Biochemical Pharmacology, Vol. 31, No. 3, pp. 359–365, 1982
Printed in Great Britain.

0006-2952/82/030359-07 $03.00/0

THE BINDING CHARACTERISTICS OF SOME ADRENERGIC BETA-RECEPTOR ANTAGONISTS TO HUMAN SERUM PROTEINS

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(*Received* 27 *March* 1981; *accepted* 30 *July* 1981)

Abstract—Binding of pindolol and 8 related compounds was studied *in vitro* by equilibrium dialysis. The overall binding in serum was compared with the binding to the main, isolated, serum proteins. Most substances show both saturable and non-saturable binding in serum. The saturable and main binding is to α_1-AGP, the low non-saturable binding corresponds to albumin and lipoprotein binding. The binding to α_1-AGP is characterized by approximately one binding site and association constants K ranging from 10^4 to $10^6\ M^{-1}$. The binding of pindolol to α_1-AGP is strongly inhibited by propranolol, lidocaine, erythromycin, imipramine and TBEP. Significant correlations were found between log NK and log partition coefficient octanol–phosphate buffer suggesting that the protein binding of the 9 adrenergic beta-receptor antagonists to all serum proteins, including α_1-AGP, is predominantly hydrophobic in nature.

Recent interest has focused on the serum protein binding of adrenergic beta-receptor antagonists and the role of proteins other than albumin [1–5]. It has been shown that the α_1-AGP avidly binds alprenolol [1] and propranolol [2, 3]. It was also found that propranolol was bound to lipoproteins [2, 3].

The purpose of this study has been to investigate the serum protein binding of pindolol, another widely used adrenergic beta-receptor blocking agent, and of 8 other chemically related beta-blocking substances. We therefore determined the main serum proteins that bind these 9 drugs. We also performed experiments to characterize the nature of the binding and to find out if some protein binding sites are shared with other drugs.

MATERIALS AND METHODS

Chemicals. All ^{14}C-labelled and unlabelled beta-blocking substances were synthetized at the Pharmaceutical Department of Sandoz Ltd., Basle. The radiochemical purities of pindolol, 17-895, 18-426, 18-502, 21-009, 34-679, 23-179, 32-468 and 18-645 were respectively 100%, 91%, 89%, 93%, 92%, 100%, 94%, 99% and 91%. The structures of the compounds are given in Table 1. Unlabelled propranolol, lidocaine, erythromycin, imipramine (all from Sigma®) and tris(2-butoxyethyl)phosphate (TBEP from Ega-Chemie®) were used for displacement studies.

Serum and purified proteins. A pooled human serum (Nabi®) stored at −20° with following characteristics: total protein 67 g/l, serum albumin (HSA) 40 g/l and α_1-acid glycoprotein (α_1-AGP) 0.9 g/l was used. The concentrations of total proteins and albumin were determined by the Biuret method and by electrophoresis; the concentration of α_1-AGP was measured by radial immunodiffusion (M-Partigen, Behring/Hoechst®).

Batch solutions of albumin (Fr. V fatty acid free, 98% pure, Miles®) and α_1-AGP (99% pure, Behring/Hoechst®) were prepared in 0.02 M phosphate buffer containing 0.6% NaCl, pH 7.4, at the former physiological concentrations and then stored at −20°. All classes of lipoproteins (very low, low and high density lipoproteins) were separated as a whole by preparative ultracentrifugation according to Sager [3]. Potassium bromide was added to human plasma (Blood Bank, Basle) to achieve a density of 1.195 g/ml and then ultracentrifuged for 45 hr at 105,000 *g* at 4°. The floating lipoproteins were withdrawn and dialyzed against phosphate buffer (pH 7.4). The protein content of this lipoprotein mixture was determined by the method of Lowry and adjusted to 2 g/l which corresponds approximately to physiological concentrations [6]. The mixture of lipoproteins was then immediately used for binding experiments.

