Disposition of Azole Antifungal Agents. I. Nonlinearities in Ketoconazole Clearance and Binding in Rat Liver

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The disposition of ketoconazole was characterized in the rat over a wide dose/concentration range. Bolus dose (0.03–10 mg/kg) studies indicate that plasma concentration–time profiles for ketoconazole are not superimposable when dose normalized because of nonlinearities occurring in both volume of distribution and clearance. The volume of distribution decreases from 3 to less than 1 L/kg, while the plasma clearance decreases 10-fold from 25 mL/min/kg as the dose is escalated. From these results, infusion rates were calculated to maintain the plasma concentrations achieved with particular bolus doses. The curvilinear relationship between steady-state plasma concentration (0.015–8.3 mg/L) and ketoconazole infusion rate (0.021–2.45 mg/hr/kg) was analyzed in terms of Michaelis–Menten kinetics. A V_{max} of 3.2 mg/hr/kg and K_m of 2.1 mg/L were obtained by nonlinear regression analysis. At the end of the ketoconazole infusion, liver, adrenals and kidneys were removed and assayed for ketoconazole. Tissue-to-plasma partition coefficients for the liver and adrenals showed a marked dependence upon steady-state plasma concentration. Both parameters (liver, 22; and adrenals, 53) showed a decrease of approximately 10-fold as the plasma concentrations were increased. In contrast, the kidney:plasma partition coefficient (1.8), blood:plasma concentration ratio (0.6), and plasma binding (96%) of ketoconazole did not show a concentration dependence over the range studied. It is concluded that the liver is an important determinant of ketoconazole's volume of distribution and that saturation of this process accounts largely for the reduction in volume of distribution with increasing dose. The characterization of ketoconazole's hepatic clearance and binding in the rat helps resolve the apparent discrepancy between *in vitro* and *in vivo* observations on this azole's interaction with cytochrome P450.

KEY WORDS: ketoconazole pharmacokinetics in rat; nonlinear clearance; nonlinear liver binding; cytochrome P450 interactions.

INTRODUCTION

Ketoconazole was the first orally active azole antifungal agent to be introduced for use against a wide variety of superficial and systemic mycoses (1). The mode of antifungal action involves inhibition of 14-demethylation of lanosterol to form ergosterol, an essential membrane component of the fungus (2). This reaction is cytochrome P450-mediated and the strong affinity of ketoconazole for this enzyme is not limited to the fungal cytochrome P450. Indeed other therapeutic uses for ketoconazole have been identified which involve its interaction with mammalian cytochromes P450. Suppression of hormone production has been achieved with ketoconazole in precocious puberty (3) and in patients with prostatic cancer (4) and Cushing's syndrome (5). In addition, the inhibition of hepatic cytochromes P450 involved in drug metabolism has also been documented (6–12) and a number of drug interactions with ketoconazole are believed to occur via this mechanism (13).

In recent years ketoconazole has been used extensively as a cytochrome P450 probe *in vitro*. Studies on its Type II spectral behavior and inhibitory properties with hepatic microsomal and adrenal mitochondrial fractions (6–12,14–17) demonstrate a strong affinity between ketoconazole and cytochromes P450. In contrast, *in vivo* studies in both animals and man have been conflicting, demonstrating either moderate or no inhibitory action (9–11,13). In a recent study (11) we have demonstrated a clear dose dependency and an unusual time dependence in the inhibition of antipyrine *in vivo* by ketoconazole. At low doses the effect was transient and the duration of effect increased disproportionately with the dose. Nonlinearity in ketoconazole pharmacokinetics was proposed to explain this relationship and other anomalies in ketoconazole inhibitory action *in vivo*.

