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BINDING OF PHENOTHIAZINE NEUROLEPTICS TO PLASMA PROTEINS*

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Abstract—The binding of chlorpromazine, trifluoperazine, perphenazine, desipramine, propranolol and salicylic acid to human plasma and isolated plasma proteins was studied using equilibrium dialysis. Unlike salicylic acid, an acidic compound only bound to human serum albumin, the basic drugs were bound to all plasma protein fractions studied (albumin, α_1-acid glycoprotein, lipoproteins, γ-globulins) with α_1-acid glycoprotein an important binding protein for each of them. The interaction of chlorpromazine, perphenazine and trifluoperazine with α_1-acid glycoprotein was studied using Scatchard analysis. The primary class of binding sites revealed a low capacity (n = 0.5–1) and a high affinity ($K = 10^5$–$10^6\ M^{-1}$) for the phenothiazines. The interaction of chlorpromazine, perphenazine and trifluoperazine with albumin was of the high capacity–low affinity type. In binding studies using plasma obtained from healthy volunteers, α_1-acid glycoprotein was found to be a very important binding protein for the basic drugs studied with the exception of desipramine. This shows that results derived from binding studies using isolated protein fractions should be interpreted with caution.

Phenothiazines are among the most widely used drugs in medical practice. They are primarily used in the management of patients with serious psychiatric illnesses. Poor clinical response to neuroleptics is a well known phenomenon [1–3]. Difficulties in predicting clinical effect from plasma levels of these antipsychotic agents may reflect human biological variability in absorption, distribution and elimination of these compounds. Binding to plasma proteins is an important pharmacological parameter, since it frequently affects drug distribution and elimination [4–6] and duration and intensity of pharmacological effects of drugs [7–9]. Therefore, intraindividual variation in the binding of phenothiazines to plasma constituents may, at least partly, explain the apparent lack of correlation between total (bound and unbound) plasma concentrations and central nervous system effects of these agents.

Phenothiazines are cationic (basic) compounds. Whereas albumin is the major binding protein for most anionic (acidic) drugs, many cationic drugs exhibit only moderate affinity for albumin and bind more avidly to other plasma proteins such as α_1-acid glycoprotein (orosomucoid) and lipoproteins [10–12]. The objective of the present study, therefore, was to investigate the relative contribution of albumin, α_1-acid glycoprotein, γ-globulins and lipoproteins to the overall binding of chlorpromazine (CPZ), perphenazine (PER) and trifluoperazine (TFP) in plasma. Comparisons were made with other basic drugs such as desmethylimipramine (DES) and propranolol (PROP) and with a prototypical acidic compound, salicyclic acid (SA).

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MATERIALS AND METHODS

Drugs. [^{3}H]Chlorpromazine hydrochloride (30.0 Ci/mmole) and [^{3}H]desipramine hydrochloride (50.0 Ci/mmole) were purchased from New England Nuclear, Lachine, Quebec, Canada. [^{3}H]Perphenazine·2HCl (10.3 Ci/mmole) and [^{3}H]trifluoperazine·2HCl (12.8 Ci/mmole) were obtained from the Nuclear Research Centre, Beer-Sheva, Israel. [^{3}H]Propranolol hydrochloride (21 Ci/mmole) and [^{14}C]salicylic acid (51.7 mCi/mmole) were purchased from the Amersham Corp., Oakville, Ontario, Canada. Radiolabeled phenothiazines were purified by high-pressure liquid chromatography at least once every month to prevent the build-up of breakdown products. The labeled salicylic acid, desipramine and propranolol were used as received from the manufacturer without further purification. In each case, radiochemical purity was periodically checked by thin-layer chromatography and found to be greater than 98%. Unlabeled drugs (CPZ, PROP and SA) were purchased from the Sigma Chemical Co., St. Louis, MO, or were provided by Smith Kline & French Canada Ltd., Mississauga, Ontario (TFP), Schering-Plough, Bloomfield, NJ (PER), and Ciba-Geigy, Summit, NJ (DES).

