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Influence of Plasma Protein Binding on the Brain Uptake of an Antifungal Agent, Terbinafine, in Rats

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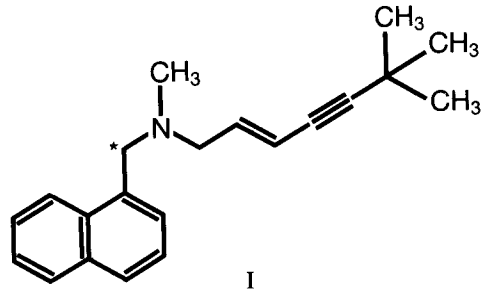
Abstract—The intracarotid injection technique has been used to determine the unidirectional brain uptake of an antifungal, lipophilic agent, terbinafine (Lamisil, Sandoz Basle), in the rat. Ultrafiltration showed it to be highly bound to human plasma, human serum albumin (HSA), α_1-acid glycoprotein (AAG) and lipoproteins (VLDL, LDL, HDL). The effect of plasma protein binding of the drug on brain uptake was also examined with the technique. The lowest brain uptake was observed in the presence of plasma (6%); it varied from 23 to 30% with physiological concentrations of VLDL, LDL and HSA and was significantly higher (43–45%) in the presence of physiological concentrations of AAG and HDL. The free fraction as determined in-vitro and the brain uptake of the drug varied inversely with the plasma protein concentrations; however, the brain uptake was higher than expected from in-vitro measurements. These data indicate that the amount of circulating Lamisil available for brain penetration exceeds its free fraction; they also show that plasma proteins differently reduce the brain transport of the drug.

The blood brain barrier (BBB) restricts the passage of polar compounds and macromolecules from the blood into the brain interstitium. The BBB is situated at the endothelial cells of the cerebral capillaries joined by tight junctions. Cerebral capillaries differ from other capillaries by the absence of fenestrae, a low vesicular transfer and pinocytosis and their close contact with astrocytes end feet (Cornford 1985). Accordingly, a substance must pass through the cells rather than between them to translocate from blood to brain. In general, uptake into the brain occurs by passive diffusion which is determined by such factors as molecular size, lipophilicity and binding to plasma proteins.

Based on the free drug hypothesis (Gillette 1973), the tissue exchangeable drug concentration which regulates drug action is believed to be directly related to the free drug measured in-vitro, whereas the plasma protein bound drug cannot cross the capillary membrane because of its molecular size. However, in-vivo studies of drug uptake into the brain (Pardridge & Landaw 1984) and liver (Pardridge 1987; Weisiger et al 1981) have shown that drug is often available for transfer into the tissue from the circulating plasma protein-bound pool. Moreover, it has been suggested that certain plasma proteins, like albumin, may be involved intimately in the transport process beyond their simple role in the binding interactions.

Terbinafine (Lamisil, TM Sandoz, Basle), an antifungal agent (I), is a highly lipophilic compound that is avidly bound to different plasma proteins such as albumin (HSA), α_1-acid glycoprotein (AAG) and lipoproteins (VLDL, LDL, HDL). The rapid intracarotid injection technique permits the examination of the effects of binding to the various plasma proteins on brain uptake of the drug and the testing of the free drug hypothesis on terbinafine transport into rat brain.



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Materials and Methods

Radiolabelled compounds

[^{14}C]Terbinafine (54·8 mCi mmol^{-1}) and [^{3}H]H_2O were supplied by Sandoz (Basle, Switzerland). The drug was stored at −20°C until use. Its radiochemical purity was higher than 97% as assessed by thin layer chromatography on silica plates.

Human plasma proteins

HSA and AAG were purchased from Behring (Marburg, FRG) and plasma lipoproteins from Sigma (St Louis, USA). Protein fractions were diluted to the desired concentration with buffered Ringer solution.

Animals

Male Wistar albino rats 200–250 g, obtained from KFM (Fullinsdorf, Switzerland) were maintained at 22°C. They were anaesthetized with 0·3 mL of a ketamine (130 mg kg^{-1} i.m.) and xylazine (1·3 mg kg^{-1} i.m.) solution.

