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Assessment of the contribution of α_1-acid glycoprotein to the serum binding of basic drugs using serum treated with sulphosalicylic acid and DEAE-cellulose

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Treatment of human serum with DEAE-cellulose in acid conditions almost completely removed α_1-acid glycoprotein (α_1-AG) with little change in the concentration of albumin and β-lipoprotein, while treatment with sulphosalicylic acid removed almost all the proteins except α_1-AG. The binding of various drugs to serum treated as above was measured by equilibrium dialysis and the contribution of α_1-AG to drug binding by human serum was assessed. Sulphosalicylic acid-treated serum exhibited a saturable binding for propranolol, which was considered to be due to the binding to α_1-AG while DEAE-cellulose-treated serum mostly exhibited non-saturable binding, for which albumin and β-lipoprotein may be responsible. With this treated serum, α_1-AG was estimated to contribute approximately 40% to the binding of therapeutic concentrations of propranolol, 15% to that of imipramine and 15–20% to that of desipramine, respectively, in serum samples pooled from healthy adults. However, no contribution of α_1-AG was observed in the binding of salicylic acid to the serum. Dissociation constants of the propranolol binding to the high affinity site in control serum, sulphosalicylic acid-treated serum and purified α_1-AG showed similar values (3·7–6·7 μM). These results suggest that treatment of serum with sulphosalicylic acid and DEAE cellulose is useful in assessing the contribution of α_1-AG to the serum binding of basic drugs.

α_1-Acid glycoprotein (α_1-AG) has been shown to be one of the proteins binding various basic drugs in human serum (Piafsky & Borga 1977; Piafsky et al 1978; Schley et al 1980; Abramson et al 1982). The concentration of α_1-AG has wide inter- and intra-individual variability and increases in various disease states and after surgery. Inter-individual variability in serum protein binding for basic drugs is explained by inter-individual variability of α_1-AG concentration (Rudman et al 1972; Roberts et al 1975; Snyder et al 1975). Therefore, there is the need for α_1-AG concentration in the serum of individual patients to be monitored (Piafsky 1980).

It has been suggested that not only α_1-AG but also albumin (Jackson et al 1982; Belpaire & Rosseneu 1982) and lipoprotein (Vallner & Chen 1977; Pike et al 1982) are concerned with the binding of basic drugs. The extent of the contribution of α_1-AG, albumin and lipoprotein in serum protein binding has been estimated for various basic drugs using two methods. An indirect method (Method I) is used to estimate the contribution of α_1-AG by plotting the binding ratio (bound/free) vs α_1-AG concentration, based on inter-individual variability of serum protein binding (Nilsen et al 1978; Sager et al 1979). A direct method (Method II) is used to estimate the contributions by measuring the binding for purified α_1-AG, albumin, lipoprotein, etc (Brinkschulte & Breyer-Pfaff 1980; Glasson et al 1980). However, since it is probable that the binding properties of purified proteins are different from those in serum, it is difficult to estimate the correct contribution with Method II, while Method I needs some assumption of binding properties.

In the present study we performed binding experiments for various drugs using sulphosalicylic acid-treated serum, from which proteins other than α_1-AG were removed, and DEAE-cellulose-treated serum from which α_1-AG was specifically removed. We then analysed the contribution of α_1-AG to serum protein binding with this method.

METHODS

α_1-Acid glycoprotein (α_1-AG), human serum albumin (Fraction V) (Sigma Chemical Co., St Louis, MO, USA), DEAE-cellulose (Type DE52, Whatman Chemical Separation Ltd, Osaka, Japan), sulphosalicylic acid (Wako Pure Chemical Industries Ltd, Osaka, Japan) and auramine O (Eastman

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Kodak Co., Rochester, NY) were purchased. The drugs used in the binding experiments were propranolol (Sumitomo Chemical Co. Ltd, Osaka, Japan), imipramine (Yoshitomi Pharmaceutical Industries Co. Ltd, Osaka, Japan), desipramine (Ciba-Geigy Japan Co., Tokyo, Japan) and salicylic acid (Koso Chemical Co., Tokyo, Japan). Labelled drugs were [^{3}H]propranolol (25 Ci mmol^{-1}, Amersham International Ltd, UK), [^{3}H]imipramine (70 Ci mmol^{-1}, New England Nuclear Co., Boston, MA, USA), [^{3}H]desipramine (89·1 or 67·8 Ci mmol^{-1}, Amersham) and [^{14}C]salicylic acid (51·7 Ci mmol^{-1}, New England Nuclear).

