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EFFECT OF ERYTHROCYTES AND PLASMA PROTEIN BINDING ON THE TRANSPORT OF PROGABIDE AND SL 75102 THROUGH THE RAT BLOOD–BRAIN BARRIER

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Abstract—Brain extraction of two antiepileptic compounds, progabide and its acid metabolite, SL 75102, was investigated using the carotid injection technique in the rat. The extent to which drug binding to plasma proteins could inhibit the brain extraction was measured. Equilibrium dialysis at 4° showed that both drugs were highly bound to human serum proteins, mainly to serum albumin. Progabide is also bound to red blood cells and to lipoproteins. The free dialyzable drug fraction was inversely related to the protein concentration. Similarly, the brain extraction of the drugs in the presence of either albumin, or red blood cells for progabide was inversely related to their respective concentrations. However, the rat brain extraction of both drugs was higher than expected from the *in vitro* measurement of dialyzable fraction. Furthermore, despite a significant degree of progabide binding to lipoproteins, no significant reduction in the brain extraction of the drug was observed. These data indicate that the amount of circulating progabide or SL 75102 available for penetration in a peripheral tissue such as brain exceeds the dialyzable fraction of drug. However, the *in vivo* exchangeable drug fraction still parallels the dialyzable fraction, except if the drug is lipoprotein-bound.

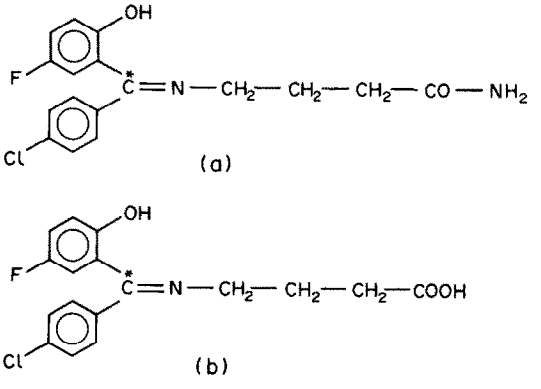

Fig. 1. Chemical structures of progabide (a) and SL 75102 (b).

Progabide and its metabolite, SL 75102, (Fig. 1) are lipophilic compounds identified as GABA receptor agonists which easily penetrate into the brain [1] and are used in the treatment of epilepsy [2, 3]. Both compounds are highly bound to isolated plasma proteins [4] with overall plasma binding percentages respectively equal to 96 and 98% for progabide and SL 75102. The free drug hypothesis assumes that only the free fraction of drug in plasma can penetrate the tissues and that the drug transport in tissues is governed by the free drug concentration gradient between plasma [5] and tissue, until an equilibrium is reached, consequently, the clinical interest of measuring the unbound drug concentration in plasma, rather than the total drug concentration, has been previously emphasized [6].

However, recent studies [7] have demonstrated that part of the plasma protein-bound drug fraction was available for tissue diffusion depending on the nature of the binding protein: propranolol bound to α_1 acid glycoprotein (AAG) is partially available for brain extraction, whereas the fraction bound to albumin was not. Similar observations have been reported regarding numerous steroid hormones [8] demonstrating that a large protein-bound fraction of hormone is available for entry in tissues. In such cases, measuring the free drug concentration *in vitro* may lead to an underestimation of the *in vivo* plasma exchangeable fraction of drug. In this work we investigated the transport of progabide and SL 75102 in the rat brain with respect to their plasma protein binding. These two antiepileptic compounds are mainly bound to human serum albumin (HSA) and to a lesser extent to the three main lipoprotein density classes and to red blood cells (RBC) [4]. Thus, the consequences of the different drug–protein interactions on the free drug fraction *in vitro* (dialyzable fraction) and *in vivo* (exchangeable fraction) could be compared.

MATERIALS AND METHODS

Radiolabeled compounds. ^{14}C progabide and ^{14}C SL 75102 were supplied by Synthelabo—L.E.R.S.

The specific activity for both drugs was 2.15 TBq/mol (58.1 Ci/mol). Their radiochemical purity was <98% as assessed by thin-layer-chromatography on silica plates in three different systems: ether/methanol (95/5, v/v), benzene/acetone (7/3, v/v) and benzene/ethanol (90/10, v/v). They were stored at −80° until use. Drugs were first solubilized in a small volume of methanol (<5% in the final solutions) and the Ringer's buffer (pH 7.4, 5 mM Hepes) was added. The ^{3}H water (C.E.A., TMM 300) specific activity was 0.37 TBq/mol (10 Ci/mol).