Brain uptake studies

The unidirectional influx for terbinafine relative to tritiated water was measured by the tissue sampling single injection technique developed by Oldendorf (1970). The right common carotid artery was surgically exposed and cannulated with a 27-gauge needle which did not occlude the vessel assuring free arterial flow. A bolus of approximately 200 μL of solution was rapidly injected ($< 0{\cdot}5$ s) so that it did not mix with the circulating plasma; mixing effects of about 5% were

found by Pardridge et al (1985b). Five seconds after injection of the bolus the animal was decapitated; its hemisphere ipsilateral to the injection was quickly removed and homogenized by extrusion through a 20-gauge needle. Approximately 40 to 60 mg of tissue were solubilized overnight at room temperature (20°C) with 2 mL of Soluene-350 (Packard) before double isotope liquid scintillation counting. An aliquot of each injected solution was similarly treated and counted.

Injection solutions

The injection bolus consisted of human plasma heparinized or isolated plasma proteins (HSA, AAG, VLDL, LDL, HDL) at physiological concentrations dissolved in Ringer solution buffered to pH 7·4 with 10 mM Hepes [4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid]. This bolus contained the freely diffusible internal reference [^{3}H]H_2O (10 μCi mL^{-1}) and the test molecule [^{14}C]terbinafine (1 μCi mL^{-1}). The determination of in-vitro and in-vivo drug binding was also investigated in the presence of various concentrations of HSA (0·15 to 605 μM), AAG (0·24 to 19·5 μM), VLDL (0·03 to 0·26 μM), LDL (0·013 to 0·70 μM) and HDL (0·11 to 5·35 μM).

Estimation of drug tissue uptake

The brain uptake index (BUI) measured the unidirectional flux of the ^{14}C-labelled test compound in comparison with that of a freely diffusible tritiated reference standard. Counts per minute were converted to disintegrations per minute (d min^{-1}) by standard quench correction and BUI was calculated as follows:

$$BUI = \frac{[^{14}C/^3H]d\ min^{-1}\ brain}{[^{14}C/^3H]d\ min^{-1}\ injectate} \times 100 \quad (1)$$

The BUI was converted to the percent extraction (E) in a single circulatory passage by multiplying by the actual percent extraction of reference tracer. Thus:

$$E_t = BUI \times E_r \quad (2)$$

Where E_t and E_r were the extraction of unidirectional influx of the test ([^{14}C]terbinafine) and reference ([^{3}H]H_2O) compounds, respectively, on a single pass. The E_r value of water in the ketamine-anaesthetized animal was 0·62 in brain (Pardridge et al 1985a).

Determination of in-vitro and in-vivo drug binding

The free fraction f_u of terbinafine was measured in-vitro by ultrafiltration using an Amicon Centrifree Micropartition System (Zysset & Zeugin 1986). An aliquot of injection solution, without the reference tracer, was incubated at 37°C during 30 min and submitted to ultrafiltration. According to the law of mass action,

$$f_u = \frac{K_d}{K_d + P_f} \quad (3)$$

Where K_d is the dissociation constant of drug protein complex and P_f is the concentration of free protein binding sites. Since we used only trace concentrations of the antifungal drug in our study, the concentration of occupied binding sites is negligible relative to the total concentration of protein binding sites (P_t) and thus $P_f = P_t$. Equation (3) becomes:

$$f_u = \frac{K_d}{K_d + P_t} \quad (4)$$

The in-vitro estimation of K_d was obtained by linearization of equation (4),

$$1/f_u = 1 + P_t/K_d \quad (5)$$

In the case of in-vivo studies, the exchangeable fraction of drug is given by the Crone equation of capillary physiology (Crone 1963):

$$E = 1 - e^{-PS/F} \quad (6)$$

where PS is the permeability capillary surface area product (mL min^{-1} g^{-1}) on the blood side of the BBB, and F is the rate of cerebral blood flow (mL min^{-1} g^{-1}). In the presence of plasma proteins, the exchangeable fraction of drug corresponds to f_u and equation (6) becomes:

$$E = 1 - e^{-fu.PS/F} \quad (7)$$

substituting equation (4) in equation (7), it becomes:

$$E = 1 - e^{-(PS/F)/(1 + P_t/K_d)} \quad (8)$$

The values of the dissociation constants K_d and of the ratios PS/F were estimated by least-square fitting to equation (8) of the experimental data, using the FIT FUNCTION option of RS/1 software (RS/1 Version 1.2, BBN Research Systems, Cambridge, USA). The expected values of brain extractions were calculated by using the previous PS/F estimate and the in-vitro measured free fraction f_u in equation (7).

