Disposition of Azole Antifungal Agents. III. Binding of Fluconazole and Other Azoles in Rat Liver

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It has been shown for the azole antifungal agents, ketoconazole and DTP, that hepatic binding, is a major determinant of their volume of distribution and this observation is of particular interest in view of the well-documented avid binding of azoles to cytochromes P450. Whilst the hepatic binding characteristics of these two compounds are similar, their hepatic clearance differs markedly in terms of the rate of metabolism and the number and nature of metabolites produced. Fluconazole is a bis-triazole drug similar in structure to DTP but not subject to metabolism in rat. We have demonstrated by means of steady-state infusion studies the clearance of this azole (1.85ml/min/kg) to be independent of blood concentration over a 0.01–50mg/L range. Also fluconazole plasma protein binding is minimal (9.5%) and its blood:plasma ratio unity over a similar concentration range. Liver:blood partition coefficients for fluconazole are concentration dependent ranging from 30 to 2. The volume of distribution term is also nonlinear with concentration and can be correlated with the liver:blood partition coefficient. These findings are discussed together with earlier documented data on ketoconazole and DTP in terms of a tissue binding role for hepatic cytochromes P450. The similarity in behaviour of the hepatic partitioning of the three azoles contrasts markedly with the nature of (or lack of) hepatic metabolism.

KEY WORDS: fluconazole pharmacokinetics in rat; nonlinear liver binding of azoles; azole-cytochrome P450 interactions.

INTRODUCTION

The success and limitations of azole antifungal therapy revolve around the cytochrome P450 enzyme system. The mode of action of these agents involves inhibition of P45051A1, the enzyme responsible for the 14α-demethylation of lanosterol to form the essential fungal membrane component ergosterol (1,2). Unfortunately, their inhibition properties extend to mammalian P450s and there is evidence of strong interactions between azoles, in particular ketoconazole, and hepatic drug metabolizing and steroidogenic P450s (3–6). Furthermore clinical studies have substantiated the effects of ketoconazole on the production of several endogenous steroids and the metabolism of co-administered drugs (7,8). Fluconazole, a new bis-triazole antifungal drug (9), whilst showing a much greater degree of P450 selectivity than the earlier imidazole antifungals, is not completely devoid of these problems (8,10).

Recently we have documented the hepatic disposition of ketoconazole (11) and a triazole probe DTP (12) *in vivo*. Michaelis Menten parameters describing the saturable hepatic metabolism of these two azoles were determined. Also characterized was the hepatic binding of these antifungals which proved to be a major determinant of their volume of distribution. The binding phenomenon proved to be quite distinct from the hepatic clearance. Whilst there are similarities in the hepatic binding characteristics of ketoconazole and DTP, their hepatic clearance differs with respect to the rate of overall metabolism and the number and nature of metabolic products (presumably reflecting interactions with different P450s). The present studies extend these earlier investigations to include fluconazole. This latter azole is subject to minimal (if any) metabolism and relies on renal clearance for its elimination in several animal species (9,13).

The hepatic binding and renal clearance of fluconazole has been investigated in the rat over a wide range of steady-state blood concentrations achieved by using a series of balanced bolus loading doses and zero order infusion regimens. The aim of these studies was to characterize the hepatic binding properties of an azole which is not subject to hepatic metabolism.

MATERIALS AND METHODS

Preliminary Bolus Studies A group of ten Sprague Dawley rats (240–290g) were fitted with cannulae, under general anaesthesia, in the carotid artery and jugular vein (14). Following a period of recovery, a single bolus dose of fluconazole (0.5–50mg/kg in PEG 400:propylene glycol, 9:1; 1–2ml/kg; 1–1.35μCi/ml) was administered intravenously and flushed through the cannula with heparin-saline (1 in 50) solution. Ten arterial blood samples (100μL) were taken over the course of the 6 hour study, and plasma fluconazole concentrations determined by radiochemical analysis.

Steady-State Infusion Studies A second group of 28 Sprague Dawley rats (240–280g) previously fitted with carotid artery and jugular vein cannulae, were used in these studies. At the start of the study the jugular vein cannula was used to administer a loading dose of fluconazole (0.02–48mg/kg in PEG 400:propylene glycol, 9:1; 1ml/kg; 0.0281–65.22μCi/mg) and immediately following a constant rate infusion designed to maintain levels at the targeted steady-state concentration (Css 0.01–48mg/L). Eq. 1 and 2 were used to calculate these dosages from clearance (CL) and volume of distribution (V) parameters obtained in the preliminary bolus studies—

$$\text{Loading dose (mg/kg)} = \text{Css}\cdot V \quad (1)$$

$$\text{Infusion rate (mg/hr/kg)} = \text{Css}\cdot CL \quad (2)$$

Samples of blood were taken arterially at specified times over the course of the 6 hour experiment and fluconazole plasma concentrations were determined by radiochemical analysis.