Human plasma proteins. Albumin (Sigma A 1887) was dissolved at 300 μM in Ringer's buffer. Lipoprotein fractions were isolated from human serum, using the method of Nelson [9] modified by Glasson *et al.* [10], and dialysed overnight at 4° against buffered Ringer's solution. The concentrations were adjusted to 14.8 μm, 1.7 μM and 0.06 μM respectively for high density (HDL), low density (LDL) and very low density (VLDL) lipoproteins in the Ringer's solution.

Red blood cells. The RBC of an healthy volunteer were washed in NaCl 9‰. Three RBC suspensions were prepared in Ringer's buffer where hematocrit values were 0.10, 0.22 and 0.30.

Brain uptake studies. The initial brain extraction of ^{14}C drug relative to the reference compound (^{3}H water) was measured according to the method of Oldendorf [11]. A 200 μl bolus of buffered Ringer's solution containing ^{14}C drug and ^{3}H water in the absence or presence of isolated RBC or proteins, was rapidly injected (<0.25 sec) via a 26-gauge needle (Microlance, 26 G 3/8, 0.45 × 10) into the common carotid artery of anesthetized (50 mg/kg sodium pentobarbital i.p.) male Wistar rats (200–220 g). After injection, the needle was left in place to prevent bleeding, and the carotid flow past the puncture site was unimpeded. At 5 sec after the injection, the rat was sacrificed. The hemisphere ipsilateral to the injection was solubilized in 2 ml Soluene 350 (Packard Instrument Co.) at 60° overnight before double isotope liquid scintillation counting. Because the rate of injection largely exceeds the rate of carotid blood flow, there is no significant mixing of the bolus with rat blood [12].

Estimation of drug tissue uptake. Brain uptake index (BUI) was calculated as follows:

$$\text{BUI} = \frac{(^{14}\text{C}/^{3}\text{H})\text{dpm in tissue}}{(^{14}\text{C}/^{3}\text{H})\text{dpm in injectate}} \quad (1)$$

The BUI represents the net uptake of the drug normalized by the net uptake of the reference compound. The BUI is therefore a direct function of the single pass extraction of the drug ($E_d = E_r \times$ BUI, the subscripts d and r refer to the drug and reference compound).

With regard to the reference compound, the maximal extraction of ^{3}H water is 0.85 under our experimental conditions [13]. The relationship between the maximal extraction $E_r(0)$ and the extraction at 5 sec, E_r (5) is defined as [7].

$$E_r(5) = E_r(0)e^{-k_{2(r)} \times t} \quad (2)$$

where $k_{2(r)}$ is the efflux rate constant for ^{3}H water (0.57 min^{-1}) [13]. Substitution of the values for $E_r(0)$ and $k_{2(r)}$, and using $t = 0.05$ min (the time between bolus entry in brain and decapitation) into the above equation indicates E (5) = 0.83 for ^{3}H water. With regard to the drugs, the E_d (5) is essentially identical to $E_d(0)$ for the efflux rate constants for the drugs are expected to be slow relative to the influx rate constants, according to the *in vivo* data which indicates that the drugs are sequestrated in brain [14].

Determination of in vitro *and* in vivo *drug binding.* According to the laws of mass action,

$$fu = \frac{K_D}{K_D + P_f} \quad (3)$$

where K_D is the dissociation constant of the drug–protein complex, P_f is the concentration of free protein binding sites, and fu is the drug free fraction measured *in vitro*. Since we used small concentrations of drugs relative to protein concentration in our experiments, the concentration of occupied binding sites (P_b) is negligible relative to the total concentration of protein binding sites (P_t) and $P_f \simeq P_t$. Then equation 3 becomes:

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

The *in vitro* drug protein binding was measured by equilibrium dialysis at 4° during 3 hr 30 as previously described in detail [4]. The *in vitro* drug RBC binding was measured after an incubation of ^{14}C drug in RBC for 1 hr at 4°. Total drug concentration and unbound drug concentration were determined respectively in the suspension (C_t) and in the supernatant (C_u) after a centrifugation at 1500 *g* at 4° for 15 min. The unbound drug fraction (mass of drug contained in buffer) was obtained by:

$$f_u = \frac{C_u}{C_t}(1 - H) \quad (5)$$

where H is the hematocrit value.