Results

Uptake of terbinafine in the presence of different plasma proteins at physiological concentrations

Fig. 1 represents the brain uptake values for the drug in the presence of human plasma and different isolated human plasma proteins at physiological concentrations. Injection of human plasma results in the lower brain uptake, i.e. 7%. The uptake varies markedly depending on which plasma proteins are added to the injected solution; it amounts to 23, 26 and 30% respectively, for VLDL, LDL and HSA. Brain uptake values are significantly higher with AAG (43%) and HDL (45%). Fig. 2 shows the free fraction of terbinafine determined in the injection solution used for the brain uptake studies depicted above. The in-vitro free fraction varies from 0·3% with plasma to 6·7% with HDL. Thus, these results show that the brain uptake values are higher than predicted values based on the free drug fraction determined in-vitro by ultrafiltration.

Influence of plasma protein binding on the uptake of terbinafine by brain

The in-vivo dissociation constants of the drug were determined with the intracarotid injection technique by changing the concentration of proteins in the injectate. The concentrations of protein vary from about 75 to 605 μM for HSA, 0·24 to 19·5 μM for AAG, 0·07 to 0·27 μM for VLDL, 0·01 to 0·7 μM for LDL and 1·2 to 5·5 μM for HDL.

As shown in Figs 3 and 4, the brain uptake data are fitted

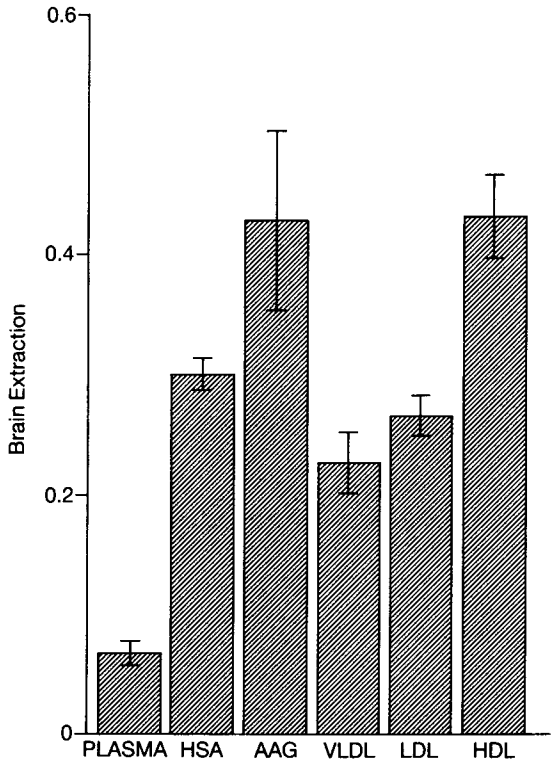

FIG. 1. Effect of human plasma and isolated plasma proteins on the brain uptake of terbinafine. The plasma protein concentrations used were the following: HSA 40 g L^{-1}; AAG 0·8 g L^{-1}; VLDL 2 g L^{-1}; LDL 2·45 g L^{-1} and HDL 1·8 g L^{-1}. Data are the mean ± s.d. (n = 6).

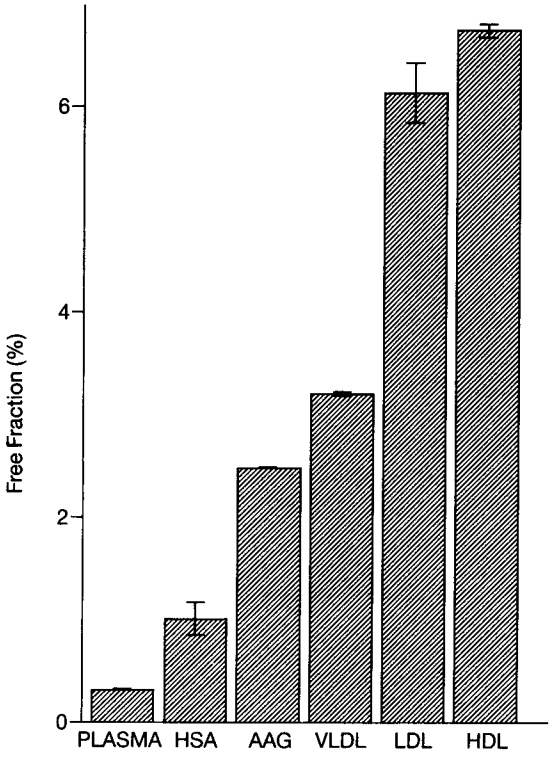

FIG. 2. Free fraction of terbinafine determined by the in-vitro ultrafiltration technique in plasma and with isolated plasma proteins. Data are mean ± s.d. (n = 3).

to equation (8) to yield PS/F and K_d in-vivo parameters. The E values markedly decrease by increasing HSA and LDL concentrations, they decrease slightly with higher AAG and HDL levels and remain constant (23%) with varying VLDL concentrations. For all proteins, the in-vivo dissociation constants of terbinafine in the brain capillaries are higher, factor 7 to 177, than those obtained in-vitro by ultrafiltration (Table 1).

