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Pipequaline Transport from Blood to Brain and Liver: Role of Plasma Protein-bound Drug

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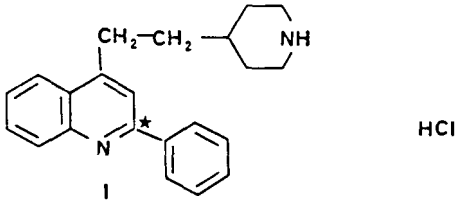
Abstract—Brain uptake of pipequaline (45319 RP) has been studied in-vivo after a single capillary transit by intracarotid injection to rats. Pipequaline is extensively bound to plasma proteins: i.e. human serum albumin (HSA), α-1-acid glycoprotein (AAG), lipoproteins and blood cells, mainly erythrocytes. The dialysable drug fraction as measured in-vitro by equilibrium dialysis at 37°C, was inversely related to the concentration of binding component. Similarly, the brain uptake of pipequaline was inversely related to the protein concentration of the injected solution. However, the measured brain uptake of pipequaline was higher than those predicted by in-vitro measurements of dialysable drug for all proteins and erythrocytes, except HSA. These results show that a fraction of bound pipequaline as measured in-vitro is available for transport through the blood brain barrier. HSA-bound pipequaline is an exception as it is restricted to the vascular space. Pipequaline was totally cleared by the liver through a single passage.

Pipequaline (I), a recently developed drug with anxiolytic properties, exhibits partial agonist activity on the central benzodiazepine receptor, although its chemical structure is not related to this class of drugs (Mizoule et al 1984).



I

1. [^{14}C]Pipequaline, a new anxiolytic drug.

However, like most benzodiazepine derivatives, it is a basic drug with a high degree of lipophilicity (hexane/water partition ratio = 5·7), and is extensively bound to plasma proteins and erythrocytes (Essassi et al 1987). In blood, pipequaline circulates highly bound to albumin (HSA) and α-1-acid glycoprotein (AAG), and bound with lower affinity to the three lipoprotein density classes (HDL, LDL, VLDL) and erythrocytes. Some recent reviews have emphasized differences between the free fraction of drug as measured in-vitro (dialysable fraction) and the fraction of drug available for tissue uptake (exchangeable fraction) (Pardridge 1981; Cornford 1985; Jones et al 1986). The exchangeable fraction of drug is generally greater than the dialysable fraction, which is in opposition to the commonly accepted hypothesis that only the free fraction of drug in blood is available for transport into tissues (Brodie et al 1960; Gillette 1973). Additionally, the binding component and the ligand are both determinants of the uptake for a given tissue. The exchangeable fraction of basic drugs such as lignocaine (lidocaine) or propranolol is equal to the dialysable fraction when bound to HSA; in contrast, it is greater when the drugs are bound to AAG (Pardridge et al 1983). Consequently, the purpose of this study was to investigate the effects of pipequaline binding to different proteins and to erythrocytes on brain uptake and additionally, on liver uptake in the rat.

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Materials and Methods

Radiolabelled compounds

[^{14}C]Pipequaline (51·5 mCi mmol^{-1}) was supplied by Pharmuka-Rhône Poulenc laboratories. Its radiochemical purity was >99% as assessed by thin-layer-chromatography on silica plates with chloroform–diethylamine 45:5 (v:v) system. The [^{3}H]water (5 mCi mL^{-1}) was purchased from Amersham (Gif-sur-Yvette, France). [^{14}C]Pipequaline was stored at −80°C until use.

Human plasma proteins

Two samples of HSA, essentially non-esterified fatty-acids-free (Sigma A-1887) (HSA), the second containing non-esterified fatty-acids (HSA-FFA) (Sigma A-2386), expressed as mole FFA/mole HSA (ratio 2) were used. Serum lipoprotein fractions, very low density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were fractionated by ultracentrifugation of normolipidic human serum pooled from healthy subjects: (4 males and 5 females: 26–40 years old) following Nelson's method (1980) modified by Glasson et al (1982).

The isolated lipoprotein fractions were dialysed overnight at 4°C against buffered Ringer's solution (pH 7·4). AAG was purchased from Behring (Marburg, FRG). Protein fractions were diluted to the desired-concentration with the buffered Ringer's solution pH 7·4.

