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α_1-ACID GLYCOPROTEIN INVOLVEMENT IN HIGH AFFINITY BINDING OF TRICYCLIC ANTIDEPRESSANTS TO HUMAN PLASMA

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Abstract—The binding of the tricyclic antidepressants imipramine (IMI) and desmethylimipramine (DMI) to human plasma and individual proteins was studied by equilibrium dialysis. Both drugs bound extensively to plasma, albumin, and α_1-acid glycoprotein, while there was very little binding to the γ-globulin fraction. The binding of both IMI and DMI to α_1-acid glycoprotein was high affinity (association constant K, 9.2×10^4/M and 4.7×10^4/M respectively) and low capacity (number of binding sites, $n = 1$ for both IMI and DMI), whereas the binding to albumin was low affinity (K for IMI, 2.3×10^2/M and for DMI, 3×10^2/M) and high capacity ($n = 7$). The binding of IMI to a mixture of human serum albumin and α_1-acid glycoprotein revealed two sets of binding sites; a high affinity binding site corresponding to α_1-acid glycoprotein and a low affinity binding site corresponding to albumin. The binding affinity and/or number of binding sites for IMI binding to albumin decreased with increasing albumin concentrations. The free fraction in plasma of nineteen normal, male controls was significantly correlated with the concentration of α_1-acid glycoprotein ($r = 0.601$, $P < 0.01$), although there was no correlation with albumin or free fatty acid concentrations in plasma.

The tricyclic antidepressant imipramine (IMI) and its desmethyl derivative, desipramine (DMI), are highly bound (85–95%) to human plasma proteins [1–4]. However, there are substantial interindividual differences in this binding. Glassman *et al.* [1] reported 5.4 to 23% free IMI in plasma from twenty-six depressed patients, and Piafsky and Borga [5] reported 6 to 11% free IMI in plasma from twenty-three healthy volunteers. Although IMI has been shown to bind to isolated human serum albumin [6, 7], lipoproteins [7, 8] and α_1-acid glycoprotein [5], few studies have characterized the binding to α_1-acid glycoprotein [9]. Since the kinetic parameters (binding affinities and the number of binding sites) can define the roles of the macromolecules involved in the binding of drugs, the purpose of this study was to determine the association constants and number of binding sites for IMI and DMI binding to human serum albumin (HSA) and α_1-acid glycoprotein.

MATERIALS AND METHODS

Materials. Imipramine hydrochloride and desipramine hydrochloride were gifts from Ciba–Geigy, Summit, NJ. Radiolabeled [^{14}C]imipramine hydrochloride (sp. act. 48 mCi/mmole) and [^{3}H]desipramine hydrochloride (sp. act. 27.6 Ci/mmole) were obtained from the Amersham Corp., Arlington Heights, IL. Human serum albumin (fatty acid free) was purchased from the Sigma Chemical Co., St. Louis, MO. γ-Globulin was obtained from Miles Laboratories, Elkhart, IN. α_1-Acid glycoprotein and radial immuno diffusion (RID) kits for albumin and α_1-acid glycoprotein determination were from CalBiochem, La Jolla, CA. All the other reagents used were of analytical grade.

Methods. Blood was collected in heparinized tubes. Contact with the rubber stopper was avoided since it decreases binding [2]. After centrifugation, binding to plasma was determined within 24 hr.

The albumin and α_1-acid glycoprotein concentrations in plasma samples were determined using RID kits, while free fatty acids were determined by a colorimetric method [10]. Total and free sialic acid was measured by the method of Aminoff [11].

Equilibrium dialysis. The binding experiments were performed by equilibrium dialysis using a multiple-cell block. Each cell had two 1-ml chambers which were separated by a cellulose membrane. The cellulose membrane was washed with distilled water and buffer before use. One side of the cell was filled with 0.9 ml of buffer of the following composition: NaH_2PO_4, 5 mM; Na_2HPO_4, 20 mM; KCl, 5 mM; NaCl, 120 mM, pH adjusted to 7.4. The unlabeled drug was dissolved in a small volume of water and diluted with buffer to give the required concentrations. In all experiments, except for Scatchard analysis, the concentration of IMI and DMI used was 250 ng/ml. The labeled drug was added to the working drug solution in buffer to give approximately 50,000 cpm/ml of solution.