Discussion

The results of the present study are, first, that terbinafine, bound to plasma proteins was partially transported through the blood-brain barrier, and second, the brain uptake varied markedly depending on which plasma proteins were added to the injection solution.

Unexpected results were obtained when uptake of the drug was studied without or with very low plasma protein concentrations in the injectate. The uptake from Ringer solution was 40% and similar results were obtained with very low plasma protein concentrations. However, this value of 40% seems not to be representative of the real transfer of the drug and its uptake must be underestimated. This could result from an overestimate of the amount of the drug arriving in the brain relative to the solution injected. Therefore, in some control experiments, the carotid artery was removed simultaneously with the brain and prepared for liquid scintillation counting. In the absence of, or with very low plasma protein concentrations, the 'carotid uptake index' of the drug was about 200-fold higher than the BUI. On the contrary, protein concentrations used in this study demonstrated insignificant carotid uptake, thus justifying our results.

With all plasma proteins used, the brain uptake of terbinafine exceeded that which would be expected if only free drug were available. Thus, the circulating drug available for entry into the brain is not restricted to the free fraction but includes the larger protein bound fraction. Our results show that a significant fraction of HSA- and AAG-bound drug is available for brain diffusion, which confirms results obtained with other basic drugs, e.g. imipramine (Riant et al 1988). In contrast, regarding the influence of lipoproteins on drug passage through the capillary membrane, it has been shown that isolated lipoproteins had no effect on imipramine's penetration of the brain (Riant et al 1988), or that of isradipine and darodipine (Urien et al 1987) or progabide (Hamberger et al 1987). However, it was found that the brain uptake of cyclosporin, a drug essentially bound to lipoproteins, was strongly reduced in the presence of plasma (Lemaire et al 1988). Our study also indicates that the brain uptake of a very lipophilic drug such as terbinafine, depends on its binding to lipoproteins. It is also interesting to note that the variations in free drug fraction measured in-vitro with plasma, VLDL, LDL and HSA still parallels the variations in exchangeable drug fraction in-vivo. Similar protein-mediated effects have been reported to occur in liver (Pardridge 1987), salivary gland (Terasaki et al 1986) and kidney (Lemaire et al 1988). These results do not support the free drug hypothesis, rather they are in agreement with the general observation that the exchangeable fraction of drug in-vivo is higher than the predictive value obtained from the

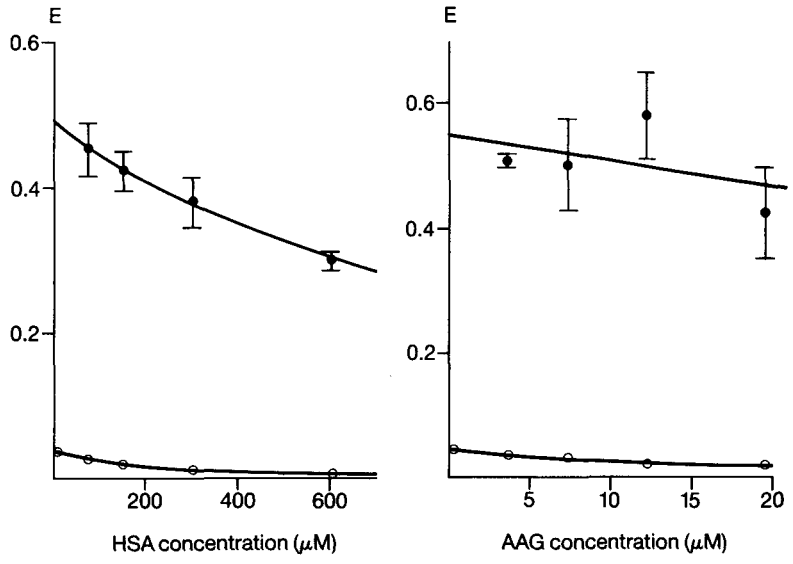

FIG. 3. Rat brain extraction (E) of terbinafine is plotted as a function of HSA and AAG concentrations in the carotid injection solution. The observed values (●) are the mean ± sd for group of 6 rats; the expected values (○) are calculated from the equation (7) using f_u values measured in-vitro and previously estimated PS/F.

