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The Distribution of Bound Propranolol between the Different Human Serum Proteins

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SUMMARY

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Propranolol binding to the main, isolated, serum proteins was measured by equilibrium dialysis and compared to its overall binding in serum measured by the same method. The results showed that both saturable and nonsaturable binding phenomena exist in serum. The saturable component involves the binding of propranolol to α_1-AGP; for this interaction $n = 1$ and $K = 30\ 450\ M^{-1}$. The other component represents multiple nonsaturable binding to HSA, VLDL, LDL and HDL. The nK values for HSA–propranolol binding decreased when the HSA concentration used was increased. Warfarin did not modify HSA–propranolol binding but palmitic acid decreased it at high concentrations. Propranolol and erythromycin were found to probably share the same or a close binding site on α_1-AGP. When the measured binding of propranolol to serum was simulated using the parameters obtained from the isolated protein components, the best fit was obtained by using an HSA concentration of 29 μM for the nK value. This discrepancy may be due to the extrapolation of binding parameters obtained at low HSA concentrations to the much higher protein concentrations encountered in serum.

INTRODUCTION

It has been previously shown that, in human plasma, propranolol is partly bound to human serum albumin (1, 2). More recently, however, it was also found that the α_1-acid glycoprotein binds nonnegligible amounts of alprenolol (3) and propranolol (4, 5). It is also well known that basic drugs can bind to plasma lipoproteins. So it appears that propranolol is probably bound to several proteins in human serum. The purpose of this work is to determine the main serum proteins that bind propranolol, to characterize their binding and to determine the fraction of propranolol that is bound to each serum component. This was done by measuring drug binding to the main, isolated, serum macromolecules and to a pooled serum. We also did other experiments to characterize the difference propranolol binding sites. Finally, starting from the results obtained, a calculation was made to estimate the quantitative distribution of bound propranolol between the various serum proteins.

MATERIALS AND METHODS

Propranolol binding was measured by equilibrium dialysis at pH 7.4 in 0.66 M phosphate buffer ($\mu = 0.284$) at 37° for 6 hr, using a Dianorm apparatus according to an experimental scheme previously described (6). Drug solutions were prepared by isotopic dilution of a constant amount of [^{3}H]propranolol (17.7 Ci/mol, Amersham) with increasing amounts of unlabeled drug. The protein solutions used were all of human origin and were as follows: pooled serum, VLDL,[2] LDL, and HDL, extracted from this pool according to Havel (7); HSA (Sigma A-1887), [FFA]/[HSA] < 0.04, α_1-AGP (Behringwerke); 99% pure, γ-globulins (Sigma); and haptoglobin (Behringwerke). The purity of these different proteins was assessed by radial immunodiffusion on plates. The influence of FFA on HSA–propranolol binding was checked using palmitic acid (Sigma P-2010). It was solubilized in ethanol, then evaporated to dryness with nitrogen flux; the HSA solution was then added and gently stirred. FFA concentration was controlled by gas chromatography. The identity of the propranolol binding sites was checked using different drugs as binding site probes for the corresponding proteins. Warfarin was employed as an acidic drug for HSA, imipramine was used as a lipo-

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[2] The abbreviations used are: VLDL, very-low-density lipoproteins; LDL, low-density lipoproteins; HDL, high-density lipoproteins; HSA, human serum albumin; FFA, free fatty acids; α_1-AGP, alpha-1-acid glycoprotein.

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philic drug for lipoproteins, while erythromycin was used as a basic drug for α_1-AGP.

When propranolol binding was found to be a saturable phenomenon, i.e., with α_1-AGP, n, the number of binding sites, and K the association constant (or affinity) were calculated. When a nonsaturable phenomenon occurred, i.e., for all other proteins studied, only the product nK was calculated. When serum was used, NK was determined, N being the total binding site concentration. N can be defined as the product (nR) of the number of binding sites (n) and the total receptor concentration (R). When both saturable (n_1 and K_1) and nonsaturable (n_2K_2) phenomena occurred, the corresponding parameters were calculated using the general equation derived from the mass action law (8).

$$\frac{\bar{B}}{F} = \frac{K_1 n_1}{1 + K_1 F} + \frac{K_2 n_2}{1 + K_2 F}. \quad [1]$$

When the affinity K_2 is so low that $F \ll 1/K_2$, this equation can be simplified to

$$\frac{\bar{B}}{F} = \frac{K_1 n_1}{1 + K_1 F} + (K_2 n_2) \quad [2]$$

where $\bar{B}$ is the ratio of bound drug (B) to total receptor concentration (R). K, n and nK were estimated by means of the nonlinear least-squares method using a Gauss–Newton algorithm. The drug nK product decreasing when inhibitor concentration T' increased, the following empirical equation was used:

$$nK = (nK)_0 e^{bT'}, \quad [3]$$

where $(nK)_0$ is the nK value corresponding with the absence of inhibitor and b is a negative rate constant.