Blood samples collected from groups of 14 and 8 healthy males were allowed to stand for 1 h and centrifuged at 2000 rev min^{-1} for 15 min. These samples were designated as pooled serum I and II, respectively. After the pooled serum was dialysed against 0·025 M acetate buffer (pH 4·1) overnight, 0·05 g DEAE-cellulose ml^{-1} serum which was swollen in the same buffer was added and kept on ice for 2 h with frequent stirring. This treated serum was centrifuged at 2000 rev min^{-1} for 3 min and the supernatant was adjusted to pH 7·4 with 2 M NaOH, and then was dialysed against 0·13 M Sörensen buffer overnight. Subsequently, it was concentrated by ultra-filtration through a Diaflo PM 10 membrane (Amicon Co., Lexington, Mass, USA) to the same volume as that initially.

Equal volumes of 6% sulphosalicylic acid and the pooled serum were mixed well and centrifuged at 3000 rev min^{-1} for 5 min. The supernatant was adjusted to pH 7·4 with 2 M NaOH and dialysed at 4 °C against 0·13 M Sörensen buffer overnight. The samples were concentrated as described above to the same volume as the initial volume.

Commercially available α_1-AG and human serum albumin were dissolved in 0·13 M Sörensen buffer at the concentrations shown in Tables 1–4. Total protein in the serum samples was determined by the method of Lowry et al (1951). The concentration of α_1-AG was determined with a radioimmunodiffusion method (M-partigen plate, Hoechst Japan, Tokyo, Japan) and with the recently developed auramine O method (Sugiyama et al 1985). The concentrations of albumin and β-lipoprotein were determined using commercially available kits (Wako Pure Chemical Industries Ltd, Osaka, Japan).

The serum protein binding of drugs was determined by equilibrium dialysis at 37 °C using 0·13 M Sörensen buffer (pH 7·4) in semimicrocells (Kokugo-Gomu Co., Tokyo, Japan) with semipermeable membranes (Spectrum Medical Industries Inc., Los Angeles, CA). After equilibration was attained at 6 h, the drug concentrations in the protein side and the buffer side were measured in a liquid-scintillation spectrometer. The concentrations of drugs (labelled and unlabelled) initially added to the serum were 0·05–15 μg ml^{-1} for propranolol, 0·3 μg ml^{-1} for imipramine and desipramine and 30 μg ml^{-1} for salicylic acid.

An ultrafiltration method using a semipermeable membrane (Spectrum Medical Industries Inc., Los Angeles, CA) was also used. The serum, to which 0·05 μg ml^{-1} propranolol was added, was incubated at 37 °C for 20 min and then 2 ml of the incubated serum was centrifugally filtered. The serum was centrifuged at 2500 rev min^{-1} for 30 min at room temperature (20 °C) and the filtrate (200 μl) collected.

The binding data obtained by equilibrium dialysis was corrected for the volume shift by the method of Tozer et al (1983). Binding data for propranolol were fitted to the following equations by a non-linear least squares method depending upon the serum or protein samples used.

$$C_b = \frac{n_1P_1 \times C_f}{Kd_1 + C_f} \quad (1)$$

$$C_b = \frac{n_1P_1 \times C_f}{Kd_1 + C_f} + \frac{n_2P_2}{Kd_2} \cdot C_f \quad (2)$$

$$C_b = \frac{n_1P_1 \times C_f}{Kd_1 + C_f} + \frac{n_2P_2 + C_f}{Kd_2 + C_f} \quad (3)$$

where C_b and C_f are the concentrations of the bound drug and the unbound drug, respectively, n_1P_1 and n_2P_2 are the capacities of the high and low affinity sites, respectively, and Kd_1 and Kd_2 are the dissociation constants for the high and low affinity sites, respectively. Equations 1, 2 and 3 were used for sulphosalicylic acid-treated serum and α_1-AG, for DEAE-cellulose-treated serum and albumin, and for control serum, respectively.