Plasma and purified proteins. Normal human plasma used in these studies was obtained from healthy volunteers (age 18–38 years). Blood was collected in evacuated glass tubes (Vacutainer, Beckton & Dickinson, Rutherford, NJ) and centrifuged (15 min, 3000 rpm, 4°); separated plasma was always used immediately. During collection of these blood samples, care was taken to avoid contact of blood with the rubber stopper of the Vacutainer tubes. Total lipoproteins were isolated from human plasma by preparative ultracentrifugation according to Sager *et al.* [13]. Potassium bromide was added to the fresh

plasma sample to achieve a density of 1.95 g/ml and then centrifuged (IEC model B-60 Ultracentrifuge, DAMON/IEC Division, Needham Heights, MA) for 45 hr at 4° (speed: 105,000 *g*). The floating lipoproteins were withdrawn and dialyzed against isotonic phosphate buffer (pH 7.4) for 24 hr at 4°. The lipoproteins were then diluted with isotonic phosphate buffer (pH 7.4) to obtain the original plasma volume and immediately used in the binding experiments. Human serum albumin (HSA; essentially fatty acid free), human α_1-acid glycoprotein (α_1-AGP; orosomucoid), and human γ-globulins (γ-GLOB; Cohn fraction II, 99% pure) were purchased from the Sigma Chemical Co. and used without further purification. These protein fractions were dissolved in isotonic phosphate buffer (pH 7.4) to give the following concentrations: 4 g/100 ml (HSA), 80 mg/100 ml (α_1-AGP), and 1.2 g/100 ml (γ-GLOB).

Equilibrium dialysis. Binding to plasma and plasma protein fractions was determined by equilibrium dialysis using a Dianorm apparatus (Spectrum Medical Industries Inc., Los Angeles, CA) with 1 ml teflon cells. One side of the dialysis cell contained 1 ml isotonic phosphate buffer (pH 7.4) and the other contained an equal volume of plasma or protein solution to which a mixture of labeled and unlabeled drug was added. The two compartments were separated by a semipermeable membrane (Spectrapor 2 membranes, Spectrum Medical Industries). The dialysis was carried out at 37° under constant stirring. The time used for equilibration was 4 hr. The cells were protected from light by wrapping them in aluminum foil to prevent breakdown of the phenothiazines. At the end of each experiment, radioactivity was determined in aliquots recovered from each compartment by liquid scintillation counting (LKB 1215 Racketbeta) using the method of external standardization.

Measurement of binding proteins. Albumin and α_1-acid glycoprotein plasma levels were measured by radial immunodiffusion [14] using commercially available kits (M-Partigen, Hoechst Canada, Montreal, Quebec). As a measure of lipoproteins, total cholesterol was assayed enzymatically in plasma samples using the Abbott ABA100 bichromatic analyzer [15].

Binding parameters. The percentage of unbound or free drug in plasma or protein solution was calculated as follows: $\%U = (C_b/C_p) \cdot 100$ where C_p = drug concentration in the protein or plasma compartment and C_b = drug concentration in the buffer compartment. The number of binding sites on the α_1-AGP protein (n) and the association constant (K) for the phenothiazine–α_1-AGP interaction were derived from Scatchard curves [16]. The molecular weights of α_1-acid glycoprotein and albumin were assumed to be 40,000 and 66,300 respectively [17]. The binding characteristics for two independent binding sites were obtained by drawing asymptotic straight lines to the curve close to the abscissa and ordinate and then moving these lines parallel so that the sum of the distances to the ordinate intercepts equalled the initial distance to the ordinate intercept. From these asymptotic lines, the curve was regenerated by drawing arbitrary straight lines through the origin of the coordinates. Along these the distance from the origin to the regenerated curve was made equal to the sum of the distances from the origin to the intercepts of the two asymptotic lines as described by Rosenthal [18]. Corrections were made on the asymptotic lines until the fit with the experimental curve was satisfactory. Each of the asymptotic lines represented one binding site on the protein molecule. The number of binding sites (n) and the association constant (K) were given by the intercept with the abscissa (n) and the ordinate (n K).

Statistical methods. To examine the effects of albumin and α_1-acid glycoprotein plasma concentrations on the unbound fraction of drug in plasma, Pearson correlation analyses (available on the Statistical Package for the Social Sciences) were performed on a DEC-20 computer system.

