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High performance liquid chromatographic determination of a new antifungal compound, ADKZ in rat plasma

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Abstract

A high performance liquid chromatography (HPLC) method was developed and validated for the determination of ADKZ (1-(1H-1,2,4-triazole)-2-(2,4-diflurophenyl) -3-[*N*-methyl-*N*-(4-iodo-benzyl)amino]-2-*p*ropanol) in rat plasma. The compound was extracted from plasma samples by liquid–liquid extraction, and an isomeric compound of ADKZ (1-(1H-1,2,4-triazole)-2-(2,4-diflurophenyl)-3-[*N*-methyl-*N*-(3-iodo-benzyl)amino]-2-propanol) was used as the internal standard (IS), which were analyzed on a reversed-phase C18 column (5 μm, 200 mm × 4.6 mm i.d.). The extracted plasma samples were eluted with acetonitrile–0.018M triethylamine solution adjusted to pH 3.2 with phosphoric acid (35:65, v/v). The effluent was monitored by a UV detector at 230 nm. The retention time of ADKZ was 7.1 min and IS 8.2 min. The calibration curves were linear in the concentration range of 0.02–2.00 μg/ml with the correlation coefficients greater than 0.999. The quantification limit of ADKZ in rat plasma was 0.02 μg/ml. Intra- and inter-day precision ranged from 2.6 to 7.9% and 3.1 to 9.6%, respectively. The extraction recovery from plasma was no less than 80%. No endogenous interferences were observed with either ADKZ or IS. The method has been successfully used to support the pre-clinical pharmacokinetic studies of ADKZ in rats.

Keywords: HPLC; Antifungal compound ADKZ; Pharmacokinetics

1. Introduction

Azole antifungal drugs play chief roles in the treatment of fungal infection, which show the advantages of stable metabolism, taken orally or injected. They have good effect both on deep and superficial fungal infections [1]. Azoles, such as fluconazole and itraconazole, act by inhibition of cytochrome P450 lanosterol 14α-demethylase, thereby preventing conversion of lanosterol to ergosterol [2]. But these agents have many drawbacks when used in clinical practice. For example, fluconazole is not active against *filamentous* fungi and emergence of fungal resistance has been reported, while the bioavailability of itraconazole is low [3,4].

Aiming to obtain new compounds with more potent activity, less toxicity and broader spectrum, some compounds were synthesized on the basis of computer-aided drug designing, according to the crystal structure of cytochrome P450 14α-sterol demethylase (CYP51) and the docking results of inhibitors to the active site of the enzyme [5]. One of these compounds is ADKZ [6,7] (1-(1H-1,2,4-triazole)-2-(2,4 -diflurophenyl)-3-[*N*-methyl-*N*-(4-iodo-benzyl) amino]-2-propanol), whose structure is shown in Fig. 1.

Besides the pharmacodynamic evaluation, it is also important to determine the pharmacokinetic properties of ADKZ. An assay for this compound in biological samples will be required to support its pre-clinical studies. Some published papers are concerned with the determination of similar azole drugs such as itraconazole, involving liquid chromatography (LC) with ultraviolet (UV) detection [8] or fluorescence detection [9] and liquid chromatography tandem mass spectrometry (LC–MS–MS) [10]. There is no article related to ADKZ determination. The subject of this paper is to develop a simple and rapid HPLC-UV assay for the determination of ADKZ in rat plasma. The method was validated and then applied to the pre-clinical pharmacokinetic studies of ADKZ in rats.

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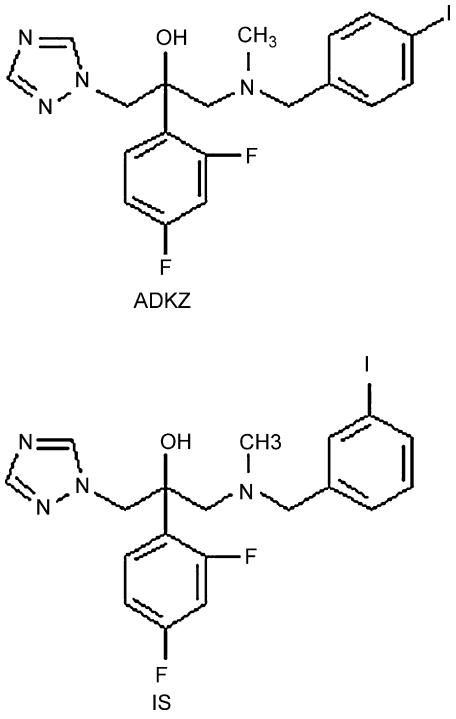

Fig. 1. Structure of ADKZ and the internal standard (IS).

