0026-895X/87/030294-07$02.00/0

MOLECULAR PHARMACOLOGY, 31:294–300

Binding of Two Anthranilic Acid Derivatives to Human Albumin, Erythrocytes, and Lipoproteins: Evidence for Glafenic Acid High Affinity Binding

EDITH ALBENGRES, SAÏK URIEN, PASCALE RIANT, GEORGES A. MARCEL, and JEAN-PAUL TILLEMENT

Service Hospitalo-Universitaire de Pharmacologie, Hôpital Intercommunal, 40, avenue de Verdun, F-94010 Creteil Cedex (E.A., S.U., P.R., J.-P.T.) and Laboratoires Roussel, 97, rue de Vaugirard, 75279 Paris Cedex 06 (G.A.M.)

Received May 2, 1986; Accepted December 15, 1986

SUMMARY

The binding of two anthranilic acid derivatives, glafenic and floctafenic acids, to human erythrocytes and plasma proteins has been investigated *in vitro* by equilibrium dialysis. Despite their close chemical structures it was shown that the binding of the two compounds to serum albumin, lipoproteins, and erythrocytes was dramatically different both in quality and quantity. Using various techniques including fluorometry and circular dichroism, it was shown that glafenic acid binds to the human serum albumin (HSA) warfarin/azapropazone site and that floctafenic acid binds to both warfarin/azapropazone and benzodiazepine sites. Glafenic acid is strongly bound to HSA with $n = 1$, $k = 2.4 \times 10^6$ liters/mol and to erythrocytes with $N = 12.4$ μmol/liter, $K = 1.7 \times 10^6$ liters/mol. Floctafenic acid is bound with a weaker affinity to HSA, $n = 2$, $k = 0.3 \times 10^6$ liters/mol and to erythrocytes, $N = 2900$ μmol/liter and $K = 0.007 \times 10^6$ liters/mol.

HSA and erythrocytes are known to bind numerous xenobiotics. The bound fraction of acidic drugs in plasma is generally greater than 80% and this binding can be reasonably ascribed to drug association with HSA. Acidic drugs are commonly bound to HSA with binding constants in the range of 10^4–10^5 M^{-1}. The unique properties of this protein are explained by the presence of at least two discrete binding areas on the molecule for acidic drugs, named sites I and II (1–3). However, the possibility that certain acidic drugs could interact with either erythrocytes or lipoproteins has not been extensively investigated. Previous studies have shown that certain acidic drugs could be retained in erythrocytes, but no binding constants, maximal erythrocyte drug sites, or association constant were derived from the data (4).

Glafenic and floctafenic acids are extensively used analgesic drugs related to anthranilic acids (Fig. 1). Some reported adverse reactions involving mainly glafenic acid and, to a lesser extent, floctafenic acid have been a subject of concern (5). Drug effects are thought to be related to their free concentration in blood which depends on the drug interaction with plasma proteins and blood cells. Therefore, we have characterized the binding of glafenic and floctafenic acids to albumin, erythrocytes, and other blood cells and plasma proteins.

This work was supported by grants from Roussel-Uclaf France and Diamant Laboratories and from la Direction de la Recherche au Ministère de l'Education Nationale.

Materials and Methods

Human serum albumin. HSA (Sigma A 1887, FFA molar ratio = 0.04) was used, dissolved in phosphate buffer (0.067 mol/liter) at pH 7.4. When pooled human serum was used, HSA concentration was estimated by the bromocresol green method (6).

Blood and cells. Blood was drawn from the antebrachial vein of four healthy volunteers and collected in test tubes containing lithium heparinate; erythrocytes were washed four times with 2.5 volumes of Ringer's bicarbonate buffer at pH 7.4. Plasma and buffer were separated from blood cells by centrifugation at 1000 × *g* for 5 min. The cells were then resuspended in buffer. For lymphocytes, 20 ml of blood were collected in EDTA-containing tubes and centrifuged on Lymphoprep density gradient (7, 8). For leukocytes, 20 ml of blood were collected in EDTA-containing tubes and mixed with 5% Dextran T 500 (Pharmacia) (9). Platelets were obtained from a platelet-rich plasma (10). For erythrocyte ghosts, 20 ml of blood were collected in ACD-containing tubes and hemolyzed in 20 mOsm of phosphate buffer at pH 7.4 (11).

Lipoproteins. Each isolated lipoprotein was obtained by ultracentrifugation from pooled normolipidemic human serum. No chylomicrons were present. Plasma lipoproteins were isolated by sequential ultracentrifugal flotation of plasma at increasing density (12). The purity of each fraction was tested according to the method of Ouchterlony and estimated to be greater than 95%. The concentration of each lipoprotein was measured by Lowry's method considering that VLDL, LDL, and HDL protein contents are 10, 20, and 50%, respectively, and the purity was checked by electrophoresis.

Other proteins. AAG (Behring, purity 99%) and γ Gs (Sigma,

ABBREVIATIONS: HSA, human serum albumin; AAG, α_1-acid glycoprotein; γG, γ-globulin; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; EDTA; ethylenediaminetetraacetate.

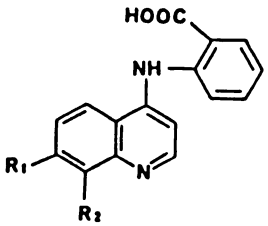

Fig. 1. Chemical structures of glafenic acid and floctafenic acid. R_1 = Cl and R_2 = H correspond to glafenic acid; R_1 = ' and R_2 = CF_3 correspond to floctafenic acid.

human Cohn II fraction, purity 99%) were used in phosphate buffer at pH 7.4. Hemoglobin A_1 was obtained by DEAE-cellulose chromatography (13).

Chemicals. [^{3}H]-(+)-Glafenic acid (18 Ci/mmol) and [^{3}H]-(+)-floctafenic acid (27 Ci/mmol) were provided by Roussel-Uclaf Laboratories. Their radiochemical purities (98.5%) were assessed by thin layer chromatography using the following solvent system: chloroform/methanol/water (85:15:0.9, v/v). Azapropazone, flurbiprofen, and phenylbutazone were provided by Siegfried SA, Boots-Dacourt, and Ciba-Geigy, respectively. Palmitic acid was purchased from Sigma (P 2010).