Binding experiments. The binding of the 9 substances was determined by equilibrium dialysis using a Dianorm apparatus containing 1 ml Teflon cells. One side of the dialysis cell contained isotonic phosphate buffer 0.02 M (pH 7.4) equal in volume to those of the protein solution in the opposite side of a Visking cellulose membrane 27/32. Drugs were added to the buffer by isotopic dilution of a constant amount of ^{14}C-labelled drug with increasing amounts of unlabelled drug. The drugs were used over a wide range of concentrations (0.1 to 200 μg/ml). Some experiments were performed using [^{14}C]pindolol with other cold compounds (propranolol, lidocaine, erythromycin, imipramine, TBEP) in order to check their effects on α_1-AGP binding. The dialysis was carried out at 37° for 4 hr under constant stirring at 8 rpm; equilibration between the free fractions of

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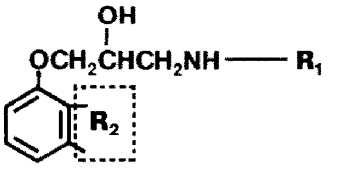
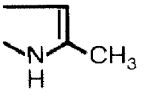
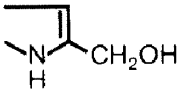
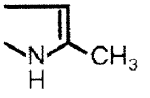
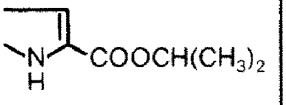
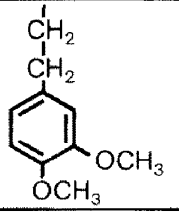
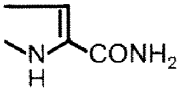
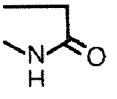
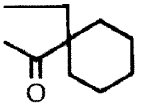
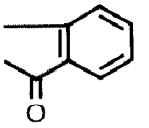
Table 1. Structure, pK_a and distribution data for the 9 adrenergic beta-receptor antagonists studied



Compound	R_1	R_2	pK_a	Distribution ratio octanol/ phosphate buffer pH : 7.4	Molecular weight
Pindolol	$-CH(CH_3)_2$		8.80	0.41	248.33
17-895	$-CH(CH_3)_2$		8.90	0.84	262.35
18-426	$-CH(CH_3)_2$		9.20	0.14	278.35
18-502	$-C(CH_3)_3$		9.40	1.11	276.38
21-009	$-C(CH_3)_3$		8.75	7.18	452.50 hydrogene malonate
34-679			7.90	3.41	931.01 malonate
23-179	$-CH(CH_3)_2$		7.10	0.05	300.79 hydro-chloride
32-468	$-C(CH_3)_3$		8.85	10.35	449.54 hydrogene malonate
18-645	$-C(CH_3)_3$		8.75	14.21	361.87 hydro-chloride

the compounds in both chambers was ascertained by the equivalence of buffer and serum concentrations after 2, 3 and 5 hr of dialysis respectively. No significant binding was observed to the dialysis membrane. At the end of each experiment, concentrations in each compartment were measured by liquid scintillation counting (Packard Tricarb Liquid Scintillation Spectrometer 3375).

Partition studies. The distribution ratio P was determined for the solvent system 1-octanol/phosphate buffer 0.1 M (pH 7.4). 25 ml of each mutually saturated phase, with the labelled drug (200 ng/ml) dissolved in the aqueous phase were shaken in stoppered flasks for 1 hour. Then the drug was left to equilibrate between the phases for 3 hr at room temperature. The concentration in both phases was then determined by liquid scintillation counting.

Binding calculations. The computation of binding parameters has been described by Zini [7]. At equilibrium the bound (B) and free (F) concentrations were measured and a curve $B = f(F)$ was plotted. When the binding was found to be a saturable phenomenon, i.e., with α_1-AGP, N the total binding site concentration of the protein solution, n the number of binding sites per receptor molecule and K the association constant were calculated according to the equation:

$$B = \frac{NKF}{1 + KF} = \frac{nRKF}{1 + KF} \quad (1)$$

where R is the α_1-AGP concentration (0.9 g/l = 20.5 μM). When a non-saturable phenomenon occurred, i.e., for all other proteins studied, only the product NK was calculated according to the equation:

$$B = NKF \quad (2)$$

When serum was used a distinction was generally made between saturable (s) and non-saturable (ns) phenomena, and the corresponding parameters NK, N_1K_1 (s), N_2K_2 (ns) were calculated according to the equations:

$$B = \frac{N_1K_1F}{1 + K_1F} + N_2K_2F \quad (3)$$

and

$$NK = N_1K_1 + N_2K_2 \quad (4)$$

All the binding parameters were estimated by means of the non linear least squares method using a Gauss Newton algorithm.

