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Hepatotoxicity and toxicokinetics of ketoconazole in rabbits

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KEY WORDS ketoconazole; hepatotoxicity; pharmacokinetics

ABSTRACT

AIM: To study the relationship between hepatotoxicity and toxicokinetics of ketoconazole in rabbits. **METHODS**: Normal rabbits were given intragastric gavage ketoconazole 40, 80, and 160 mg/kg. Ketoconazole plasma concentrations were measured by high performance liquid chromatography. Toxicokinetic parameters were determined from the plasma concentration-time data with the 3P97 software package. Activities of serum glutamate-pyruvate-transaminase and glutamate-oxalate-transaminase and hepatic histopathological changes were observed at 36 h after administration. The relationship between hepatotoxicity and toxicokinetic parameters of ketoconazole was analyzed by linear correlation. **RESULTS:** The concentration-time curves of three doses of ketoconazole fitted well into a two-compartment model. The proportional increase in the area under the plasma concentration-time curve (AUC) was more than that in the dose after the dose reached 80 mg/kg. Ketoconazole resulted in a marked elevation in the enzyme activities and significant damage of hepatocytes. Hepatotoxicity induced by ketoconazole was correlated to the dose, clearance (CL), maximum plasma concentration (C_{max}), and most closely correlated to AUC when it was assessed by elevation transaminases in serum. **CONCLUSION:** The severity of ketoconazole-induced hepatotoxicity was closely related to the exposure level (AUC) of the drug.

INTRODUCTION

The synthetic imidazole drugs exert their antifungal actions by blocking the conversion of lanosterol or 24-methylene-dihydrolanosterol to ergosterol in fungi. They are prominent oral antifungal agents with broad spectrum used in the treatment of systemic mycoses in clinic. But the drugs may result in liver injury[1-3]. The incidence of hepatotoxicity induced by ketoconazole was the highest among the imidazole antifungals[4], and may even cause death due to severe hepatic necrosis[5-7]. However, there are few studies exploring the relationship between the hepatotoxicity and dose, plasma concentration of ketoconazole. Little is known about how the pharmacokinetic parameters change when liver injury occurs.

Ketoconazole-induced hepatotoxicity is probably not mediated through an immunoallergic mechanism, but might be related to a reactive metabolite[1,8], *N*-deacetyl ketoconazole (DAK). The latter appears to be the major metabolite[9] which is further metabolized by rat hepatic microsomal flavin-containing monooxygenase (FMO)[10]. DAK was more cytotoxic than ketoconazole *in vitro* in rat hepatocytes[11]. Rodriguez and Miranda proved that there was a similar metabolic pathway of ketoconazole in rats and in rabbits or in human by cDNA-expressed human and rabbit FMOs[12]. We speculated that there was a similar hepatotoxicity of ketoconazole in rabbits and human, but there was no

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report about hepatotoxicity of ketoconazole in rabbits *in vivo* up to now, so that we investigated the relationship between hepatotoxicity and toxicokinetics of ketoconazole in rabbits.

MATERIALS AND METHODS

Drugs and reagents Ketoconazole was purchased from Shanghai Zhaohui Pharmaceutical Factory. The internal standard diazepam was a product of Sigma Chemical Co, USA. Methanol of HPLC grade and other reagents of analytical grade were purchased from Wuhu Chemical Reagent Company.

Rabbits New Zealand rabbits of either sex, weighing 2-3 kg were used, and were fasted for 12 h before experiment.

Chromatographic condition The Agilent HPLC 1100 system consisted of G1312A binary pump, G1314 UV detector and G2170 instrument workstation. The analytical column was Agilent Hypersil ODS C_{18} column (5 μm, 250 mm×4.6 mm) with Agilent Zorbax ODS C_{18} precolumn (4.6 mm×12.5 mm). The mobile phase was composed of methanol-water-triethylamine (79.1:19.8:1.1, v:v:v) at pH 6.6. The flow rate was 1.0 mL/min, the column temperature was kept at 30 ° C, and the ultraviolet wavelength was set at 241 nm.

Assay of ketoconazole in plasma An aliquot (0.2 mL) of plasma was transferred to a 15-mL test-tube, diazepam as an internal standard and 3 mL ether was added. Tube was vortex-mixed for 3 min. After centrifugation (500×*g*) for 10 min, the organic phase was transferred to a tapered tube and evaporated to dryness on 50 ° C water bath. The residue was redissolved in 100 mL mobile phase and 20 μL was injected into the chromatograph.

