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Short communication

Determination of AKF-PD in whole blood of rat by HPLC-UV

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Abstract

An HPLC-UV method was developed and validated for the determination of AKF-PD in whole blood of rat. Phenacetin was chosen as the internal standard, and the separation was achieved on a C_{18} column with methanol and 0.02 M phosphate buffer (pH 3.2) as mobile phase. The obtained calibration graphs were linear ($r = 0.9999$, $n = 9$) in the range of 0.203–52.0 μg ml^{-1}. The low limit of quantitation was 0.203 μg ml^{-1}. This method can be used to study the pharmacokinetics of AKF-PD in rat.

Keywords: AKF-PD; HPLC; Pharmacokinetics

1. Introduction

AKF-PD, 5-methyl-1-(3-fluorophenyl)-2-[1H]-pyridone, which has been synthesized by the Department of Pharmacochemistry, School of Pharmaceutical Sciences, Central South University, has the similar chemical structure with pirfenidone (PFD) (Fig. 1). As a novel antifibrotic drug, phase II clinical trials of PFD has been finished and phase III clinical trials will be started in March 2006. The antifibrotic effect of AKF-PD has been demonstrated in several animal models and cellular experiments. The finding indicated that the antifibrotic activity of AKF-PD was stronger than that of PFD while the toxicity of AKF-PD was poorer. State Food and Drug Administration, PR China has licensed AKF-PD to be used as new antifibrotic drug. Unfortunately, there was no reported method for analysis of AKF-PD in animals' blood. Therefore, the objective of this study was to develop a simple HPLC-UV method for AKF-PD.

2. Experiment

2.1. Chemicals and reagents

AKF-PD standard (purity: 99.38%) was synthesized by the Department of Pharmacochemistry, School of Pharmaceutical Sciences, Central South University. Phenacetin (purity: >99%) was purchased from Sigma (Missouri, USA). Acetonitrile and methanol were HPLC grade (Tedra, USA). Water was distilled, and zinc sulfate heptahydrate, potassium dihydrogen phosphate, phosphoric acid were analytical grade.

Stock solutions of AKF-PD (1 mg ml^{-1}) and phenacetin (315.6 μg ml^{-1}) were prepared by dissolving in water or acetonitrile, respectively. The solution of zinc sulfate (0.2 M) was dissolved in water. The standard solutions for calibration were prepared by diluting stock solution of AKF-PD with water. All the solutions were stored at 4 °C.

2.2. High-performance liquid chromatography (HPLC)

2.2.1. HPLC conditions

Shimadzu 2010C_{HT} HPLC system with an SPD-M10A photodiode array detector (Shimadzu, Kyoto, Japan) was used in this study. The separations were performed on a Thermo C_{18} column (150 mm × 4.6 mm, 5 μm, Hypersil Gold) (Thermo Electron, USA) with phosphate buffer (0.02 M, pH 3.2)–methanol (60:40 v/v) being mobile phase. The flow rate was 1.0 ml min^{-1} and the detector wavelength was 310 nm. A 20-μl volume was injected onto the LC-column [1].

2.2.2. Sample preparations for HPLC

Fifty microliters of whole blood, together with 50 μl zinc sulfate solution and 200 μl acetonitrile (includes phenacetin

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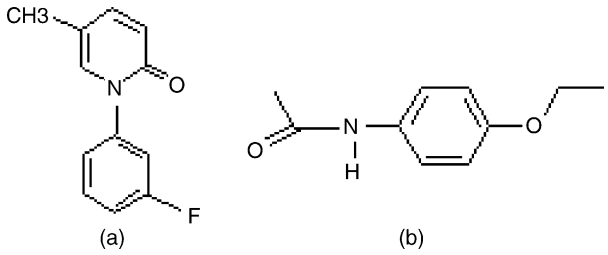

Fig. 1. Chemical structures of AKF-PD (a) and phenacetin (b).