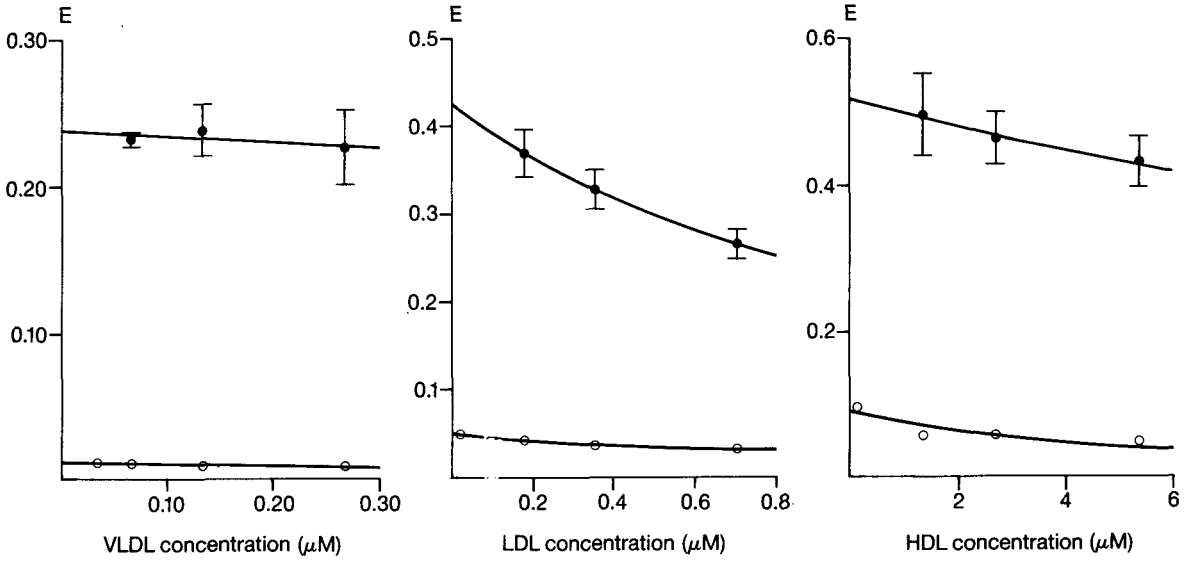

FIG. 4. Rat brain extraction (E) of terbinafine's is plotted as a function of VLDL, LDL and HDL concentrations in the carotid injection solution. ●, experimentally measured values n = 6; ○, expected values calculated from the in-vitro free fraction of drug. See legend of Fig. 3 for details.

Table 1. Comparison of estimated in-vivo and measured in-vitro dissociation constants for the drug with various plasma proteins. K_d in-vivo and PS/F values are obtained from observed brain extraction curves. K_d in-vitro are obtained from free fraction curves (not shown).

Proteins	PS/F	K_d in-vivo (μM)	K_d in-vitro (μM)	K_d in-vivo/in-vitro
HSA	0·671	702·07	7·96	88·20
AAG	0·790	83·70	0·89	94·04
VLDL	0·272	5·30	0·03	176·67
LDL	0·550	0·88	0·13	6·77
HDL	0·727	17·80	0·86	20·70

in-vitro measurement of the free drug fraction. Because the plasma proteins themselves are not significantly transported out of the brain on a single pass, the process represents a mechanism of enhanced dissociation of drug from the binding site in the microcirculation. The mechanism(s) responsible for such enhanced uptake by any organ are not well understood and several alternative hypotheses have been suggested. The capillary endothelial cells could have a specific surface binding site for the drug-protein complex, resulting in enhanced dissociation, possibly by causing a change in the protein conformation. There are suggestions that albumin receptors may exist in the liver (Weisiger et al

1981), however, studies with the isolated brain capillary preparation have not yet demonstrated the existence of such sites (Pardridge et al 1985a). The inhibitor model has been suggested by Pardridge (1986); this mechanism includes the presence within the capillary bed of a non-competitive inhibitor of ligand binding to the plasma protein.

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