To date, pharmacokinetic information on ketoconazole is limited. As no intravenous formulation is available for this drug, human studies have been limited to oral administration. The primary pharmacokinetic parameters, clearance and volume of distribution, have been determined in rat (18) and dog (19) following intravenous administration but at only one dose level. To aid our understanding of ketoconazole–cytochrome P450 interactions, it is important to characterize, over a wide range of plasma concentrations, the pharmacokinetics of this drug. Therefore we have investigated the pharmacokinetics of ketoconazole in the rat under two conditions: first, following administration of a range of intravenous bolus doses and, second, using various intravenous infusion rates to achieve a range of steady-state concentrations. Both studies demonstrate marked nonlinearities in the clearance and binding of ketoconazole in rat liver, which are of importance in understanding the mechanisms of azole–cytochrome P450 interactions.

MATERIALS AND METHODS

Bolus and Infusion Studies. Male Sprague Dawley rats (Charles Rivers, Manston, UK; 230–280 g) were fitted with cannulae in the right carotid artery and jugular vein (20) under halothane or ether anaesthesia. On recovery the following day ketoconazole was administered intravenously via the jugular vein as a bolus (0.03–10 mg/kg, 1–2 μCi/kg; in polyethylene glycol 400:propylene glycol, 9:1, 1 mL/kg) over 1 min. In the steady-state infusion studies a zero-order infusion (0.021–2.45 mg/hr/kg, 1–2 μCi/kg; in polyethylene glycol 400:propylene glycol, 9:1, 0.15 mL/hr) was started at the same time as the bolus administration using a Sage Infusion Pump Model 351 (Arnold R Horwell Ltd., London). In the latter experiments the initial plasma concentrations achieved from the bolus were maintained by the zero-order infusion. The rate of infusion (R) was calculated from the target

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steady-state concentration (C_{ss}) and the clearance (CL) (obtained from the bolus studies):

$$R = C_{ss} \cdot CL \quad (1)$$

Blood samples (250 μL; $n = 6$) were collected at various times over 2 hr in the bolus studies (1–10 mg/kg) and 3 hr in the infusion studies. In the lowest bolus dose study the blood samples were larger in volume and fewer in number. Plasma concentrations were analyzed following chloroform extraction either by HPLC based on the method of Riley and James (21) or via a specific radiometric procedure. The solvent extraction procedure, common to both assay methods, involved basifying the plasma sample (100 μL) with sodium hydroxide (1 *M*, 100 μL), adding chloroform (5 mL), rotary mixing, and removing the aqueous layer by aspiration. The organic layer was transferred to clean tubes and evaporated to dryness and the residue was stored at −20°C. Samples were reconstituted in either mobile phase (100 μL, potassium monophosphate (0.05 *M*), pH 6:acetonitrile:triethylamine (50:50:05), or liquid scintillation fluid (Optiphase MP).

The HPLC method was used for samples taken in the higher bolus (5–10 mg/kg) and infusion (1.87–2.45 mg/hr/kg) studies and employed a Spherisorb S5C1 (Hichrom, Reading, UK) column, UV dectection at 230 nm, and clotrimazole as an internal standard. Ketoconazole and internal standard were well resolved from each other and extraneous peaks within a run time of 8 min. Recovery was good (96%), and reproductibility acceptable (<5%). The sensitivity of the procedure allowed a minimum detection plasma concentration of 0.2 μg/mL.

The radiometric method was used for samples taken in the lower bolus (0.03–3 mg/kg) and infusion (0.02–155 mg/hr/kg) studies and for some sets of samples taken from the higher bolus and infusion studies. There was excellent agreement between sets of samples for the same dosage assayed by the two methods. Further validation of the specificity of the radiometric procedure was carried out in preliminary studies where plasma samples were divided into two portions to allow assay of ketoconazole by both methods. Once more, excellent agreement was observed between the two analytical methods.