RESULTS

Binding to serum proteins

The saturable and non-saturable bindings of pindolol are shown in Fig. 1 where binding percentages observed in serum were compared with those obtained for each isolated protein: the binding of pindolol to albumin and lipoproteins was almost constant in the concentration range studied whereas the binding to serum and α_1-AGP was quickly decreasing for concentrations higher than 5 μg/ml.

NK values observed in serum were compared with those obtained for each group of isolated proteins (Table 2). Isolated α_1-AGP was the only serum protein that showed saturable binding when the drug concentrations were increased. The other proteins bound lower drug amounts according to a non-saturable phenomenon. For 6 drugs it was feasible to decompose the NK values in serum as the sum of one saturable (N_1K_1) and one non-saturable (N_2K_2) phenomenon using equation (3). Although being lower, N_1K_1 was of the same magnitude as NK of α_1-AGP. Likewise N_2K_2 was not greatly different from the sum of NK corresponding to HSA and lipoproteins. This suggests that the saturable binding in human serum corresponds to the α_1-AGP binding.

Characteristics of the binding to α_1-AGP

Except for the substance 23-179 which has the lower serum protein binding, all the substances show a saturable binding with α_1-AGP. The calculated binding parameters (Table 3) show that the binding to α_1-AGP is characterized by the presence of approximately one single binding site with association constants ranging from $0.8 \cdot 10^4$ to $130.2 \cdot 10^4\ M^{-1}$.

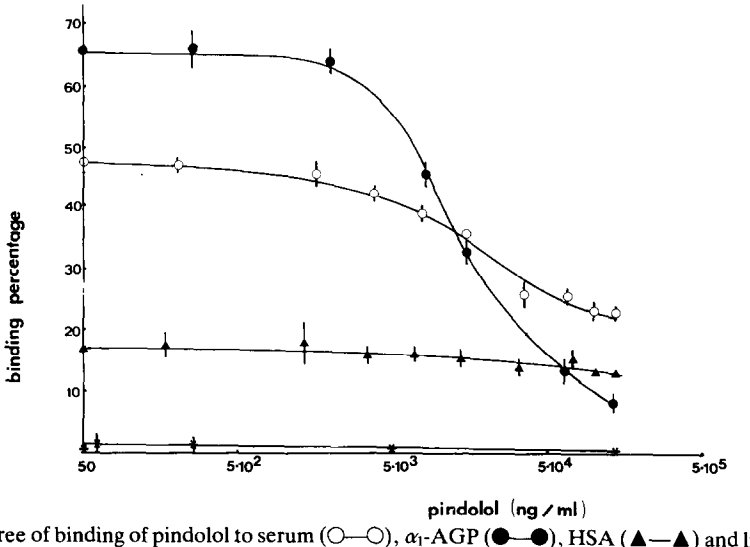


Fig. 1. Degree of binding of pindolol to serum (○—○), α_1-AGP (●—●), HSA (▲—▲) and lipoproteins (*—*) at different total concentrations of drug. Not represented standard deviations are included in symbols.

Table 2. Binding parameters NK of the 9 adrenergic beta-receptor antagonists studied to serum proteins

Proteins Solutions used	Binding Parameters	Pindolol	17–895	18–426	18–502	21–009	34–679	23–179	32–468	18–645
Serum	NK	0.97	0.61	0.31	1.56	12.60	8.50	0.11	7.70	25.10
	N_1K_1 (s)	0.70	–	–	1.23	11.49	6.91	–	6.19	19.70
	N_2K_2 (ns)	0.27	–	–	0.33	1.11	1.59	–	1.51	5.40
α_1-AGP	NK (s)	2.05	0.67	0.30	4.85	13.58	19.47	0.08	16.39	26.38
HSA	NK (ns)	0.16	0.37	0.19	0.42	0.68	0.68	0.06	0.80	1.96
Lipoproteins	NK (ns)	0.01	0.03	0.00	0.07	0.21	0.11	0.00	0.54	1.14

(s): saturable binding; (ns): non-saturable binding. Protein concentrations: HSA 40 g/l; α_1-AGP 0.9 g/l; lipoproteins 2 g/l. —: binding constants not determined.