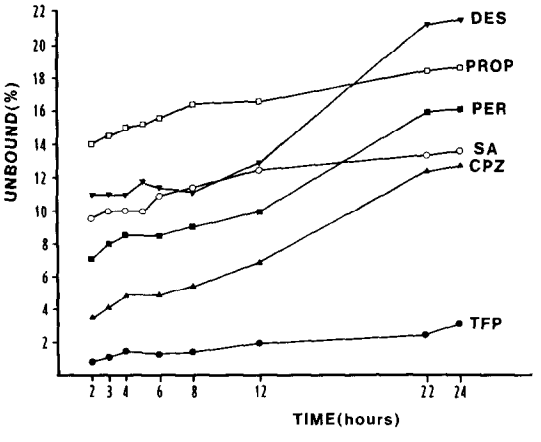

Fig. 1. Influence of equilibration time on the percent of unbound drug in plasma for chlorpromazine (CPZ), perphenazine (PER), trifluoperazine (TFP), desipramine (DES), propranolol (PROP) and salicylic acid (SA).

RESULTS

Equilibration time studies. The influence of equilibration time on the apparent plasma protein binding of the involved drugs was determined. Radioactive plus cold drug was added to fresh plasma samples to obtain therapeutic concentrations (CPZ: 200 ng/ml; PER: 50 ng/ml; TFP: 5 ng/ml; DES: 200 ng/ml; PROP: 20 ng/ml; SA: 200 μg/ml). The dialysis was stopped at regular intervals (2, 3, 4, 6, 8, 12, 22 and 24 hr) and the binding was determined. The results are shown in Fig. 1. They indicate that for CPZ, PER, TFP, DES and PROP there was a pseudo-equilibrium between 4 and 6 hr. Dialysis beyond 8 hr led to an increased free fraction with time. For SA, binding to plasma was constant between 3 and 5 hr and then slowly decreased. The increase in free SA fraction in plasma with time beyond 8 hr was not as pronounced as with the basic drugs. The same time-dependent *in vitro* binding was also observed when studying the interaction of these drugs with purified HSA and α_1-AGP. In all subsequent studies, equilibrium dialysis was carried out for 4 hr for all drugs.