In the bolus studies, the elimination rate constant (k) was calculated by a log-linear regression of the concentration–time data. The half-life was calculated as $0.693/k$, the total clearance by Dose/AUC (where AUC is the area under the plasma concentration–time curve, itself calculated as C_0/k, where C_0 is the concentration at time 0 obtained by extrapolation of the log-linear regression line to time 0), and the volume of distribution as Dose/C_0. In the infusion studies, the apparent clearance was calculated from the ratio of infusion rate and steady-state plasma concentration achieved. In all infusion studies the average plasma concentration over the 1- to 3-hr time period was taken as steady state. V_{max} and K_m parameters to describe the clearance process were obtained by nonlinear regression using the equation

$$C_{ss} = \frac{K_m \cdot R}{V_{max} - R} \quad (2)$$

Binding Studies. Rat blood containing heparin (25 U/mL) was spiked with [^{3}H]ketoconazole and cold carrier to give concentrations of either 1, 5, or 10 g/L and a radioactivity concentration of 0.5 μCi/mL. Following thorough mixing, aliquots of blood were removed for assay and the remainder was subjected to centrifugation to obtain plasma. Plasma aliquots were also taken for assay, and the blood:plasma ratio was determined from the ratio of the drug concentrations in each matrix.

Plasma protein binding was determined by ultracentrifugation (22) using polyallomer centrifuge tubes at 37°C for 15 hr (180,000*g*; Sorvall OTD 65D). Plasma was spiked with [^{3}H]ketoconazole and cold carrier to give concentrations identical to those used for blood binding. Preliminary studies established no loss of ketoconazole due to tube adsorption or instability.

Whole-blood, total plasma, and plasma water concentrations of ketoconazole were determined by liquid scintillation counting. Blood:plasma concentration ratios and unbound fraction (plasma water:total plasma concentration ratio) were then calculated.

Tissue concentrations of ketoconazole were determined following administration of ketoconazole as described above for the steady-state experiment. Following homogenization and chloroform extraction, liquid scintillation counting was used to determine ketoconazole concentrations in adrenals, liver, and kidney. Data were expressed per gram of original organ weight and divided by the corresponding plasma concentration (per mL) to provide a tissue:plasma partition coefficient (K_p). Liver partition coefficients were corrected for metabolism (23) by the equation

$$K_p = K_p(\text{observed}) \frac{(1 + CL_{int})}{Q} \quad (3)$$

where Q is plasma flow to the liver [48 mL/min/kg (26,27)] and CL_{int} is intrinsic clearance, calculated by

$$CL_{int} = \frac{V_{max}}{K_m + C_{ss}} \quad (4)$$

Unbound fractions for tissues (fu_t) were derived from partition coefficients and plasma unbound fractions (24)

$$fu_t = fu/K_p \quad (5)$$

Chemicals. Ketoconazole (19.9 mCi/mmol; ^{3}H-phenyl ring adjacent to piperazine ring; radiochemical purity, >99%) and cold ketoconazole were obtained from Janssen Life Science Products (Wantage, UK). Clotrimazole was obtained from Sigma Chemical Co. (Poole, Dorset, UK).

RESULTS

Profiles Following Bolus Administration

Figure 1 illustrates typical plasma concentration–time profiles from rats who had received ketoconazole intravenously at doses of 0.03, 1, 3, 5, and 10 mg/kg. A number of features in these profiles indicate nonlinearities in both the distribution and the elimination of ketoconazole.

At the lowest dose studied (0.03 mg/kg) the plasma concentration decline is monoexponential, with a half-life of 70 min. However, at higher doses the half-lives are considerably shorter (30–40 min at doses of 1 and 3 mg/kg), and at the

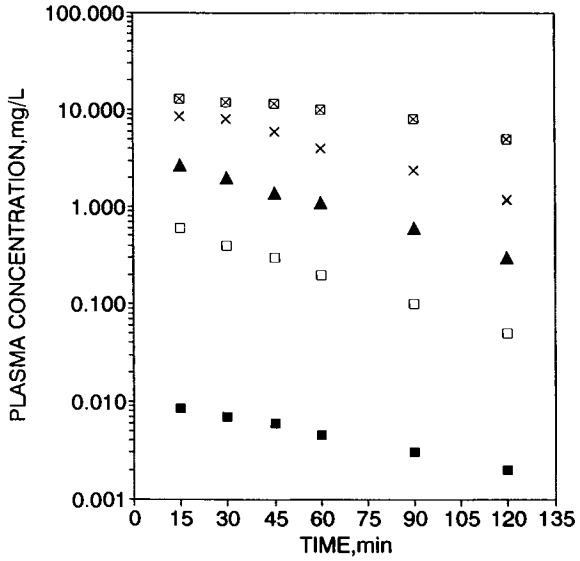

Fig. 1. Plasma concentration–time profiles for ketoconazole following intravenous bolus administration of either 0.03 (■), 1 (□), 3 (▲), 5 (×), or 10 (⊠) mg/kg.

top doses of 5 and 10 mg/kg, convex profiles are apparent, with no log-linear phase in the sampling period (Fig. 1).