2. Experimental

2.1. Reagents and chemicals

ADKZ and IS (Fig. 1, purity of both ≥98.0%) were synthesized at the laboratory in the Pharmacy School of Second Military Medical University (Shanghai, China). Acetonitrile and *n*-hexane of HPLC grade were purchased from Merck (Darmstadt, Germany). Phosphoric acid and triethylamine of HPLC grade were purchased from Tedia (Fairfield, USA). Water was redistilled in our laboratory. All other reagents were of analytical grade.

2.2. Instrumentation and chromatographic conditions

A Shimadzu HPLC system equipped with LC-10AD VP pump, SIL-10AD VP automated sample injector, CTO-10AS VP thermostatted column compartment and SPD-10A UV–vis detector was used. N2000 workstation (Zhejiang University, Hangzhou, China) was used for data acquisition and mathematical calculations.

The chromatography was performed on a Elite HPLC C18 column (5 μm, 200 mm × 4.6 mm i.d.) supplied by Elite Scientific Instruments Co. (Dalian, China).

For plasma samples, the mobile phase consisted of acetonitrile–0.018M triethylamine solution adjusted to pH 3.2 with phosphoric acid (35:65, v/v) and was delivered at a flow-rate of 1.0 ml/min. The injection volume was 20 μl. The whole chromatographic system was operated at 25 °C. Detection was performed at 230 nm, at the absorbance maximum of ADKZ.

2.3. Preparation of stock solution and validation samples

The ADKZ stock solution was prepared by dissolving 25 mg in methanol to achieve a concentration of 1 mg/ml. Appropriate dilutions of the stock solution were made with distilled water. The internal standard (IS) was prepared at a concentration of 1.824 μg/ml in distilled water. A series of ADKZ standard solutions for plasma were prepared by spiking blank rat plasma with appropriate stock solutions in order to construct the calibration curves. The final concentrations were 0.02, 0.05, 0.10, 0.20, 0.50, 1.00 and 2.00 μg/ml for rat plasma. These standard solutions prepared independently from the calibration standards were also used for the validation of the method.

2.4. Sample preparation

Two hundred microliters aliquot of the plasma sample was accurately measured into a 5 ml stoppered glass tube, followed by the addition of 40 μl IS solution (1.824 μg/ml), 100 μl of 4% sodium hydroxide solution and 2 ml *n*-hexane. The mixture was then vortexed for 3 min and centrifuged at 2891 × *g* for 10 min. The organic layer (1.6 ml) was collected and evaporated to dryness at 45 °C under a gentle stream of nitrogen gas. The residue was reconstituted with 80 μl of the mobile phase and transferred into HPLC auto-injector vials for analysis.

2.5. Validation procedure

2.5.1. Linear range and limit of quantification (LOQ)

The linear equation was assessed by injecting extracted biological samples with known amount of ADKZ continuously. The calibration curves were constructed by plotting the peak area ratios of ADKZ to IS versus the concentrations spiked and generated on 5 consecutive days.

The limit of quantification was defined as the lowest concentration of ADKZ giving a signal to noise of 10:1 with a precision less than 15%.

2.5.2. Precision and accuracy

Validation samples were prepared and analyzed to evaluate the intra-day and inter-day precision and accuracy, which were determined by quantitating five replicates at concentrations of 0.02, 0.20 and 2.00 μg/ml on the same day and 5 consecutive days. Mean, standard deviation and relative standard deviation (R.S.D.) were calculated from validation samples values and used in the estimation of intra- and inter-day precision. Accuracy was assessed by comparison of the calculated mean concentrations to nominal concentrations.

2.5.3. Extraction recovery

The recoveries of ADKZ from plasma were determined by spiked samples at three concentrations, 0.02, 0.20 and 2.00 μg/ml. The extraction recoveries were calculated by comparing mean peak areas of five low, medium and high spiked samples with mean peak areas of five the same amounts of unextracted ADKZ solutions. The internal standard at the concentration of 0.304 μg/ml was determined in the same way.

2.5.4. Stability

Sample stability in plasma was investigated as follows using validation samples. Short-term stability was examined by analyzing samples at room temperature for 24 h. Long-term stability study was performed by analyzing samples stored for 2 months at −20 °C. For freeze-thaw stability study, the samples were left for 1 h to thaw, then refrozen for 24 h. This cycle was repeated three times and analysis was done after the third freeze-thaw cycle. The stability of ADKZ in reconstituted extracts during run-time in the HPLC auto-injector was tested performing a second analysis of the same extracts left 8 h at room temperature.

