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# In-vitro distribution of terbinafine in rat and human blood

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**Abstract**

The association of drugs with plasma lipoproteins has the potential to influence drug action and disposition. In this study, the uptake and distribution of the lipophilic antifungal drug, terbinafine, was investigated in rat and human blood and plasma. Fresh plasma was incubated with terbinafine (200–1000 ng $mL^{-1}$), then subjected to vertical spin density gradient ultracentrifugation to separate protein fractions. The concentrations of terbinafine in each fraction was determined using a validated reversed-phase HPLC method. The association of terbinafine with very-low-density lipoproteins (15.5±7.1% of total concentration) in human plasma was significantly lower than that associated with fractions containing soluble proteins (28.0±6.2%), high- (26.8±7.7%) and low-density lipoproteins (31.6±4.6%). In rats terbinafine was found to be distributed evenly through plasma protein fractions. The association of terbinafine in lipoproteins was independent of concentration (over the range 200–1000 ng $mL^{-1}$) and species. The distribution of terbinafine was examined in human and rat blood and the blood-to-plasma ratio of terbinafine was 0.70±0.09 and 1.01±0.20, respectively, indicating higher association of terbinafine with plasma components than erythrocytes in humans. This study suggests that in humans and rats, terbinafine associates with a number of plasma proteins independently of terbinafine concentration. Alteration in plasma lipoprotein concentrations are therefore likely to influence terbinafine binding in blood and distribution in the body.

## Introduction

The association of drugs with plasma lipoproteins has the potential to modify the pharmacokinetics, including tissue distribution and pharmacological activity of lipophilic compounds (Wasan 1996; Wasan & Cassidy 1998). For example, an increase in the association with low-density lipoproteins (LDL) has been linked to an increase in nephrotoxicity associated with amphotericin B (Wasan et al 1993) and ciclosporin (Luke et al 1992). Plasma proteins, including albumin and lipoproteins, have a significant role in the transport of lipophilic drugs throughout the body as has been shown for annamycin and nystatin (Wasan 1996). There is limited data available on the distribution of terbinafine, a lipophilic antifungal drug, in the blood. Information about the major binding proteins or components of blood and their concentration dependency are lacking for terbinafine. Knowledge of the distribution of terbinafine in the blood can provide an insight into the factors governing the pharmacokinetics, pharmacological activity and potential toxicity of the drug. Schafer-Korting et al (1995) have shown that the in-vitro antifungal activity of terbinafine depends on the unbound fraction of terbinafine available in

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the media. Furthermore, Machard et al (1989) showed that uptake of terbinafine into the brain depends on the drug's association with lipoproteins. Based on these observations it is clear that alterations in lipoprotein concentrations may have significant effect on terbinafine pharmacokinetics and pharmacodynamics. The aim of this study was to investigate the association of terbinafine with human and rat blood constituents including lipoproteins. This study will explore the major binding proteins for terbinafine in blood and their concentration dependency.

## Materials and Methods

### Materials

Terbinafine was provided as a gift from Novartis Pharmaceuticals Australia Pty Ltd. The internal standard (clotrimazole) was purchased from Sigma Chemicals (Sydney, Australia). All organic solvents were HPLC grade and obtained from Selby Biolab (Sydney, Australia). Borate buffer consisted of 0.2 M boric acid and potassium chloride and 0.2 M sodium hydroxide (Ajax Chemicals, Sydney, Australia). Potassium bromide was obtained from Ajax Chemicals.

### Plasma samples

This investigation had the ethics approval of the University of Sydney's Animal Ethics Committee and Human Ethics Committee for the collection of blood samples from rats and human subjects, respectively.

Blood samples from healthy human subjects (n = 4) and male Sprague-Dawley rats (n = 3) were used for in-vitro distribution studies. The blood samples from healthy subjects were collected using venipuncture, into 10-mL Vacutainer tubes containing heparin sodium (Becton Dickson Vacutainer Systems, Franklin Lakes, NJ). Rat blood was collected from the posterior vena cava under halothane anaesthesia, into 10-mL plain tubes or tubes containing EDTA. Plasma was separated by centrifugation at 1500 rev $min^{-1}$ for 20 min.

