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BINDING *IN VITRO* OF PIPEQUALINE (45319 RP) ONTO PLASMA PROTEINS AND BLOOD CELLS IN MAN

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Abstract—Serum binding of pipequaline, a new anxiolytic drug, was studied *in vitro* by equilibrium dialysis. The percent binding in serum is high, 96.3%, and remains constant within the range of therapeutic concentrations. Investigations performed on isolated proteins with a wide range of concentrations showed one site with a high affinity constant ($K_a = 450{,}000\ M^{-1}$) for α_1-acid glycoprotein and two sites with a lower affinity constant ($K_a = 58{,}000\ M^{-1}$) for human serum albumin. Binding to lipoproteins was saturable, with an affinity constant of $22{,}000 \leq K_a \leq 35{,}000\ M^{-1}$. Over the range of therapeutic concentrations, the ratio of pipequaline concentrations in serum and red blood cells remained constant (14.4%) and was shown to be dependent on the free fraction of pipequaline in serum.

Binding of a drug onto macromolecules and/or particulate elements of blood may influence its pharmacological effects. For this reason, the binding of any new molecule should be studied and the binding parameters determined.

The purpose of this work was to determine *in vitro* the characteristics of binding of pipequaline (45319 RP), a new anxiolytic drug, to human plasma proteins, erythrocytes and lymphocytes. The numbers of binding sites on these proteins and cells, the corresponding affinities and the variation of the percentage of drug bound in plasma within the therapeutic range of concentrations, were determined using a ^{14}C-labelled molecule.

Pipequaline (45319 RP) (Pharmuka-Rhône-Poulenc), or (Piperidyl 4)2-ethyl 4-phenyl 2-quinoline, hydrochloride, a new quinoline derivative, shows a high affinity for brain-type benzodiazepine binding sites and an anticonflict activity in the Vogel Test [1]. However, unlike the classical benzodiazepines, it is devoid of anticonvulsant and sedative effects [1].

This compound is very lipophilic and the partition coefficient of the molecule (hexane/water) is 5.7. It is a basic molecule, the pK_a of quinolinic group is 5.0.

Pipequaline and ^{14}C-pipequaline (4-piperidyl 2-ethyl 4-phenyl ^{14}C-2 quinoline) were provided by Pharmuka-Rhône-Poulenc Laboratories.

MATERIALS AND METHODS

Binding of pipequaline has been first studied in human serum obtained from healthy volunteers (4 men, 3 women) ranging in age from 25 to 40 years. These donors were drug free and fasted before blood collection. Serum samples were pooled and stored at −30°. The concentration of human serum albumin (HSA) was 560 μM. The concentration of free fatty acids (FFA) was such that the molar ratio FFA/HSA was 1.8 [2]. The concentration of α_1-acid glycoprotein (α_1-AGP) measured by radial immunodiffusion (M-Partigen, Behringwerke) was 20 μM. Two samples of HSA, one essentially FFA-free (Sigma A—1887), the second containing FFA (Sigma A—2386), expressed as mole FFA/mole HSA (ratio = 2) were used to study binding of pipequaline to HSA and the possible influence of FFA on it. Lipoproteins were fractionated by ultracentrifugation of normolipidic human serum following Nelson's method [3] modified by Glasson *et al.* [4]. Protein concentration was measured according to the method of Lowry *et al.* [5]. Lipoproteins like α_1-AGP, HSA and γ-globulins (Sigma HGII, 99% purity) were dissolved in phosphate buffer pH 7.4. Human erythrocytes were washed in 0.9% NaCl and adjusted to a haematocrit of 0.45 in phosphate buffer (0.067 M) containing 0.9% NaCl, pH 7.4. In addition, the same haematocrit (0.45) was reconstituted in the plasma of the same donor with the washed human erythrocytes.

Pipequaline was incubated for 15 min at 37° with erythrocytes and the mixture then centrifuged at 1500 *g* and +4° for 10 min. An aliquot of the supernatant was assayed in order to estimate the total fraction of pipequaline in the buffer or in the serum.