Following the final blood sampling (6 h), the animal was sacrificed by cervical dislocation. Liver, kidneys and adrenals were quickly removed and frozen in liquid nitrogen prior to storage at −75°C. At the time of analysis, the organs were thawed, cleaned and weighed. Each was then hand homogenised in a known volume of S.E.T. buffer (Sucrose 0.25M, EDTA 5.4mM, Tris 20mM; pH 7.4; approximately

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30ml, 10ml and 1ml for liver, kidneys and adrenals respectively) at 4°C. An aliquot of each was then taken for radiochemical analysis. Tissue partition coefficient (Kp) was calculated as the ratio of organ fluconazole concentration (per g of tissue) to blood fluconazole concentration (per ml) at the time of sacrifice. Unbound fractions for tissues (fu_T) were derived from the partition coefficient and blood unbound fraction (15)—

$$fu_T = fu_b/Kp \quad (3)$$

Determination of Blood to Plasma Concentration Ratio and Plasma Protein Binding These were determined as detailed previously (12) over a fluconazole concentration range of 0.01–100mg/L (0.075μCi/ml). The blood:plasma ratio was calculated from the radioactivity per ml of blood and plasma respectively. The fractions unbound (fu) determined by ultracentrifugation were calculated from the ratio of the radioactivity per ml of plasma water and the radioactivity per ml of total plasma.

Radiochemical Assay The selectivity of the radiolabelled assay for the analysis of fluconazole in plasma and tissue homogenates was assessed by radio-TLC. An aliquot of each sample (50μL) was spotted onto a TLC plate (silica gel 60 F_{254}, 20cm² by 0.25mm, Merck 5715) and developed with chloroform/methanol/ammonia 0.88 (80/20/1) solvent system (13). In each case a single peak was observed which corresponded to fluconazole.

Chemicals Fluconazole and [^{14}C-3,5-triazole]-fluconazole (specific activity 198mCi/mmole; radiochemical purity >98%) were supplied by Pfizer Central Research, Sandwich, Kent.

RESULTS

Preliminary Bolus Studies Clearance and volume of distribution terms were calculated from the fluconazole plasma concentration-time profiles following administration of 0.5, 2.4, 5, 24 and 50mg/kg. Dose normalized area under the curves indicated that clearance was linear over this dose range—mean 1.8 ± 0.5ml/min/kg. In contrast the volume of distribution was dose dependent; the lower two doses showing a statistically significantly ($p<0.01$ by ANOVA) larger volume than the higher three doses. Fluconazole volume of distribution ranged from 1–2.6 L/kg. There was a corresponding reduction in half-life from 27±8 to 8±2h ($p<0.01$ by ANOVA) over the dose range.

Binding within the blood matrix Over the 10,000-fold DTP concentration range studied (0.01–100mg/l), the blood to plasma ratio was calculated to be approximately one (mean 0.93, range 0.88–0.97 for the six concentrations studied, largest coefficient of variation 3%). This indicates that fluconazole does not bind extensively to erythrocytes, but is associated mainly with the erythrocyte water.

Similarly, no significant (one way ANOVA) concentration dependency was evident in the binding of fluconazole to plasma proteins over the concentration range studied (0.01–100mg/l). Approximately 10% of DTP was bound to plasma proteins (mean unbound percentage 90.5%, range 89.3–91.8% for the six concentrations studied, largest coefficient of variation 2.4%) reflecting a low degree of vascular binding. Hence there is a high percentage of fluconazole unbound in whole blood (mean fu_b = 0.97).

Steady-state studies A plasma steady-state concentration range of 0.01–50mg/L was selected for study. Using the clearance and volume of distribution parameters obtained from the preliminary bolus studies appropriate loading doses to attain, and infusion rates to maintain, these targeted concentrations were calculated. Fig. 1 illustrates the fluconazole plasma concentration-time profiles from six of these studies. The excellent agreement between the targeted and observed steady state concentrations, and the constancy in the levels achieved, shown in this figure were typical of the entire study. For each set of bolus and infusion doses, concentration was time invariant with minimal fluctuations which averaged 5.4% (range 2–11%).