Equilibrium dialysis experiments. Drug binding was studied with equilibrium dialysis for isolated proteins, erythrocytes, and ghosts. The experiments were carried out at 37°, pH 7.4, for 4 hr under constant rotation at 20 rpm (Dianorm apparatus). Preliminary distribution studies showed that an equilibrium between the two sides of the dialysis membrane was achieved within 3.5 hr. No significant binding to the dialysis tubing (Visking) or cell walls of the apparatus was observed. Two- or 0.2-ml dialysis cells were used. Glafenic acid was used at concentrations of 0.335–335 μmol/liter (0.1–100 μg/ml) with a [^{3}H] glafenic acid concentration of 33.5 nmol/liter. Floctafenic acid was used at concentrations of 0.3–1480 μmol/liter (0.1–500 μg/ml) with a [^{3}H] floctafenic acid concentration of 29.5 nmol/liter. At the end of each experiment, concentrations in each compartment were measured with a liquid scintillation counter (SL 3000 Inter-Technique Liquid-Scintillation Spectrometer). HSA was used at either 580 μmol/liter (40 g/liter), 10 μmol/liter (0.66 g/liter), or 15.2 μmol/liter (1 g/liter), and AAG was used at 20 μmol/liter (0.9 g/liter). The following molecular weights were used: 7.5×10^6 for VLDL, 3.5×10^6 for LDL, and 3×10^5 for HDL. The concentration of these lipoproteins was 4 g/liter. The γ G concentrations was 14 g/liter and a mean molecular weight of 180,000 was used for the calculations. The hematocrit value was 0.45. The ghost-protein concentration (method of Lowry) was 2.86 g/liter.

Optical absorption spectroscopy. Absorption spectra were recorded at room temperature with a Jobin-Yvon JY 201 spectrophotometer.

Circular dichroism measurements. The spectra were recorded at room temperature using a Jobin-Yvon Mark III dichrograph equipped with a Nicolet 1171 signal averager. A rectangular cell with path-length of 1 cm was used. The absorbance was kept below 1.4 CD is expressed in terms of the molar dichroism absorbance $\Delta\epsilon$, based on the bound ligand concentration.

Fluorescence studies. Fluorescence measurements were made at room temperature in an Aminco SPF 500 spectrofluorometer. The absorbance was kept below 0.069 to minimize the inner filter effect. A rectangular cell with path-length of 1 cm was used. Slit widths were fixed at 5 nm for both emission and excitation.

Direct incubation of blood cells with glafenic and floctafenic acids. Isotopic dilutions of glafenic or floctafenic acid were added to whole blood or to a suspension of lymphocytes, leukocytes, or platelets to achieve final concentrations between 3.35 and 3350 nmol/liter for glafenic acid and between 2.95 and 2950 nmol/liter for floctafenic acid. Preliminary distribution studies showed that an equilibrium between cells and plasma or buffer was achieved within 30 min at 37° and was constant for a least 3 hr.

Determination of drug binding parameters and characterization of their binding sites. The methods and calculations used have been reported previously elsewhere (14). The binding characteristics were expressed as follows: n is the number of binding sites per mole of protein and was used for isolated plasma proteins; N is the binding site concentration of blood, plasma, and any solution or suspension and was applied particularly to isolated blood cells; K is the corresponding association constant. The HSA-binding sites were investigated by fluorescence spectroscopy and CD was investigated by using specific probes, such as azapropazone and phenylbutazone for the HSA drug site I or warfarin site, and flurbiprofen for the HSA drug site II or benzodiazepine site (1–3). The effect of palmitic acid was then also investigated.

Simulations. The data obtained with a ligand and an inhibitor have been ascribed to the site-binding model as a phenomenological description of the interaction. The equations describing the binding of a ligand (L) to an n site model in the presence of a competitor (I) are (15):

$$L_b = \sum_{i=1}^{n} \frac{K_{Li} \cdot L_f}{1 + K_{Li} \cdot L_f + K_{Ii} \cdot I_f} \quad (1)$$

$$I_b = \sum_{i=1}^{n'} \frac{K_{Ii} \cdot I_f}{1 + K_{Ii} \cdot I_f + K_{Li} \cdot L_f} \quad (2)$$

where the subscripts b and f denote bound and free concentrations, and K is the association constant for drug (L *or* I). In a dialysis system, the mass conservation equations are:

$$L_{total} = 2 \cdot L_f + L_b \quad (3)$$

$$I_{total} = 2 \cdot I_f + I_b \quad (4)$$

According to the estimated binding parameters for glafenic and floctafenic acid interactions with albumin, the bound concentrations of ligand and competitor can be calculated from their total concentrations using Eqs. 1–4 by numerical estimation. The numerical approximation was terminated when the calculated values of L_{total} and I_{total} were within 1% of the corresponding experimental values.

Results

Equilibrium dialysis studies. Glafenic and floctafenic acids were found to bind to the same plasma proteins, HSA and lipoprotein, but with different characteristics (Tables 1 and 2). The main binding protein for the two drugs was HSA; the corresponding binding processes were found to be quickly saturable and involved two classes of binding sites. The association constant was dramatically high in the case of glafenic acid, at least for the first class of sites. The number of available binding sites was different for the two drugs, whatever the considered class.