Finally, lymphocytes were isolated from the blood of a healthy 25 year old volunteer and adjusted to a concentration of 3.10^6 cells/ml and a protein-content of 0.8 mg/ml. They were isolated by the density gradient method using Ficoll-Paque (Pharmacia) according to Boyum [6].

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Protein binding measurement. The method used was equilibrium dialysis with a Dianorm® apparatus comprising 20 pairs of chambers each of 0.2 ml capacity, maintained under constant stirring, 20 rpm at 37°. The semi-permeable membrane (SPECTRAPOR 2) had a molecular weight cut off limit of 12,000–14,000 daltons. Equilibrium was reached after three hours. After this equilibrium, the percentage of bound pipequaline was calculated from the concentrations measured in the two parts (A and B) of the chamber, according to the formula

$$\text{bound product (\%)} = \frac{B - A}{B} \times 100 \qquad (1)$$

A and B are respectively representing the concentrations of free and total drug (free + bound).

At equilibrium, the concentrations in each compartment were measured by liquid scintillation counting (Packard Tricarb 460 CD). Free (F) and bound (B) molar concentrations of ^{14}C-pipequaline were calculated. The data obtained at equilibrium (B and F) were fitted according to

$$B = \sum_{j=1}^{m} \frac{N_j K_{aj} F}{1 + K_{aj} F} = \sum_{j=1}^{m} \frac{n_j R K_{aj} F}{1 + K_{aj} F} \qquad (2)$$

where N_j, n_j, K_{aj} denote, respectively, the molar binding sites concentration of the jth class, the numbers of binding sites and the affinity constant to this specific protein; R denotes the concentration of the protein.

The parameters n and K_a were calculated by a non-linear method using a Gauss–Newton algorithm.

Statistics for the numbers of binding site (n) and the affinity constants (K_a) were calculated using the Student's test with $(a - b)$ degrees of freedom where a was the number of experimental points and b the number of estimated parameters.

The concentrations of pipequaline in the phosphate buffer, pH 7.4, serum and erythrocytes were measured after centrifugation. The concentration of ^{14}C-pipequaline in the erythrocytes (C_E) was obtained by the following equation

$$C_E = \frac{C_s - C_p (1 - H)}{H} \qquad (3)$$

where H represents the value of the haematocrit, C_s and C_p, respectively represent the total blood and plasma concentrations of pipequaline.

The erythrocyte total bound fraction f_E is given by:

$$f_E = \frac{C_E}{C_S} \times H \qquad (4)$$

The fraction of drug bound to erythrocytes (f'_E) in whole blood when there is only passive diffusion, is given by:

$$f'_E = \frac{H}{1 + \frac{f_b}{f_u} - H \times \frac{f_b}{f_u}} \qquad (5)$$

where f_u and f_b respectively represent the free and bound fractions of pipequaline in the total plasma.

RESULTS

Within the range of concentrations from 0.15 μM to 1.5 μM (50–500 ng/ml), the percentage of binding of ^{14}C-pipequaline in the serum, to each of the circulating proteins and blood components remained constant (Table 1).

At higher concentrations, from 1.5 μM to 120 μM, the percentage of pipequaline binding remained constant in the serum 96.3 ± 0.8 with a binding coefficient $NK_A = 32.5 \pm 0.3$ (Table 2). Binding of pipequaline to α_1-AGP (75 μM) over loaded serum showed a higher binding percentage ($P < 0.1$) and also a higher binding coefficient ($P < 0.001$) (Table 2).

At concentrations of pipequaline between 0.15 μM and 90 μM, there was a saturation of HSA at 10 μM (Fig. 1) with a number of sites: $n = 1.86 \pm 0.16$ and