2.6. Pharmacokinetic studies in rats

Sprague-Dawley rats (200 ± 20 g, male and female) were divided into three groups (60 rats/per group) and received 10, 20 and 40 mg/kg oral doses of ADKZ in a suspension using 0.5% carboxymethyl cellulose after an overnight fast. Animals had access to water and food 4 h after drug administration. Blood samples (3 ml) were collected into heparinized tubes before administration and at different time points (0.083, 0.25, 0.5, 0.75, 1, 1.5,2, 3, 5, 8 and 12 h) after administration (5 rats/time point). Immediately after sampling, the plasma was separated from heparinized blood by centrifugation at 2124 × *g* for 10 min. Plasma (200 μl) samples were prepared as described under Section 2.4.

3. Results and discussion

3.1. Optimization of ADKZ separation in rat plasma

3.1.1. Mobile phase

The simple mobile phase was found to be appropriate for the analysis of ADKZ. The retention time for ADKZ in plasma samples was 7.1 min and IS 8.2 min.

Different ratios of the mobile phase as well as pH and column temperatures were studied in order to shorten the retention time of ADKZ and to avoid the interference of the endogenous substances. It was found that when increasing the pH value, the retention time of ADKZ was prolonged and the resolution from the endogenous substances was improved.

The optimal assay condition for plasma samples were found when using a ratio of 35:65(v/v) for acetonitrile–0.018 M triethylamine, with the pH adjusted to 3.2, and the column temperature at 25 °C.

3.1.2. Choice of internal standard

In the course of the selection of the internal standard other azole drugs such as itraconazole, ketoconazole and fluconazole had been tried. These compounds can be separated from ADKZ with good resolution, but failed partly for the low sensitivity of itraconazole and fluconazole at 230 nm and low extraction recovery of ketoconazole from plasma samples. An isomeric compound of ADKZ (1-(1H-1,2,4-triazole)-2-(2,4- diflurophenyl)-3-[*N*-methyl-*N*-(3-iodo-benzyl)amino]-2-propanol) was finally selected as the internal standard, because the maximum absorbance of this compound was also at 230 nm and it had a suitable retention time. Increasing the pH value, the retention time of IS was also delayed and a good resolution from ADKZ was achieved.

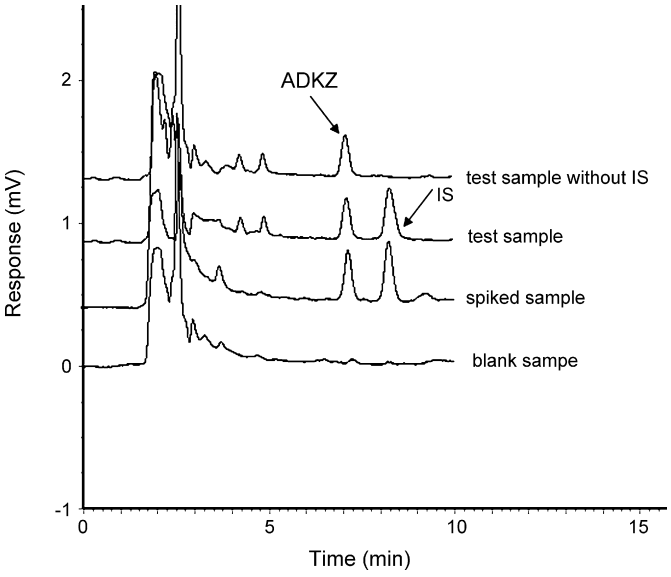

Fig. 2. Representative chromatograms of extracted blank plasma, plasma spiked with 0.200 μg/ml of ADKZ and IS, test plasma spiked with IS at 0.5 h after the dose of 20 mg/kg (concentration 0.384 μg/ml), and test plasma without IS at 0.5 h after the dose of 20 mg/kg.

Typical chromatograms of the blank, spiked samples and test samples with or without IS in plasma are shown in Fig. 2, respectively. The results showed that the chromatograms of blank samples were free from any endogenous substances and metabolites and the chromatograms of the post-dose samples were also free from metabolites at the retention time of IS, thereby confirming the selectivity and specificity of the method.

3.2. Validation

3.2.1. Linearity and LOQ

Calibration curves were performed by the internal standard technique following linear regression analysis by plotting plasma concentrations of ADKZ (0.020–2.004 μg/ml) against peak area ratio of ADKZ to IS. The linear regression equation was $y = 3.7468x + 0.0321$ ($r = 0.9999$). The limit of quantification of ADKZ in plasma was 0.02 μg/ml.

3.2.2. Precision and accuracy

The intra- and inter-day precision data for ADKZ in rat plasma are listed in Table 1. Intra- and inter-day precision ranged from 2.6 to 7.9% and 3.1 to 9.6%, respectively, and accuracy ranged from 97.3 to 103.5%. The results of intra- and inter-day analysis indicated that the method was accurate, reliable and reproducible.