The human erythrocytes from a healthy volunteer (female, 30 years old) were washed in 0·9% NaCl and adjusted to a haematocrit of 0·45 in a Ringer's buffer pH 7·4.

Brain uptake studies

The first-pass brain uptake of [^{14}C]pipequaline was measured according to Oldendorf's method (Oldendorf 1970). This

study was carried out with male Wistar rats (200–250 g) anaesthetized with pentobarbitone (500 mg kg^{-1} sodium pentobarbitone i.p.). Drug was first solubilized in a small volume of methanol (< 1% in the final solution), then, a mixture of approximately 0·8 μCi ($1{\cdot}77 \times 10^6$ d min^{-1}) of [^{14}C]pipequaline and 4 μCi ($8{\cdot}8 \times 10^6$ d min^{-1}) of [^{3}H]water in a 200 μL bolus of Ringer solution (mM: NaCl 150, KCl 5·2, $CaCl_2$ 2·2, $MgCl_2$ 0·2, $NaHCO_3$ 6, glucose 2·8) buffered to pH 7·4 with 5 mM HEPES buffer (H-Sigma) was injected rapidly (0·25 s) into the common carotid artery through a 26-gauge needle (Microlance, 26G318, 0·45 × 10 mm).

After injection, the needle was left in place to prevent bleeding and the carotid flow past the puncture site was unimpeded. The solution containing [^{14}C]pipequaline and ^3H_2O was injected in the absence or the presence of human isolated plasma proteins and erythrocytes. Five seconds after the bolus injection, the rat was decapitated and the hemisphere ipsilateral to the injection was cut off. The time of death was as early as possible to minimize tissue washout of drug and reference compound, and late enough to assume all of the non-extracted substance had passed out of the brain (Oldendorf 1970). Tissue samples were solubilized in 2 mL Soluene 350 (Packard) at 60°C overnight in an incubator, and decolourized with 0·5 mL H_2O_2 (33%). Then, 15 mL of Pico-Fluor 40 (Packard) was added to the vial. This treatment allowed double isotope liquid scintillation counting with standard quench correction. An aliquot of the injection solution was spiked to the control tissue and treated similarly. The number of d min^{-1} was generally between 150 000 and 500 000 for ^{3}H counting, and between 2000 and 10 000 for ^{14}C counting.

Liver uptake studies

For this purpose, the same injection solutions were injected as a 200 μL bolus in the portal vein, after ligation of the hepatic artery. At 18 s after injection, the right major lobe was removed and processed like the brain samples.

Estimation of drug-tissue uptake

Brain uptake index (BUI) or liver uptake index (LUI) were calculated as follows:

$$\text{BUI (LUI)} = \frac{(^{14}C/^3H)\text{d min}^{-1}\text{ tissue}}{(^{14}C/^3H)\text{d min}^{-1}\text{ injection}} \quad (1)$$

The BUI or LUI represents the net uptake of the drug normalized by the net uptake of the reference compound (here ^3H_2O).

The BUI or LUI is a direct function of the single pass extraction (E) of the drug ($E_d = E_r \times BUI$), where the subscripts d and r refer to the drug and reference compound.

With regard to the reference compound, the maximal extraction (E_{r0}) of tritiated water under the experimental conditions is 60% approximately (Pardridge et al 1982, 1983, 1984; Pardridge & Landaw 1984); the tissue-to-blood transport of the drug and ^3H_2O (measured as the remaining tissue radioactivity normalized by the injected dose and tissue weight) was determined at various times following the injection. The data were analysed using an iterative non-linear program to the formula:

$$E(t) = E(o) \times e^{-k \times t} \quad (2)$$

where E(t) and E(o) stand, respectively, for tissue extraction at time t and time zero, and k is the efflux rate constant. The uptake index at time t, BUI (t), is related to the efflux rate constants and initial uptake, BUI (o), by the following relation:

$$BUI(t) = BUI(o) \times e^{-(kd-kr)\times t} \quad (3)$$

The maximal extraction of the drug, $E_d(o)$, can then be calculated from the BUI (t) as follows:

$$E_d(o) = e^{kd \times t}\, BUI(t) \times E_r(t) \quad (4)$$

where kd is the efflux rate constant for the drug. Since the correction factor was negligible ($E_d(o) = 1{\cdot}009\ E_d(5s)$) we assumed that $E_d(5s)$ was essentially identical to $E_d(o)$.