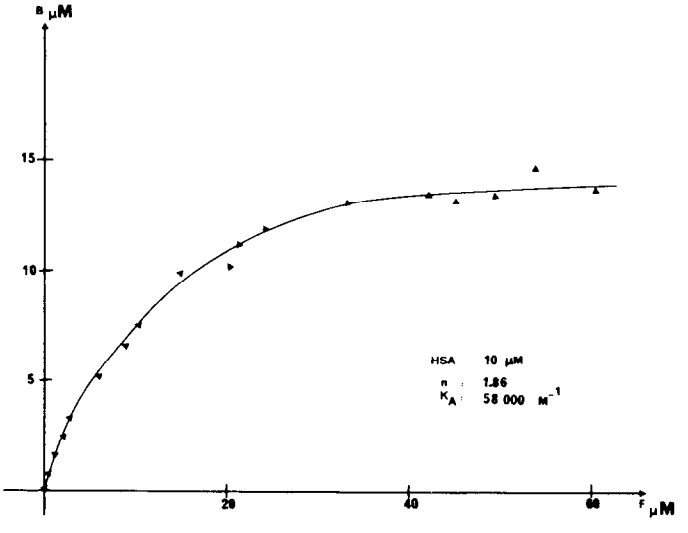

Fig. 1. Binding of pipequaline to HSA. The HSA concentration is 10 μM. B and F are respectively the concentration of bound and free pipequaline. Each point represents the mean ± SD of three determinations.

Table 1. Percentage of binding pipequaline to plasma proteins and to particulate components of the blood at therapeutic concentrations

Proteins	(μM)	^{14}C pipequaline (μM)					
		0.15	0.25	0.50	1	1.5	M ± SD
Serum I	HSA = 560						
	α_1-AGP = 20	94.8 ± 0.7	96.4 ± 1.2	96.7 ± 0.8	96.8 ± 2.3	96.7 ± 0.9	96.3 ± 0.8
Serum II	HSA = 560						
	α_1-AGP = 75	97.7 ± 0.23**	97.0 ± 0.8	96.8 ± 1.7	97.0 ± 3.1	97.2 ± 0.5	97.1 ± 0.3*
	HSA 575	87.6 ± 1.7	87.0 ± 0.85	85.0 ± 2.8	88.2 ± 4.4	89.5 ± 1.56	87.5 ± 4.65
HSA + FFA	575–1200	78.3 ± 2.3**	75.6 ± 2.3**	77.8 ± 4.1**	82.4 ± 2.1**	79.9 ± 0.6**	78.8 ± 2.5**
	α_1-AGP 10	86.2 ± 0.9	85.3 ± 1.6	88.8 ± 1.2	88.3 ± 1.5	89.0 ± 1.0	87.5 ± 1.7
	VLDL 0.2	20.0 ± 2.0	19.8 ± 1.5	21.0 ± 2.8	18.9 ± 3.0	19.8 ± 1.8	19.9 ± 0.7
	LDL 0.5	83.8 ± 0.2	81.4 ± 0.8	81.1 ± 0.1	82.1 ± 2.1	82.5 ± 0.7	82.2 ± 1.1
	HDL 5	89.0 ± 0.8	89.5 ± 0.4	90.1 ± 0.7	88.4 ± 0.9	89.3 ± 0.9	89.3 ± 0.6
γ-globulins	100	25.0 ± 4.2	30.2 ± 2.9	28.6 ± 2.3	24.9 ± 4.9	28.9 ± 2.3	27.5 ± 2.4
Erythrocytes	Ht = 0.45	94.8 ± 1.3	95.0 ± 3.1	96.0 ± 2.1	97.0 ± 1.8	96.2 ± 2.1	95.8 ± 0.9
Lymphocytes	3.10^9 cells/l	63.7 ± 1.6	62.9 ± 1.1	63.4 ± 0.6	63.6 ± 0.3	64.3 ± 0.9	63.6 ± 0.6

*$P < 0.10$; ** $P < 0.001$. (Student's test.)
Each value represents the mean of three to five measurements with the standard deviation (M ± SD)

Table 2. Binding parameters of pipequaline to plasma proteins and to the particulate components of the blood