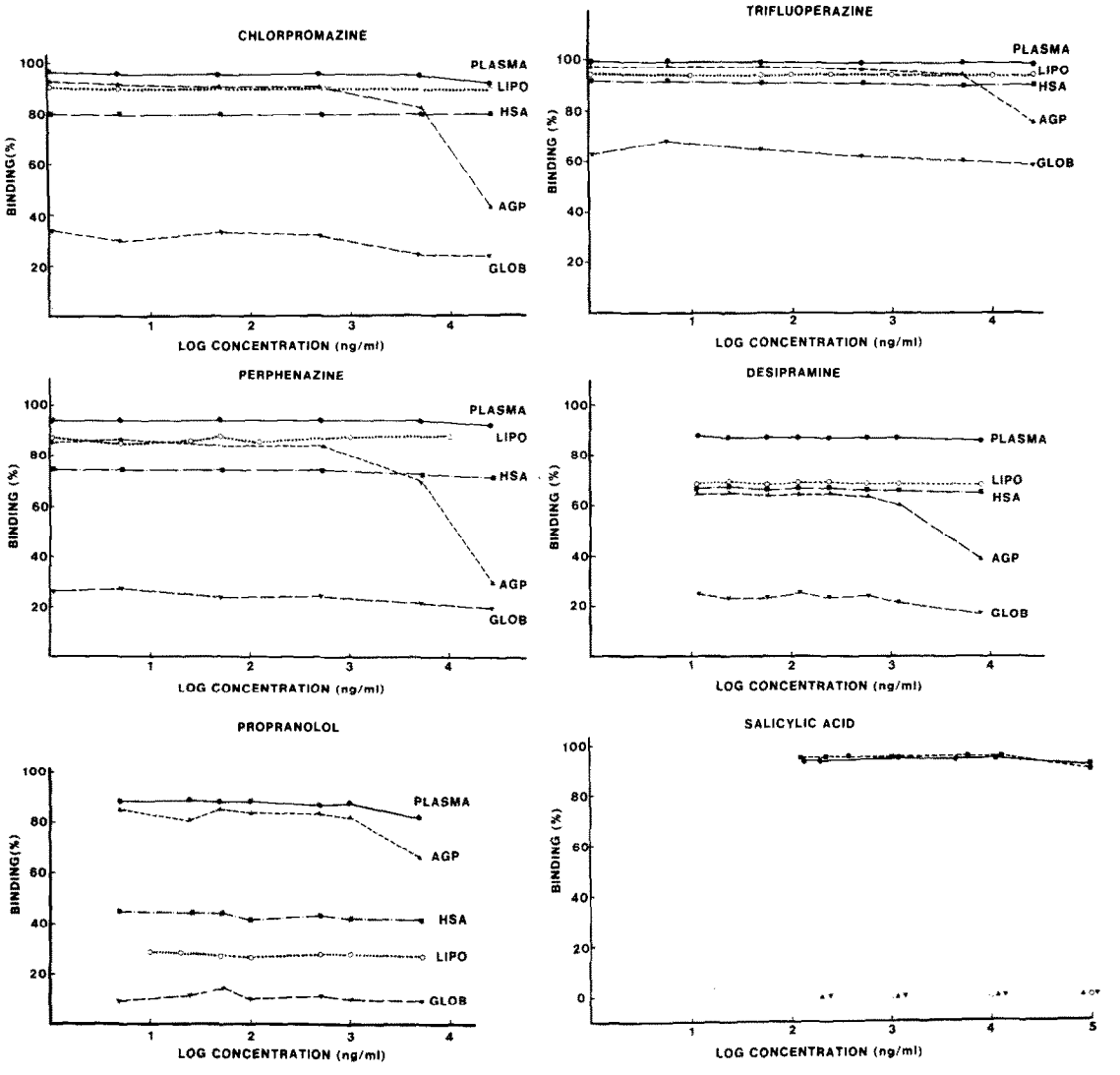

Fig. 2. Binding of chlorpromazine, perphenazine, trifluoperazine, desipramine, propranolol and salicylic acid to human plasma and isolated plasma protein fractions (HSA: 4 g/100 ml; α_1-AGP: 80 mg/100 ml; and γ = globulins: 1.2 g/100 ml).

Binding to plasma and plasma protein fractions. Figure 2 shows the binding of CPZ, PER, TFP, DES, PROP and SA to human plasma, lipoproteins isolated from the same plasma sample, HSA, α_1-AGP and γ-globulins. The binding pattern was very similar for the three phenothiazines: binding to HSA, lipoproteins and γ-globulins was non-saturable over the drug concentration range studied. Isolated α_1-AGP was the only protein fraction that showed saturable binding characteristics when the drug concentration was increased. The binding of PER and TFP to plasma was non-saturable over the drug concentration range studied, whereas CPZ binding to plasma showed a slight decrease at the upper concentration range. The binding characteristics of the other two basic drugs studied (DES and PROP) were similar to those of the three phenothiazines (Fig. 2), except for the finding that the binding of PROP to lipoproteins was not as pronounced as for the other basic drugs studied. For the one acidic drug studied i.e. SA, however, the binding to HSA accounted for the overall binding to plasma. Binding of SA to AGP, lipoproteins and γ-globulins was negligible. These experiments indicate that HSA, lipoproteins and α_1-AGP may all contribute significantly to the overall plasma binding of the basic drugs studied.

Characteristics of the phenothiazine–α_1-AGP interaction. Figure 3 shows the Scatchard curves for the interactions of CPZ, PER and TFP with α_1-AGP. The curves indicate the existence of two sets of binding sites characterized by number (n_1 and n_2) and association constant (K_1 and K_2). The number of binding sites with high affinity (n_1) on α_1-AGP was approximately 0.5 for CPZ and PER. TFP, on the other hand, had about one primary binding site. The association constants of the first class of binding sites were found to be $9.4 \times 10^5\,M^{-1}$, $3.4 \times 10^5\,M^{-1}$ and $6.0 \times 10^5\,M^{-1}$ for CPZ, PER and TFP respectively (Table 1).