Compared to HSA, the lipoprotein binding was weak although significant. Binding to AAG, γG, and apolipoproteins A and B was negligible. Glafenic acid was shown to inhibit significantly the HSA binding of floctafenic acid (Fig. 2A). Separate analysis of each set of binding data provided the following estimates: no inhibitor, $n_1 = 2.2 \pm 0.3$, $K_1 = 0.45 \pm 0.17$ liter/μmol, $n_2 = 6.9 \pm 0.7$, $K_2 = 0.005 \pm 0.001$ liter/μmol; plus 15 μM glafenic acid, $n_1 = 2.3 \pm 0.4$, $K_1 = 0.15 \pm 0.02$ liter/μmol, $n_2 = 7.1 \pm 0.5$, $K_2 = 0.005 \pm 0.001$ liter/μmol; plus 60 μM glafenic acid, $n_1 = 2.4 \pm 0.5$, $K_1 = 0.04 \pm 0.01$ liter/μmol, $n_2 = 7.5 \pm 1.0$, $K_2 = 0.003 \pm 0.001$ liter/μmol. Obviously, the inhibition involves a net decrease in the K values whereas the n values are not decreased or are slightly modified. The binding isotherms of floctafenic acid in the absence or presence of 15 and 60 μmol/liter of glafenic acid were simultaneously analyzed assuming either a competitive or a noncompetitive model of inhibition. The competitive model was chosen, since it provided

TABLE 1

Binding parameters of glafenic and floctafenic acids to HSA, lipoproteins, erythrocytes, and ghosts

n, *N*, and *K* values are the mean (± standard deviation) of five determinations. For erythrocytes, the binding parameters reported are those of the first, saturable process. The binding parameters to HSA are derived from the simultaneous analysis of data according to the competitive model that was chosen. No other blood proteins or cells but those included in the table bind the two drugs to a significant extent.

Plasma proteins	Glafenic acid		Floctafenic acid	
	n	$K \times 10^{-6}$ M^{-1}	*n*	$K \times 10^{-6}$ M^{-1}
HSA				
First class	1.10 ± 0.01	2.36 ± 0.37	2.10 ± 0.27	0.317 ± 0.042
Second class	3.40 ± 0.01	0.052 ± 0.004	6.94 ± 0.43	0.012 ± 0.002
HDL	4.40 ± 0.85	0.11 ± 0.03	15.42 ± 0.72	0.0213 ± 0.0012
LDL	23.5 ± 1.9	0.021 ± 0.003	147.9 ± 14.9	0.0057 ± 0.0011
VLDL	7.6 ± 0.7	0.58 ± 0.06	8.54 ± 1.36	0.949 ± 0.188
	N (μM)	$K \times 10^{-6}$ M^{-1}	*N* (μM)	$K \times 10^{-6}$ M^{-1}
Erythrocytes	12.4 ± 1.7	1.72 ± 0.22	9.59 ± 2.24	1.090 ± 0.245
Ghosts	13.7 ± 0.7	0.171 ± 0.010	17.0 ± 0.6	0.1504 ± 0.0092

TABLE 2

Lipoprotein total binding constants (*nK*) and partition coefficients of the two drugs

	nK ($\times 10^{-6}$ liters/mol)	
	Glafenic acid	Floctafenic acid
HDL (lipid content = 50%)	0.328	0.484
LDL (lipid content = 80%)	0.843	0.493
VLDL (lipid content = 90%)	8.104	4.408
Partition coefficient[a]	61	45

[a] Partition coefficients were determined using octanol/phosphate buffer, pH = 7.4 at room temperature.

the smallest residual sum of squares. Conversly, the inhibition of glafenic acid binding to 7.5 μM HSA by floctafenic acid could also be ascribed to a competitive model (Fig. 2B). Obviously, addition of 10 μM floctafenic acid involved a net decrease in the association constants of glafenic acid to HSA, $n_1 = 1.2 + 0.2$, $K_1 = 1.2 \pm 0.3$ liters/μmol, $n_2 = 3.4 \pm 0.2$, $K_2 = 0.04 \pm 0.01$ liter/μmol versus values without inhibitor, $n_1 = 1\ 0.2$, $K_1 = 2.4 \pm 0.5$ liters/μmol, $n_2 = 3.2 \pm 0.2$, $K_2 = 0.06 \pm 0.02$ liter/μmol. Surprisingly, when the floctafenic acid concentration was 40 μM, glafenic acid binding to HSA could not be ascribed any more to two classes of binding sites. Then, the binding was ascribed to a single class of sites with $n = 4.0 + 0.1$ and $K = 0.06 + 0.02$ liter/μmol. This result may be reasonably interpreted as follows: the apparent association constant of the first class of sites is diminished to a value close to the association constant of the second class of sites, so that two distinct classes could not be derived from the binding data. Moreover, the simultaneous analysis of the binding data in the absence and presence of floctafenic acid showed that the competitive model (*n* value is fixed, *K* values may vary) leads to the smallest residual sum of squares.

Interactions with erythrocytes. In response to the protein binding profile, erythrocytes were the only blood cells that bound glafenic and floctafenic acids (Fig. 3, Table 1). No binding was observed with leukocytes, lymphocytes, and platelets except for floctafenic acid that bound to platelets in a saturable process with very few sites ($N = 44.09 \pm 9.26$ nmol/liter, $K = 16.1 \pm 5.5 \times 10^6$ liters/mol). Glafenic acid bound to erythrocytes following two saturable processes whereas floctafenic acid exhibited only one saturable process with a much weaker affinity. The ghosts contributed to this binding whereas hemoglobin A_1 binding was found to be negligible.

