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Binding of [^{3}H]mianserin to bovine serum albumin, human serum albumin and α_1-acid glycoprotein

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Scatchard plots which were curvilinear with negative slopes were obtained when the binding of [^{3}H]mianserin to bovine serum albumin (BSA), human serum albumin (HSA), defatted human serum albumin (D-HSA) and α_1-acid glycoprotein (α_1-AGP) was studied with equilibrium dialysis with constant protein concentrations and various ligand concentrations. Binding parameters were estimated graphically and with a non-linear least-squares computer program, assuming two classes of independent binding sites. α_1-AGP had the highest binding affinity (K) and binding capacity (nK). The binding parameters, n and K were not independent of protein concentration when the BSA concentration was varied. Linear atypical Scatchard plots with positive slopes were obtained when the protein concentration was varied for BSA, HSA and D-HSA, at a fixed ligand concentration.

Mianserin (1,2,3,4,10,14b-hexahydro-2-methyldibenzo[c,f]pyrazine [1,2-a]-azepin) is a basic tetracyclic antidepressant. Very little is known about its binding to blood proteins apart from a brief report that approximately 90% is bound to human plasma (Brogden et al 1978).

In this present investigation the binding of [^{3}H]mianserin to bovine serum albumin (BSA), human serum albumin (HSA) and defatted human serum albumin (D-HSA) was initially examined with the aid of equilibrium dialysis, using the traditional approach of keeping the protein concentration constant and varying the ligand concentration. In a second series of experiments the ligand concentration was kept constant and the concentration of protein was varied.

Since it has been shown that α_1-acid glycoprotein (α_1-AGP) is an important determinant in the binding of basic drugs, such as propranolol (Glasson et al 1980) and perazine, amitriptyline and nortriptyline (Brinkshulte & Breyer-Pfaff 1980) the binding of [^{3}H]mianserin to this protein was also examined.

MATERIALS AND METHODS

Materials

Crystalline lyophilized BSA, fraction V; crystalline lyophilized HSA, and α_1-AGP were obtained from Sigma Chemical Co., Poole, Dorset. Defatted HSA was prepared according to the method of Fiehn & Hasselbach (1970). Mianserin hydrochloride was a gift from Organon Laboratories Ltd., Morden, Surrey. [8-^{3}H]Mianserin hydrochloride (specific activity 49mCi mg^{-1}) was obtained from Amersham International Ltd., Amersham, Bucks. Scintillant NE-260 for liquid scintillation counting was obtained from Nuclear Enterprises, Edinburgh. All solutions and buffers were prepared with distilled water.

Binding Studies

Equilibrium dialysis was performed with a Dianorm Equilibrium Dialyser (Diachema AG, Rushlikon-Zurich, Switzerland) equipped with Macro-15 Teflon cells (total working volume 2·38 ml) containing high permeability Spectrapor membranes (Diachema, molecular weight cut-off 2000 to 14 000) which had been prepared in accordance with the manufacturer's instructions.

Solutions of mianserin containing [^{3}H]mianserin as a tracer were prepared in a Krebs buffer (0·154M) pH 7·4. BSA, HSA, D-HSA and α_1-AGP were also dissolved in Krebs buffer. Aliquots (1 ml) of protein solutions containing [^{3}H]mianserin at various concentrations were dialysed against Krebs buffer (1 ml) for 17 h at 37 °C using the Dianorm Equilibrium Dialyser. Alternatively aliquots (1 ml) of [^{3}H]mianserin solutions containing protein at various concentrations were dialysed against Krebs buffer (1 ml) for 17 h at 37 °C. [^{3}H]Mianserin and protein concentrations are shown in Table 1.

After dialysis, duplicate aliquots were taken from both the protein and buffer compartments for liquid scintillation counting.

The rate of equilibration had been determined by dialysing mianserin solutions containing bovine serum albumin for between 1 and 22 h. No changes in the volume of the protein solutions during dialysis were observed.

All the binding data were plotted according to the

* Correspondence.

Table 1. Protein and initial [^{3}H]mianserin concentrations for binding experiments.