### Density gradient ultracentrifugation

Plasma lipoproteins were separated using the temperature-controlled vertical spin density gradient ultracentrifugation technique (Chung et al 1986). The density of fresh plasma samples incubated (at 37°C for 2 h) with a range of concentrations of terbinafine (200, 600 and 1000 ng $mL^{-1}$) was adjusted with potassium bromide to a density of 1.21 g $mL^{-1}$ by addition of 0.3265 g KBr per mL plasma (Chung et al 1986). A discontinuous density gradient was then achieved by gently overlayering saline buffer on the density-adjusted plasma using a pipette, in Polyallomer centrifuge tubes (8.9 mL capacity, Optiseal; Beckman Instruments Inc., Spinco Division, CA). Samples were then placed in the vertical rotor (Type 90Ti, Beckman Instruments Inc., CA) and centrifuged in a Beckman XL-90 Ultracentrifuge (Optima; Beckman Instruments Inc., CA) at 37°C for 210 min at a speed of 70000 rev $min^{-1}$. After ultracentrifugation, each lipoprotein fraction was sharply banded, with very-low-density lipoprotein (VLDL) at the top, low-density lipoprotein (LDL) in the upper middle, high-density lipoprotein (HDL) in the lower middle portion of the tube and lipid-depleted plasma fraction undisturbed in the lower portion of the tube. The separated lipoprotein fractions were collected by downward drop fractionation of the gradient samples (representing approximately 18 fractions). Each fraction was assayed for the concentration of terbinafine, cholesterol and albumin.

### Blood-to-plasma ratio

The in-vitro blood-to-plasma ratio of terbinafine was determined for both humans and rats over the concentration range 100–600 ng $mL^{-1}$. These concentrations were selected based on the plasma steady-state concentration of terbinafine observed in humans (Matsumoto et al 1995). Known concentrations of terbinafine were added to samples of fresh blood. After vortex mixing, the spiked blood was incubated at 37°C for 1 h to allow for distributional equilibrium to be achieved. The sample was then divided into two portions, one of which was assayed directly for determination of terbinafine concentration in whole blood and the other was centrifuged at 8000 *g* for 20 min. The plasma was separated and stored frozen pending HPLC analysis. The blood-to-plasma ratio was calculated as the ratio of the concentration of terbinafine in blood and plasma.

### Analytical methods

*HPLC analysis*

Plasma samples were analysed for terbinafine concentrations using a validated high-performance liquid chro-

matography (HPLC) method (Hosseini-Yeganeh & McLachlan 2000). Briefly, terbinafine and internal standard (clotrimazole) were extracted from plasma, blood or ultracentrifuge fractions (0.5 mL) by hexane liquid–liquid extraction and back extracted into a mixture of 0.5 M sulphuric acid and isopropyl alcohol (85:15 v:v) before injection onto the column. The inter- and intra-day variability for terbinafine was in the range of 0.2–16%. The limit of detection of terbinafine was 1 ng $mL^{-1}$, when 200 $\mu$L of reconstituted sample was injected into the HPLC system.

*Determination of cholesterol and albumin*

The concentration of total cholesterol and albumin in plasma ultracentrifuge fractions was determined by enzymatic methods (Doumas et al 1971; Tietz & Andresen 1986) using a commercially available enzymatic colorimetric reagent kit (Boehringer-Mannheim, Mannheim, Germany).

### Statistical analysis

The one-way analysis of variance was used for comparison of terbinafine concentrations in different fractions. The post-hoc Fisher's LSD test was then employed to determine which group differed from each other. The unpaired Student's *t*-test was used for determination of any differences in two groups. In all statistical analyses $P < 0.05$ was considered to be statistically significant.