Protein solutions identical to those injected to rats were used, and K_D was estimated by using equation 4. In the case of *in vivo* studies, the exchangeable fraction of drug in brain capillary when no binding protein is present is given by the Crone equation of capillary physiology [15]:

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

where PS is the permeability-capillary surface area product and F the rate of cerebral blood flow. In a presence of plasma protein, the exchangeable fraction of drug (f) is diminished because of protein binding and equation 6 becomes [4]:

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

Substituting equation 4 in equation 7 it becomes:

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

when the drug extraction is measured at a series of protein concentrations, PS/F and K_D can be estimated by fitting the data to equation 9.

Analysis of data. The binding parameters and PS/F

values were estimated by the mean of a non-linear regression program using a Gauss–Newton algorithm. Preliminary estimations of the parameters were obtained by linearization of equation 6, $1/f_u = 1 + P_t/K_D$ and equation 5, $-1/\log(1-E) = 1/(PS/F) + P_t/(K_D \times PS/F)$. All values are presented in the form of a mean ($\bar{X}$), standard deviation (SD) and sample number (N). Means were compared by ANOVA and estimated parameters by their 95% confidence interval.

RESULTS

Effect of HSA binding on progabide and SL 75102 brain uptake

As shown in Figs 2 and 3, the brain extraction of either progabide or SL 75102 was decreased in the presence of HSA and the decrease in extraction was related to the HSA concentration in the injection solution. The measured brain extraction *in vivo* was greater than that predicted on the basis of *in vitro* measurement of dialyzable fraction. For each drug the concentration of HSA which caused a 50% reduction in the brain extraction (dissociation constant K_D, *in vivo*) was significantly higher ($P < 0.001$) than that estimated from *in vitro* studies (Table 1). The PS/F values were respectively 2.0 ± 0.3 and 0.22 ± 0.04 for progabide and SL 75102.

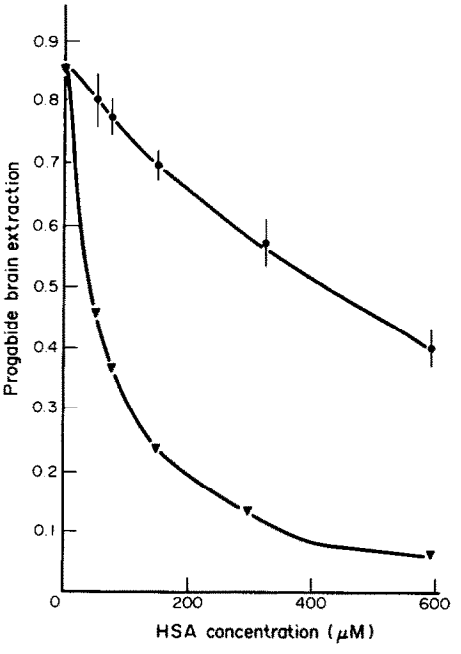

Fig. 2. The rat brain extraction of progabide is plotted as a function of HSA concentration in the carotid injection solution. The observed values (●) are the mean ± SD of 5 rat groups. The expected values (▲) are calculated from the equation $E = 1 - e^{-fu \times PS/F}$ where fu is the measured dialyzable drug fraction (N = 5) and PS/F is estimated from the above equation. The observed curve is drawn by using PS/F and K_D values derived from non linear regression of the data to equation 7 and the expected curve by using the previous PS/F estimate and K_D value estimated from *in vitro* studies. $PS/F = 1.99 \pm 0.71$, K_D *in vivo* = 224.1 ± 18.0 μM, N = 5; K_D *in vitro* = 22.6 ± 0.8 μM, N = 5. K_D values are significantly different ($P < 0.001$).