Fig. 2 shows the relationship between the ratio of infusion rate/concentration and concentration for all the animals in this study. This plot confirms the lack of concentration dependence in fluconazole clearance (the ratio infusion rate/concentration equals clearance). Over the concentration range investigated fluconazole clearance averaged 1.85±0.25ml/min/kg (sd, n = 28) which compares well with that determined previously in the bolus studies.

Fig. 3 shows the relationship between the ratio of loading dose/concentration and concentration for these investigations. It illustrates the concentration dependence in this ratio which, in view of the time constancy previously noted for the individual profiles, can be regarded as a reasonable estimation of volume of distribution. This parameter decreased by approximately two-fold (2.2–0.9L/kg) over the concentration range investigated. The trend in distribution volume is consistent with that observed in the bolus studies.

Tissue Binding In order to assess the relative tissue and plasma binding of fluconazole, partition coefficients between total drug concentrations in liver (Kp_H), kidney (Kp_K) and adrenals (Kp_A), and total drug concentration in blood were

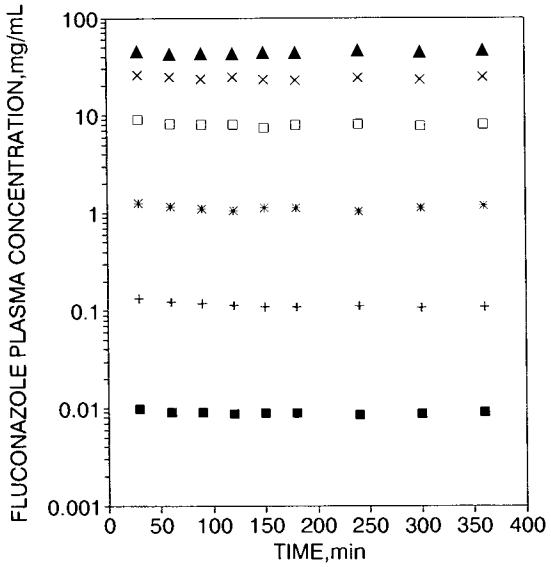

Figure 1. Typical fluconazole plasma concentration-time profiles following intravenous loading doses and intravenous infusions of fluconazole. Targeted steady-state concentrations were 0.01 (■), 0.1 (+), 1 (*), 9 (□), 24 (×) and 48 (▲) mg/L.

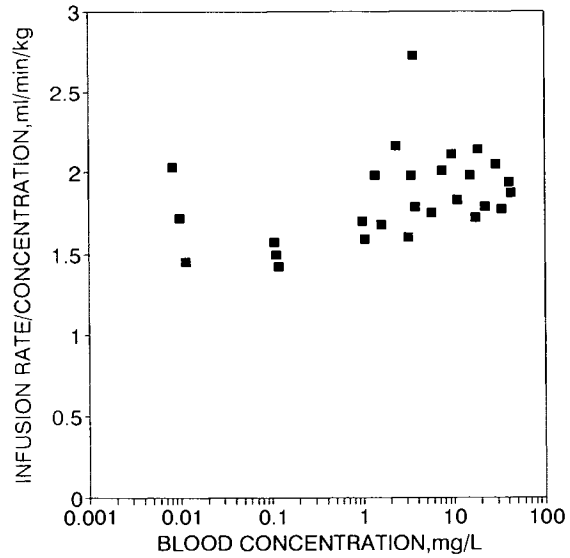

Figure 2. Relationship between the ratio of infusion rate/concentration and concentration for fluconazole in the 28 animals investigated. There is no statistically significant relationship between this ratio and concentration. Therefore clearance averaged 1.85±0.25 ml/min/kg.