Determination of AUC shows a disproportionate increase with escalating dose (Table I), which is consistent with a saturation of the clearance process(es) at higher doses. The intercepts in Fig. 1 allow estimation of volume of distribution and indicate a dose-dependent change in this parameter (Table I). Over a 100-fold increase in dose, the volume of distribution of ketoconazole changes by 4-fold, the largest value being observed at the lowest dose. This nonlinearity may be rationalized by a saturation of tissue binding sites for the drug.

Steady-State Concentrations Following Infusion

Based on the single-dose studies, loading doses and zero-order infusion rates to achieve and to maintain a range of steady-state concentrations were calculated using the volumes of distribution and clearance terms, respectively. Table II shows the increase in steady-state ketoconazole plasma concentrations as the zero-order infusion rate is increased by 100-fold. The disproportionate increase is indicative of saturation of clearance above infusion rates of 0.32 mg/hr/kg.

Table I. Parameters Describing Ketoconazole Plasma Concentration–Time Profiles Following Intravenous Bolus Administration of Various Ketoconazole Doses[a]

Dose (mg/kg)	Volume of distribution (L/kg)	Area under curve, 0–120 (mg/L · min)	Half-life (min)
0.03	3.03 ± 0.41	0.71 ± 0.10	70 ± 3
1	1.25 ± 0.14	32.3 ± 4.2	30 ± 3
3	0.81 ± 0.11	150 ± 10	35 ± 4
5	0.63 ± 0.09	447 ± 50	—
10	0.76 ± 0.08	2020 ± 120	—

[a] Mean values ± SD (N = 5–8).

Table II. Ketoconazole Steady-State Plasma Concentrations and Apparent Plasma Clearance Following Zero-Order Infusion Doses of Ketoconazole[a]

Infusion rate (mg/hr/kg)	Steady-state concentration (mg/L)	Apparent clearance (ml/min/kg)[b]
0.021	0.015 ± 0.008	23.9
0.32	0.198 ± 0.085	26.9
0.71	1.56 ± 0.41	7.6
1.55	3.30 ± 2.36	7.8
1.87	8.27 ± 2.19	3.8
2.30	8.35 ± 2.42	4.6
2.45	12.74 ± 2.03	3.2

[a] Mean values ± SD (N = 4–7).
[b] Calculated from infusion rate/steady-state concentration.

At low infusion rates plasma clearance is constant at approximately 25 mL/min/kg but it decreases to one-tenth of this value over the series of infusion experiments. Nonlinear regression analysis of the relationship between steady-state concentrations and infusion rate gave Michaelis–Menten parameters to describe this process. A V_{max} of 3.2 mg/hr/kg and a K_m of 2.1 mg/L were obtained. The ratio of these parameters (V_{max}/K_m) gives a clearance term describing elimination under nonsaturating conditions of 25.4 mL/min/kg. The latter value agrees well with the clearances observed at low infusion rates (Table II) and low bolus doses (Table I).

Binding to Plasma Albumin and Red Blood Cells

Ultracentrifugation was used to determine ketoconazole binding in rat plasma. Over the concentration range of 0.1–10 mg/L, binding was linear, with an average unbound fraction of 0.037 ± 0.003 (SD) as illustrated in Fig. 2.

Blood cell partitioning was measured over a similar ketoconazole concentration range and found to be very low. A blood-to-plasma concentration ratio of 0.6 ± 0.07 (SD), which is approximately equivalent to (1 − hematocrit), was established. This value indicates that ketoconazole is largely restricted to the plasma within the blood matrix. Such a phenomenon is not unexpected for such a highly plasma protein-bound drug.