The other side of the cell was filled with 0.9 ml of plasma or protein solution in the buffer. The dialysis was carried out in a shaking water bath at 37°. Under these conditions equilibrium was reached in 16–18 hr. Duplicate aliquots of 100 μl from each chamber were pipetted into scintillation vials, and 15 ml of counting fluid were added. Quenching of the solutions was evaluated by the channel ratio method

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and found to be insignificant at the protein concentrations used. All experiments were carried out in duplicate.

Stability of α_1-acid glycoprotein. To determine the stability of α_1-acid glycoprotein, free and total sialic acid and protein concentrations were determined before and after the 24 hr incubation. There were no differences in the concentrations of α_1-acid glycoprotein and total sialic acid contents before and after incubation. There was no detectable free sialic acid after incubation. These results suggest that α_1-acid glycoprotein was chemically stable during the incubation.

Calculations. The percentage of drug bound to protein was calculated from the radioactivities in the buffer chamber (free drug concentration) and the protein chamber (total drug concentration). The concentration of bound drug, B, in μmoles/liter was calculated by the following equation as described by Sandberg *et al.* [12]:

$$B = \frac{\% B \times T_D}{100 + \% F}$$

where T_D = total drug concentration in μmoles/liter and $\% F$ = percentage of free drug in the protein chamber at equilibrium.

Binding parameters. The association constants and the number of binding sites were obtained by plotting r/F against r (moles of ligand/mole of protein) according to the method of Scatchard [13]. Least-square linear regression analysis was used to determine the slope and the intercepts for the albumin and α_1-acid glycoprotein data. In the experiments involving the binding of different ligand concentrations to plasma or protein mixture, the B/F ratio was plotted against B, according to the method of Sandberg *et al.* [12].

RESULTS

Figure 1 shows the time course of dialysis for IMI equilibration when the drug was added either to the buffer chamber or the plasma chamber of the dialysis cell. Equilibrium was reached within 16–18 hr of dialysis. There were no differences in the binding at equilibrium whether the drug was added to the buffer or plasma. Furthermore, the binding remained constant for more than 24 hr of incubation, indicating the stability of plasma proteins that contribute to the binding. Hence, all experiments were carried out for 24 hr to ensure equilibrium. The reliability of the method was calculated by determining IMI binding to HSA in nine samples ($\% F$, $\bar{X} = 41.5$; standard deviation, $\pm$ 1.14). The coefficient of variation for the free fraction was 2.8%.

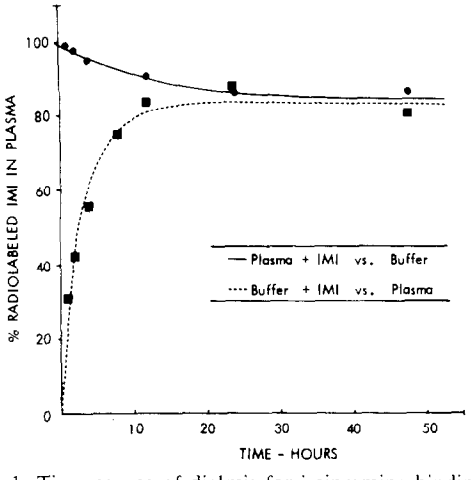

Fig. 1. Time course of dialysis for imipramine binding at 37°. The drug (250 ng/ml) was added either to the buffer compartment (■·····■) or to the plasma compartment (●—●). The results are expressed as percents of total radioactivity in the plasma.

Table 1. Binding of IMI and DMI to human plasma, serum albumin, α_1-acid glycoprotein and γ-globulin*

	Protein concn (mg/ml)	% Bound IMI	% Bound DMI
Plasma		88.5	82.5
Albumin	48	54.0	61.9
α_1-Acid glycoprotein	1.0	88.0	67.6
γ-Globulin	7.5	10.3	13.1

* The concentration of IMI and DMI was 250 ng/ml.