	Protein ($\times 10^{-4}$M)	[^{3}H]mianserin ($\times 10^{-4}$M)
BSA	0·456	0·1 to 5·4
	0·650	0·1 to 5·4
	0·850	0·1 to 5·4
	0·0968 to 0·968	0·0366
	0·0968 to 0·968	0·305
	0·0968 to 0·968	1·22
HSA	0·456	0·111 to 4·45
	0·0968 to 0·968	0·0336
D-HSA	0·495	0·265 to 6·61
	0·0495 to 0·495	0·106
α_1-AGP	0·360	0·347 to 6·34

Scatchard method (Scatchard 1949). The protein binding parameters, association constant (K) and number of binding sites (n) were obtained by curve stripping the Scatchard plots to obtain graphical estimates of K and n, and then fitting the equation:

$$R = \frac{n_1K_1[D_f]}{1 + K_1[D_f]} + \frac{n_2K_2[D_f]}{1 + K_2[D_f]} \quad (1)$$

to a non-linear least-squares computer program. Equation 1 assumes that there are only 2 classes of independent binding sites, and R is the degree of binding and $[D_f]$ is the free drug concentration.

Preliminary recovery experiments demonstrated that no more than 0·2% of the total radioactivity was associated with the dialysis membrane.

The calculated values of n and K were obtained from the final radioactive concentrations of [^{3}H]mianserin, not from the initial concentrations.

RESULTS

The extent of mianserin binding to HSA and D-HSA was lower than for BSA at similar protein concentrations (Fig. 1). Moreover the extent of binding to α_1-AGP ($0{\cdot}366 \times 10^{-4}$M) at low mianserin concentrations ($0{\cdot}1 \times 10^{-4}$M) was 74% which was considerably greater than for either BSA (52%), HSA (43%) or D-HSA (33%) at similar protein concentrations (Fig. 1). With increasing concentrations of BSA the extent of binding increased (Fig. 1A). However at BSA concentrations of $0{\cdot}65$ and $0{\cdot}85 \times 10^{-4}$M the extent of binding was similar at all concentrations of mianserin.

Scatchard plots of the binding data are shown in Fig. 2. The graphical estimates of the binding parameters are shown in Table 2. At a BSA concentration of $0{\cdot}248 \times 10^{-4}$M the error in the duplicate measurements increased resulting in poor reproducibility, making it impossible to obtain reliable binding constants. The values of K_1 and K_2 decreased with increasing BSA concentration (Table 2), as did the values for n_1, while the values for n_2 increased. Although the extent of mianserin binding to HSA was markedly lower than for BSA, the K_1 value for HSA was higher than for BSA at the same protein concentration (Table 2). Moreover, the K_1 value for D-HSA and the binding capacity (nK) value were lower than the corresponding values of K and nK for BSA and HSA. Of all the proteins studied α_1-AGP gave the highest value for K_1, which was 10 to 20 fold higher than the K_1 values for BSA, HSA and D-HSA.

When the binding of mianserin to BSA and HSA was studied with varying protein concentrations (range $0{\cdot}0968 \times 10^{-5}$ to $0{\cdot}968 \times 10^{-4}$M) at a constant ligand concentration, the extent of mianserin binding increased with increasing protein concentration (Fig. 3). Transformation of the binding data into Scatchard plots resulted in plots with positive slopes (Figs 4, 5).

Table 2. Graphical estimates of the binding parameters for binding of mianserin to BSA, HSA, D-HSA and α_1-AGP at constant protein concentration and various ligand concentrations.

Protein	($\times 10^{-4}$M)	K_1($\times 10^3$M^{-1})	n_1	n_1K_1 ($\times 10^3$M^{-1})	K_2($\times 10^3$M^{-1})	n_2	n_2K_2 ($\times 10^3$M^{-1})
BSA	0·456	8·5	0·90	7·65	2·1	8·9	18·7
		(12·9)	(0·89)	(11·5)	(1·46)	(8·4)	(12·26)
	0·650	5·5	1·0	5·5	1·6	10·9	17·4
		(8·5)	(0·91)	(7·73)	(1·09)	(11·3)	(12·32)
	0·850	4·5	0·70	3·2	0·86	14·8	12·7
		(7·8)	(0·56)	(4·37)	(0·75)	(16·0)	(12·0)
HSA	0·456	12·8	0·85	10·9	2·33	3·7	8·6
		(20·7)	(0·56)	(11·59)	(1·18)	(5·3)	(6·25)
D-HSA	0·495	5·6	0·74	4·14	0·88	8·7	7.66
		(8·2)	(0·48)	(3·94)	(0·64)	(9·0)	(5·76)
α_1-AGP	0·366	114·1	1·25	142·6	3·37	5·8	19·5
		(191·9)	(0·94)	(180·4)	(1·32)	(5·9)	(7·79)

BSA, HSA & D-HSA data are mean of 3 to 4 experiments. Figures in parentheses are the computer estimates.
α_1-AGP data are mean of 2 experiments.

