98	90.93
7F	347.46	0.37	4.15	50.29	0.84	1.50	404.61	455.79	88.77
1M	263.24	0.74	5.34	20.59	0.30	0.55	290.76	341.19	85.22
2M	362.05	1.63	6.61	14.98	1.63	0.61	387.51	485.20	79.87
3M	379.96	0.54	3.48	53.80	0.92	0.90	439.60	507.18	86.68
4M	344.76	1.14	6.51	80.07	2.51	2.26	437.25	475.03	92.05
5M	330.87	0.75	7.97	100.93	1.56	2.51	444.60	488.21	91.07
6M	299.40	0.45	4.92	79.42	0.84	1.00	386.03	404.45	95.45
7M	354.53	0.38	7.16	53.46	0.64	1.00	417.18	473.02	88.19
Mean	341.15	0.80	4.86	46.62	1.10	1.16	395.69	454.87	87.07
SD	32.60	0.56	1.77	28.34	0.56	0.58	44.92	49.40	4.72

Material Balance

Total excretion was calculated as the total amount of AKF-PD and its metabolites M1 and M2 excreted in the urine and feces. Total excretion divided by actual dose and multiplied by 100% was the total recovery. The calculation results are shown in Table 3. The urinary and fecal excretion rates of AKF-PD and its main metabolites M1 and M2 are shown in Table 4.

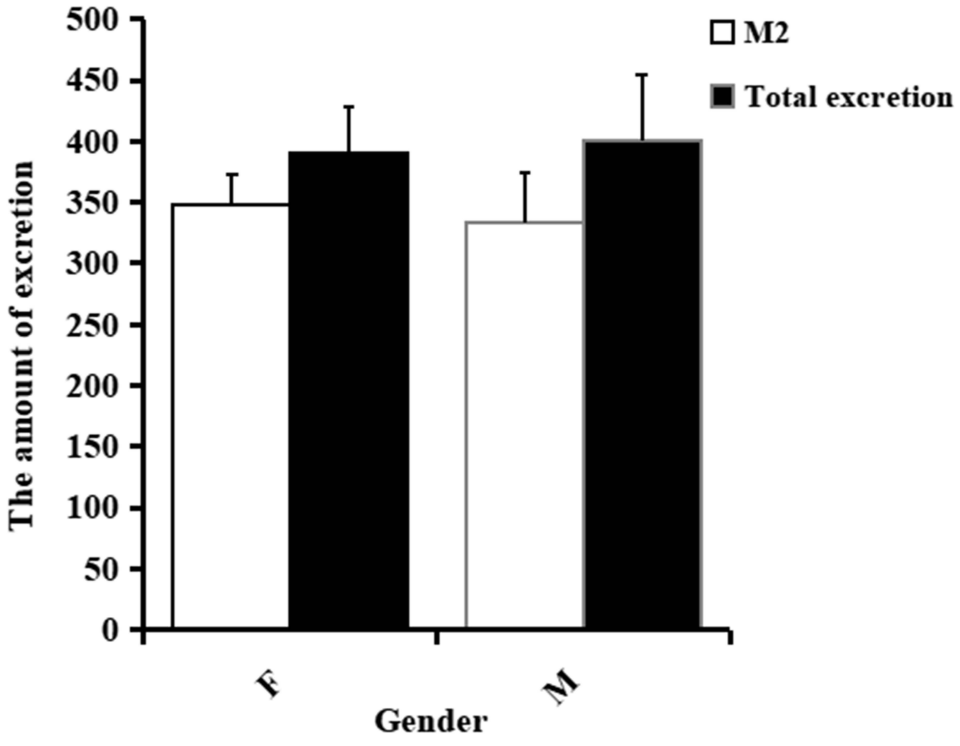

Figure 2 The amount of excretion in rat urine (μmoL) the total excretion of rats.

Table 4 Excretion Rates (%) of AKF-PD and Its Metabolites M1 and M2 in Urine and Feces of Rats

Animal Number	The Amount of Excretion in Rat Urine (μmoL)			The Amount of Excretion in Rat Feces (μmoL)			Recovery Rate %
	M2	AKF-PD	M1	M2	AKF-PD	M1	
1F	72.09	0.07	0.40	13.26	0.15	0.19	86.17
2F	77.64	0.21	0.77	9.91	0.31	0.27	89.09
3F	73.33	0.08	0.62	6.33	0.13	0.23	80.71
4F	75.82	0.19	0.91	2.32	0.21	0.18	79.62
5F	80.31	0.52	1.36	2.54	0.27	0.17	85.17
6F	81.09	0.09	0.82	8.36	0.32	0.26	90.93
7F	76.23	0.08	0.91	11.03	0.18	0.33	88.77
1M	77.15	0.22	1.57	6.04	0.09	0.16	85.22
2M	74.62	0.34	1.36	3.09	0.34	0.13	79.87
3M	74.92	0.11	0.69	10.61	0.18	0.18	86.68
4M	72.58	0.24	1.37	16.86	0.53	0.48	92.05
5M	67.77	0.15	1.63	20.67	0.32	0.51	91.07
6M	74.03	0.11	1.22	19.64	0.21	0.25	95.45
7M	74.95	0.08	1.51	11.30	0.14	0.21	88.20
Mean	75.18	0.18	1.08	10.14	0.24	0.25	87.07
SD	3.39	0.13	0.40	5.96	0.12	0.12	4.72

Discussion and Conclusion

AKF-PD is a pyridone compound, which is a newly discovered anti-organ or tissue fibrosis drug. It has a good effect on various organ fibrotic diseases such as liver fibrosis, kidney fibrosis and so on. However, there are few reports on the AKF-PD, metabolites of AKF-PD and the quality control methods of their preparations by combining animal experiments with HPLC-MS at home and abroad. In this paper, experiment combined with HPLC-MS was established to determine the content of AKF-PD in rats after administration. The method was simple, specific, good separation and accurate, it can effectively study the effects of AKF-PD on metabolism and excretion pathway in rats.

After the rats were given AKF-PD intragastrically, 98% of the total excretion was excreted in the form of carboxylated metabolites and there were few prototype drugs and hydroxylated metabolites from the results that combines animal experiments with HPLC-MS. And the main excretion route was kidney excretion, with bile excretion only accounting for approximately 11% of the total excretion. Which was no significant difference between male and female rats. The total recovery rate in this study was 87.07%, below 90%, but the prototype drug excreted from the stool was 0.3% below the dose. In addition, AKF-PD belongs to a compound with high permeability and liposolubility, which is mainly oxidized into 2-hydroxymethyl and 5-carboxyl AKF-PD through the phase I metabolic reaction in rats. Therefore, the influence of liver drug enzyme activity on drugs should be further considered.

The metabolism and excretion mechanism of AKF-PD in rats was studied by substance balance and corresponding mechanism, in order to provide theoretical reference for further study of AKF-PD.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Ethics Approval

Institutional ethical issues: The study protocol for animal experiments was approved by the Institutional Animal Care and Use Committee of Xiangya School of Pharmeceutical Science, Central South University and conformed to the guidelines of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Every effort was made to minimize their pain, suffering, and death.

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Disclosure

The authors report no personal, financial, commercial, or academic conflicts of interest in this work.

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