Table 1 shows the binding of IMI and DMI to human plasma, HSA, α_1-acid glycoprotein, and γ-globulin. The concentration of IMI and DMI used was 250 ng/ml. Both IMI and DMI bound extensively to plasma, HSA and α_1-acid glycoprotein, whereas there was very little binding to the γ-globulin fraction. The binding to HSA was significantly lower than to plasma. Also, when plasma was diluted 3-fold with a 4.8% solution of HSA, the binding decreased from 85 to 70%. In other words, there was a 2-fold increase in free fraction (from 15 to 30%), even though the albumin concentration was not changed.

Effect of drug concentration on binding. Table 2 shows the percentage of IMI bound to plasma, and the physiological concentrations of HSA and α_1-acid glycoprotein, when the drug concentration was varied from 0.25 to 10 μg/ml. Although the percent binding of IMI to α_1-acid glycoprotein was significantly reduced, the fraction bound to plasma and HSA was relatively unchanged over the above drug concentration range. At higher drug concentrations (50–1000 μg/ml) the free fraction increased signifi-

Table 2. Effect of IMI concentration on its binding to plasma, albumin and α_1-acid glycoprotein

Drug concn (μg/ml)	% IMI bound to Plasma	HSA (48.0 mg/ml)	α_1-Acid glycoprotein (0.67 mg/ml)
0.25	84.7	58.2	62.4
0.50	85.7	56.5	62.6
1.0	84.0	58.7	51.6
5.0	84.5	59.0	52.3
10.0	82.2	57.3	44.5

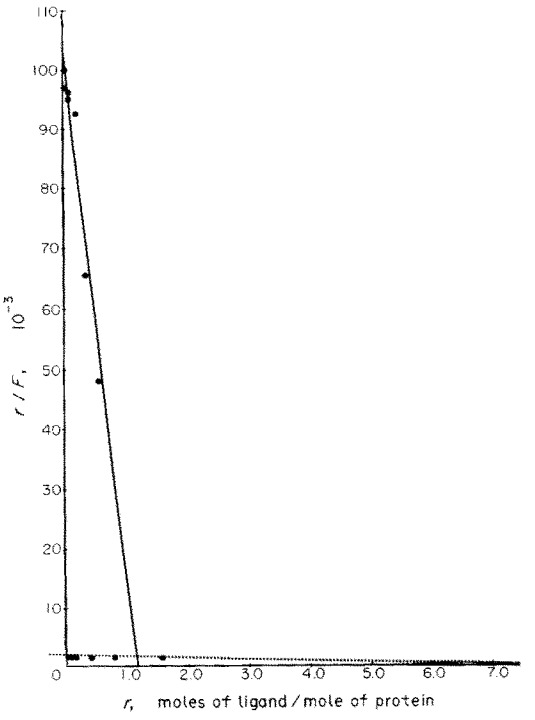

Fig. 2. Scatchard plots of imipramine binding to HSA (●·····●) and α_1-acid glycoprotein (●—●). The lines were fitted by linear regression with the method of least squares.

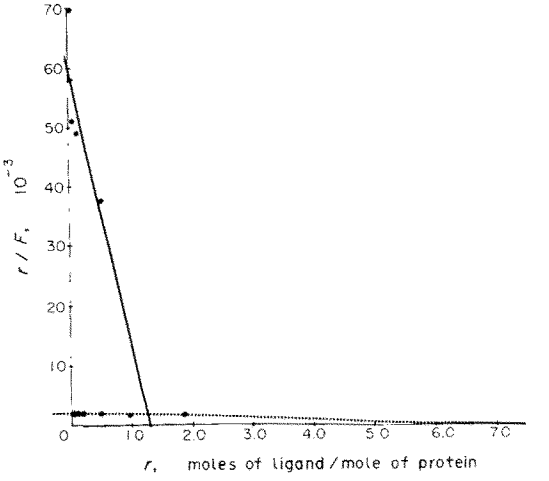

Fig. 3. Scatchard plots of desipramine binding to HSA (●·····●) and α_1-acid glycoprotein (●—●). The lines were fitted by linear regression with the method of least squares.

cantly. Brinkschulte and Breyer-Pfaff [3] have similarly shown that the fraction of amitriptyline and nortriptyline bound to plasma was constant over the drug concentration range of 0.129 to 39 μg/ml.