Determination of drug binding in-vitro and in-vivo

According to the mass action law,

$$fu = Kd/(Kd + Pf) \quad (5)$$

where: Kd is the dissociation constant governing the binding reaction between drug and plasma protein, Pf is the concentration of the free protein binding sites, and fu is the drug-free fraction measured in-vitro. Since we used small concentrations of drug relative to the protein concentration in our experiments, the concentration of occupied binding sites (Pb) is negligible relative to the total concentration of the protein binding sites (Pt) and so Pf = Pt.

Equation (5) becomes:

$$fu = Kd/(Kd + Pt) \quad (6)$$

The drug protein binding was measured in-vitro by equilibrium dialysis at 37°C, for 3 h, as previously described (Essassi et al 1987). Protein solutions identical to those injected into the rats were used, and Kd was estimated with equation (6).

The binding of drug to erythrocytes was measured in-vitro after incubation of [^{14}C]pipequaline with erythrocytes for 1 h at 4°C. Total drug concentration and unbound drug concentrations were determined respectively, in the suspension (Ct) and in the supernatant (Cu) after a centrifugation at 1500 *g* for 15 min. The unbound drug fraction (mass of the drug contained in buffer) was obtained by

$$fu = (Cu/Ct) \times (1\text{-}H) \quad (7)$$

where H represents the haematocrit value (H = 50%). With the in-vivo studies, the extractable fraction of the drug in brain capillary when no binding protein is present is given by the Crone equation of capillary physiology (Crone 1973):

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

where the PS product is the permeability capillary surface area product, and F the rate of cerebral blood flow.

When a protein is present, the exchangeable fraction of drug (fu) can be diminished because of protein binding, and in this case equation (8) becomes:

$$E = 1 - e^{-fu \times PS/F} \quad (9)$$

Rearranged algebraically, and in consideration of equation (6), equation (9) is equivalent to:

$$E = 1 - e^{-(PS/F)/(1 + Pt/Kd)} \quad (10)$$

So, Kd and PS/F were estimated by fitting of the experimental extraction data to equation (10). The application of

equation (9) to the transport of plasma protein-bound ligands assumes that the ligand-protein binding reaction is maintained in near equilibrium as the bolus passes through the microcirculation (Terasaki et al 1986).

Analysis of data

The binding parameters and PS/F values were estimated by means of a non-linear regression program using a Gauss-Newton algorithm. Preliminary estimations of the parameters were obtained by linearization of equation (6).

$$1/fu = 1 + Pt/Kd$$

and equation (9),

$$-1/\text{Log}(1\text{-}E) = 1/(PS/F) + Pt/(Kd \times (PS/F)).$$

All values are presented in the form of a mean (m), standard deviation (s.d.) and sample number (n).

Results

Uptake and washout from brain

The extraction values for [^{14}C]pipequaline at various times after carotid injection in non-protein Ringer's solution are shown in Fig. 1. The drug present in trace concentration in the injection solution was rapidly cleared from the brain microcirculation with $E_d(o)$ value of $29{\cdot}5 \pm 7{\cdot}92\%$ (n = 6).

As shown in Fig. 1, the drug was retained by the brain and washed out from the brain at a slow rate compared with tritiated water. The efflux rate-constants (k efflux) calculated from the slopes were of $3{\cdot}24 \pm 1{\cdot}14$ min^{-1} and $0{\cdot}582 \pm 0{\cdot}096$ min^{-1} for [^{14}C]pipequaline and ^3H_2O, respectively. These results suggest that the drug is sequestrated in brain tissues.

Table 1 summarizes the brain uptake values measured for pipequaline in the presence of human serum proteins. As shown, addition of each of them to the injected solution decreases brain extraction. However, the reduction in brain extraction was less than the predicted values based on the free drug fraction in-vivo.