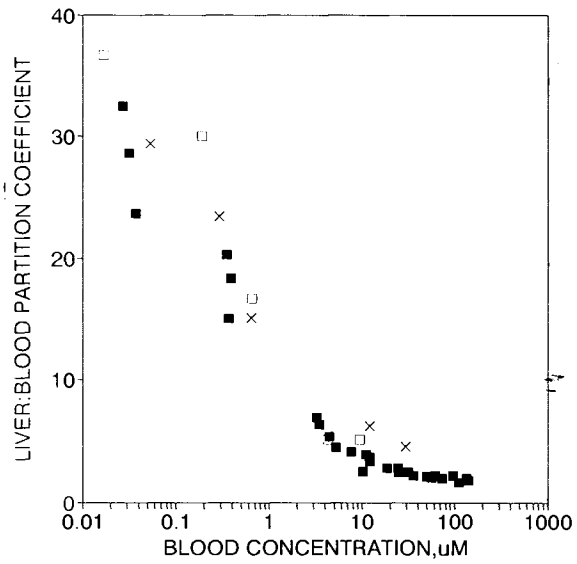

Figure 4. Relationship between liver:blood partition coefficient and blood concentration for three azoles. Each point (■) represents an individual animal from the fluconazole study. Also shown are mean data from previous publications on ketoconazole (×) and DTP (□).

determined. Over the wide blood concentration range covered (0.01–50mg/L), all three tissues showed greater affinity for fluconazole than the blood. There is a significant relationship (one way ANOVA) between both Kp_H and Kp_A and fluconazole blood concentration. The effect is most pronounced in the liver (Fig. 4) where a maximum Kp of approximately 30 is observed, whereas the corresponding maximum Kp_A is 7 (data not shown). For both tissues the Kp decreases with increasing concentration to a minimum value of approximately 2. In contrast, as the concentration of fluconazole in blood increased by over three orders of magnitude, Kp_K values are maintained at a mean value of 1.6 (range 1.2–2.2).

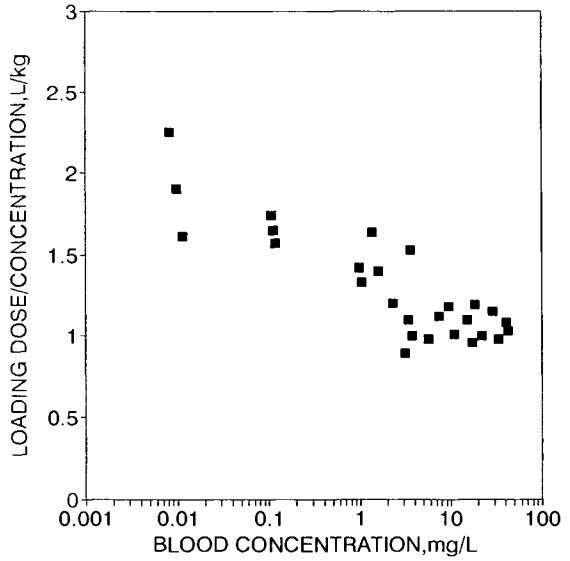

Figure 3. Relationship between the ratio of loading dose/concentration and concentration for fluconazole in the 28 animals investigated. There is a statistically significant ($p<0.01$) negative relationship between the ratio and concentration.

The unbound fractions of fluconazole were calculated for each tissue. In the liver this fraction increased from 0.03 to 0.47, in the adrenals from 0.14 to 0.48 and in the kidneys it was maintained at 0.6 over the concentration range covered.

DISCUSSION

Fluconazole is more polar than the imidazoles (ketoconazole, clotimazole, miconazole) and, in contrast to these latter azole antifungal drugs, is eliminated almost entirely by renal clearance (13). We report a value for fluconazole clearance of 1.8ml/min/kg which is constant over a wide blood concentration range (0.01–50mg/L). The pharmacokinetic behaviour of fluconazole is extremely predictable giving excellent agreement between steady-state fluconazole concentrations observed following an infusion/bolus dosage regimen and calculated steady state concentrations using pharmacokinetic parameters from single bolus studies.

A low degree of plasma protein binding for fluconazole is observed over four orders of magnitude in concentration. A similar lack of nonlinearity is evident in the blood:plasma concentration ratio for this azole which is close to unity. In contrast there is extensive and saturable uptake by both liver and adrenals (maximal Kp of 30 and 7, respectively). The liver partitioning is similar in nature to that previously reported with ketoconazole (11) and DTP (12) which are also shown in Fig. 4. (The previously published Kp data for ketoconazole was based on plasma concentrations, these have been converted to blood concentrations for the present comparison). All three compounds have been shown to give the classic Type II difference spectra with hepatic microsomes, however, the magnitude of this spectra is less with fluconazole than the other azoles (8,12). The extent of partitioning of fluconazole into the adrenals is much reduced when com-

pared to that previously reported (11) for ketoconazole. At equimolar blood concentrations there is a ten-fold difference between the two azoles (7-88 for ketoconazole, 2-6 for fluconazole) over a 0.01–10μM range.