Blood distribution of the drugs. According to a mathe-

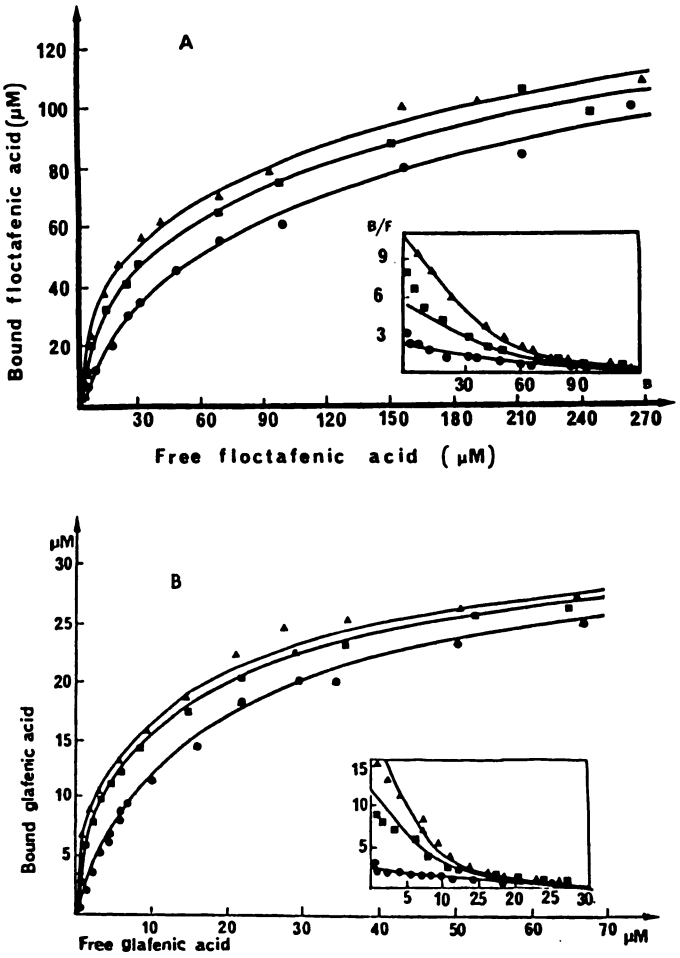

Fig. 2. A. Displacement of floctafenic acid (▲) from HSA by glafenic acid, 15 μmol/liter (■) and 60 μmol/liter (●). B. Displacement of glafenic acid (▲) from HSA by floctafenic acid, 10 μmol/liter (■) and 40 μmol/liter (●). Data were obtained over a broad concentration range of ligand (1–500 μg/ml). HSA concentration was 15 μmol/liter. Glafenic acid displaced the ligand but the percentage of bound ligand varied only for the lowest concentrations since it was 91.6–28.7 for ligand at 1 and 500 μg/ml and then became 88.5–28.5 with glafenic acid at 15 μmol/liter, and 77–27.5 with glafenic acid at 60 μmol/liter as inhibitor. *Insets*, Scatchard plots.

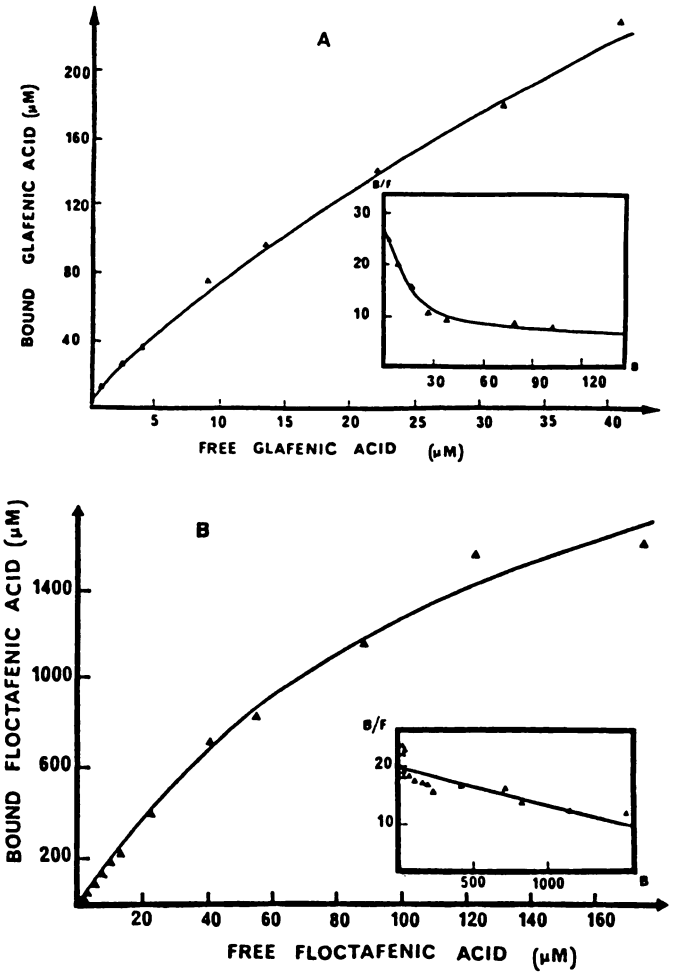

Fig. 3. Binding of glafenic acid (A), saturable plus nonsaturable, and of floctafenic acid (B), saturable, to erythrocytes (Ht = 0.4). *Inset*, Scatchard plots.

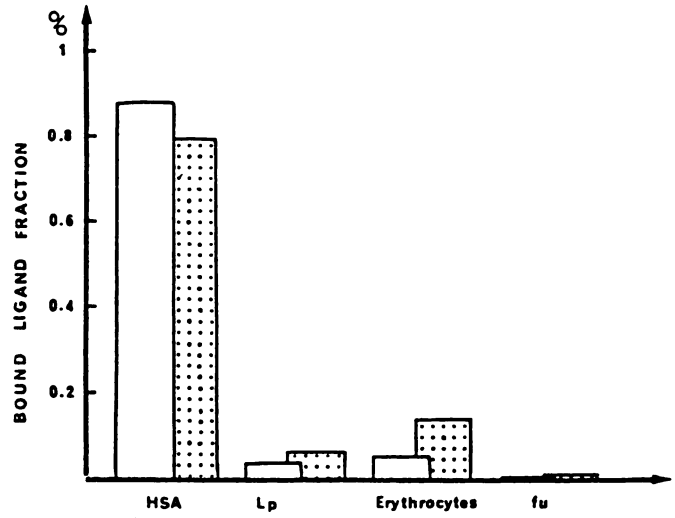

Fig. 4. Simulated distribution of glafenic and floctafenic acids between HSA, lipoproteins (*Lp*), and erythrocytes. HSA is the major binding structure. Erythrocytes are the only cells to which the drugs bind significantly. Membranes are a main binding structure of erythrocytes. □, floctafenic acid. *fu* is the free remaining fraction of each drug.

matic model previously described (14), we have simulated the distribution of the drugs between the various proteins and blood cells (Fig. 4). It is clear that HSA for proteins and erythrocytes for blood cells are the two major remaining binding structures. Free blood concentration of drugs accounts for about 1%.