Binding percentages were compared using Student's t test for independent samples. Finally, computer simulations were made for evaluating the respective bound drug concentrations to each serum protein. Over the range of propranolol concentrations 20 to 200 ng/ml, none of the serum proteins can be saturated. So the general equation used was

$$B_i = K_i n_i R F \quad [4]$$

applied to each drug–protein binding of the ith class, R being the total receptor concentration and F the total free drug concentration. For several nonsaturable receptors, the total bound ligand concentration (B) was

$$B = \sum B_i. \quad [5]$$

Then,

$$\frac{B}{T} = \frac{(\sum K_i n_i) R}{1 + (\sum K_i n_i) R}, \quad [6]$$

with $T = (F + B)$ being the total drug concentration.

RESULTS

1. Propranolol Binding to Serum Proteins

Propranolol binding percentages observed in serum were compared with those obtained for each isolated protein (Fig. 1). Isolated α_1-AGP was the only serum protein that showed saturable binding when the propranolol concentration was increased. The relevant binding parameters were $n = 1$, and $K = 30,450 \pm 7700$ M^{-1}. The other proteins bound lower propranolol amounts according to nonsaturable phenomena whose characteristics are listed on Table 1.

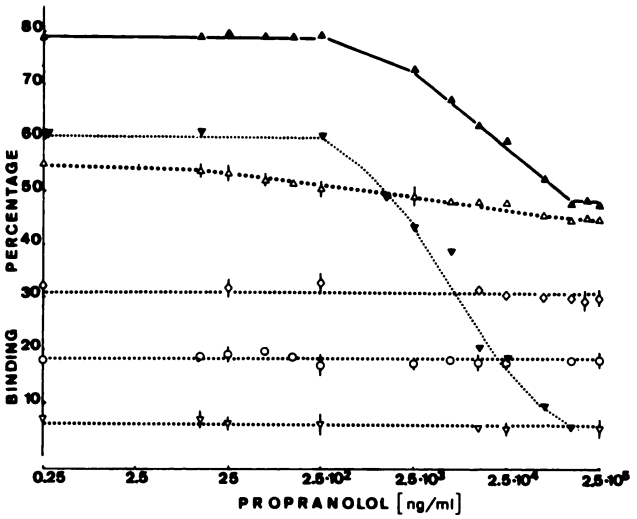

FIG. 1. *Propranolol binding to different receptors*
Serum at 70 g/liter (▲—▲); α_1-AGP at 0.9 g/liter or 22.5 μM (▼·····▼); HSA at 40 g/liter or 580 μM (△·····△); HDL at 3 g/liter or 13 μM (◇·····◇); LDL at 3 g/liter or 1 μM (○·····○); VLDL at 1.5 g/liter or 0.2 μM (▽·····▽). Not represented standard deviations are included in symbols.

Using measured propranolol binding percentages on serum, i.e., the upper curve of Fig. 1, NK is 1.49 ± 0.05 which can be decomposed as the sum of one saturable ($N_1K_1 = 0.70 \pm 0.02$) and one nonsaturable phenomenon ($N_2K_2 = 0.82 \pm 0.04$) using Eq. [2]. The term N_1K_1 is not statistically different from that of α_1-AGP ($NK = 0.69 \pm 0.01$), showing that in serum α_1-AGP has the same binding characteristics as when it is alone (Table 1). This also explains why propranolol binding to pooled serum is a saturable phenomenon.

2. The Propranolol Binding Sites of Serum Proteins

2.1. Characteristics of propranolol binding to HSA.

TABLE 1

Propranolol binding parameters to isolated serum proteins

Protein concentrations used, as indicated in brackets, are physiological ones, expressed in μM. With α_1-AGP, binding of propranolol follows a saturable phenomenon where $n = 1$ and $K = 30,450$ M^{-1}. With the other proteins, propranolol binding follows nonsaturable phenomenon. nK is the product of the number of binding sites (n) of a definite protein and its corresponding affinity constant (K). The binding site concentration (N) of a solution containing proteins multiplied by the corresponding affinity constant (K) provides the NK product.

Receptor	Concentration (μM)	$nK \times 10^{-3}$ (M^{-1})	NK
α_1-AGP	22.5	30.45 ± 7.70	0.69 ± 0.01
HSA	580	1.80 ± 0.02	1.01 ± 0.01
VLDL	0.2	287.0 ± 28.0	0.043 ± 0.004
LDL	1	176.0 ± 0.6	0.21 ± 0.01
HDL	13	16.0 ± 1.4	0.25 ± 0.02
γ-Globulins	75	1.640 ± 0.004	0.120 ± 0.003
Haptoglobin	10	No binding	No binding

The product nK of propranolol binding to HSA varies with the protein concentration. A significant decrease in nK is observed when the HSA concentration is increased (Table 2). This unusual finding has also been observed for acidic drugs and it has been suggested that it may be due to the presence of bound FFA (9, 10).