RESULTS

The effect of buffer type on the binding of propranolol to human serum was investigated using equilibrium dialysis. The four buffer types used here have been commonly used in equilibrium dialysis experiments (Schley et al 1980; Sager et al 1979; Glasson et al 1980; Yoshikawa et al 1984). The results are listed in Table 1 together with the values obtained by an ultrafiltration method which can be used without buffer. The binding ratios (C_b/C_f) obtained using Sörensen buffer and phosphate buffered isotonic saline were close to those obtained by an ultrafiltra-

Table 1. Influence of buffer on serum protein binding of propranolol.[a]

Method	C_b/C_f
Equilibrium dialysis[b]	
Sörensen buffer (0·13 M phosphate buffer	7·54
Krebs–Ringer phosphate buffer	9·15
Phosphate buffered isotonic saline	7·16
0·28 M Tris-HCl buffer	4·45
Ultrafiltration[c]	7·75 ± 1·61

[a] Initial concentration of propranolol is 0·4 μM.
[b] Equilibrium dialysis was performed at 37 °C. The average of two experimental data.
[c] At room temperature. The mean ± s.e. for three experimental results.

tion method while the binding ratios obtained in Krebs–Ringer phosphate buffer and 0·28 M Tris-HCl buffer were larger and smaller, respectively. Consequently, in the present study, all the equilibrium dialysis experiments were performed using Sörensen buffer (pH 7·4).

The pH change after dialysis using various buffers was checked. The serum pH after dialysis was between 7·5 and 7·6 regardless of the kind of buffer used. The serum pH after ultrafiltration was also checked and was between 7·7 and 7·8. We then determined the effect of pH on the binding of propranolol to human serum, α_1-AG and albumin. In any protein sample, the binding increased as the pH (the final pH after dialysis) increased (Fig. 1). Considering that propranolol is a basic drug (pK_a = 9·6), such pH profiles suggest that the non-ionic form has higher affinity for proteins than the cationic form.

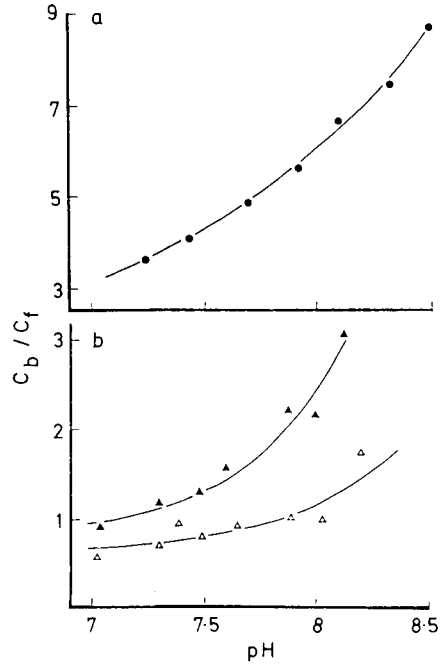

FIG. 1. The pH profile for the protein binding of propranolol; (a) human serum (●), (b) albumin (▲) and α_1-AG (△). Binding was determined by an equilibrium dialysis at 37 °C using Sörensen buffer (the mixture of 0·13 M Na_2HPO_4 and KH_2PO_4). Pooled serum from 6 healthy adults which was different from pooled serum I and II was used. The pH was varied by changing the ratio of the two buffer components.

Table 2. Effect of temperature on serum protein binding of propranolol.[a]

Temperature (°C)	C_b/C_f
37	7·20
25	9·62
4	13·25

[a] Binding was determined by an equilibrium dialysis using Sörensen buffer (pH 7·4). The average of two experimental results is shown.

Table 3. Concentration of various serum proteins in differently treated sera, used in the binding studies.