Assay validation Known amounts of ketoconazole were added into blank plasma to achieve the standard samples at 0.5, 1, 2, 5, 10, 20, and 50 mg/L. Replicate samples (n=5) in each concentration were extracted and injected for analysis by HPLC as described above. The peak area ratios of ketoconazole to the internal standard were plotted against concentration of ketoconazole and analyzed by linear regression with weighing coefficient (w=1/c) to generate calibration curve. The limit of detection was defined as the concentration at which the signal-to-noise ratio was 3. The solutions containing known amounts of ketoconazole were compared with spiked plasma standards undergoing analysis to calculate the percent recovery at 0.5, 5, and 50 mg/L. Interday and intraday variability was determined by analyzing ketoconazole standard plasma at 0.5, 5, and 50 mg/L for 5 times in one day or in 5 consecutive days, respectively.

Toxicokinetics study Sixteen rabbits were divided into four groups. Each group, which consisted of 2 males and 2 females, received single intragastric gavage dose of kecotonazole 40, 80, and 160 mg/kg and normal saline, respectively. Blood sample was drawn into a heparinized tube before and at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 12.0, 24.0, and 36.0 h after administration. The plasma was isolated and kept at -20 ° C until analysis. The plasma concentration of ketoconazole was measured as described above.

Assessment of liver function Blood was drawn from rabbits having received normal saline or ketoconazole before administration and at 36.0 h after administration. The activities of serum glutamate-pyruvate-transaminase (sGPT) and glutamate-oxalate-transaminase (sGOT) were measured to assess the liver function.

Histopathological observation Liver sections were taken from one lobe of the organ at 36.0 h after administration of ketoconazole. The tissue was fixed in 10 % neutral-buffered formalin for 12 h, dehydrated with 75 % ethanol, and embedded in paraffin. Sections (5 mm) from the tissue were cut, stained with haematoxylin-eosin, and observed by light microscopy. The morphological changes of hepatocytes were scored: 0=normal, 1=slight cloudy swelling, 2=moderate cloudy swelling, 3=serious cloudy swelling even ballooning degeneration, 4=degeneration and punctate necrosis.

Data analysis Ketoconazole plasma concentration-time data were assessed with the 3P97 software package to determine toxicokinetic parameters. The relationship between toxicokinetic parameters and hepatotoxicity in terms of increase in sGPT and sGOT activities after administration of ketoconazole, as well as morphological changes, was analyzed by least square linear correlation. Data were expressed as mean±SD, The differences between groups and between sexes were tested by two-way analysis of variance using SPSS software. P<0.05 was considered as significant.

RESULS

Quality control of HPLC assay Retention times for internal standard and ketoconazole were 3.80 and 4.92 min, respectively. The endogenous materials in

plasma and the metabolite of ketoconazole did not interfere with either ketoconazole or internal standard peaks. The linearity was obtained over the range of 0.5-50.0 mg/L (r=0.9997). The limit of detection was 0.2 mg/L. Absolute recoveries of ketoconazole were 91.1 %±4.2 %, 96.9 %±5.6 %, and 98.6 %±2.2 % at 0.5, 5, and 50 mg/L, respectively. Interday and intraday variability was 4.89 % and 8.47 % at 0.5 mg/L, 4.66 % and 6.95 % at 5 mg/L, and 2.35 % and 6.48 % at 50.0 mg/L, respectively.

Concentration-time profile The average concentration-time profiles of ketoconazole 40, 80, and 160 mg/kg were well described with two-compartment model (Fig 1). The concentration at 36 h after administration of ketoconazole 40 and 80 mg/kg were below the quantification limit.

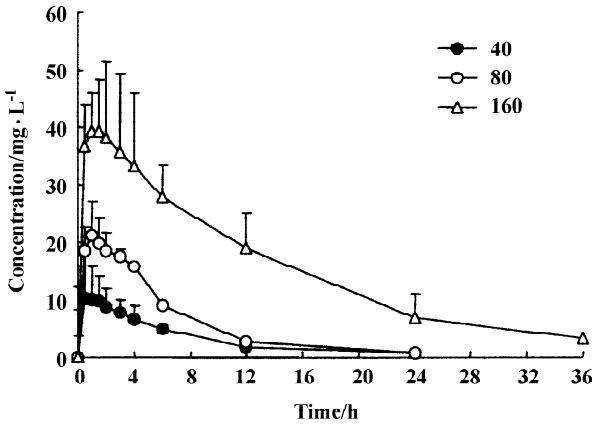

Fig 1. Profiles of mean concentration-time of ketoconazole after intragastric gavage 40, 80, and 160 mg/kg in rabbits. *n*=4. Mean±SD.