Despite the fact that the HSA sample used was practically free from FFA, this possibility was checked by measuring propranolol binding percentages after adding increasing amounts of palmitic acid. The results showed (Table 3) that this FFA did not modify propranolol binding to HSA except at very high concentrations, when respectively the molar ratios were (palmitic acid/propranolol) = 100 and (palmitic acid/HSA) = 10. Variations of FFA concentrations cannot explain the observed nK variations.

Warfarin, an acidic drug, did not modify the binding of propranolol, even at high molar ratios (warfarin/propranolol) = 10^5.

2.2. Characteristics of propranolol binding to α_1-AGP. The effect of erythromycin and propranolol on their respective binding to α_1-AGP is shown in Fig. 2. The results indicate that an inhibition takes place between these two drugs according to the following equations:

$$nK = 31{,}100\ e^{-0.0269\ T'} \qquad (r = 0{,}9996) \qquad [7]$$

for the inhibition of propranolol binding by erythromycin, and

$$nK = 36{,}710\ e^{-0.0279\ T'} \qquad (r = 0{,}9991) \qquad [8]$$

for the inhibition of erythromycin binding by propranolol. This inhibition does not follow the classical competitive binding equations.

2.3. Characteristics of propranolol binding to lipoproteins. Imipramine did not modify propranolol binding to VLDL, LDL and HDL.

3. Attempts to Quantify the Distribution of Bound Propranolol between the Different Serum Proteins

The simulation results are shown in Table 4. As nK values of propranolol binding to HSA vary according to the HSA concentration used, calculations were done with values obtained at 580 μM (i.e., plasma normal range) and at 29 μM. Simulation with nK measured at 29 μM HSA agreed better with the total binding percentage measured with pooled serum than did nK measured at 580 μM HSA.

TABLE 2

nK variation of HSA–propranolol binding according to HSA concentration used

The different nK values are compared to the nK value obtained for HSA, 29 μM.

HSA (μM)	nK (M^{-1})
29	3770 ± 71
72.5	2668 ± 4*
145	2107 ± 32*
290	1827 ± 8*
580	1600 ± 20*

* $P < 0.001$ according to Student's t test.

TABLE 3

Variations of HSA–propranolol binding percentages with increasing palmitic acid concentrations

Propranolol = 7.25 μM.; HSA = 72.5 μM.

[Palmitic acid] / [Propranolol]	[Palmitic acid] / [HSA]	Binding percentage
1	0.1	17.8 ± 1.4
5	0.5	18.7 ± 0.7
10	1	19.9 ± 2.4
100	10	12.3 ± 1.6*

* $P < 0.001$ compared to the three other values.

DISCUSSION

1. The Propranolol Binding Proteins in Human Serum

Numerous studies already showed that propranolol is bound to HSA, but the extent of binding of this drug in plasma could not be accounted for by albumin alone (11, 12). It was also previously demonstrated that α_1-AGP binds alprenolol (3) and propranolol (4, 5). Our results agree in principle with these findings; however, they also show that lipoproteins bind nonnegligible amounts of this drug. This has already been shown for other basic drugs (13–17). So it is clear that, in serum, propranolol is bound to HSA, α_1-AGP, VLDL, LDL, and HDL, and that the only saturable binding is to α_1-AGP.

2. The Propranolol Binding Sites in Human Serum

The failure of warfarin to displace HSA–propranolol strongly suggests that these two drugs have different and independent binding sites on HSA. This is in accordance with previous findings showing that HSA binding sites for acidic and basic drugs are independent (13). However, propranolol can be displaced by high concentrations of palmitic acid which is known to share some anionic drug binding sites (18–21). A structural change of HSA in-