Proteins (μM)	Parameters				
	N (μM)	n	K_a (M^{-1})	NK_a	nK_a (M^{-1})
Serum I	—	—	—	32.57 ± 0.35	—
Serum II	—	—	—	39.8 ± 0.36**	—
HSA 10	18.6 ± 1.17	1.86 ± 0.16	58,000 ± 13,000	0.892 ± 0.057	116,000 ± 35,000
α_1-AGP 10	8.44 ± 0.72	0.84 ± 0.11	454,000 ± 56,000	3.83 ± 0.80	383,000 ± 97,000
VLDL 0.2	100 ± 7.6	500 ± 35	35,000 ± 4500	3.50 ± 0.70	(17.5 ± 3.5) × 10^6
LDL 0.14	77.1 ± 7.7	550 ± 55	26,000 ± 4000	2.00 ± 0.51	(14.3 ± 3.6) × 10^6
HDL 1.63	78.2 ± 15.5	48 ± 10	22,000 ± 7000	1.75 ± 0.40	1.07 ± 0.22
γ-globulins 100	—	—	—	0.118 ± 0.010	1180 ± 120
Erythrocytes (Ht = 0.45)	—	—	—	14.9 ± 1.2	—
Mb Membranes	—	—	—	16.3 ± 0.61	—
Hb Hemoglobins	—	—	—	2.26 ± 0.07	—
Lymphocytes 3.10^9 cells/l	20.9 ± 1.7	—	80,000 ± 13,000	2.33 ± 0.51	—

N, concentration of binding sites; n, number of binding sites per protein molecule; K_a, association constant or affinity at equilibrium of drug; NK_a, binding coefficient (product of the number of protein sites (N) and the association constant at equilibrium (K_a) of a drug); nK_a, total affinity of a drug (product of the intrinsic site number (n) and its binding constant K_a); **$P < 0.001$. (Student's test.)

association constant: $K_a = 58{,}000 \pm 13{,}000\ M^{-1}$ (Table 2).

When the concentration of α_1-AGP was 10 μM, there was saturation with a number of sites: $n = 0.84 \pm 0.11$ and affinity: $K_a = 450{,}000 \pm 56{,}000\ M^{-1}$ (Fig. 2).

The presence of FFA in HSA (FFA/HSA ratio = 2) induced a significant decrease in binding of pipequaline to HSA ($P < 0.001$) (Table 1).

Higher concentrations of pipequaline (10–800 μM) showed a saturable binding to the lipoproteins with affinity: $K_a = 22{,}000 \pm 7000\ M^{-1}$ for HDL, $26{,}000 \pm 4000\ M^{-1}$ for LDL and 35,000 ± 4500 for VLDL (Table 2).

Binding of pipequaline to γ-globulins did not reach saturation and the total affinity was: $nK_a = 1180 \pm 120\ M^{-1}$ (Table 2).

At therapeutic concentrations (0.15–1.5 μM), the

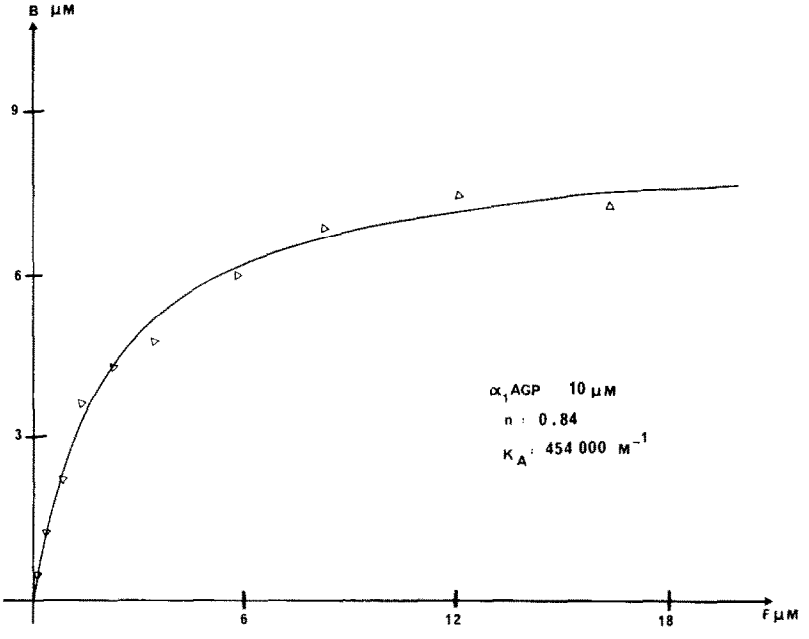

Fig. 2. Binding of pipequaline to α_1-AGP. The α_1-AGP concentration is 10 μM. B and F are respectively the concentration of bound and free pipequaline. Each point represents the mean ± SD of three determinations.