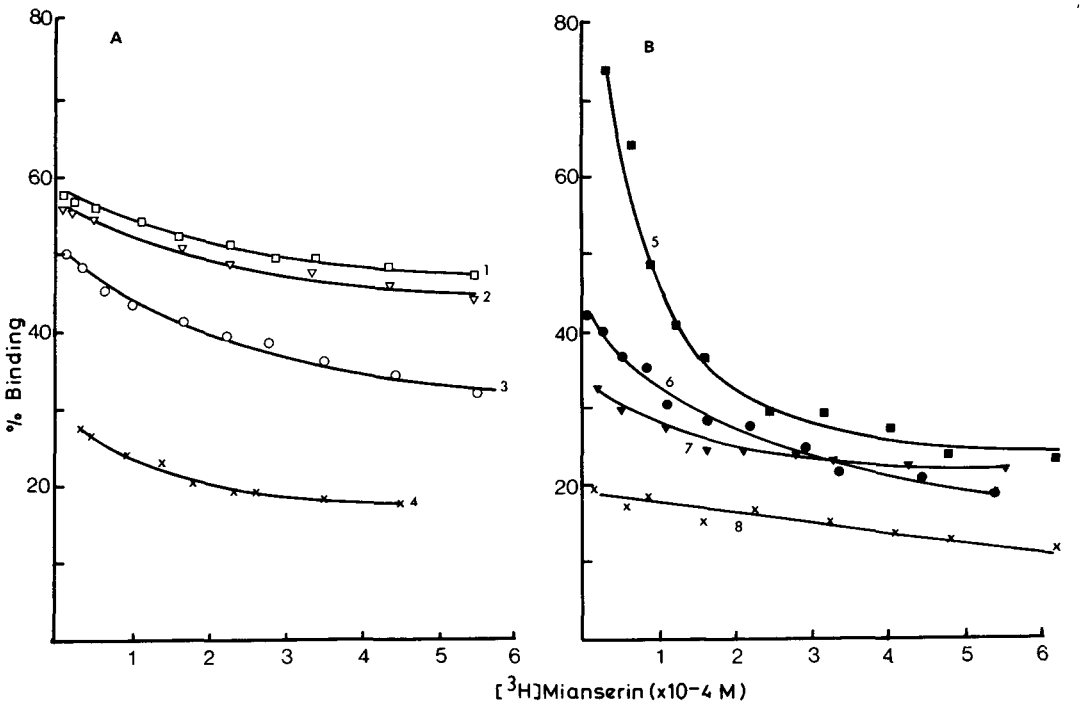

FIG. 1. Percentage binding of [^{3}H]mianserin to proteins. A. BSA, 0·85 × 10^{-4}M(1); 0·65 × 10^{-4}M(2); 0·456 × 10^{-4}M(3); 0·248 × 10^{-4}M(4) B. α_1-AGP, 0·336 × 10^{-4}M(5); HSA, 0·456 × 10^{-4}M(6); D-HSA, 0·495 × 10^{-4}M(7); HSA, 0·366 × 10^{-4}M(8)

DISCUSSION

Association constants (K) and the number of binding sites (n) of a protein-ligand interaction are usually determined either graphically (Klotz & Huntson 1971; Weder et al 1974) or by using non-linear least-squares fitting procedures (Fletcher et al 1973). In both methods, values for K and n are obtained by employing a chemical protein-ligand interaction model (site approach) which assumes that the values of K and n are independent of protein concentrations and that the classes of binding sites are independent of each other.

One graphical method used to determine n and K is that of Scatchard where the ratio of the degree of binding (R) and the free ligand concentration (D_f) are plotted against the degree of binding (R). Moreover, if a non-linear least squares fitting procedure is adopted, graphical estimates for K and n are still required as starting values for the fitting procedure. Various methods, which can be separated into two main categories, have been described whereby K and n values are obtained from Scatchard plots.

Firstly K and n are obtained from the slopes and intercepts of the extrapolated tangents drawn at the extremities of the plot (Klotz & Huntson 1971), when it is assumed that there are no interactions between the classes of binding sites. Alternatively there are methods available which allow for interacting binding sites, which are unfortunately subject to considerable variation since they rely on the subjective estimation of K and n (Weder et al 1974).