CD studies. Glafenic and floctafenic acids are not chiral. The CD difference spectra of the complexes between HSA and glafenic or floctafenic acid are depicted in Fig. 5, A and B. In both cases, new CD bands are generated in the wavelength regions where the ligands exhibit absorption bands (spectra not shown). Several features of these spectra are noteworthy. The CD difference spectrum of the glafenic acid-HSA complex consists of a CD doublet including a positive component at 360 nm and a negative one at 315 nm, and of a negative CD band at 265 nm. The CD difference spectrum of the floctafenic acid-HSA complex has a roughly symmetrical pattern relative to

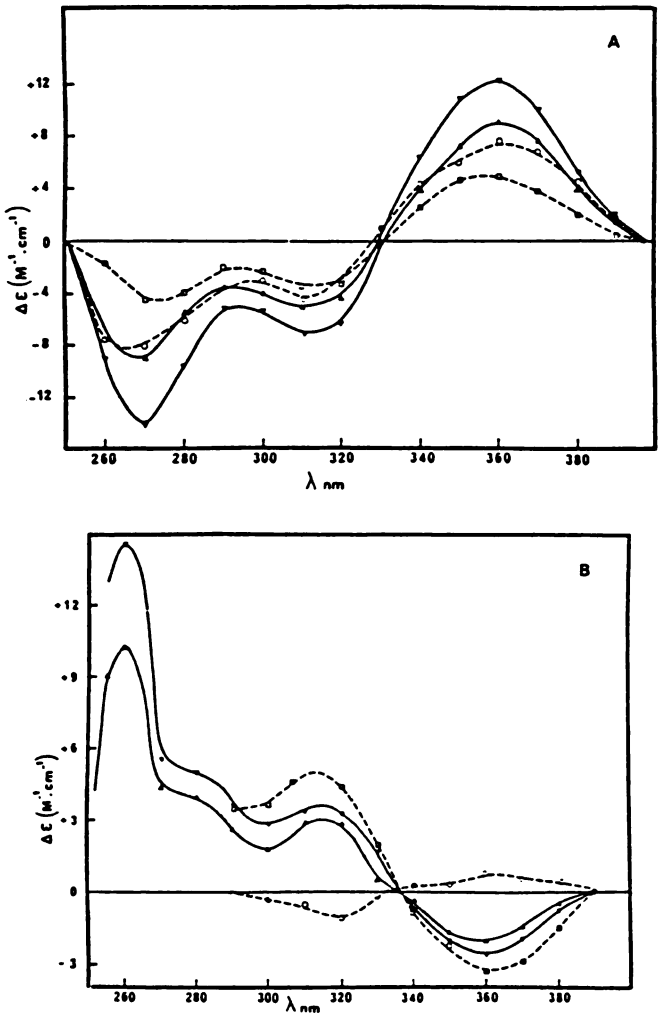

Fig. 5. Difference CD spectra of glafenic acid-HSA (A) and floctafenic acid-HSA (B) at drug/HSA molar ratios of 1:1 (▲) and 2:1 (▼) after subtraction of the HSA spectrum, and difference CD spectra obtained in the presence of the following binding site probes (– – –), azapropazone (□) or flurbiprofen (○), after subtraction of the contribution of each corresponding probe-HSA spectrum. The molar ratio of drug/probe/HSA was 1:1:1. Experimental conditions were: HSA, 15 μmol/liter in phosphate buffer; cell path-length, 1 cm; scale setting 2×10^{-6} mm^{-1}; room temperature. Because of the high level of noisy baseline for floctafenic acid-probe-HSA spectra in the shorter UV range, the difference CD spectra could not be satisfactorily determined.

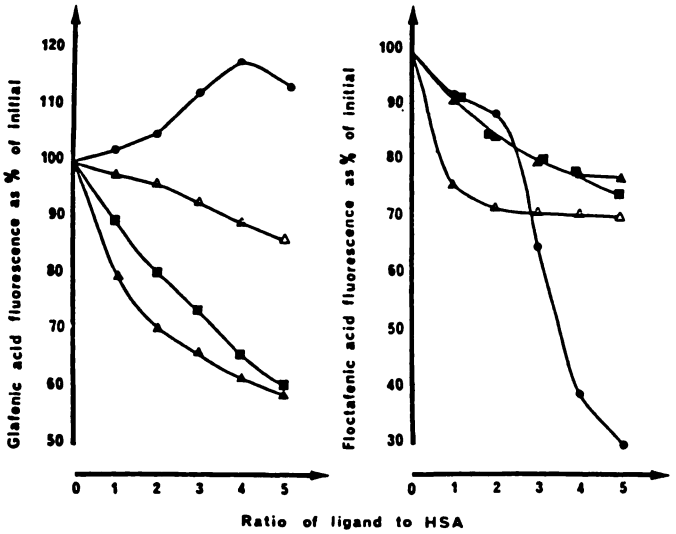

Fig. 6. Ligand-induced changes in fluorescence of glafenic and floctafenic acids bound to HSA. The fluorescence of solution containing 10 μmol/liter HSA and 10 μmol/liter glafenic or floctafenic acid in phosphate buffer, pH 7.4, was measured before and after the addition of ligands. Fluorescence was measured at 425 or 450 nm with excitation at 350 or 365 nm for glafenic and floctafenic acids, respectively. No significant variation of the optical density occurred at any of these wavelengths during the titration by the ligands. The ligands used were: azapropazone (■); phenylbutazone (▲); flurbiprofen (Δ); palmitic acid (●).

that of the glafenic acid-HSA complex, thus suggesting qualitative differences in the respective HSA drug binding.

To locate the glafenic and floctafenic acid-binding sites, two drugs, azapropazone and flurbiprofen were used as probes of sites I and II. Their association constants are high, in the range of 10^6 liters/mol. Evidently, flurbiprofen does not affect significantly the CD spectrum obtained from the binding of glafenic acid to HSA, whereas azapropazone involves a fair decrease in the amplitude of the CD bands (Fig. 5A). Thus, one may assume that glafenic acid shares its primary high affinity binding site with azapropazone.