Binding of CPZ, PER and TFP to HSA. Figure

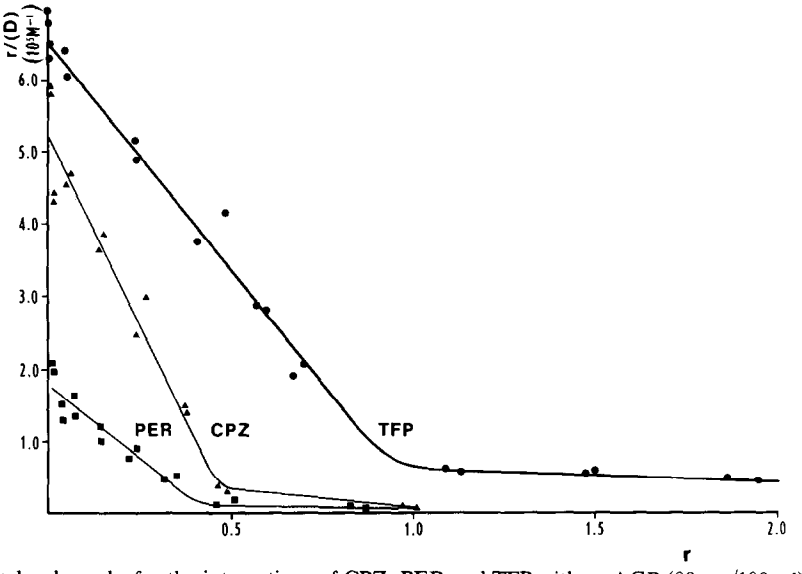

Fig. 3. Scatchard graphs for the interactions of CPZ, PER and TFP with α_1-AGP (80 mg/100 ml). Key: r = number of moles of drug bound per mole of protein, and (D) = molar concentration of unbound drug.

4 shows the Scatchard curves for the interactions of CPZ, PER and TFP (1 ng/ml to 25,000 ng/ml) to HSA (4 g/100 ml). Over the drug concentration range studied, which was the same as in the phenothiazine–α_1-AGP binding studies, there was no clear declining trend in the Scatchard curves. The intercepts with the y-axis (measure of the association constant) were 0.66×10^4 M^{-1}, 0.46×10^4 M^{-1} and 3.30×10^4 M^{-1} for the CPZ, PER and TFP binding with HSA respectively. At very low drug plasma concentrations (region close to y-axis in Figs. 3 and 4), the y-intercept represented n K since the contribution of the secondary binding sites is usually negligible. Therefore, assuming $n \geqslant 1$ for the phenothiazine–HSA interaction, the affinity between the phenothiazines and albumin was at least 100 times smaller than the affinity between phenothiazines and α_1-AGP.

Table 1. Association constants (K) and number of binding sites (n) for the interaction of CPZ, PER and TFP with isolated α_1-AGP

Drug	n_1	K_1 (10^5 M^{-1})	n_2	K_2 (10^4 M^{-1})
CPZ	0.5	9.4	1.0	5.0
PER	0.5	3.4	1.0	1.0
TFP	1.0	6.0	3.0	2.0

Binding of CPZ, PER, DES, PROP and SA to human plasma. CPZ, PER, DES, PROP and SA binding was determined in plasma from twenty-four subjects (forty-nine for DES) using therapeutic concentrations for each drug (see "Equilibration time studies"). The unbound plasma fraction of CPZ, PER

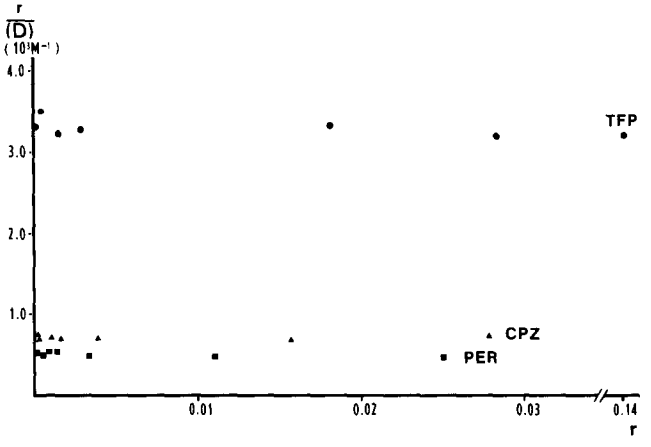

Fig. 4. Scatchard graphs for the interactions of CPZ (▲), PER (■), and TFP (●) with HSA (4 g/100 ml). Key: r = number of moles of drug bound per mole of protein, and (D) = molar concentration of unbound drug.