315.6 μg ml^{-1}) were added to polypropylene tubes. After vortex mixed for 30 s, all the samples were centrifuged for 10 min at 27,000 × *g* at room temperature. Twenty-microliter aliquot of the supernatant of each sample was injected into the system.

2.3. Calibration graphs

A standard curve was obtained by adding different concentration of AKF-PD standard solutions into blank whole blood to get nine different concentrations (52.0, 26.0, 13.0, 6.5, 3.25, 1.625, 0.812, 0.406 and 0.203 μg ml^{-1}). Calibration curves ($y = ax + b$) were represented by plotting the peak area ratios (y) of AKF-PD to internal standard versus the concentrations (x) of the calibration standards.

2.4. Quality control samples

Quality control samples were prepared at three different concentration levels, the lowest (0.203 μg ml^{-1}), near the middle (1.625 μg ml^{-1}) and the higher (13.0 μg ml^{-1}).

The stability of AKF-PD was investigated during one sample run in the autosampler at room temperature after 48 h of reconstitution following sample preparation. Stability of AKF-PD through three freeze–thaw cycles (the interval is 5 days) was also investigated.

3. Results

3.1. Chromatography

The retention time of AKF-PD and IS were 9.1 and 7.2 min, respectively. Typical chromatograms are shown in Fig. 2. These profiles showed no endogenous interferences were observed.

3.2. Calibration graphs

The regression equation for calibration curves at the range of 0.203–52.0 μg ml^{-1} was $y = 50.3917x - 0.0799$ ($r = 0.9999$, $n = 9$) for AKF-PD, indicating a good linearity. The analyte response at the LLOQ (the lower limit of quantitation) should be at least five times the response of blank baseline and the R.S.D. ≤20%, as we found that the LLOQ of AKF-PD in whole blood was 0.203 μg ml^{-1}. The analyte response at the limit of detection (LOD) should be reliably differentiated from background noise. The LOD of AKF-PD was 0.05 μg ml^{-1}.

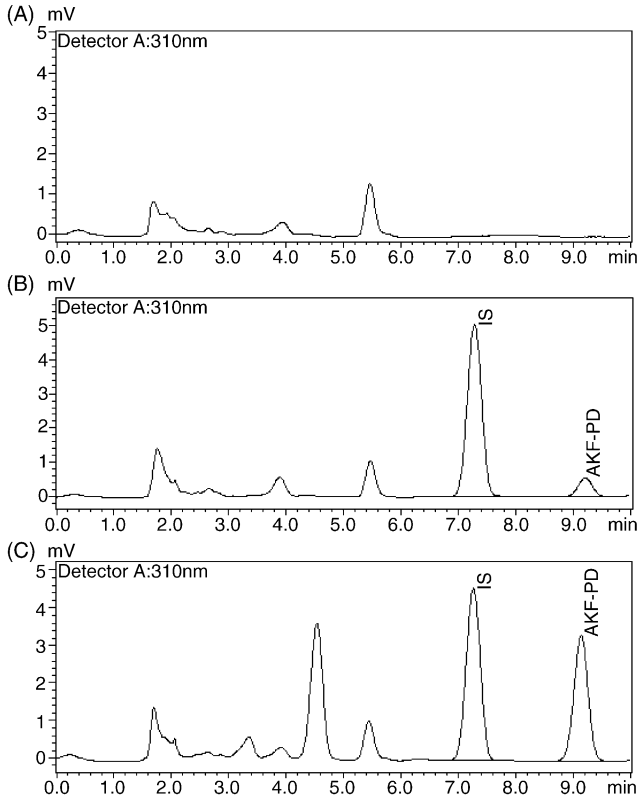

Fig. 2. HPLC chromatograms of blank (A), blank with IS (phenacetin, 315.6 μg mL^{-1}) and AEF-PD (1.625 μg mL^{-1}) (B), sample with IS (C) AKF-PD: 10.040 μg mL^{-1}) under the wavelength of 310 nm.