Tissue Partition Coefficients

At the end of the infusion studies, liver, kidneys, and adrenals were removed to estimate ketoconazole concentrations. These data are expressed as tissue-to-plasma concentration ratios in Table III. In contrast to the kidney, both liver and adrenal partitioning is substantial and concentration dependent. Over a plasma concentration range of 0.025–13.9 mg/L, there is a 10-fold decrease in the partitioning parameter for these two tissues. The fractions unbound in plasma, kidney, liver, and adrenals are shown in Fig. 2. There is a similar nonlinear trend for liver and adrenal unbound fractions, and this contrasts with the linear binding apparent in plasma and kidneys.

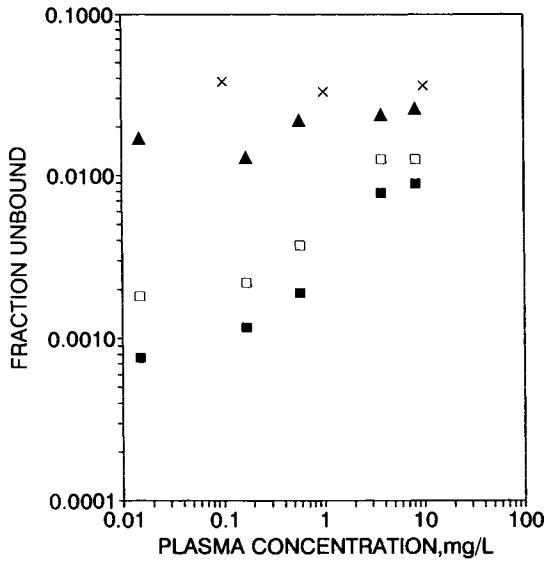

Fig. 2. Relationship between ketoconazole unbound fraction in adrenals (■), liver (□), kidney (▲), and plasma (×) and plasma concentration. Tissue unbound fractions were calculated from tissue:plasma partitions coefficients as described under Materials and Methods.

DISCUSSION

Ketoconazole shows strong affinity for a variety of cytochromes P450 involved in drug metabolism and steroidogensis (6,7,14,16). It is believed that this drug interacts with the ferric form of the heme group as a sixth ligand via one of the nitrogens of the imidazole group, while the hydrophobic tail consisting of the phenyl and piperazine rings stabilizes the interaction by its affinity for the apoprotein. There is much interest in this azole as a probe for cytochrome P450 interactions (6–17). However, the pharmacokinetics of ketoconazole have been previously ill defined and *in vivo* concentration–response relationships have not been elucidated. Hence the *in vivo* relevance of much of the *in vitro* findings remains unexplored.

No urinary excretion of unchanged ketoconazole has been detected (18), indicating that hepatic clearance (by metabolism and possibly biliary excretion) represents the mode of elimination of this drug. Numerous metabolites resulting from oxidation of the imidazole, piperazone, and phenyl rings, scission of imidazole, piperazine, and dioxolane rings, and *O*-dealkylation are thought to be formed (25). We report a plasma clearance of 25 mL/min/kg under linear conditions (dose, <1 mg/kg). Remmel *et al.* (18) report a value of 14 mL/min/kg, but the dose used (5 mg/kg) would have resulted in partial saturation at the early stages of the study. When clearance is expressed in terms of blood concentrations, a value of 42 mL/min/kg is obtained. Compared to hepatic blood flow [80 mL/min/kg (26,27)], a hepatic extraction ratio of 0.52 and a hepatic availability of 0.48 are obtained. Thus contrary to the statements of others (18), ketoconazole shows only an intermediate first-pass effect, and the low bioavailability encountered in certain studies may indicate incomplete absorption in addition to first-pass hepatic loss. Certainly the oral absorption process is slow, as the plasma concentration–time profiles are absorption rate limited following this route of administration (18).