Values of K and n obtained by non-linear least-squares fitting procedures are also dependent on the weighting techniques used for computer fitting. These are usually chosen so that the error in the experimental data is accounted for. In this present study the weighting procedure employed only accounted for variations in R/D_f values perpendicular to the R-axis, since averaged R/D_f values were used. Unfortunately this resulted in marked variations in the computed values of K_2 and n_2, which in turn influenced the estimates for K_1 and n_1 (Table 2 values in parentheses). An alternative weighting procedure has been proposed (Lutz et al 1975), where the slopes of the Scatchard plot are constrained within a band whose width is not uniform on either side of the plot since the band is also influenced by variations in R/D_f along the free ligand concentration line as well as variations in R/D_f perpendicular to the R axis. However since the authors did not indicate how this band was constructed, no comment can be made on the validity or

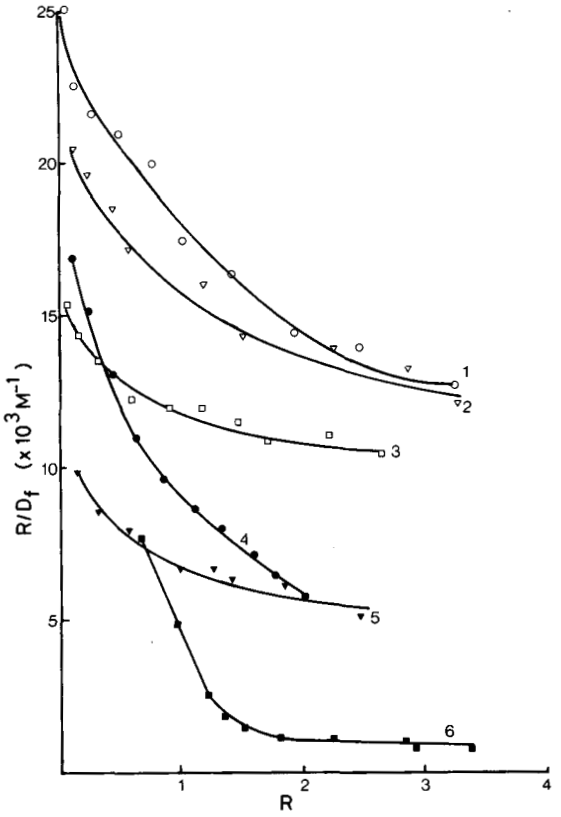

FIG. 2. Scatchard plots. BSA, 0·45 × 10^{-4}M(1); BSA, 0·65 × 10^{-4}M(2); BSA, 0·85 × 10^{-4}M(3) HSA, 0·456 × 10^{-4}M(4); D-HSA, 0·495 × 10^{-4}M(5); α_1-AGP, 0·366 × 10^{-4}M(6).

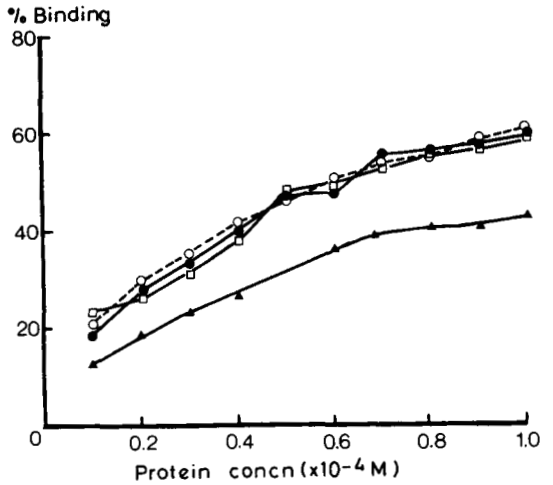

FIG. 3. Percentage binding of [3H]mianserin to BSA and HSA at a constant mianserin concentration and varying protein concentrations. BSA-Mianserin concentration 0·366 × 10^{-5}M (●); 0·305 × 10^{-4}M (○); 1·22 × 10^{-4}M (□). HSA-Mianserin concentration 0·366 × 10^{-5}M (▲).

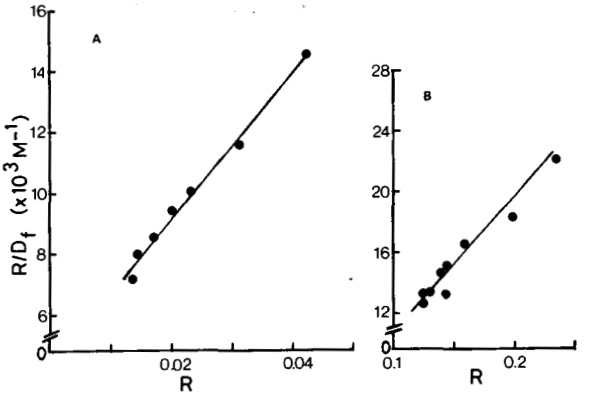

FIG. 4. Scatchard plots for the binding of [3H]mianserin to HSA(A) and D-HSA(B) at varying protein concentrations. (A) Mianserin concentration 0·034 × 10^{-4}M. (B) Mianserin concentration 0·106 × 10^{-4}M.

statistical significance of this weighting procedure.