Toxicokinetics The toxicokinetic parameters were listed in Tab 1. Significant differences were found between three groups for maximum plasma concentration (C_{max}), the area under the plasma concentration-time curve (both AUC_{0-24} and $AUC_{0-\infty}$) (P<0.01). The proportional increase in AUC_{0-24} and $AUC_{0-\infty}$ was more than that in the dose after the dose reached 80 mg/kg.

Tab 1. Toxicokinetic parameters of ketoconazole after intragastric gavage 40, 80, and 160 mg/kg in rabbits. *n*=4. Mean±SD. ^{c}P<0.01 *vs* 160 mg/kg.

Parameter	40 mg/kg	80 mg/kg	160 mg/kg
$T_{1/2\alpha}$/h	1.5±1.0	2.0±0.8	1.3±0.8
$T_{1/2\beta}$/h	7.1±1.8	6.4±1.0	10±5
$T_{1/2(Ka)}$/h	0.5±0.4	0.47±0.29	0.6±0.3
V_1/L· kg^{-1}	3.2±2.0	2.7±1.1	2.6±0.3
CL/L· h^{-1}· kg^{-1}	0.54±0.20	0.54±0.05	0.34±0.18
T_p/h	1.6±1.1	1.8±1.6	1.2±1.2
C_{max}/mg· L^{-1}	13.1±10.3^{c}	22±5^{c}	44±5
AUC_{0-24}/mg· L^{-1}· h	78±33^{c}	150±12^{c}	497±170
$AUC_{0-\infty}$/mg· L^{-1}· h	85±35^{c}	158±10^{c}	623±315

$T_{1/2\alpha}$: distribution half-life; $T_{1/2\beta}$: elimination half-life; $T_{1/2(Ka)}$: absorption half-life; V_1: apparent volume of distribution of central compartment; CL: clearance; T_p: the time to reach the peak plasma concentration; C_{max}: maximum plasma concentration; AUC_{0-24}: the area under the plasma concentration-time curve from 0 h to 24 h; $AUC_{0-\infty}$: the area under the plasma concentration-time curve from 0 h to infinity.

Hepatotoxicity Administration of ketoconazole resulted in a significantly dose-dependent increase in serum transaminase activities (GPT P<0.01, GOT P <0.05, between groups) as well as cloudy swelling (Fig 2B), ballooning degeneration (Fig 2C), and necrosis (Fig 2D) of the hepatocytes in rabbits (Tab 2).

Relationship between toxicokinetics and hepatotoxicity The correlation coefficients between increase of serum transaminase activities or morphological changes of hepatocytes and toxicokinetic parameters were obtained by least square linear correlation (Tab

Tab 2. Effect of intragastric gavage 40, 80, and 160 mg/kg ketoconazole on serum GPT and GOT level and morphological changes of hepatocytes in rabbits. *n*=4. Mean±SD. ^{b}P<0.05, ^{c}P<0.01 *vs* Normal saline. ^{e}P<0.05 *vs* 80 mg/kg. ^{h}P<0.05, ^{i}P<0.01 *vs* 160 mg/kg.

Dose /mg· kg^{-1}	GPT/IU Before	GPT/IU After	GOT/IU Before	GOT/IU After	Morphological score of hepatocytes
Normal saline	4.2±2.9	4±4	3.6±3.0	2.8±2.4	0.00±0.00
40	3.9±2.2	68±87^{i}	2.6±1.8	42±58^{h}	2.2±1.0^{ceh}
80	5±6	188±93^{bh}	5±4	217±193^{b}	3.5±0.6^{ch}
160	8±8	377±164^{c}	3.8±2.6	291±143^{c}	3.5±0.6^{c}