The effects of the two probes on the CD spectrum of floctafenic acid are more complex (Fig. 5B). It is obvious that the CD bands induced by floctafenic acid at equimolar amounts relative to HSA are enhanced by azapropazone. By contrast, in the presence of flurbiprofen, CD bands of opposite signs and smaller amplitude are induced by floctafenic acid at equimolar amounts relative to HSA. These results may be interpreted as follows: the primary floctafenic acid-binding site is that of flurbiprofen (site II) since the CD bands are blocked in the presence of flurbiprofen; the small CD bands of opposite sign that are then produced are probably induced by the binding of floctafenic acid to a secondary binding site. Thus, the CD bands obtained from the binding of floctafenic acid to HSA result from the addition of two CD spectra of opposite signs induced by the occupation of two distinct binding sites. Accordingly, the blockade of the secondary binding site should induce an increase in the amplitude of the resulting CD spectrum, both by blocking the opposite CD contribution of this site and by increasing the concentration of floctafenic acid to the primary site, as is observed in the presence of azapropazone. Moreover, the binding of floctafenic acid to its secondary binding site (in the presence of flurbiprofen) induces a CD spectrum that resembles the CD spectrum induced by the binding of glafenic acid.

Fluorescence studies. Mixtures of glafenic or floctafenic acid and HSA excited at 350 or 365 nm emit light at 425 or 450 nm (spectra not shown), whereas glafenic or floctafenic acid does not exhibit fluorescence in the free state in buffer at these wavelengths.

Fig. 6 shows the effects of binding site probes and palmitic acid on the fluorescence of glafenic and floctafenic acids. Phenylbutazone and azapropazone caused a marked displacement of glafenic acid, whereas their effect on the HSA-bound floctafenic acid fluorescence was much less evident. By contrast, flurbiprofen did not change significantly the HSA-bound glafenic acid fluorescence but involved a net decrease in the floctafenic acid fluorescence. These results support the above observations on the location of glafenic and floctafenic acid-binding sites. Moreover, the HSA-bound glafenic acid fluorescence is increased to a 5:1 ratio of palmitic acid to HSA, whereas the fluorescence of floctafenic acid is decreased.

Simulations of drug displacement interactions. According to the dialysis and optical experiments, the binding of glafenic and floctafenic acid to HSA could be ascribed to the following model: the two drugs bind to one common high affinity site, floctafenic acid binds to one supplementary and

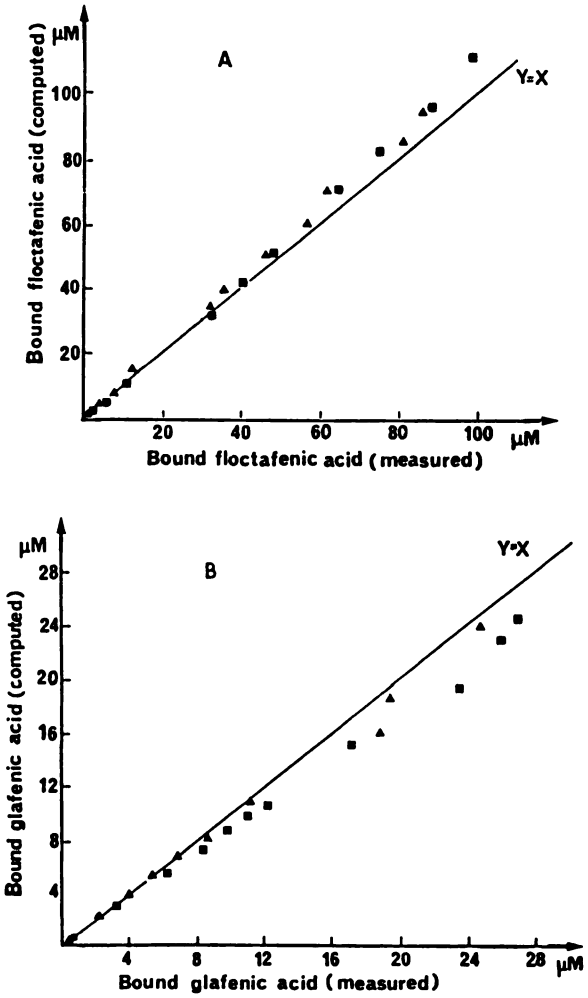

Fig. 7. Plot of simulated versus measured concentrations of bound ligand. Simulated concentrations are derived from Eqs. 1–4 according to the binding site model defined in Results. A. Bound concentrations of floctafenic acid in the presence of 15 (■) and 60 (▲) μM glafenic acid; B, bound concentration of glafenic acid in the presence of 10 (■) and 40 (▲) μM floctafenic acid.

distinct binding site of comparable affinity, the two drugs share at least three (3.4) binding sites of weaker affinity, and floctafenic acid binds to three (3.6) supplementary and distinct binding sites of weak affinity.

Simulated bound drug concentrations were calculated by using Eqs. 1–4 and are presented as a function of measured concentration of bound drug (Fig. 7, A and B). The calculated values agree closely with the experimental data at low ligand values. However, we observe a systematic deviation from the theoretical line at higher ligand values.

Discussion

Characteristics of HSA binding of the two drugs. Glafenic and floctafenic acids are mainly bound to HSA, the binding process being quickly saturable and involving two classes of binding sites. These results are in agreement with current knowledge about HSA binding of acidic drugs (16, 17) but, in this case, raise several questions.

The first question comes from the apparently discrepant results of equilibrium dialysis: the calculation of binding parameters fits with one and two main binding sites for glafenic and floctafenic acids, respectively, whereas displacement studies strongly suggest competitive mechanisms, i.e., common sites. Therefore, CD and fluorescence studies were performed to examine in greater detail the possibility of common sites. The two drugs interact with HSA in such a way that large amplitude CD bands are generated. The mechanisms to be considered as the source of these new CD bands have been discussed in detail by Strickland (18). Two main mechanisms were considered by Hood *et al.* (19) for CD bands of this size: (a) immobilization of a chromophore within the molecule in a stable chiral conformation with CD generated by an intermolecular polarizability mechanism localized essentially within the chromophoric ligand, and (b) interaction of amino acid residues with the chromophores of the ligand, including the possibility of coupling between the strongly allowed electronic transitions of the chromophore and those of nearby amino acids.

Both mechanisms can contribute simultaneously, although not necessarily additively. Since the absorption bands of HSA and the two ligands overlap in the near UV range, the CD band around 265 nm originating from the interaction of HSA with one of these ligands cannot be interpreted in terms of HSA conformational changes.