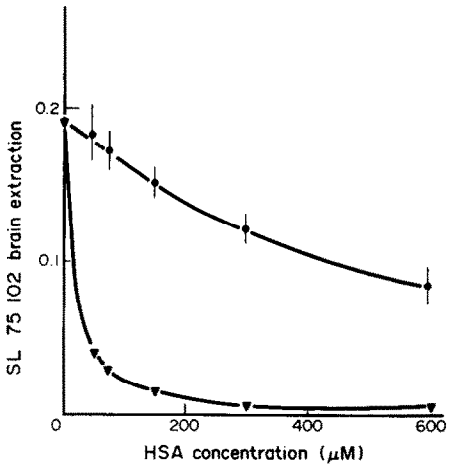

Fig. 3. The rat brain extraction of SL 75102 is plotted as a function of HSA concentration in the carotid injection solution. Experimentally measured values (●), mean ± SD, N = 5, expected values from the measured dialyzable fraction of drug (▲), mean ± SD, N = 5. $PS/F = 0.2 \pm 0.08$, K_D *in vivo* = 395.1 ± 10.4 μM, K_D *in vitro* = 10.9 ± 0.5 μM. K_D values are significantly different ($P < 0.001$). See legend of Fig. 1 for details.

Effect of RBC and lipoproteins on progabide brain uptake

As the contribution of RBC and lipoproteins in the blood–binding of SL 75102 was negligible, lower than 1% [4], only the consequence of progabide binding to RBC and lipoproteins on the exchangeable drug fraction was studied. Despite the fact that progabide was bound to a significant extent to the physiologic concentrations of lipoproteins ($82.5\% \pm 0.3\%$), the brain extraction of the drug was not impaired, as compared to the extraction in the absence of proteins (Table 2). As shown in Fig. 4, the brain extraction of progabide in the presence of RBC was higher than that predicted from the *in vitro* measurement of dialyzable fraction indicating that the exchangeable fraction was higher than the dialyzable fraction.

DISCUSSION

The two main results provided by our investigations are, first, the brain capillary permeability area product is dramatically higher for progabide than for SL 75102 and second a part of protein or RBC-bound drug is available for entry into the brain.

The high PS/F value found for progabide relative

Table 1. Apparent dissociation constants in μM to HSA, established from *in vitro* and *in vivo* studies

Drug	Progabide	SL 75102
In vitro	22.6 ± 0.8	10.9 ± 0.5
In vivo	224.1 ± 18.0	395.1 ± 10.4

In vivo values are significantly different from *in vitro* values ($P < 0.001$).

Table 2. Effect of lipoproteins on the rat brain extraction and the percentage of free progabide (dialyzable *in vitro*)

Protein	Dialyzable (%) N = 5	Brain extraction expected	(ED × 100) measured N = 5
None (control)	100		58.2 ± 1.5
Lipoproteins	17.5 ± 0.2	7.6 ± 1.7	60.2 ± 6.5

The brain extraction in presence of lipoproteins is not significantly different from control.

to that observed for SL 75102 was not unexpected, since progabide is an unionized lipophilic compound, it is able to diffuse across biological membranes, whereas SL 75102 is an acidic molecule ionized at pH 7.4. The *PS/F* values indicate that the clearance of progabide through the capillary membrane is ten times greater than that of SL 75102. Accordingly, it has been reported that progabide penetration into the rat brain was fast and brain-to-plasma concentrations ratio was rapidly superior to 1. By contrast, the brain-to-plasma concentrations ratio of SL 75102 slowly increased to reach a value of 1 after 5 hr [14]. These observations strongly suggest that a part of progabide in brain is then hydrolyzed to form SL 75102 *in situ*.

Our studies have shown that SL 75102, as progabide, bound to HSA, are partially transported through the blood–brain barrier (BBB) in contrast to the free drug hypothesis which states that the dialyzable fraction of drug is the only one available for tissue diffusion.

Although the contribution of lipoproteins (10.7%) was greater than that of RBC (4.3%) in the computed distribution of bound progabide in whole blood [4], lipoproteins had no effect on the progabide transport through the BBB. The fractions of progabide bound to lipoproteins and to RBC were respectively totally and partially available for entry into brain. Other studies have shown that albumin-bound steroid or thyroid hormones, which are also neutral compounds, were partially available for diffusion into the brain [16, 17, 18], as found for progabide, in contrast to basic drugs, as and lidocaine and propranolol [7].