Table 1. Effect of human serum, HSA, AAG, HDL, LDL and VLDL on the rat brain extraction of pipequaline. Expected brain extraction (values are derived from equation (9) using fu values measured in-vitro). The numbers in parentheses are the standard deviation. HDL: high-density lipoprotein, LDL: low-density lipoprotein, VLDL: very low-density lipoprotein, HSA: human serum albumin, AAG: α-1-acid glycoprotein.

Protein	Dialysable (n = 3) fu (%)	Brain extraction Measured (n = 4) Ed × 100	Brain extraction Expected
None	100	29·5 (7·92)	
HSA 39·6 g L^{-1}	34·7 (1·5)	13·2 (1·49)	11·0
AAG 0·6 g L^{-1}	29·5 (3·5)	13·9 (3·69)	8·5
HDL 2 g L^{-1}	23·3 (1·7)	11·5 (0·71)	5·30
LDL 2 g L^{-1}	19 (1·4)	15·5 (2·12)	6·00
VLDL 1·5 g L^{-1}	40 (2·8)	14·0 (0·85)	9·00
Human serum	1·3 (0·28)	14·0 (1·2)	0·40

Figs 2 and 3 show the effects of different AAC and HSA concentrations on the brain extraction of pipequaline. As shown, the measured values were similar to the predictive values for HSA, in contrast to AAG, LDL and HDL for which the measured extraction was higher than that predicted.

Table 2 indicated that the concentration of either AAG or lipoproteins that caused a 50% reduction in the brain extraction (Kd in-vivo) was higher than that estimated from in-vitro studies (Kd in-vitro). The mean PS/F value was $0{\cdot}29 \pm 0{\cdot}065$ (n = 20).

Except for HSA, these results clearly suggest that the in-vivo exchangeable fraction is higher than the in-vitro dialysable fraction, i.e. some protein-bound fraction of pipequaline is partly available for transport into the brain.

The effects of erythrocytes on pipequaline brain extraction are shown in Table 3; their presence in the injection solution

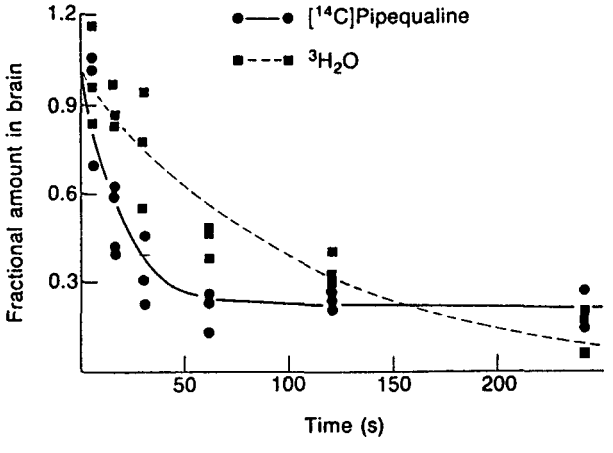

FIG. 1. Time course of rat brain extraction ratio of pipequaline after bolus injection in carotid artery of [^{14}C]pipequaline (●) and ^3H_2O (■) in Ringer solution, pH 7·4. Ordinate: fraction of initial amount per unit of brain weight; arbitrary units. Results are shown as the mean ± s.d. for groups containing three to four rats at various times after bolus injection. Analysis of variance showed that time had a significant effect on the brain radioactivity amount ($P < 0{\cdot}001$). The curves are drawn according to the efflux rate constant derived from non-linear analysis of the data. The efflux rate constant estimated for ^3H_2O washout is $0{\cdot}58 \pm 0{\cdot}096$ min^{-1}. The difference between pipequaline and ^3H_2O washout suggests tissue binding of the drug.