In agreement with ketoconazole and DTP, the liver is a major determinant of the volume of distribution of fluconazole. At low concentrations the effective volume of distribution within the liver ($Kp_H \cdot V_H$ where V_H is the volume of liver—40ml/kg) is 1120ml/kg which is approximately one-half of the total volume of distribution of fluconazole. When similar calculations are performed for ketoconazole (11) and DTP (12), the liver's contribution to the total volume of distribution for these azoles is 32% and 50% respectively. As hepatic binding becomes saturated the importance of this organ in the overall distribution decreases to 8, 12 and 18% for fluconazole, DTP and ketoconazole, respectively.

Previously (12) we have reported a strong relationship between the progressive decrease in Kp_H observed as the dose of DTP is escalated and the decrease in V. This observation was rationalized using a physiological modelling approach to drug distribution (16,17) in which the liver was isolated from the other tissue terms

$$V = V_b + Kp_H \cdot V_H + Kp_T \cdot V_T \quad (4)$$

where V_b, V_H and V_T are the volumes of blood (80ml/kg), liver (40ml/kg) and other body water compartments (480ml/kg) respectively (the sum of these is total body water—600ml/kg) and Kp_T is an average tissue partition coefficient. Fig. 5 illustrates the relationship between volume of distribution and Kp_H for all of the animals involved in the fluconazole study. There is a strong ($r = 0.946$) statistically significant ($p < 0.01$) correlation with a slope of 36ml/kg which is not significantly different from 40ml/kg (V_H). Also shown on this figure are the previously reported data for DTP and ketoconazole which are in good agreement with the general trend.

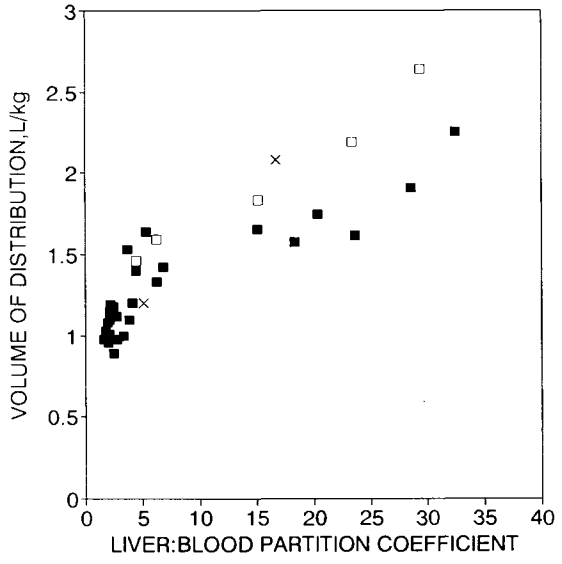

Figure 5. Relationship between volume of distribution and liver: blood partition coefficient for three azoles. Each point (■) represents an individual animal from the fluconazole study. Also shown are mean data from previous publications on ketoconazole (×) and DTP (□). One datum point for ketoconazole corresponding to a Kp_H of 36.7 and a V of 5.05 L/kg has been omitted, see text. Linear regression of all data gives a slope of 38 ml/kg (liver volume).

It should be realised that both terms, volume of distribution and Kp, are measures of relative binding of azoles in tissue with respect to blood. On these relative scales there are similarities between the three compounds and probably other azoles. In absolute terms the liver binding of the three azoles are very different as can be seen in the fu_H terms. For ketoconazole, DTP and fluconazole the minimal fractions are 0.0017, 0.019 and 0.03 and the maximal fractions are 0.012, 0.12 and 0.47, respectively. Hence for hepatic binding a clear rank order of ketoconazole> > DTP > fluconazole is evident.

In conclusion the data presented herewith demonstrate that the volume of distribution of fluconazole, in keeping with DTP and ketoconazole, is substantially influenced by liver partitioning. The strong positive relationship between volume of distribution and liver:blood partition coefficient is unlikely to be unique for azoles but is of particular interest for these compounds in view of their avid P450 interactions. The precise role of P450s in defining azole hepatic binding remains to be elucidated. It is evident however that the hepatic partitioning of azoles is independent of their susceptibility to metabolism. In the rat, ketoconazole is rapidly metabolized to numerous products (18), DTP is slowly metabolized via a single pathway (12) and fluconazole is not subject to metabolism (13).