Table 3. Percentage of binding of pipequaline to erythrocytes at therapeutic concentrations

^{14}C pipequaline (μM)	0.15	0.25	0.50	1	1.5	M ± S.D.
f_{ET} (%)	94.8 ± 1.3	95.0 ± 3.1	96.0 ± 2.1	97.0 ± 1.8	96.2 ± 2.1	95.8 ± 0.9
f_{ES} (%)	39.8 ± 1.0	38.8 ± 1.2	40.0 ± 0.7	44.0 ± 1.0	40.0 ± 1.0	40.5 ± 2.0
f'_E (%)	2.8	2.8	2.8	2.8	2.8	2.8
f_{ES}/f'_E	14.2 ± 0.4	13.9 ± 0.4	14.3 ± 0.3	15.7 ± 0.4	14.5 ± 0.4	14.4 ± 0.7

f_{ET}, erythrocyte fraction of pipequaline in the buffer; f_{ES}, erythrocyte fraction of pipequaline in the blood; f'_E, erythrocyte fraction of pipequaline in the blood during passive diffusion.
Each value represents the mean of three to five measurements with the standard deviation (M ± SD).

binding of pipequaline to erythrocytes showed no saturation and remained significantly high (95.8 ± 0.9%) (Table 1) with a binding coefficient $NK_a = 14.9 \pm 1.2$ (Table 2). This binding was rather to the membrane of erythrocytes than to hemoglobin showing a binding coefficient eight times higher in membranes than in hemoglobin. In addition, the erythrocyte pipequaline bound fraction is higher in presence of phosphate buffer than in the presence of plasma (40.5 ± 2.0%) (Table 3), indicating a retention of pipequaline in the plasma proteins.

For lymphocytes, the binding of pipequaline reached a saturation with an affinity constant: $K_a = 80{,}000 \pm 13{,}000\ M^{-1}$ (Table 2) and a concentration of sites $N = 9.6$ nmoles/10^6 cells (or 20.9 ± 1.7 μM in a suspension of 3×10^9 cells per liter) (Table 2). However, at concentrations from 0.15 μM to 1.5 μM, binding of pipequaline did not reach saturation and the percentage of binding was of 63.6 ± 0.5 (Table 1).

Pipequaline binding to HSA in the presence of diazepam and warfarin. Within the range of concentrations from 1 to 100 μM, the binding of ^{14}C-pipequaline to HSA (10 μM) was characterized by an affinity constant, K_a, of 66,000 ± 7400 M^{-1}, with a number of binding sites, n, equal to 2.03 ± 0.07 (Fig. 3). In the presence of 200 μM of diazepam or warfarin, there was a significant competitive inhibition of pipequaline (Fig. 3), since the affinity constant became approximately twice lower in presence of diazepam ($K_a = 35{,}900 \pm 3500\ M^{-1}$) or warfarin ($K_a = 34{,}300 \pm 3200\ M^{-1}$), whereas the number of binding sites was constant ($n = 2.03 \pm 0.07$) (Table 4).

On the other hand, the two curves showing the pipequaline binding in presence of diazepam or warfarin were not statistically different (Fisher test) (Fig. 3 and Table 4).

DISCUSSION

These results show that the total blood binding of pipequaline is higher than 99%. This high percentage results essentially from its binding to HSA, α_1-AGP, lipoproteins and particulate elements of blood (erythrocytes and lymphocytes) (Fig. 4). Addition of free fatty acids (FFA) to HSA leads to a decreased binding of pipequaline (Table 1). These FFA are transported by HSA [7–9], their binding to this protein is accompanied by a change in HSA-configuration and a decrease of the affinity of the binding sites, especially of the site II [10]. The longer the