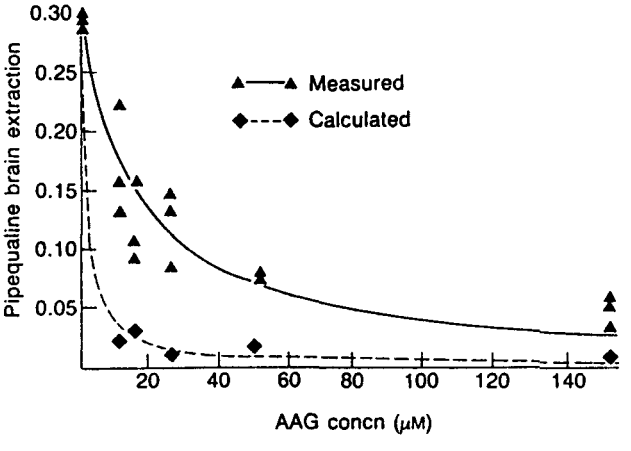

FIG. 2. Predicted ◆ and observed brain ▲ extraction for various concentrations of proteins in carotid solution. AAG: α-1-acid glycoprotein. The solid line represents the observed values (mean ± s.d., n = 4 rats per point), and the dotted line was simulated according to equation $E = 1 - e^{-fu \times PS/F}$, where fu is the measured dialysable drug fraction (n = 4 rats) and PS/F is estimated from the above equation.

Table 2. Apparent dissociation constants (Kd μM) estimated from in-vitro and in-vivo studies. Numbers in parentheses, s.d. For abbreviations, see Table 1. (Significant difference between in-vitro and in-vivo values, * = $P < 0.05$, *** $P < 0.001$).

Proteins	HSA	AAG	HDL	LDL	VLDL
In-vivo	381 (82)*	13·2 (1·9)***	4·74 (0·45)***	9·74 (1·69)***	1·35 (0·48)***
In-vitro	312 (9)	1·16 (0·13)	2·86 (0·16)	0·16 (0·01)	0·085 (0·028)

Table 3. Effect of human erythrocytes on the rat brain extraction and the free dialysable in-vitro percentage of pipequaline. Numbers in parentheses (s.d.). (Significant difference between human serum and Ringer solution, *** $P < 0.001$).

Samples	Dialysable (n = 4) (%)	Brain extraction	
		Measured (n = 4) Ed × 100	Expected
Control	100	26 (0·81)	
Erythrocytes (Haematocrit = 0·50)			
Human serum	12·3 (0·6)***	5·43 (0·08)***	3·80***
Ringer solution	24·3 (1·5)	10·2 (0·17)	7·30

at a haematocrit of 0·50 results in a decreased brain uptake, especially when they were diluted in serum.

Even at very low concentrations (0·50 to 1·50 ng mL^{-1}), pipequaline was highly bound to serum erythrocytes (Essassi et al 1987) and their contribution in the blood transport of pipequaline (12%) was not negligible (Essassi et al 1987). So, it is worth noting that their inhibitory effect on brain uptake is important.

Liver uptake studies

Table 4 summarizes the effect of the different serum proteins studied on the liver extraction of pipequaline. As shown in Fig. 4, the drug was not retained in the liver capillary space and freely transferred into hepatic cells, and the presence of

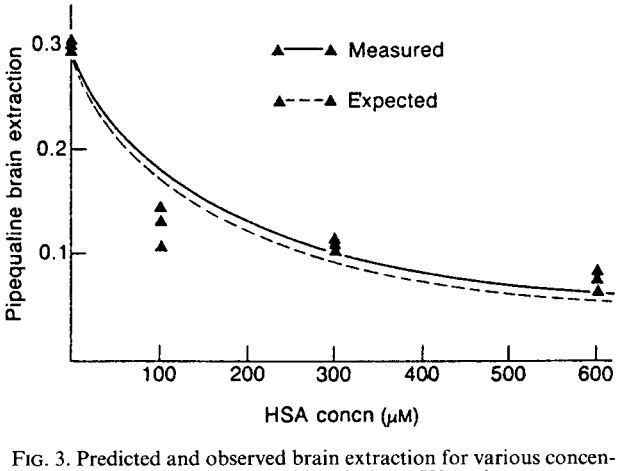

FIG. 3. Predicted and observed brain extraction for various concentrations of proteins in carotid solution (HSA: human serum albumin). The solid line represents the observed values (mean ± s.d., n = 4 rats per point), and the dotted line was simulated according to equation $E = 1 - e^{-fu \times PS/F}$ where fu is the measured dialyzable fraction (n = 4 rats) and PS/F is estimated from the above equation.