Table 3. Affinity (K) and number of binding sites (n) on α_1-AGP of the 8 adrenergic beta-receptor antagonists studied

Compound	n	$K.10^{-4}$ (M^{-1})
Pindolol	1.4 ± 0.0	7.1 ± 1.0
17–895	1.8 ± 0.2	1.8 ± 0.5
18–426	1.8 ± 0.2	0.8 ± 0.3
18–502	1.4 ± 0.1	17.0 ± 4.2
21–009	1.3 ± 0.0	50.0 ± 8.0
34–679	0.7 ± 0.0	130.2 ± 35.6
32–468	1.6 ± 0.0	51.3 ± 8.6
18–645	1.6 ± 0.0	80.9 ± 4.8

Results are the mean (±S.D.) of 5 determinations.

Propranolol, lidocaine, erythromycin, imipramine and tris(2-butoxyethyl)phosphate (TBEP) are known to bind to α_1-AGP [1–3, 8–10].

As shown in Table 4 and Fig. 2 the binding of pindolol to α_1-AGP is strongly inhibited by these 5 substances. All the displacing drugs produce a decrease of K; however, they differ from one another in their effect on n in that n remains virtually constant with lidocaine, erythromycin and imipramine suggesting a direct competition phenomenon while n is reduced in the presence of propranolol and TBEP.

The effect of 10, 50 and 100 μg/ml propranolol or lidocaine on the binding of pindolol to α_1-AGP is shown in Table 5: nK significantly decreases as the inhibitor concentration increases. For inhibitor concentrations higher than 40 μM, an increase of n or even a non-saturable binding are observed which correspond to a non-competitive interaction.

Correlation of hydrophobicity with serum protein binding

The correlation coefficients for the graphs (Fig. 3) of log NK against log partition coefficient are 0.97 for serum, 0.95 for α_1-AGP, 0.95 for HSA and 0.97 for lipoproteins. This suggests that the binding of the 9 adrenergic beta-receptor antagonists to the different serum proteins is predominantly hydrophobic in nature.

DISCUSSION

As demonstrated by Borga, Sager and Glasson [1–3], propranolol and alprenolol are mainly bound in serum to α_1-AGP and less to other serum proteins. We have also confirmed that the serum protein binding of pindolol and 8 other chemically related beta-blockers is also essentially due to α_1-AGP. Contrary to the α_1-AGP binding, the binding to albumin and lipoproteins is non-saturable and of less importance. Unpublished results also show that the binding of the 9 compounds to other serum proteins, i.e., haptoglobin, gammaglobulins and transferrin has no major significance. Comparing the saturable and non-saturable binding in serum with the binding to isolated proteins (Table 2, Fig. 1) it can be noted that N_1K_1 related to the saturable phenomenon is lower than the corresponding values observed with

Table 4. Values of n and K for the binding of pindolol to α_1-AGP with and without displacing substances

Compound	Pindolol	Pindolol + Propranolol 10 μg/ml	Pindolol + Lidocaine 10 μg/ml	Pindolol + Erythromicin 10 μg/ml	Pindolol + Imipramine 10 μg/ml	Pindolol + TBEP 10 μg/ml
n	1.4 ± 0.0	0.9 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.3 ± 0.2	0.5 ± 0.1
K. 10^{-4} (M^{-1})	7.1 ± 1.0	2.4 ± 1.1	1.1 ± 0.3	3.5 ± 1.2	1.7 ± 0.8	3.5 ± 1.6

Results are the mean (±S.D.) of 3 determinations.

α_1-AGP. This difference may be explained by different hypotheses including heterogeneity and purity of the α_1-AGP preparation, displacement by endogenous inhibitors present in the serum and molecular aggregation of the different plasma proteins. On the other hand the values of N_2K_2 related to the non-saturable phenomenon do not differ greatly from the corresponding sums of NK related to albumin and lipoproteins binding; this better correlation is possibly due to the nature of the unspecific binding being less sensitive to the different endogenous substances influencing the α_1-AGP binding.