The specificity of at least two binding sites on HSA, namely the azapropazone/warfarin site, or site I, and the benzodiazepine site, or site II (1–3), is now well documented for anionic drugs. Azapropazone and flurbiprofen are bound to just their primary site, I and II, respectively, when present in equimolar amounts relative to HSA (20). Evidently, the structure of the different binding sites of a macromolecule varies from one site to another. Thus the interaction of different sites with a ligand will lead to qualitative and quantitative differences in the binding. The asymmetric conformation of the site around the chromophore will be different, which may generate different extrinsic Cotton effects, as previously shown for indomethacin (21). In the present study, we have shown that specific binding site probes modified or blocked the amplitude of the specific Cotton effects generated by the interaction of glafenic and floctafenic acids with HSA, and it was concluded that glafenic acid was primarily bound to HSA drug site I and floctafenic acid to both site I and site II on HSA. Glafenic and floctafenic acids induced comparable CD spectra when bound to their common site (HSA site I), whereas floctafenic acid bound to HSA site II produced a CD spectrum of opposite signs. Since the patterns of the difference CD spectra produced by the interaction of the anthranilic acids with HSA were not altered (only the amplitudes were), it is unlikely that significant conformational changes of the HSA molecule would have occurred in the presence of the probes.

Glafenic and floctafenic acids exhibit fluorescence in an aqueous solution only when bound to HSA, suggesting that the binding site of these drugs is situated in a hydrophobic area of the protein with limited access to solvent molecules around the binding region (22). Also, the studies on displacement of the HSA-bound anthranilic acid fluorescence by specific probes supported the previous binding site location from the CD studies. The effect of palmitic acid on the fluorescence of the bound drugs is also very suggestive of the sites involved in the binding. Sudlow *et al.* (1) showed that the fluorescence of markers bound to the HSA site I was enhanced by the presence of stearic acid, as observed for glafenic acid, whereas the fluorescence of markers bound to the HSA site II was generally decreased, as occurred for floctafenic acid. This last observation suggests that the primary binding site of floctafenic acid is HSA site II, the secondary binding site being HSA site I. Indeed, if HSA site I had been the primary site of floctafenic acid (at equimolar amounts relative to HSA, floctafenic acid is only bound to its primary site), palmitic acid would have enhanced its fluorescence.

From these results, we may reasonably conclude that the two drugs share one common HSA-binding site, namely, site I, and that floctafenic acid binds also to site II. This last characteristic was recently described for ethacrynic acid by Fehske and Muller (23). It is also tempting to speculate about the acidic drugs previously reported to have two main HSA-binding sites without further site identification (24): they bind perhaps simultaneously to the two classical sites I and II.

Another remaining problem now is to acertain the compatibility of this conclusion with the previous binding displacement studies. The analysis of binding data obtained by dialysis experiments according to our binding site model for the two drugs (see simulation, Fig. 7, A and B) indicates that the displacement may be considered as a purely competitive phenomenon at low ligand level, involving the common sites. However, the situation is more complicated at high ligand concentrations, where both competitive and noncompetitive interactions, as well as the possibility of coupling between distinct binding sites, may occur (1, 25, 26).

A more general question is to ask whether a simpler way might lead to the same conclusions. It includes both methodological and calculation choices. Considering our first goal, the localization of high affinity sites by equilibrium dialysis is obviously of limited value as compared to optical methods. But these need appropriate signals brought only by selected structures and, thus, not of general value. Regarding calculation methods, it might also be possible to analyze the results according to the stoichiometric model proposed by Honoré and Brodersen (27) to determine whether such a model could discriminate the two main affinities of floctafenic acid. These questions are still open.

Binding to the other plasma proteins. The two drugs do not bind to AAG to a significant extent. Despite the fact that

some acidic drugs do so and that the relevant bond is mainly hydrophobic, it may be related to their free carboxylic groups, which impair such binding (28). In contrast, the binding to lipoproteins was unexpected. Since the fundamental paper of Vallner and Chen (29), only cationic drugs were thought to be bound to lipoproteins. However, in our experiments we found significant binding to the three lipoprotein fractions; the acidic drug diclofenac has also been recently reported to interact with these macromolecules (30). As apolipoproteins A and B do not bind the drugs, a solubilization in the lipid core of the lipoproteins seems likely to occur, as it was previously observed for basic drugs, providing their partition coefficients were rather high (12, 14). Accordingly, the total binding constant nK increases for each drug with the lipid content of the lipoprotein (Table 2).

Binding to erythrocytes and platelets. The erythrocyte binding of the two drugs was unexpected. As erythrocyte intracellular pH is about 7.20, cationic drug penetration inside the cell is more likely to occur than that of acidic drugs.

The erythrocyte binding of glafenic acid is qualitatively and quantitatively different from that of floctafenic acid. Glafenic acid exhibited a high affinity binding to erythrocytes, whereas that of floctafenic acid was found to be very low. Such a result was not expected since their chemical structures are closely related: it may suggest a certain degree of specificity for glafenic acid.

Such binding of drugs to erythrocytes has already been described and recently reviewed by Ehrnebo (31). Three major components may be involved: hemoglobin, carbonic anhydrase, and the cell membrane. As no binding to the last two components was observed, binding to the cell membrane is likely to occur. However, the fact that the association constant decreases dramatically from the total erythrocyte to the relevant ghost suggests that a binding factor exists in the intact cell and is lost during the ghost preparation.

The binding of floctafenic acid to platelets exhibited a surprisingly high affinity but associated with a very low concentration of sites. It is thus of no quantitative significance, whereas a possible qualitative significance is still unclear.

Acknowledgments

We gratefully acknowledge Mr. Jacques Bolard for helpful criticism in the optical studies. The circular dichroism studies have been performed at the Department de Recherches Physiques of the Université Pierre et Marie Curie (Paris).