Initially, the binding of mianserin to the proteins was studied employing equilibrium dialysis using the customary method of keeping the protein concentration constant whilst varying the ligand concentration. In these experiments the extent of binding was observed to decrease as the concentration of mianserin increased (Fig. 1). Transformation of the binding data into Scatchard plots resulted in non-linear plots with negative slopes (Fig. 2). Plots with non-linear slopes are often interpreted, using the classical site approach, to indicate the presence of two classes of binding sites or interacting sites in the protein molecule (Scatchard 1949). This model, however, assumes that the binding parameters n and K are independent of protein concentration, which

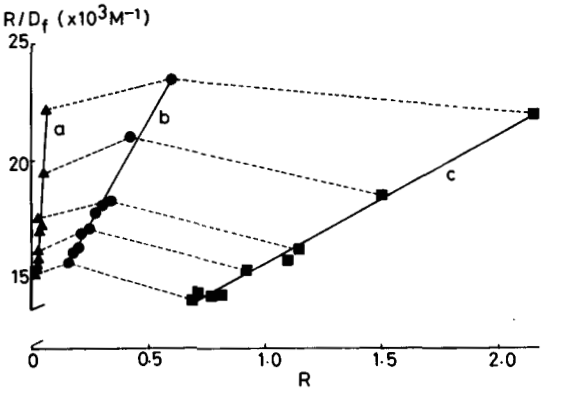

FIG. 5. Scatchard plots for the binding of [3H]mianserin to BSA at varying protein concentrations. Solid lines represent Scatchard plots obtained from the experimental data. Broken lines join equal protein concentrations and represent the predicted Scatchard plot at constant protein concentration. Mianserin concentrations 0·0366 × 10^{-4}M (line a); 0·305 × 10^{-4}M (line b); 1·221 × 10^{-4}M (line c).

was contrary to what was observed when the BSA concentrations were varied (Table 2). The dependence of protein-binding capacity (nK) on protein concentration was also observed in studies on phenytoin binding to HSA (Bowmer & Lindup 1978). Various explanations for nK being dependent on protein concentration have been proposed. One is that this decrease in binding capacity with increasing protein concentration is attributed to protein-protein interactions due to either non-specific molecular aggregation or polymer formation (Brock 1976; Teller 1976).

Although the n_1K_1 values were the same for BSA and HSA at the same protein concentration, the affinity constant for HSA was nearly double that for BSA (Table 2), whilst the n_2K_2 value for HSA was nearly half that of the BSA value. At a slightly lower protein concentration than for HSA, the K_1 value for α_1-AGP was nearly 10 times that of the K_1 value for HSA, with a binding capacity (n_1K_1) nearly 15 times greater than for HSA. These observations are consistent with the view that this blood protein is an important determinant in the binding of basic drugs. The binding affinities of prazosin (Rubin & Blaschke 1980), diazepam and lignocaine (Routledge et al 1981) to α_1-AGP have been shown to be at least 15 times higher than for HSA, as has the nK value of propranol for this protein (Glasson et al 1980).

When the binding experiments were performed at a constant ligand concentration with the concentrations of either BSA, HSA or D-HSA being varied, the extent of mianserin binding increased with increasing protein concentration (Fig. 3). Moreover, transformation of the data into Scatchard plots resulted in plots with positive slopes (Figs 4, 5). There have been several reports of Scatchard plots with positive slopes when these experimental conditions have been used (Shen & Gibaldi 1974; Judis 1980; Mueller & Potter 1981), and it has been suggested that such plots may indicate co-operativity especially if a positive slope is also obtained when the protein concentration is kept constant whilst varying the ligand concentration (Mueller & Potter 1981). However since the traditional site approach predicts that Scatchard plots have negative slopes and that the binding parameters are independent of protein concentration, the values for n and K were not determined. These findings must also cast some doubt on the validity of using the site approach to determine the values of n and K shown in Table 2.

It has been proposed (Mueller & Potter 1981) that it is possible to predict the shape of the typical Scatchard plot from plots obtained when the concentration of protein is varied at two or more ligand concentrations, by joining points at the same protein concentration. However it is evident (see Fig. 5) that care should be taken if this approach is adopted when only two drug concentrations are used while varying the protein concentration, since plots with positive slopes could be predicted (broken line joining a & b, Fig. 5).

Attempts have been made to use the data obtained from in-vitro binding experiments in physiologically and anatomically based pharmacokinetic models (e.g. Wilkinson & Shand 1975). It would appear however that there are difficulties if such extrapolations are made, since protein binding parameters are subject to considerable variations related to the experimental conditions and the methods used to determine them.

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