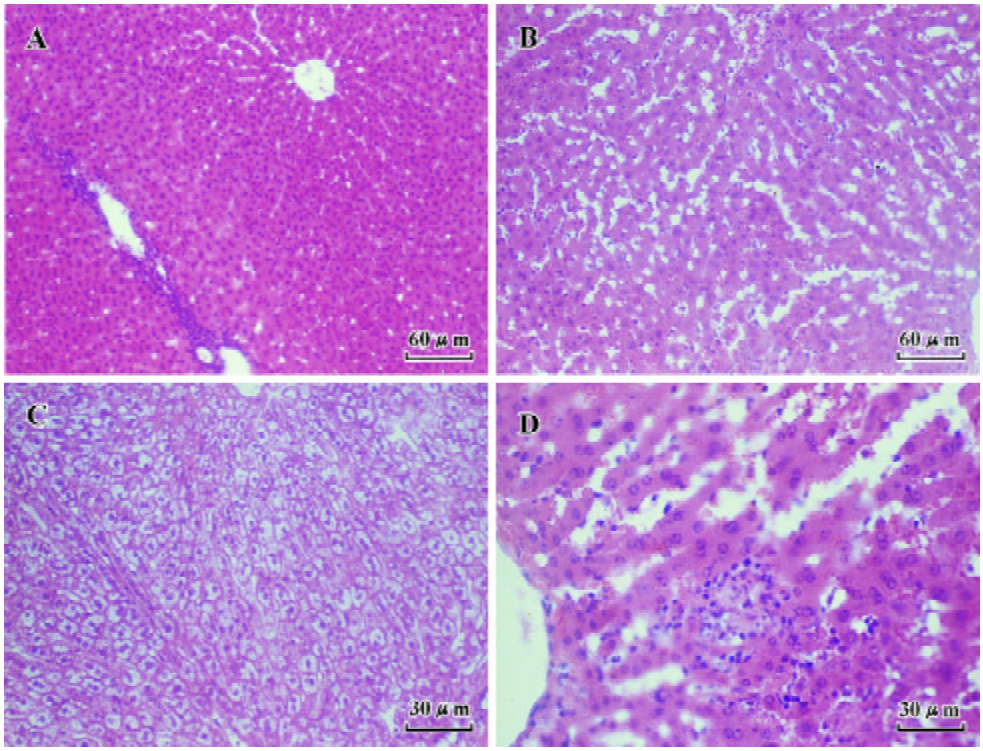

Fig 2. The photomicrographs of liver section from rabbits after ig ketoconazole. A: normal saline. B: 40 mg/kg. C: 80 mg/kg. D: 160 mg/kg. HE stain, A and B ×165, C and D ×330.

3). Hepatotoxicity of ketoconazole was more closely related to its AUC than to either its C_{max} or dose, as revealed by the correlation coefficients.

Tab 3. Relationship between serum transaminase activities or morphological changes of hepatocytes and toxicokinetic parameters after intragastric gavage 40, 80, and 160 mg/kg ketoconazole in rabbits.

Parameter	GPT		GOT		Morphological changes of hepatocytes	
	r	*P*	*r*	*P*	*r*	*P*
Dose	0.773	<0.01	0.585	<0.05	0.517	>0.05
$T_{1/2\beta}$	0.458	>0.05	0.459	>0.05	0.137	>0.05
V_1	-0.166	>0.05	-0.300	>0.05	0.028	>0.05
CL	-0.605	<0.05	-0.479	>0.05	-0.287	>0.05
C_{max}	0.654	<0.05	0.535	>0.05	0.389	>0.05
AUC_{0-24}	0.836	<0.01	0.637	<0.05	0.490	>0.05
$AUC_{0-\infty}$	0.821	<0.01	0.647	<0.05	0.472	>0.05

r: correlation coefficient; *P*: the probability value of significance test of correlation coefficient; see also the legend of Tab 1 for definition of toxicokinetic parameters.

DISCUSSION

In this study a single dose of 40 mg/kg ketoconazole resulted in hepatotoxicity in rabbits. Through calculation of interspecies conversion of dose by body surface area[13] the equivalent toxic dose is about 800 mg in human, four times as high as usual therapeutic dose. This investigation revealed that the hepatotoxicity of ketoconazole was related more closely to its AUC than its C_{max} or dose. Because AUC is determined by both concentration and duration of the drug in body it may be considered that hepatotoxicity of ketoconazole was related not only to its concentration but also to its duration. Though the clinical therapeutic dose, 200 mg daily, of ketoconazole is lower than those used in the present study and usually dose not cause hepatotoxicity after single dose in human, the accumulative AUC of ketoconazole after repeated administration may reach the exposure level (AUC) to generate hepatotoxicity. This reasoning is consistent with the situation that ketoconazole may cause damage of liver by usual therapeutic multi-dose in clinic[1,4-7].

There is gender-related difference of the DAK's metabolism in rats[14], possibly due to the fact that male rats have higher FMO activity than female[15]. Because

the metabolism of DAK was mainly mediated by human and rabbit FMO3[12] and the activity of FMO3 are gender-independent[16], we speculate that the gender-related difference of ketoconazole metabolism may not be significant in rabbits, so that rabbits of either sex were used in the present investigation. Our result also made clear that there was no gender-related difference in the toxicokinetic parameters of ketoconazole.