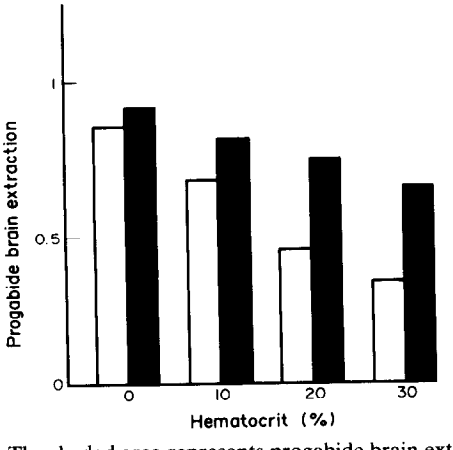

Fig. 4. The shaded area represents progabide brain extraction compared to the expected brain extraction (non-shaded area) in the presence of different hematocrit values in the carotid injection solution. See legend of Fig. 1 for explanation.

The influence of RBC binding on the brain uptake was rarely investigated. Zonisamide has been reported to be bound to RBC and to be partially available for diffusion through BBB [19].

Up to now, regarding the influence of drug binding to lipoproteins on drug passage through the capillary membrane, it has been shown that labeled cholesterol integrated in lipoproteins was not able to cross the BBB, in contrast to progabide [20]. In our study, binding to lipoproteins did not affect brain extraction of progabide. Gillette [21] has emphasized that the rate of dissociation of a drug–protein complex is a limiting factor for tissue extraction of the bound form only if the half-time of the complex dissociation is equal or greater than the mean circulatory transit time in the tissue. Our results suggest that the half-time of progabide–lipoprotein complex dissociation is very short relative to the brain capillary transit time. Accordingly, one can calculate that the rate constant of dissociation is at least 70 sec^{-1}.

The availability of HSA-bound acidic drug for transport through the BBB was reported [13, 22]. The brain extraction of SL 75102 was lower (14%) than that of phenobarbital (24%), phenytoin (28%) and valproic acid (40%) [22]. This low brain extraction enforces our previous suggestion that the SL 75102 concentrations in the brain mainly result from a hydrolysis of progabide in the brain.

The decrease of the drug brain extraction with increasing concentrations of HSA was observed with other drugs [23]. A possible mechanism by which the dissociation of drug from protein is enhanced, is the release from the endothelial surface of non competitive plasma protein binding inhibitors [23], that may be free fatty acids [24]. This mechanism could explain the increase of the dissociation constant of progabide binding reactions, since previous studies have shown *in vitro* a significant inhibition of progabide serum binding by high concentrations of stearic, palmitic and oleic acids (unpublished results). Our results agree with the general observation that the exchangeable fraction of drug *in vivo* is superior to that predicted from *in vitro* measurement of the free drug fraction. This may be explained by the "free intermediate" model described and discussed in detail by Pardridge and Landaw [23]:

$$DP \underset{k2}{\overset{k1}{\rightleftharpoons}} P_f + D_m \overset{k3}{\rightarrow} D_b \qquad (9)$$

where DP is the drug–protein complex, D_m and D_b are the free drug in the microcirculation and in brain respectively and k_1, k_2 and k_3 are the dissociation, association and plasma-to-brain transport rate constants respectively ($Kd = k_1/k_2$). In this model, the capillary transit time (1 sec in brain), the rate of unidirectional dissociation from the protein and the rate of drug diffusion through the biological membrane lining the blood compartment are the three main determinants. Therefore, protein-bound drugs may enter the brain provided drug–protein binding reactions are fast relative to the membrane transport reaction. This condition most likely prevails, owing to the high rates of ligand dissociation that have been reported [4]. Our observation that the rate of drug dissociation from the binding protein is faster *in vivo* than *in vitro*, may come about by specific interactions between the drug–protein complex and components lining the surface of the microcirculation.

Finally, in the present study we observed that progabide binding to HSA and RBC, and SL 75102 to HSA partially inhibited the drugs entry into the brain, but the inhibition was less than would have been expected from the *in vitro* measurement.

Interestingly, lipoproteins had no effect on the drug transport process. Hence it is concluded that the dissociation of these two drugs from their binding proteins is enhanced in the brain microcirculation and the measurement of free drug fraction *in vitro* may underestimate the *in vivo* exchangeable drug fraction. However, the variations in free drug fraction measured *in vitro* will still parallel the variations in exchangeable drug fraction *in vivo*, provided these variations are not due to lipoprotein binding.

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