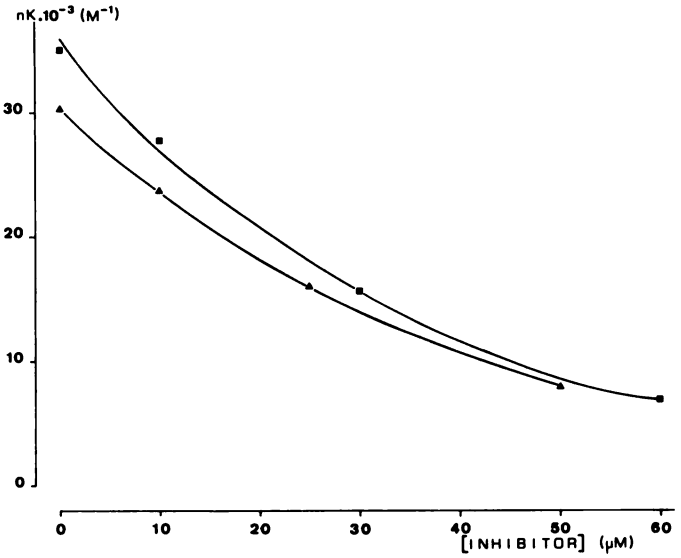

FIG. 2. *nK variations of propranolol and erythromycin bindings to α_1-AGP*

α_1-AGP concentration is 0.9 g/liter or 22.5 μM. nK propranolol variations (▲—▲) without and with 10, 25, and 50 μM erythromycin as inhibitor; nK erythromycin variations (■—■) without and with 10, 30, and 60 μM of propranolol as inhibitor.

duced by FFA can explain the binding of acidic drugs (22–24).

To explain FFA–propranolol interaction, two hypotheses can be proposed. First, one class of low-affinity binding sites of palmitic acid is common to propranolol, leading to a competitive inhibition. Second, the binding of palmitic acid to high-affinity sites induces an HSA structure modification at molar ratios FFA/HSA > 1, leading to a negative cooperative effect.

Propranolol and erythromycin each inhibit the binding of the other drug, the inhibition phenomenon being best described by an exponential curve. Moreover, using high concentrations (>100 μM), the binding of propranolol can be inhibited by erythromycin, and vice versa (unpublished results). The inhibition process involved therefore can neither be *noncompetitive* nor *competitive* because the relevant equations, derived from the mass action law, do not fit conveniently to our data. Indeed, the number of binding sites of each drug increased from 1 to 3, respectively, without and with 50 μM inhibitor, whereas the corresponding association constants decreased continuously. Nevertheless, a possible hypothesis is a competitive inhibition between these two drugs, associated with a structural modification of α_1-AGP, but this has yet to be demonstrated.

Both propranolol and imipramine have a great number of binding sites on lipoproteins, so, even with high drug concentrations, lipoproteins are not saturated. Therefore they can bind large amounts of each drug simultaneously without any mutual inhibition. This phenomenon is perhaps best described as a liposolubility (16) rather than a classical binding situation despite the fact it follows a reversible equilibrium.

3. *The Distribution of Bound Propranolol between the Different Serum Proteins*

The results obtained on every isolated fraction of human serum were used to simulate binding percentages of a "restored" serum over a therapeutic range of propranolol concentrations. Two different values of *nK* were obtained using two HSA concentrations (29 and 580 μM). It seems obvious that for this simulation, the *nK* value measured at 580 μM, i.e., at normal serum concentration, must be used. However, the best fit was observed with this obtained at 29 μM HSA. According to the mass action law, *n* and *K* are independent of both ligand and protein concentrations. However, variations of *nK* with the protein concentration have been already reported (25, 26).

It is difficult to explain these unusual variations. Different hypotheses have been proposed including the presence of contaminants in commercial albumin preparations (27), cooperativity in ligand–protein interactions (28), protein–protein interactions (10, 28, 29) and a loss of accessible protein surface at high HSA concentrations (30). Although not completely excluded, the presence in human serum of another protein binding propranolol is very improbable, the sum of *NK* related to the all serum proteins exhibiting a nonsaturable phenomenon ($\sum NK = 1.63$) being higher than the corresponding value observed with serum ($NK = 0.82$). However, the saturable phenomenon observed in serum can be exclusively related to α_1-AGP binding.

At this time, none of these can be selected. According to the observed binding percentage, the best simulated distribution was obtained with *nK* of low HSA concentration. This is the experimental condition in which the determination of binding parameters can be considered the most accurate, the use of diluted protein solutions allowing the measurement of a large range of propranolol binding percentages which include eventual saturation concentrations. Nevertheless, technical considerations cannot explain the highly significant difference between the two *nK* values obtained. Probably it lies in the anomalous binding behavior of highly concentrated protein solutions as compared to low ones. This requires further experimentation.

TABLE 4

Computed distributions of bound propranolol in serum

The range of propranolol concentration is 20–200 ng/ml.

Receptor	Concentration (μM)	Computed binding percentage	
		If nK_{HSA} = 1600	If nK_{HSA} = 3770
α_1-AGP	22.5	22	16
HSA	580	29	49
VLDL	0.2	2	1
LDL	1	6	4
HDL	13	7	5
γ-Globulins	75	4	3
Total computed		70*	78
Total measured		79.2 ± 0.6	

* $P < 0.001$ as compared to the total measured binding percentage.

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