Binding parameters [the association constant (K) *and the number of binding sites* (n)]. The binding constants for each drug–protein interaction were determined by measuring the binding at various concentrations of IMI and DMI to physiological concentrations of HSA (48 mg/ml) and α_1-acid glycoprotein (0.67 mg/ml). The drug concentrations for binding of either drug to α_1-acid glycoprotein ranged from 0.25 to 10 μg/ml, while for binding to HSA drug concentration was varied from 12.5 μg/ml to 1.0 mg/ml. Figure 2 shows the Scatchard plot for IMI binding to HSA and α_1-acid glycoprotein. There was a single class of high affinity binding of IMI to α_1-acid glycoprotein. On the other hand, the binding of IMI to HSA represented a single class of low affinity binding sites. Similarly, the binding of DMI to α_1-acid glycoprotein was high affinity and low capacity, whereas binding to HSA was low affinity and high capacity (Fig. 3).

The binding parameters for IMI and DMI to HSA and α_1-acid glycoprotein are summarized in Table 3. The association constant for IMI binding to α_1-acid glycoprotein was 400 times greater than its binding to HSA. Approximately 1 mole of IMI was bound to 1 mole of α_1-acid glycoprotein, whereas there were 7 moles of IMI bound per mole of HSA. Similarly, DMI binding to α_1-acid glycoprotein was stoichiometric and the association constant was 150 times greater than its binding to HSA. There were 6.8 moles of DMI bound per mole of HSA.

In one experiment, the effect of albumin concentration on the binding of IMI was also studied. Figure 4 shows the Scatchard plot for IMI binding (at 250 ng/ml concentration) to five different HSA concentrations (1.45×10^{-4} M to 1.45×10^{-3} M). The positive slope indicates an increase in association constant and/or the number of binding sites with decreasing protein concentrations. Such positive slopes of Scatchard plots with different protein concentrations have also been shown for codeine, morphine and methadone [14] and cortisol [15].

In another experiment, binding at various concentrations of IMI (0.5 to 1000 μg/ml) to a mixture of HSA (4.8%) and α_1-acid glycoprotein (0.067%) was also determined. The binding of IMI to the mixture of proteins revealed two sets of binding sites: a high affinity binding site corresponding to α_1-acid glycoprotein and a low affinity binding site corresponding to albumin. The binding of IMI to plasma also revealed two sets of binding sites (results not shown).

α_1-Acid glycoprotein concentrations and imipramine binding to plasma from normal controls. The binding of IMI to plasma obtained from nineteen normal, healthy males was determined. The free fraction in these individuals varied 5-fold (4.5 to 22.9%). There was no significant correlation in percent binding and albumin concentration. Similarly,

Table 3. Association constants (K) and number of binding sites (n) for IMI and DMI binding to human serum albumin and α_1-acid glycoprotein

	K (M^{-1})		n	
	IMI	DMI	IMI	DMI
HSA (48 mg/ml)	2.3×10^2	3.0×10^2	7	6.8
α_1-Acid glycoprotein (0.67 mg/ml)	9.2×10^4	4.7×10^4	1.1	1.3

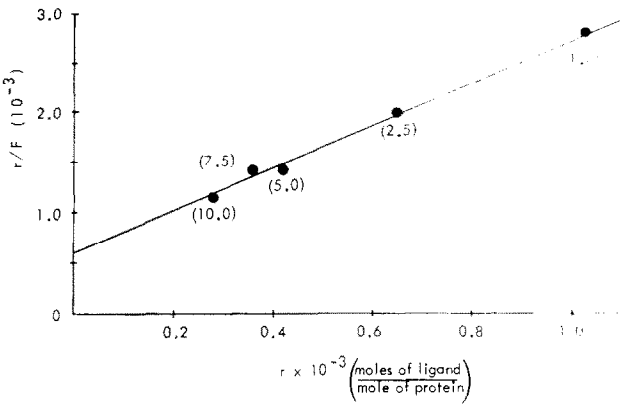

Fig. 4. Scatchard plot of imipramine binding (250 ng/ml) to various concentrations of HSA. The line was fitted by linear regression with the method of least squares. The numbers in parentheses represent the concentration of HSA in g/100 ml solution.

concentration of free fatty acids in plasma did not correlate with IMI binding. However, there was a significant inverse correlation ($r = -0.601$, $P < 0.01$) in free fraction of IMI and α_1-acid glycoprotein concentrations. These results are similar to those previously reported by Piafsky and Borga [5].