Table 2. Correlation between α_1-AGP and albumin plasma levels and percent unbound CPZ, PER, PROP, DES and SA in human plasma samples (N = 24)

Drug	α_1-AgP		Albumin	
	r	P	r	P
CPZ	−0.64	0.004	0.30	0.079
PER	−0.50	0.006	0.44	0.026
DES*	0.03	0.414	−0.38	0.005
PROP	−0.78	<0.001	0.32	0.062
SA	−0.21	0.157	−0.43	0.018

* N = 49.

and PROP was negatively correlated with α_1-AGP plasma levels (Table 2). There was no significant correlation between unbound fraction of DES and SA and α_1-AGP levels in plasma. Furthermore, the unbound fraction of the basic drug PER was positively correlated (P = 0.03) with albumin plasma levels, whereas CPZ and PROP plasma free fractions showed a positive correlation with plasma albumin levels but the correlation did not reach statistical significance (P = 0.08 and P = 0.06 respectively). SA free fractions were negatively correlated (P = 0.02) with plasma albumin as were DES free plasma fractions (P = 0.005).

DISCUSSION

The plasma protein binding of the phenothiazines chlorpromazine, perphenazine and trifluoperazine was studied and compared with the binding characteristics of other basic drugs (desipramine and propranolol) and salicylic acid. While establishing the conditions for the equilibrium dialysis experiments, it was found that a temporary plateau in the unbound plasma fraction of all the basic drugs studied existed between 4 and 6 hr following the start of the dialysis. For salicylic acid a constant binding was noted between 3 and 5 hr. A noticeable reduction of the plasma protein binding took place for all drugs (though more pronounced for the basic drugs) when dialysis extended beyond 8 hr. These findings suggest that equilibrium is reached after 4 hr and that beyond 6–8 hr secondary effects occur that decrease the binding. The same phenomenon has been observed for quinidine [19] and several tricylic antidepressant drugs [20, 21]. The exact mechanism involved, however, is not known, but volume changes due to colloidal osmotic pressure may be responsible, as recently shown by Kristensen and Gram [21]. Denaturation of the plasma proteins involved in the binding after 8 hr of dialysis at 37° may also contribute to the decrease in binding with time.

In vitro plasma protein binding studies are to some extent dependent on the method used [22] and, therefore, deviate from the real, *in vivo*, plasma binding for most drugs. Some investigators have evaluated their *in vitro* methods for protein binding determination by comparing the results with the cerebrospinal fluid (CSF)/plasma drug concentration ratio. However, it is not always certain that this so-called *in vivo* plasma binding value is the real plasma protein binding of a particular drug. Distribution of drugs into CSF is not only dependent on the plasma binding of the drug but also its lipophilicity, degree of ionization at physiological pH, and possible active transport of drug in and/or out of the CSF. In addition, CSF contains small amounts of proteins, and this may result in CSF/plasma drug concentration ratios higher than *in vivo* free plasma fraction of drug. All this makes it almost impossible to know which of the available methods will most closely correlate with the *in vivo* plasma protein binding. However, introduction of methodological errors should be kept minimal. When using equilibrium dialysis for example, equilibration times should be kept short as is illustrated by this study. Bertilsson *et al.* [23] also noted a time-dependent decrease in the plasma binding of demethylchlorimipramine when using equilibrium dialysis. However, these authors routinely use long dialysis times (19 hr) and, when comparing the *in vitro* plasma protein binding values (92%) with CSF/plasma ratios (97%), conclude that equilibrium dialysis underestimates binding and is not a good method. A shorter dialysis time would have resulted in *in vitro* plasma binding values (94%) closer to the so-called *in vivo* value (CSf/plasma ratio) as shown by these investigations in a preliminary time course experiment. In the present study, highly significant correlations (P < 0.01) could be demonstrated between the *in vitro* determined free fraction of drug in plasma and α_1-AgP plasma concentrations for CPZ, PER and PROP. This is an indication that the *in vitro* determined free plasma fractions of these drugs are closely related to the *in vivo* free plasma fraction.