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REFERENCES

1. D. R. Kirsch, M. H. Lai and J. O'Sullivan. Isolation of the gene for cytochrome P450L1A1 (lanosterol 14α-demethylase) from *Candida albicans*. *Gene* 68:229–237 (1988).
2. Y. Yoshida and Y. Aoyama. Interaction of azole fungicides with yeast cytochrome P450 which catalyzes lanosterol 14α-demethylation. In: *In vitro* and *In vivo* Evaluation of Antifungal Agents. K. Lawata and H. Van den Bossche (Eds.) Elsevier, Amsterdam, pp. 123–134 (1986).
3. J J. Sheets and J. I. Mason. Ketoconazole—a potent inhibitor of cytochrome P450 dependent drug metabolism in rat liver. *Drug Metab. Dispos.* 12:603–606 (1984).
4. M. Pasanen, T. Taskinen, M. Iscan, E. A. Sotaniemi, M. Kairaloma and O. Pelkonen. Inhibition of human hepatic and placental xenobiotic monooxygenases by imidazole antimycotics. *Biochem. Pharmacol.* 37:3861–3866 (1988).
5. J. B. Houston, M. J. Humphrey, D. E. Matthew and M. H. Tarbit. Comparison of two azole antifungal drugs, ketoconazole and fluconazole, as modifiers of rat hepatic monooxygenase activity. *Biochem. Pharmac.* 37:401–408 (1988).
6. I. Shuster. The interaction of representative members from two classes of antimycotics—the azoles and the allylamines—with cytochrome P450 in steroidogenic tissues and liver. *Xenobiotica* 15:529–546 (1985).
7. R. C. Heel, R. N. Brogan, A. Cormine, P. A. Morley, T. M. Speight and G. S. Avery. Ketoconazole: a review of its therapeutic efficacy in superficial and systemic fungal infections. *Drugs* 23:1–36 (1982).
8. J. T. B. Shaw, M. H. Tarbit and P. F. Troke. Cytochrome P450 mediated sterol synthesis and metabolism: Differences in sen-

sitivity to fluconazole and other azoles. In: Recent Trends in the Discovery, Development and Evaluation of Antifungal Agents. R. A. Fromtling (Ed). J R. Prous Science Publishers, S. A., pp. 125–139 (1987).
9. S. M. Grant and S. P. Clissold. Fluconazole: A review of its pharmacodynamic and pharmacokinetic properties and therapeutic potential in superficial and systemic mycoses. *Drugs*, 39:877–920 (1990).
10. J. D. Lazer and K. D. Wilner. Drug interactions with fluconazole. *Rev. Infect. Dis.* 12:5327–5333 (1990).
11. D. Matthew, B. Brennan, K. Zomorodi and J. B. Houston. Disposition of azole antifungal agents I. Nonlinearities in ketoconazole clearance and binding in rat liver. *Pharm. Res.* 10:418–422 (1993).
12. H. L. Bomont, M. H. Tarbit, M. J. Humphrey and J. B. Houston. Disposition of azole antifungal agents II. Hepatic binding and clearance of the probe compound DTP. *Pharm. Res.* 11: 951–960 (1994).
13. M. J. Humphrey, S. Jevons and M. H. Tarbit. Pharmacokinetic evalution of UK-49,858, a metabolically stable triazole antifungal drug in animals and humans. *Antimicrob. Agents. Chemother.* 28:648–653 (1985).
14. P. G. Harms and S. R. Ojeda. A rapid and simple procedure for chronic cannulation of the rat jugular vein. *J. Appl. Physiol.* 36:391–392 (1974).
15. J. R. Gillette. Factors affecting drug metabolism. *Ann. N.Y. Acad. Sci.* 179:43–66.
16. G. R. Wilkinson and D. G. Shand. A physiological approach to drug clearance. *Clin. Pharmac. Ther.* 18:377–390 (1975).
17. S. Oie and T. N. Tozer. Effect of altered plasma protein binding on apparent volume of distribution. *J. Pharm. Sci.* 68:1203–1205 (1979).
18. E. W. Gascoigne, G. J. Barton, M. Michaels, W. Mueldermans and J. Heykants. The kinetics of ketoconazole in animals and man. *Clin. Res. Rev.* 1:177–187 (1981).
19. S. A. Ballard, A. Lodola and M. Tarbit. A comparative study of 1-substituted imidazole and 1,2,4-triazole antifungal compounds as inhibitors of testosterone hydroxylations catalysed by mouse hepatic microsomal cytochromes P450. *Biochem. Pharmac.* 37:4643–4651 (1988).