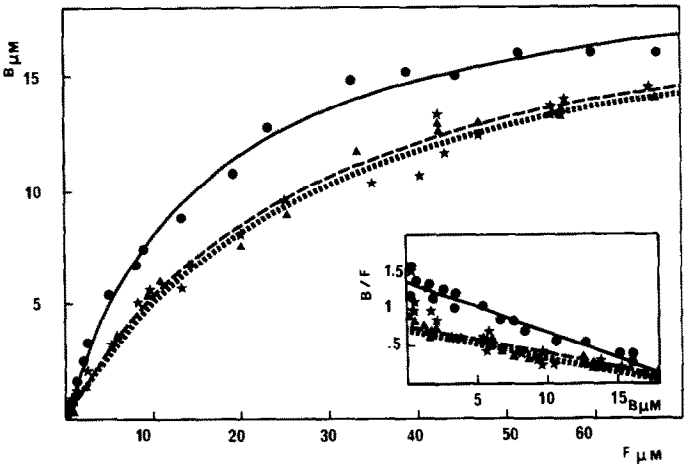

Fig. 3. Binding of ^{14}C-pipequaline to HSA in presence and absence of diazepam or warfarin. Plot showing the binding of ^{14}C-pipequaline to human serum albumin (10 μM) alone (●), and in the presence of 200 μM of diazepam (▲) or 200 μM of warfarin (★). B and F are the concentrations of bound and free drug, respectively. Each point represents the mean ± SD of three determinations. The insert is the Scatchard plot.

Table 4. Pipequaline affinity constant for HSA in the presence of diazepam or warfarin

Drugs (μM)	K_a (M^{-1})
Pipequaline alone (1–100)	66,000 ± 7400
Pipequaline + diazepam (200)	*35,900 ± 3500
Pipequaline + warfarin (200)	*34,300 ± 3200

^{14}C-pipequaline concentration varied from 1 to 100 μM. HSA was 10 μM and the number of binding sites, n, was constant, at 2.03 ± 0.07. Mean ± SD of three determinations; differences tested by the Fisher test.
*$P < 0.01$.

hydrocarbon chain of the FFA and the higher its extent of unsaturation, the more extensively will it affect the binding of drugs by HSA.

In general, the FFA do not promote a competitive displacement at sites I and II, but they are able, at physiological concentrations, to modify the binding of some drugs by allosteric mechanisms [10, 11]. This may have clinical significance [12] with some drugs such as binedaline [13], chlorpromazine [14], amitriptyline and nortriptyline [15], propranolol [16], erythromycin [17] and lidocaine [18]. That is also the case of pipequaline.

Although the affinity of pipequaline for α_1-AGP is higher than its affinity for HSA, the higher concentrations of HSA in serum in relation to those of α_1-AGP (30 times lower) suggest a quantitatively more important binding of pipequaline to HSA than to α_1-AGP.

Indeed, HSA binds about 56% of pipequaline (Fig. 3) in total blood. Contrary to the majority of lipophilic basic drugs (neuroleptic, antidepressive, β blocking etc.), pipequaline shows a saturable binding to HSA. Its behaviour to α_1-AGP is comparable to that of other basic drugs, i.e. one saturable site [14, 19, 20–24].

On the other hand, it is worthy to note that the affinity of pipequaline for HSA is weak in relation to that of other drugs such as diazepam [25–27] or warfarin [28–30]. Binding of pipequaline at low concentrations of lipoproteins reaches saturation but shows a weak affinity and a very high number of sites ($n \geq 500$ for LDL and VLDL, equal to 48 for HDL). These results are in accordance with a possible solubilization of pipequaline in the lipid core of lipoproteins rather than a binding to specific sites [31], especially as there is no binding to apoproteins (concerning apoproteins A, the percentage of binding is low, 5.9 ± 1.9 for concentrations of pipequaline from 20 to 120 μM).

These results may be compared with those obtained with other lipophilic basic drugs such as imipramine, chlorpromazine, propranolol and nortriptyline [4, 32].