DISCUSSION

The major finding of the present studies is the high affinity binding of tricyclic antidepressants IMI and DMI to α_1-acid glycoprotein. Although these drugs also bind to HSA, the affinities were 2–3 orders of magnitude lower than their binding affinities to α_1-acid glycoprotein.

The time course of dialysis (Fig. 1) showed that equilibrium under the described experimental conditions was attained only after 16–18 hr of incubation at 37°. Although Bertilsson *et al.* [16] have reported that binding of desmethylchlorimipramine (DMCI) decreased after 6 hr of dialysis, we did not find any decrease in IMI binding to plasma for up to 48 hr of incubation. Our results would suggest that the IMI binding components of plasma are stable for 24 hr at 37°. The fact that Bertilsson *et al.* [16] found a decrease in binding of DMCI to plasma over time would suggest that either some DMCI binding component of plasma does not bind IMI or DMCI itself is not stable during incubation.

The association constant for IMI binding to HSA (2.3×10^2/M) reported here was similar to that reported by Bickel (4.9×10^2/M) [7], although Weder and Bickel [17] have reported a higher association constant (5×10^3/M). This may be due to the fact that we used physiological concentrations of albumin (4.8%) as did Bickel [7], whereas Weder and Bickel [17] used 1% albumin solution. Recently, Judis [14] reported that the product, nK, for codeine, morphine and methadone binding to HSA decreased with increasing protein concentrations. Mueller and Potter [15] found similar results for cortisol binding with increasing concentrations of albumin. We observed a similar decrease in the binding affinity and/or number of binding sites for IMI binding to HSA with increasing protein concentrations (Fig. 4). Since the number of binding sites for IMI per mole of HSA ($n = 7$) was similar to that reported by Weder and Bickel ($n = 6$), the decrease in nK with increasing protein concentrations may have been primarily due to a decrease in association constant. In the present studies we used 4.8% albumin solution to determine the binding constants under physiological conditions.

Desipramine binding to HSA was also low affinity ($K = 3 \times 10^2$/M) and high capacity ($n = 6.8$).

On the other hand, the binding of both IMI and DMI to α_1-acid glycoprotein was high affinity and approximately one mole of each drug was bound per mole of protein. Although the association constants for IMI and DMI binding to α_1-acid glycoprotein have not been reported previously, Borga *et al.* [18] found that the Scatchard plots for DMI binding to plasma revealed high affinity and low affinity binding sites. In the present studies, Scatchard analysis of IMI binding to plasma also revealed high affinity and low affinity binding sites. Similarly, Brinkschulte and Breyer-Pfaff [3] reported two binding sites in plasma for both amitriptyline and nortriptyline, the high affinity site becoming apparent only at low drug concentrations. They reported these association constants to be 6×10^4/M and 2×10^4/M for amitriptyline and nortriptyline respectively. Most likely, these association constants represent the binding to α_1-acid glycoprotein, since they are of the same order of magnitude as IMI (9.2×10^4/M) and DMI (4.7×10^4/M) reported here.

Although it has generally been assumed that most of the ligand–macromolecule interactions involve the albumin as the primary macromolecule, recently more attention is being focused on α_1-acid glycoprotein as the binding protein for basic drugs. For example, α_1-acid glycoprotein has been shown to bind imipramine [2], ciclazindol [19], lidocane [20], perazine [21], methadone [22] and desmethylimipramine (present studies). The binding of drugs to α_1-acid glycoprotein may have important clinical significance. The concentration of α_1-acid glycoprotein in plasma from nineteen control males varied more than 3-fold (0.5 to 1.6 mg/ml). Furthermore, its concentrations in plasma are markedly elevated in

inflammatory diseases, stress, and in the last trimester of pregnancy (for review see Ref. 23). Since the free fraction of the drug is a pharmacologically relevant concentration, the differences in α_1-acid glycoprotein could affect the clinical outcome.

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