References

1. Sudlow, G., D. J. Birkett, and D. M. Wade. Further characterization of specific drug binding sites on human serum albumin. *Mol. Pharmacol.* **12**:1052–1061 (1976).
2. Fehske, K. J., W. E. Muller, and U. Wollert. The location of binding sites in human serum albumin. *Biochem. Pharmacol.* **30**:687–692 (1981).
3. Sjöholm, I., B. Ekman, A. Kober, I. Ljungstedt-Pahlman, B. Seiving, and T. Sjödin. The specificity of three binding sites as studied with albumin immoblized in microparticules. *Mol. Pharmacol.* **16**:767–777 (1979).
4. Kornguth, M. L., and C. M. Kunin. Uptake of antibiotics by human erythrocytes. *J. Infect. Dis.* **133**:175–184 (1976).
5. Cheymol, G., M. Biour, M. Bruneel, E. Albengres, and J. D. Hamel. Bilan d'une enquête nationale prospective sur les effets indésirables de la glafénine, de l'antrafénine et de la floctafénine. *Thérapie* **40**:45–50 (1985).
6. Sigma Technical Bulletin No. 630 (1980).
7. Boyum, A. Isolation of leukocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97):77 (1968).
8. Patrick, C. C., C. D. Graber, and C. B. Loadholt. Evaluation of four methods for separation of lymphocytes from normal individuals and patients with cancer and tuberculosis. *J. Immunol. Methods* **11**:321–323 (1976).
9. Wildfener, A. Action of anti-rheumatic drugs on the function of human leukocytes. *Arzneim. Forsch. Drug Res.* **33**:780–783 (1983).
10. Caen, J., M. J. Larrieu, and M. Samama. *l'Hemostase.* Expansion Scientifique, Paris, 72 (1975).
11. Dodge, J. T., C. Mitchell, and D. J. Hanahan. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**:119–130 (1963).
12. Glasson, S., R. Zini, and J. P. Tillement. Multiple human serum binding of two thienopyridinic derivatives, ticlopidine and PCR 2362, and their distribution between HSA, α_1 acid glycoprotein and lipoproteins. *Biochem. Pharmacol.* **31**:831–835 (1982).
13. Abraham, C. V., and S. Bakerman. Isolation and purification of the Rh (D) blood group receptor component from human erythrocyte membrane. *Clin. Chem. Acta* **60**:33–43 (1975).
14. Albengres, E., S. Urien, J. F. Pognat, and J. P. Tillement. Multiple binding of bepridil in human blood. *Pharmacology* **28**:139–149 (1984).
15. Meisner, H., J. Stair, and K. Neet. Quantitative assessment of the competitive binding of anionic ligands to albumin. *Mol. Pharmacol.* **18**:230–236 (1980).
16. Tillement, J. P., G. Houin, R. Zini, S. Urien, E. Albengres, J. Barré, M. Lecomte, Ph. D'Athis, and B. Sébille. The binding of drugs to blood plasma macromolecules: recent advances and therapeutic significance. *Adv. Drug Res.* **13**:59–94 (1984).
17. Gillette, J. R. Overview of drug protein binding. *Ann. N. Y. Acad. Sci.* **226**:6–17 (1973).
18. Strickland, E. H. Aromatic contributions to circular dichroism spectra of proteins. *Crit. Rev. Biochem.* **2**:113–175 (1974).
19. Hood, K., P. M. Bayley, and G. C. K. Roberts. Circular dichroism studies of ligand binding of dihydrofolate reductase from *Lactobacillus casei* MTX/R. *Biochem. J.* **177**:425–432 (1979).
20. Kōber, A., and I. Sjöholm. The binding sites on human serum albumin for some nonsteroidal antiinflammatory drugs. *Mol. Pharmacol.* **18**:421–426 (1980).
21. Ekman, B., T. Sjödin, and I. Sjöholm. Characterization and identification of the binding sites of indomethacin. *Biochem. Pharmacol.* **29**:1759–1765 (1982).
22. Sudlow, G., D. J. Birkett, and D. N. Wade. Spectroscopic techniques in the study of protein binding: the use of 1-anilino-8-naphtalenesulphonate as a fluorescent probe for the study of the binding of iopanoic acids to human serum albumin. *Mol. Pharmacol.* **9**:649–657 (1973).
23. Fehske, K. J., and W. E. Muller. High affinity binding of ethacrynic acid is mediated by the two most important drug binding sites of human serum albumin. *Pharmacology* **32**:208–213 (1986).
24. Birkett, D. J., and S. Wanvimolruk. Albumin as a specific binding protein for drugs and endogenous compounds, in *Protein Binding and Drug Transport.* Symposia Medica Hoechst 20. Symposium Alvor, Portugal. 24th–28th September 1985 (J. P. Tillement and E. Lindenlaub, eds.). F. K. Schattauer Verlag, Stuttgart, 11–27, (1986).
25. Drōge, J. H. M., L. H. M. Jansen, and J. Wilting. A study on the allosteric interaction between the major binding sites of human serum albumin using microcalorimetry. *Biochem. Biophys. Acta* **827**:396–402 (1985).
26. Kragh-Hansen, U. Relations between high-affinity binding sites for L-tryptophan, diazepam, salicylate and phenol red on human serum albumin. *Biochem. J.* **209**:135–142 (1983).
27. Honoré, B., and R. Brodersen. Albumin binding of anti-inflammatory drugs. Utility of a site-oriented versus a stoichiometric analysis. *Mol. Pharmacol.* **25**:137–150 (1983).
28. Urien, S., E. Albengres, R. Zini, and J. P. Tillement. Evidence of binding of certain acidic drugs to α_1-acid glycoprotein. *Biochem. Pharmacol.* **31**:3687–3689 (1982).
29. Vallner, J. J., and L. Chen. Beta lipoproteins: possible plasma proteins for basic drugs. *J. Pharm. Sci.* **66**:420–421 (1977).
30. Chamouard, J. M., J. Barré, S. Urien, G. Houin, and J. P. Tillement. Diclofenac binding of albumin and lipoproteins in human serum. *Biochem. Pharmacol.* **34**:1695–1700 (1985).
31. Ehrnebo, M. Drug binding to erythrocytes, in *Protein Binding and Drug Transport* pp 49–61. Symposia Medica Hoechst 20. Symposium Alvor, Portugal 24th–28th September 1985 (J. P. Tillement and E. Lindenlaub, (eds.). F. K. Schattauer Verlag, Stuttgart, 49–61 (1986).

Send reprint requests to: Dr. Edith Albengres, Service Hospitalo-Universitaire de Pharmacologie, Hôpital Intercommunal, 40, avenue de Verdun, F-94010 Creteil Cedex, France.