3.3. Precision and accuracy

The precision of the method obtained by QC samples was evaluated by the inter- and intra-day ($n = 6$) assays at three different concentrations of AKF-PD. The repeatability for inter-day and intra-day was 1.0–3.7% R.S.D. The accuracy of the method obtained by QC samples was studied by calculating the mean recovery of the target compound by adding standards known concentrations to the samples. As a conclusion, the mean recovery for AKF-PD was 97.9–109.5%. As listed in Table 1, the precision and accuracy of the method met the acceptable criteria.

Table 1
Inter- and intra-day precision and accuracy of the method for determination of AKF-PD in whole blood of rat ($n = 6$)

Added (μg mL^{-1})	Intra-day		Inter-day	
	Precision[a] R.S.D. (%)	Mean accuracy[b] (%)	Precision[a] R.S.D. (%)	Mean accuracy[b] (%)
0.203	3.4	109.5	3.4	103.8
1.625	1.0	102.7	2.2	102.3
13.000	1.6	97.9	1.0	99.0

[a] Expressed as relative standard derivation.
[b] Expressed as [(mean observed concentrations/nominal concevtrations) × 100] ($n = 6$).

Table 2
Stability of AKF-PD in plasma QC samples

Added ($\mu g\,mL^{-1}$)	Short-term stability (48 h)		Freeze–thaw stability (3 cycles)	
	Mean ± S.D	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)
0.203	0.197 ± 0.008	4.00	0.198 ± 0.005	2.70
1.625	1.684 ± 0.030	1.80	1.667 ± 0.036	2.20
13.000	12.977 ± 0.132	1.00	12.966 ± 0.061	0.50

$n = 6$ for short-term stability; $n = 4$ for freeze–thaw stability.

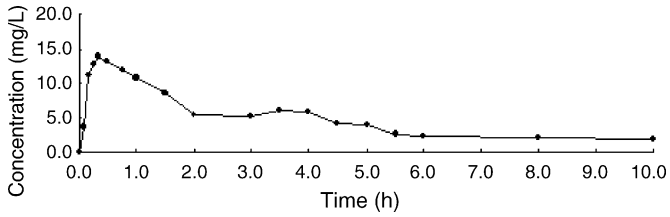

Fig. 3. Concentration–time profiles of AKF-PD after AKF-PD suspension ($50\,mg\,mL^{-1}$) was intragastrically administered to one Sprague-Dawley rat (218 g, female, dosage $500\,mg\,kg^{-1}$).

3.4. Stability

The stock solutions of AKF-PD and phenacetin were discovered to be stable at 4 °C for at least 1 month (difference between fresh and test solutions, AKF-PD: 4.6%, phenacetin: 0.6%). The stability of AKE-PD at −20 °C in the whole blood was also assessed in QC samples after storage at room temperature for 48 h, after three freeze and thaw cycles. The stability data of AKF-PD are displayed in Table 2. As Table 2 listed, AKF-PD was very stable under these conditions.

3.5. Application of the method

The described method was applied to animal experiment [2]. Typical plasma concentration–time profiles of AKF-PD after intragastric administrating AKF-PD suspension ($50\,mg\,ml^{-1}$) to one Sprague-Dawley rat (218 g, female, dosage $500\,mg\,kg^{-1}$) were shown in Fig. 3.

4. Discussion

The samples were deproteinized by acetonitrile and only 50 μl of whole blood was needed for analysis. This simplified sample preparation procedure is very suitable for pharmacokinetics study of AKF-PD in rodents. As this method is very sensitive and no endogenous interferences were observed, it also can be used with human blood. When the administrated dosage is very low, AKF-PD can be extracted by chloroform and ethyl ether.

The retention time of AKF-PD was not affected by pH, but peak shape of AKF-PD was significantly affected by pH. The symmetric peak shape of AKF-PD was improved with lower pH. But there was no difference observed between pH 3.2 and 2.0. There was no difference detected for the retention time and peak shapes of AKF-PD and phenacetin after 600 samples have been loaded onto the column.

5. Conclusions

The reported method is suitable for assessing the pharmacokinetics of AKF-PD in rodents.

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