Table 4. Liver extraction of [^{14}C]pipequaline. Effect of proteins, erythrocytes and serum binding. There was no significant difference between extraction in the absence of protein, erythrocytes and serum and the extraction in the presence of any of them. Mean ± s.d., n = 4 rats per point. For abbreviations, see Table 1. FFA = free fatty acids.

Protein	Extraction (LUI × 0·82) (n = 4)	
Buffer	1·28 (0·19)	
Human serum	1·18 (0·025)	
Rat serum	1·11 (0·013)	
Buffer	1·003 (0·56)	
HSA 100 μM	HSA-FFA	HSA
300	0·73 (0·053)	0·915 (0·054)
600	1·11 (0·34)	1·09 (0·20)
	1·15 (0·053)	0·74 (0·35)
Buffer	0·986 (0·35)	
AAG 10 μM	0·84 (0·011)	
15	0·88 (0·039)	
25	0·94 (0·079)	
30	1·48 (0·38)	
Buffer	1·15 (0·077)	
Pool of lipoproteins (LDL 3·5 g L^{-1} + HDL 4 g L^{-1})	1·06 (0·077)	
LDL 5 g L^{-1}	1·13 (0·083)	
Buffer	0·86 (0·020)	
Human erythrocytes (haematocrit = 50)		
—Human serum	0·88 (0·056)	
—Ringer solution	0·94 (0·12)	

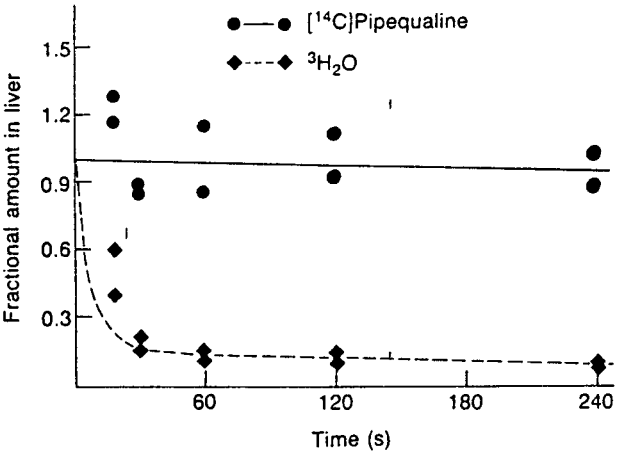

FIG. 4. Extraction (mean ± s.d., n = 4 rats per point) of [^{14}C]pipequaline (●) and tritiated water (◆) at various times (18 s—240 s) after rapid portal injection of 200 μL bolus of Ringer solution. Ordinate: fraction of initial amount per unit of liver (weight 120 mg, arbitrary unit). The curves are drawn according to the efflux rate constant derived from non-linear analysis of the data. The efflux rate constant estimated for ^3H_2O washout is equal to: k efflux = 0·186 ± 0·063 and for [^{14}C]pipequaline, the efflux rate constant is equal to: k efflux = 0·018 ± 0·0013. The t 1/2 values are: t 1/2 ^3H_2O = 0·41 min, comparable with that found by Pardridge (25 s) (Pardridge & Mietus 1979), t 1/2 [^{14}C]pipequaline = 0·17 min.

proteins or erythrocytes had no effect on the liver extraction. Because the maximal extraction of ^3H_2O at 18 s is 82% (Pardridge & Mietus 1979), the net extraction of [^{14}C]pipequaline at 18 s ($E_d(18\ s) = LUI \times 0{\cdot}82$) is approximately 100%. These results indicate that pipequaline is completely cleared through a single circulatory passage in the liver (Table 4, Fig. 4).

Discussion

The main result provided by our investigations is that the drug binding to plasma proteins had an inhibitory effect on the brain extraction of pipequaline, while it had no effect on its liver uptake. However, a part of the drug bound to proteins or erythrocytes was availabe for transport through the blood brain barrier (BBB).