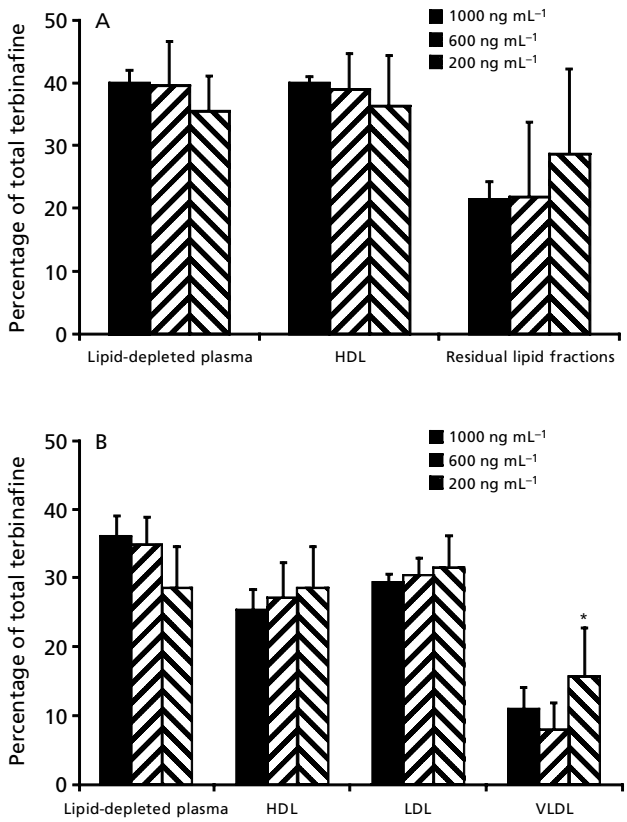


**Figure 1** Association of terbinafine with rat (A) and human (B) plasma proteins at three different concentrations, generated from pooled fractions. The error bars show s.d. (for human, n = 8 replicates from 4 healthy subjects; for rat, n = 3). *$P < 0.05$ compared with other concentration groups.

## Results and Discussion

### Density gradient ultracentrifugation

The fractions associated with soluble proteins (e.g. albumin) and lipoproteins were clearly identified as separate bands using the density gradient ultracentrifugation technique. The density profile of lipoproteins in rats is distinct from that found in human plasma (Terpstra et al 1982) showing a broad lipid band with a density between the HDL and LDL fractions observed in humans. This intermediate band observed in lipid fractions from rat plasma in this study was referred to as HDL according to the density of the lipoprotein (Terpstra et al 1982) and the remaining fractions were referred to as residual lipid fraction. There was no distinct band of LDL or VLDL observed in rat plasma in this study. It was found that terbinafine did not associate specifically with any particular protein in human or rat plasma but was evenly distributed throughout different fractions (Figure 1).

Figure 1A shows the distribution of terbinafine in protein and lipoprotein fractions in rat plasma and indicates that there were no significant differences in the association with lipid-depleted plasma and HDL fractions. However, the association of terbinafine with residual lipid fraction lipoprotein ($21 \pm 3\%$) in rat plasma was significantly lower ($P < 0.05$) than that in the lipid-depleted plasma ($40 \pm 2\%$) and HDL ($40 \pm 1\%$).

In human plasma, terbinafine was found to be evenly distributed in plasma fractions (Figure 1B). However, terbinafine association with VLDL appeared to differ from its association with HDL and LDL. Approximately, 8–16% of terbinafine was associated with VLDL, whereas 28–31%, 25–27% and 27–36% of terbinafine was associated with the LDL, HDL and

**Table 1** Association of terbinafine with plasma proteins and lipoproteins in humans and rats.

| Concentration (ng mL$^{-1}$) | Percent of total terbinafine in human plasma fractions | | | | Percent of total terbinafine in rat plasma fractions | | |
|---|---|---|---|---|---|---|---|
| | Lipid-depleted plasma | HDL | LDL | VLDL* | Lipid-depleted plasma | HDL | Residual lipid fractions |
| 1000 | 36.0±3.0 | 25.4±2.9 | 29.4±1.9 | 11.0±3.1 | 39.9±2.1 | 39.6±1.4 | 21.3±2.6 |
| 600 | 34.7±4.1 | 26.9±5.4 | 30.4±2.5 | 7.9±3.9 | 39.6±6.8 | 38.8±5.7 | 21.6±11.9 |
| 200 | 28.0±6.2 | 26.8±7.7 | 31.6±4.6 | 15.5±7.1 | 35.3±5.7 | 36.2±8.0 | 28.5±13.7 |

Data are presented as means±s.d. For humans, n = 8 replicates from 4 healthy subjects; for rats, n = 3. *$P < 0.05$, comparing terbinafine conentrations, assessed using analysis of variance.

lipid-depleted plasma fraction, respectively (Table 1). The significantly lower association of terbinafine with VLDL could possibly be due to the difficulty in fractionation of this lipoprotein or due to errors in the measurement of terbinafine in each fraction. In this study the concentrations of triglyceride have not been investigated but could have provided an insight into VLDL distribution as this triglyceride-rich lipoprotein consists of 60% triglyceride and only about 10–15% cholesterol. The distribution of terbinafine in human plasma reported in this study is in agreement with the literature (Jensen 1989). Jensen (1989) reported non-specific distribution of terbinafine throughout plasma fractions including lipid depleted plasma, HDL, LDL and VLDL. However, the report by Jensen (1989) did not provide sufficient experimental detail to allow direct comparisons between these studies.