Unlike acidic compounds which are almost exclusively bound to plasma albumin, basic compounds also bind to other plasma proteins such as α_1-acid glycoprotein and lipoproteins [10, 11]. This has been confirmed in the present study (Fig. 2 and Table 1). However, studies involving isolated protein fractions may give misleading results regarding the relative affinity of the protein fraction for basic drugs in plasma. This is illustrated very well in the present study for CPZ. According to binding studies performed using isolated protein fractions, the percent bound CPZ to HSA (80%) is only slightly lower than the percent bound CPZ to α_1-AGP (90%). However, when more detailed experiments concerning the CPZ-albumin and CPZ-AGP interaction were performed, such as Scatchard analysis, it became clear that α_1-acid glycoprotein has a much larger affinity for the phenothiazines than HSA (Figs. 3 and 4). When the saturable binding of CPZ, PER and TFP to α_1-AGP is represented by a Scatchard curve, the existence of two different binding sites on α_1-AGP with different affinities is apparent. The number of drug molecules bound to the first site was approximately 0.5 for CPZ and PER. Non-integral numbers of binding sites are often seen when proteins contain impurities. In the present study, the α_1-AGP was used as obtained from the manufacturer, and small quantities of impurities may have been present. However, an alternative explanation for this observation may be aggregation of α_1-AGP in such a way that some sites were not reached by the ligand. Furthermore, it has been shown that α_1-acid glyco-

protein is heterogeneous in the native state [24]. Recently, Tinguely *et al.* [25] were able to determine six to eight polymorphic forms of α_1-acid glycoprotein in plasma obtained from depressive patients. It is possible that one polymorphic form has binding characteristics different than those of another polymorphic form, which may lead to fractional numbers of binding sites. TFP has one primary binding site on α_1-AGP, suggesting that TFP may be bound at a different site or to different polymorphic forms.

Unusual Scatchard curves were obtained for the phenothiazine–HSA interaction. In these studies the same very low concentrations of the phenothiazines (therapeutic concentrations) were used to construct the Scatchard curves for both the albumin and α_1-AGP interaction. Therefore, the molar ratio of drug/protein was much lower for albumin, since the protein concentration was much higher (4 g/100 ml as compared to 80 mg/100 ml for α_1-AGP), resulting in Scatchard plots which apparently have no negative slope. However, several studies have been published in which saturable binding has been clearly shown for phenothiazines [26]. Assuming that albumin has at least one primary binding site for the phenothiazines, it is clear that the affinity of the phenothiazines for α_1-AGP is at least 100 times greater than their affinity for albumin.

Other reports have indicated recently that α_1-acid glycoprotein is an important binding protein in plasma for phenothiazines [27, 28]. Significant negative correlations were found in the present study between CPZ, PER and PROP free fraction in plasma and α_1-AGP plasma concentration (Table 2). A significant negative correlation was also found between SA free fraction and albumin plasma concentration, which is consistent with albumin being the only binding protein. Interestingly, no significant correlation was found between the unbound plasma fraction of DES and α_1-AGP plasma levels. Similarly, Bertilsson *et al.* [23], studying the binding of demethylchlorimipramine, did not find a correlation between the free plasma fraction of this antidepressant (structurally related to DES) and the concentration of plasma α_1-acid glycoprotein. However, a very significant negative correlation ($P = 0.005$) was found between the unbound plasma fraction of DES and albumin plasma concentrations. This finding suggests that albumin influences the *in vivo* free plasma fraction of the basic drug desipramine. Unexpectedly, a positive correlation exists between the unbound fraction of CPZ, PER and PROP and plasma albumin levels. This correlation is statistically significant ($P = 0.03$) in the case of PER and approaches significance for CPZ ($P = 0.08$) and PROP ($P = 0.06$). The same observation has been described for CPZ and PROP [29]. Piafsky *et al.* explained this by the negative correlation they observed between albumin and α_1-AGP plasma levels and which could also be found in our data.

Numerous studies have documented the increase of α_1-AGP in a variety of pathological conditions such as inflammation, infection, myocardial infarction and cancer [30–33]. But little is known about the effect of factors such as age and sex on the plasma concentration of this acute-phase protein. Since interindividual differences in the plasma protein binding of psychotropic drugs may be an important factor in the determination of clinical response, the binding of these drugs to α_1-AGP clearly deserves more attention. Furthermore, the possible effect of psychiatric illness and therapy on α_1-AGP plasma levels and, therefore, plasma free fraction should be examined more closely.

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