The binding of 8 substances to α_1-AGP is characterized by approximately one single binding site and a wide range of association constants. The existence of the single binding site in the α_1-AGP was also proved for propranolol [2]; the corresponding association constant $K = 3 \cdot 10^4\ M^{-1}$ was lower than for most pindolol derivatives. Although α_1-AGP is also the main propranolol binding protein in serum, it seems that the binding of propranolol to lipoproteins is relatively more important than the corresponding lipoproteins binding of the 9 substances studied. Propranolol, lidocaine, erythromycin, imipramine and TBEP inhibit the binding of pindolol to α_1-AGP. It is difficult to characterize the inhibition process: on one side the number of binding sites remains approximately constant in the presence of each displacing drug at low concentration (10 μg/ml) suggesting a competitive displacement, on the other

Table 5. Values of n and nK for the binding of pindolol to α_1-AGP with increasing propranolol and lidocaine concentrations

Inhibitor	(μM)	nK.10^{-3} (M^{-1})	n
	0	100.1	1.4
	39	20.8	0.9
propranolol	193	1.6	n.s.
	386	0.4	n.s.
	43	13.4	1.2
lidocaine	213	2.7	2.2
	427	1.3	n.s.

n.s.: non-saturable binding.

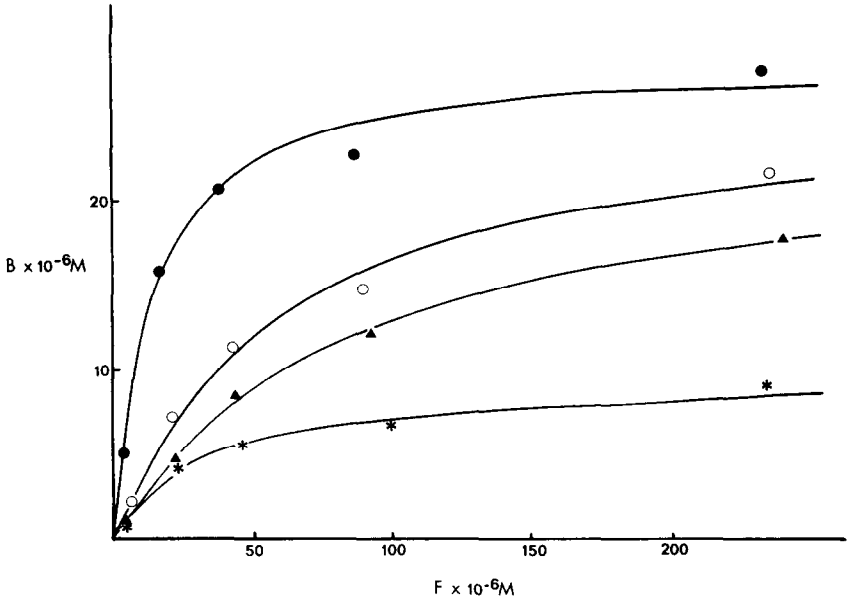


Fig. 2. Binding of pindolol (5–500 μM) to α_1-AGP (20.5 μM) alone (●—●), with imipramine (○—○), with lidocaine (▲—▲) and with TPEB (*—*) at a displacer concentration of 10 μg/ml. Results are the mean of 3 determinations.