At physiological concentrations, lipoproteins are able to bind about 22% of pipequaline bound in total

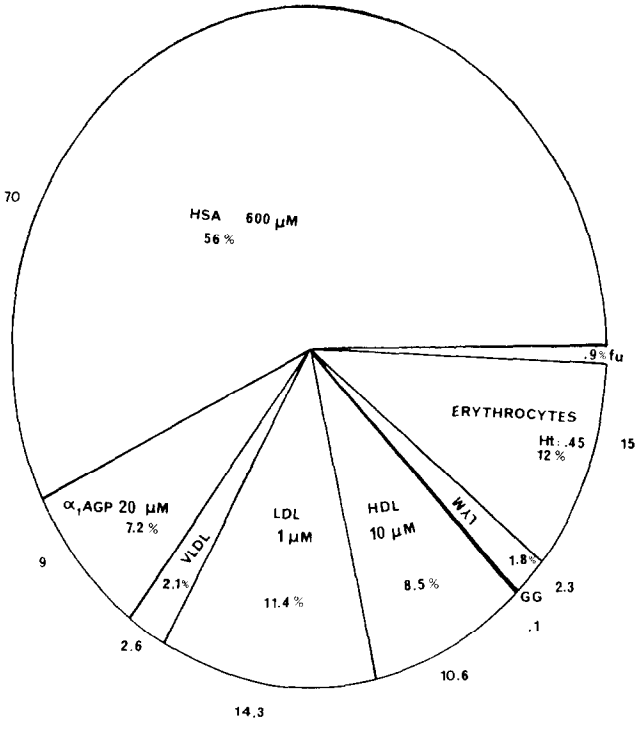

Fig. 4. Distribution of pipequaline in whole blood (%). The concentration of the various components is as follows: HSA = 600 μM; α_1-AGP = 20 μM; VLDL = 0.15 μM; LDL = 1 μM; HDL = 10 μM; γ-globulins (GG) = 100 μM; erythrocytes Ht = 0.45; lymphocytes = 3.10^9 cells/l. The values outside the circle correspond to the binding coefficient NK_a. The values inside the circle correspond to the % of binding of pipequaline in whole blood. f_u = fraction of free pipequaline in whole blood. The total pipequaline concentration was 1 μM.

blood (Fig. 3), this value is superior to that of α_1-AGP and erythrocytes. This effect could be raised in case of hyperlipoproteinemia.

In contrast, binding of pipequaline to isolate γ-globulins is weak (25%) and becomes negligible (0.1%) in relation to binding to total blood (Fig. 3).

Binding of pipequaline to erythrocytes is high (≈95%) in buffer but lower in the presence of serum (Table 3). In fact, the concentration of pipequaline in erythrocytes is 14 times more important than the free form of pipequaline in the plasma indicating an accumulation of pipequaline in erythrocytes (Table 3). However, the participation of erythrocytes in total blood binding is weak and represents only 12% of total binding (Fig. 3). As the binding of pipequaline to erythrocytes takes place essentially to the lipid fraction of the membrane (Table 2), it could be due mainly to the lipid fraction of the membrane. In total blood, the binding of pipequaline is preferentially to circulating lipoproteins: this explains the weak participation of erythrocytes in total blood binding.

Unlike erythrocytes, lymphocytes are able to bind pipequaline in a saturable manner, with an affinity comparable to that of HSA (Table 2) but therapeutic concentrations do not saturate these cells.

A remaining question is to analyze the effect of the plasma binding on the pharmacokinetic parameters of this drug. The apparent distribution volume is high, 11 l/kg (A. Uzan, personal communication) showing that blood binding has no major effect on tissue distribution. However, the fact that an important fraction of the blood bound drug was found in lipid structures, i.e. lipoproteins and erythrocyte membranes, suggests that tissue distribution involves lipidic structures, probably adipose tissue.

Pipequaline can be displaced from the HSA by diazepam or warfarin. The affinity constant of pipequaline was significantly lowered in the presence of these drugs, but the number of binding sites was not modified (Table 4 and Fig. 3). These results reveal that pipequaline displaced warfarin and diazepam from this protein by a significant competitive process. So, pipequaline could be both bound to the sites I and II on HSA [33–35] with an affinity constant relatively identical.

In therapeutics, these inhibitions observed on the isolated HSA, have little chance of occurring since the relevant clinical concentrations of pipequaline (0.5–1.5 μM), diazepam (0.5–3 μM) and warfarin 2–10 μM) are always below saturation values. However, if a displacement occurred, the excess of free drug would be in part, or completely, bound by other proteins (AAG, lipoproteins and erythrocytes membranes) according to the ratio of the respective affinity constants of these drugs to the different proteins.

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