The present study revealed that proportional increase in AUC was more than that in the dose after the dose reached 80 mg/kg. The similar phenomenon was also found in rats[17] and human[18]. The reason of disproportionate increase in AUC with dose needs further investigation.

The serum transaminase activities increased with the AUC and dose of ketoconazole, but the morphological changes of hepatocytes were not significantly different between 80 mg/kg and 160 mg/kg ketoconazole, suggesting that there is a time lag between the serum transaminase activities and the morphological changes.

REFERENCES

1 Stricker BH, Blok AP, Bronkhorst FB, Van Parys GE, Desmet VJ. Ketoconazole-associated hepatic injury. A clinicopathological study of 55 cases. J Hepatol 1986; 3: 399-406.
2 Jacobson MA, Hanks DK, Ferrell LD. Fatal acute hepatic necrosis due to fluconazole. Am J Med 1994; 96: 188-90.
3 Lavrijsen APM, Balmus KJ, Nugtern-Huying WM, Robdaan AC, van' t Wout JW, Stricker BHC. Hepatic injury associated with itraconazole. Lancet 1992; 340: 251-2.
4 Garcia Rodriguez LA, Duque A, Castellsague J, Perez-Gutthann S, Stricker BH. Acohort study on the risk of acute liver injury among users of ketoconazole and other antifungal drugs. Br J Clin Pharmacol 1999; 48: 847-52.
5 Fu HG, Zhang T. Acute hepatic failure- induced death due to ketoconazole. Adverse Drug Reactions J 2001; 3: 159.
6 Deng PY. Ketoconazole and hepatotoxic damage. Chin Pharm Affair 1995; 9: 49-51.
7 Findor JA, Sorda JA, Igartua EB, Avagnina A. Ketoconazole-induced liver damage. Medicina (B Aires) 1998; 58: 277-81.
8 Lake-Bakaar G, Scheuer PJ, Sherlock S. Hepatic reactions associated with ketoconazole in the United Kingdom. Br Med J 1987; 294: 419-22.
9 Whitehouse LW, Menzies A, Dawson B, Zamecnik J, Sy WW. Deacetylated ketoconazole: a major ketoconazole metabolite isolated from mouse liver. J Pharm Biomed Anal 1990; 8: 603-6.
10 Rodriguez RJ, Acosta D Jr. Metabolism of ketoconazole and deacetylated ketoconazole by rat hepatic microsomes and flavin-containing monooxygenases. Drug Metab Dispos 1997; 25: 772-7.
11 Rodriguez RJ, Acosta D. *N*-deacetyl ketoconazole-induced hepatotoxicity in a primary culture system of rat hepatocytes. Toxicology 1997; 117: 123-31 .
12 Rodriguez RJ, Miranda CL. Isoform specificity of *N*-deacetyl ketoconazole by human and rabbit flavin-containing monooxygenases. Drug Metab Dispos 2000; 28: 1083-6.
13 Sun RY, Ma YM, Hong ZY. Design of pharmacological experiment and statistic analysis. In: Xu SY, Bian RL, Chen X. Methodology of pharmacological experiment. 3rd ed. Beijing: People' s Medical Publishing House; p195-223.
14 Odriguez RJ, Proteau PJ, Marquez BL, Hetherington CL, Buckholz CJ, O' Connell KL. Flavin-containing monooxygenase-mediated metabolism of *N*-deacetyl ketoconazole by rat hepatic microsomes. Drug Metab Dispos 1999; 27: 880-6.
15 Lemoine A, Williams DE, Cresteil T, Leroux JP. Hormonal regulation of microsomal flavin-containing monooxygenase: tissue-dependent expression and substrate specificity. Mol Pharmacol 1991; 40: 211-7.
16 Cherrington NJ, Cao Y, Cherrington JW, Rose RL, Hodgson E. Physiological factors affecting protein expression of flavin-containing monooxygenases 1, 3, and 5. Xenobiotica 1998; 28: 673-82.
17 Sjoberg P, Ekman L, Lundqvist T. Dose and sex-dependent disposition of ketoconazole in rats. Arch Toxicol 1988; 62: 177-80.
18 Huang YC, Colaizzi JL, Bierman RH, Woestenborghs R, Heykants J. Pharmacokinetics and dose proportionality of ketoconazole in normal volunteers. Antimicrob Agents Chemother 1986; 30: 206-10.