3.2.3. Recovery and stability

In Table 2 are listed the mean extraction recoveries and the coefficients of variation (R.S.D. %) for ADKZ at each concentration, and the recoveries ranged from 83.7 to 85.4%.

The stability was tested by comparing results under different storage conditions with those for validation samples freshly

Table 1
Accuracy and precision of ADKZ in spiked rat plasma ($n = 5$)

Concentration (μg/ml)		Precision (R.S.D.,%)	Accuracy (%)
Spiked	Observed (mean ± S.D.)		
Intra-assay precision			
0.020	0.021 ± 0.002	7.9	103.5
0.200	0.202 ± 0.005	2.6	100.9
2.004	2.041 ± 0.136	6.7	101.9
Inter-assay precision			
0.020	0.021 ± 0.002	9.6	102.5
0.200	0.195 ± 0.006	3.1	97.3
2.004	2.010 ± 0.163	8.1	100.3

Table 2
Extraction recoveries of ADKZ and IS ($n = 5$)

Drug	Concentration (μg/ml)	Recovery (%) (mean ± S.D.)	R.S.D. (%)
ADKZ	0.020	83.7 ± 6.7	8.0
	0.200	84.5 ± 4.1	4.8
IS	2.004	85.4 ± 5.7	6.7
	0.304	89.3 ± 4.7	5.3

Table 3
Stability of ADKZ in rat plasma ($n = 3$)

Concentration (μg/ml)	Mean recovery (%)			
	Short-term stability	Long-term stability	Freeze-thaw cycles	Extract stability
Plasma				
0.020	96.9 ± 3.2	98.5 ± 3.8	97.3 ± 3.5	99.2 ± 2.7
0.200	97.5 ± 2.1	99.2 ± 2.3	97.9 ± 2.8	100.6 ± 1.1
2.004	97.9 ± 1.9	100.2 ± 2.9	98.3 ± 1.8	101.3 ± 1.1

prepared. The results of the stability study are summarized in Table 3. ADKZ were found to be stable in rat plasma samples for at least 24 h when stored at room temperature and for 2 months when stored at −20 °C. The results of three freeze-thaw cycles and stability test in reconstituted extracts also indicated that ADKZ was stable under these conditions.

3.3. Pharmacokinetic analysis

The method described here was successfully applied to the pharmacokinetic studies of ADKZ in rats. Plasma concentration-time profiles of ADKZ following three oral doses in rats were shown in Fig. 3. The peak plasma concentration was 0.182, 0.729 and 0.885 μg/ml, both obtained at 0.75 h and the estimated elimination half-life was 1.2, 1.3 and 1.6 h, for 10, 20 and 40 mg/kg

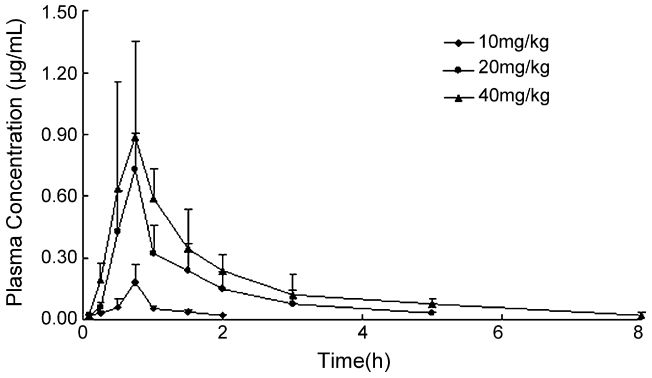

Fig. 3. Mean plasma concentration-time curves of ADKZ following three doses of 10 mg/kg (♦), 20 mg/kg (●) and 40 mg/kg (▲) in rats (5 rats/time point).

doses, respectively. ADKZ was eliminated rapidly from the plasma. Accordingly, plasma concentrations of ADKZ were detectable only up to 2 and 5 h for 10 and 20 mg/kg doses, respectively, in rats. The AUC values were 0.36, 1.18 and 1.47 μg h/ml for 10, 20 and 40 mg/kg, respectively.

4. Conclusion

An HPLC method with UV detection has been developed for quantification of ADKZ in rat plasma samples. The chromatographic condition employed provided good separation of the compound without interfering peaks from endogenous substances. The method was validated and all results were within the acceptable ranges for bio-analytical purposes. This assay is simple, reproducible, accurate and time effective. It has been successfully applied to the pre-clinical pharmacokinetic studies of ADKZ in rats.

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