The association of terbinafine with HDL, LDL and the lipid-depleted plasma fraction, in the concentration range of 0.2–1.0 $\mu$g mL$^{-1}$, was independent of drug concentration (Table 1). In an in-vitro study by Ryder & Frank (1992), the effect of serum and isolated serum proteins on the action of terbinafine against several pathogenic fungi was investigated. These researchers also found that terbinafine binding to serum components was independent of concentration over the range 0.1–3.0 $\mu$g mL$^{-1}$. This study provides preliminary evidence of concentration dependent association with VLDL. The reason for this difference in association of terbinafine with VLDL is unclear but could be due to experimental factors such as the large variability in the amount of VLDL observed in individual subjects or as a result of the difficulty in quantifying the VLDL fraction. The association of terbinafine with lipid-depleted plasma, HDL and residual lipid fractions in rats was concentration independent over the range 200–1000 ng mL$^{-1}$ (Table 1). These results also show that approximately 65% of terbinafine in plasma is associated with lipoproteins whereas the remainder (35%) is associated with lipid-depleted plasma fractions containing soluble proteins such as albumin.

The unbound fraction of terbinafine in human plasma has been reported to be approximately 1% (Jensen 1989; Machard et al 1989). The uptake of $^{14}$C-terbinafine into rat brain has been shown to be influenced by the binding of the drug to plasma lipoproteins (Machard et al 1989). Machard et al (1989) showed that in the presence of plasma, terbinafine uptake into the brain is higher than that expected from the available unbound drug concentration based on in-vitro estimates of unbound fraction of drug. This finding does not support the widely held hypothesis that only unbound drug is available for distribution into tissues. The higher uptake of terbinafine by the brain indicates that circulating terbinafine available for entry into the brain is not restricted to the free fraction of drug. In contrast, Schafer-Korting et al (1995) showed that the in-vitro antifungal activity of terbinafine decreased as the amount of human serum albumin was increased in the media, indicating that antifungal activity of terbinafine depends on the unbound fraction of terbinafine available in the media.

### Blood-to-plasma ratio

It was found that in humans, the blood-to-plasma ratio using different concentrations of terbinafine (100, 200 and 600 ng mL$^{-1}$) was less than unity (0.70±0.09) (Table 2) indicating terbinafine has a higher affinity for plasma proteins than blood cells. There was no significant difference between the blood-to-plasma ratio for the three concentrations of terbinafine assessed using analysis of variance ($P > 0.05$). In rats, the blood-to-plasma ratio was somewhat different from that in humans. Using three different concentrations of terbinafine, there was a significant difference in the ter-

**Table 2** Whole-blood-to-plasma concentration ratios of terbinafine in human and rat.

| Terbinafine concn (ng mL$^{-1}$) | Blood-to-plasma ratio in human | Blood-to-plasma ratio in rat |
|---|---|---|
| 100 | 0.80±0.12 | 0.90±0.07 |
| 200 | 0.70±0.07 | 0.90±0.08 |
| 600 | 0.70±0.09 | 1.30±0.19* |

Data are presented as mean±s.d. of six replicates. *$P < 0.05$, compared with 100 and 200 ng mL$^{-1}$ group (Fisher's LSD test).

binafine blood-to-plasma ratio at a concentration of 600 ng mL$^{-1}$ compared with at 100 and 200 ng mL$^{-1}$ (analysis of variance, $P < 0.05$). The reason for this significant difference was not clear. The overall blood-to-plasma ratio of terbinafine in rats was found to be 1.0±0.2, indicating equal distribution of terbinafine in plasma and red blood cells. These results suggest a concentration dependency in partitioning or membrane binding of terbinafine into red blood cells at a high concentration of terbinafine (600 ng mL$^{-1}$). However, there is not sufficient evidence in the literature to support concentration-dependent uptake or binding of terbinafine by red blood cells and this investigation does not support saturable binding to plasma proteins over this concentration range.

## Conclusion

The results from these in-vitro investigations indicate that terbinafine associates with lipoprotein and soluble protein fractions in plasma in humans and rats in a concentration-independent manner which is supportive of partitioning rather than specific binding. The blood-to-plasma ratio of terbinafine in humans and rats was less than unity indicating that terbinafine does not accumulate in blood cells. The distribution of terbinafine in blood is important to understand factors that influence drug distribution and elimination in the body which, in turn, influence the drug concentration at the site of action or toxicity.

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