Previous reports have demonstrated that a significant blood fraction of protein-bound ligand may be available for tissue diffusion (Pardridge 1981). Nevertheless, this assumption depends largely on the particular tissue as well as on the plasma protein. Studies with basic drugs like lignocaine or propranolol (Pardridge et al 1983) indicated that a part of the AAG-bound drug fraction could diffuse through the BBB but not the HSA-bound fraction, which is analogous to our results. Other reports have showed that HSA-bound steroid hormones (Pardridge & Mietus 1979), zonisomide (Cornford & Landon 1985) and valproate (Cornford et al 1985) were partially available for diffusion into the brain, as for pipequaline.

Concerning the influence of drug association with lipoproteins on their tissue extraction, there is little information at the moment. Some studies (Urien et al 1987; Hamberger et al 1987) have shown that drug binding to lipoproteins did not limit the transfer of drug into brain or liver. Our results show that pipequaline binding to lipoproteins inhibited to approximately 50% the brain transfer while no effect was observed with the liver uptake.

Up to now, the influence of erythrocytes binding on the brain uptake has rarely been investigated. Zonisomide and progabide have been reported to be unbound to erythrocytes and to be partially available for diffusion through the BBB (Cornford & Landon 1985; Hamberger et al 1987). Concerning pipequaline, its transport into the brain was inhibited by binding to erythrocytes, but the bound fraction remained partially available for brain extraction.

So, even if the transport of drug into the brain was inhibited by serum proteins (except HSA) and erythrocytes, the extent of inhibition was less than the predicted value based on the 'free drug hypothesis' (Fig. 2). This result indicates that there seems to be no need to take into account only the unbound drug concentration.

Thus, our results are in agreement with the general observation that the exchangeable fraction in-vivo is higher than the dialysable fraction (in-vitro) of drug (Tables 2, 3). This may be explained by the 'free intermediate' model described and discussed by Pardridge & Landaw (1984):

$$DP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Pf + Df \overset{k_3}{\rightleftharpoons} Db \qquad (15)$$

where DP is the drug-protein complex, Df and Db are respectively, the free drug in the microcirculation and in the brain, and k_1, k_2 and k_3 are respectively, the dissociation, association and plasma to brain transport rate constants $Kd = k_2/k_1$.

In this model, the capillary transit time (1 s in brain, 10 s in liver), the unidirectional dissociation from the protein, and the rate of drug diffusion through the biological membranes lining the blood compartment are the three primary determinants. Additionally, it is assumed that the rate of drug dissociation from the binding protein is higher in-vivo than in-vitro, owing to specific interactions between the plasma protein and components lining the surface of the microcirculation (Pardridge & Landaw 1984). Also, the model assumes that there is minimal mixing of the carotid injection bolus with the circulating rat blood as the bolus courses through the capillary network. This parameter was measured recently in the cerebral microvasculature and was 5% or less (Pardridge et al 1985). An alternative is that the binding proteins act as donor proteins delivering drug to cells, which implies the existence of plasma protein receptors on the capillary endothelium (Urien et al 1987). There have been reports suggesting the serum proteins mediated the transport of hormones (Pardridge 1981) and drugs (Pardridge et al 1983) into liver and brain. Accordingly, a part of the protein-bound ligand is available for entry into the brain provided drug-protein binding reactions are fast relative to the membrane transport reaction.

Concerning the liver uptake, the drug under study was totally cleared by the liver through a single passage. Neither plasma proteins nor cells had an effect on the hepatic uptake. This suggests that the intrinsic clearance largely exceeds the hepatic blood flow. Some studies have shown that physiological substances such as free fatty acids (Weisiger et al 1981) or bile salts (Forker & Luxon 1983) bound to HSA may enter the liver via an HSA-receptor mediated mechanism. However, there is, as yet, no evidence of an AAG-receptor at the surface of liver cells.

In conclusion, the major finding of this study is that blood protein bound pipequaline is partially available for transport through brain capillaries and completely available for transport through the liver. Therefore, the measurement of the free drug fraction in-vitro would underestimate the in-vivo exchangeable drug fraction. However, the exchangeable drug fraction remains roughly parallel to the dialysable drug fraction, which may still be considered as a useful parameter.

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