Table III. Ketoconazole Tissue:Plasma Partition Coefficients Following Zero-Order Infusion of Ketoconazole to Attain Steady-State Conditions[a]

Steady-state plasma concentration (mg/L)	Tissue:plasma partition coefficient		
	Liver[b]	Adrenals	Kidney
0.015	22 ± 4	53 ± 9	2.3 ± 0.7
0.17	18 ± 3	35 ± 7	3.0 ± 0.5
0.58	10 ± 2	21 ± 3	1.8 ± 0.3
3.8	3.2 ± 1.1	5.1 ± 1.5	1.6 ± 0.2
8.3	3.2 ± 1.2	4.5 ± 1.3	1.6 ± 0.3

[a] Mean values ± SD (N = 4).
[b] Corrected for hepatic clearance as described under Materials and Methods.

The hepatic clearance of ketoconazole is readily saturable (K_m of approximately 2 mg/L). Therefore any dose resulting in concentrations above 0.5 mg/L would be anticipated to show marked nonlinearity. However, the contribution of the other saturable effect, tissue binding, may confound this effect. Nonlinear pharmacokinetic behavior was predicted by our earlier ketoconazole–antipyrine inhibition study (11). Both the time- and the dose-dependent effects observed in this study are consistent with saturation of clearance. Dose-dependent clearance has also been reported in man (28).

Ketoconazole clearance is restricted by the strong binding in the plasma, presumably to albumin. When intrinsic clearance is calculated and expressed in turns of unbound plasma concentration, a value of 675 mL/min/kg is obtained, which is more representative of this drug's strong affinity for cytochromes P450. Similarly, when the K_m for ketoconazole elimination is expressed as an unbound plasma concentration, a markedly reduced value is obtained—0.078 mg/L (0.14 μ*M*). It is reasonable to assume that these unbound plasma terms are more representative of the ketoconazole concentration available for cytochrome P450 interaction within the liver.

The volume of distribution of ketoconazole is also dose dependent, with marked differences between the low and the high doses studied. Binding within plasma is substantial (fraction unbound, 0.037), which agrees with observations in human plasma (13), and no concentration dependence was observed. Binding in liver and adrenals is also avid, and this binding phenomenon does show concentration dependence. Saturation of these tissue sites and others would explain the reduction in volume of distribution as the dose is elevated. In contrast to the kidney, ketoconazole binding in liver and adrenals results in extremely low unbound fractions (Fig. 2). Due to the large mass of the liver (44 g/kg) relative to the adrenals (200 mg/kg), the former organ can be considered the more important in the distribution of the azole. At the lowest steady-state concentration studied, the liver:plasma partition coefficient is 22 and the effective volume of distribution within the liver is 0.97 L/kg (22 × 44/1000). This accounts for approximately one-third of the total volume of distribution. As liver binding saturates the role of this tissue decreases

substantially, however, even at the highest steady-state concentration studied, the unbound fraction in the liver is only 0.01 and the partition coefficient 3.25. The total volume of distribution observed at high plasma concentrations is in agreement with that reported by others in rat and dog (18,19).

Saturation of azole binding in the liver is demonstrable at lower concentrations than those responsible for saturation of hepatic clearance. Hence when comparing lower and intermediate doses (0.03 and 1 mg/kg), there is a reduction in volume of distribution, and as clearance is constant, the half-life decreases. At the higher doses (5 and 10 mg/kg) the saturation of hepatic clearance becomes evident and convex plasma concentration–time profiles result. This marked dose dependence in ketoconazole plasma concentration profiles is consistent with the complex *in vivo* inhibitory action described earlier with antipyrine (11).

The nonlinearities in hepatic clearance and binding reported here are consistent with the high affinity this azole shows for cytochromes P450 *in vitro*. Both processes are saturable in the submicromolar range when data are expressed in terms of unbound plasma concentrations. There is little doubt that cytochromes P450 are responsible for the metabolic clearance of ketoconazole; however, the extent to which this hemoprotein is involved in the liver binding of the azole has yet to be addressed. The characterization of this process, in addition to the hepatic clearance of ketoconazole, will be of value in the interpretation of future studies employing ketoconazole as a probe for cytochrome P450 interactions.

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