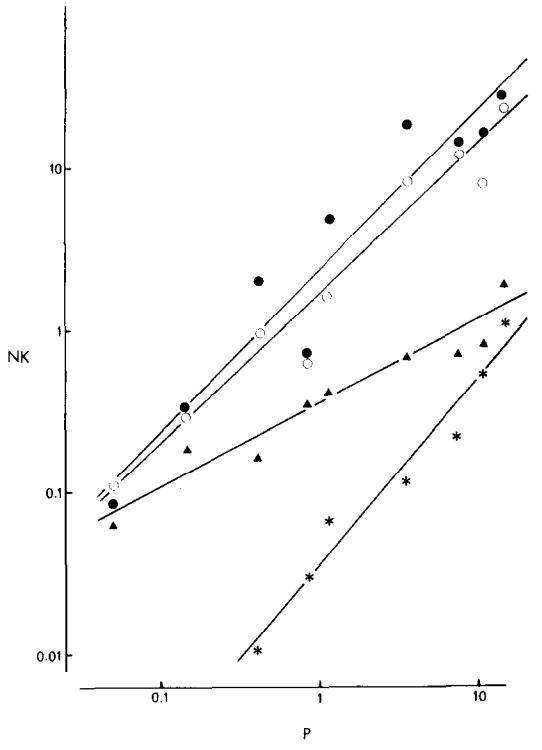


Fig. 3. Correlation between the partition coefficients P of the 9 adrenergic beta-receptor antagonists and the binding constants NK obtained respectively for serum (○—○), α_1-AGP (●—●), HSA (▲—▲) and lipoproteins (*—*).

side using higher concentrations of displacing drugs (50 and 100 μg/ml) an inhibition is found which does not follow the classical competitive binding equations. It is possible, therefore, that at low therapeutic concentrations a competitive inhibition takes place. However, as displacing drug concentration increases, a configurational change of the α_1-AGP takes place which corresponds to a non-competitive displacement mechanism.

In this study a good correlation ($r > 0.95$) between the degree of binding to the different serum proteins and the partition coefficient (measured between 1-octanol and phosphate buffer pH 7.4) was found, indicating the observed binding forces to be mainly hydrophobic in nature. Correlations were also found previously between partition coefficient and binding of different adrenergic beta-receptor antagonists to albumin [11] and plasma proteins [12]. The hydrophobic nature of the binding forces to HSA and lipoproteins was to be expected; it is noteworthy that the binding of the 9 beta-blocking drugs to α_1-AGP also shows an hydrophobic character. In fact pindolol and its 8 derivatives are basic drugs that are almost completely ionized at pH 7.4, thus one can expect that all these substances bind ionically to α_1-AGP. However, the different compounds have similar pK_a values and different lipophilicities (Table 1); therefore the important differences in binding between the 9 drugs may rather be due to their different hydrophobic characteristics than to their similar degrees of ionization. This suggests that, beside the necessary ionic character of the binding, the hydrophobic forces are of importance. As expressed by Schley [13], it seems that the lipophilic character of the drug molecule is an important factor in the specific binding to α_1-AGP.

The amount of data is still too small to permit any definite conclusions concerning the effect of substituents on binding parameters. However some observations may be made. The introduction of one or two methyl groups increases the binding tendency (pindolol, 17-895 and 18-502). By comparing pindolol with 18-426 and 23-179, it can be seen that the binding tendency decreases when an hydroxymethyl group or an oxygen atom is introduced in the position 2 of the indole ring. On the contrary the introduction of an ester group (compound 21-009) at the same position favours the binding. These effects, which have been discussed by Scholtan [14], may be due to hydrophobic forces.

This study shows that α_1-AGP is responsible for the major proportion of the binding of the 9 compounds in serum. This high affinity binding is saturated at serum concentrations over 5 μg/ml, thus the non-linear binding observed at these supratherapeutic concentrations should not be responsible for a dose-dependent disposition of these drugs. Nevertheless, in certain disease states large changes in α_1-AGP can occur [15] that can modify the saturation range and consequently the protein binding of these beta-blockers. In practice, however, the binding alterations induced by displacement or saturation phenomena may be reduced by a redistribution to the other serum proteins. Some experiments were performed with pindolol at lower concentrations than those used in this study, and all showed that the different binding percentages at these low concentrations were similar to those found at 0.1 μg/ml (Fig. 1). Moreover, a high affinity site on human erythrocytes has been reported for propranolol [16]; unpublished results have not permitted us to detect such a specific binding at therapeutic concentrations of pindolol (10–50 ng/ml).

In conclusion, the α_1-AGP is the main binding protein for the 9 beta-blockers studied so that serum binding mainly reflects α_1-AGP binding characteristics.

Acknowledgements—We wish to thank Mr. R. Zini and Mr. J. Barre for assistance in calculation of binding parameters.

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