	Total protein (mg ml^{-1})	α_1-AG (mg ml^{-1})	Albumin (mg ml^{-1})	β-lipoprotein (mg ml^{-1})
Pooled serum I				
Serum	72·6	0·55	55·2	4·0
DEAE-cellulose-treated	63·0	0·0	45·1	4·7
Sulphosalicylic acid-treated	1·6	0·52	0·3	0·3
Pooled serum II				
Serum	59·7	0·41	40·1	2·3
DEAE-cellulose-treated	63·9	0·0	40·1	1·5
Sulphosalicylic acid-treated	0·59	0·39	0·3	0·2
Commercial protein				
Albumin	n.d.[a]	n.d.	40·0	n.d.
α_1-AG	n.d.	0·50	n.d.	n.d.

[a] Not determined.

The dependency of serum binding of propranolol on temperature was determined (Table 2). As the temperature increased, the binding decreased as is often the case in the drug and protein binding. The calculated enthalpy change (ΔH) was approximately −13 kJ mol^{-1}.

The concentrations of several serum protein components in differently treated serum and protein solutions of commercially available α_1-AG and albumin are listed in Table 3. In both pooled sera (I and II) the treatment with DEAE-cellulose almost completely removed α_1-AG with little change in the concentration of albumin and β-lipoprotein, while

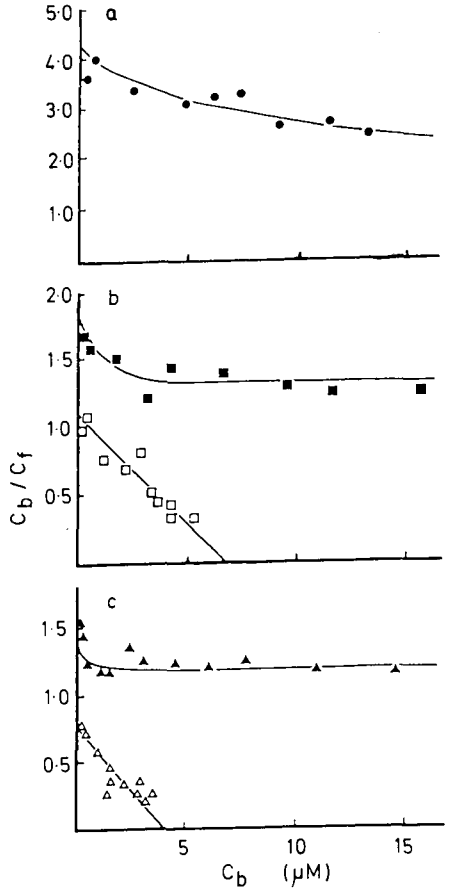

FIG. 2. Scatchard plots of propranolol binding to human serum. (a) Serum pooled from 14 healthy adults, corresponding to pooled serum II in Table 2, were used. The line was fitted by a non-linear least squares method. (b) DEAE-cellulose-treated serum (■) and sulphosalicylic acid-treated serum (□) were used. The contributions to binding of various proteins included in the treated serum are listed in Table 2. (c) Commercially available human serum albumin (▲) and α_1-AG (△) were used. The protein concentrations are 50 mg ml^{-1} for albumin and 0·55 mg ml^{-1} for α_1-AG.

treatment with sulphosalicylic acid removed almost all the proteins in serum except α_1-AG.

Fig. 2 shows the binding of propranolol to differently treated human serum, α_1-AG and albumin. The results were plotted according to Scatchard (1949). Binding parameters calculated by a non-linear least squares method are in Table 4. As shown in Fig. 2, the control serum has more than one group of binding components, while sulphosalicylic acid-treated serum or purified α_1-AG binds to a single high affinity binding component. For this, the dissociation constant (4–7 μM) and the binding capacity expressed as by α_1-AG (0·3–0·7 μM/μM) differ little among the control and sulphosalicylic acid-treated serum and purified α_1-AG.

Table 4. Dissociation constants (Kd) and capacities (nP) for the binding of propranolol to differently treated serum (Pooled I), α_1-AG and albumin.

	High affinity site			Low affinity site		
Samples[a]	Capacity n_1P_1 (μM)	Dissoc. const. Kd_1 (μM)	n_1P_1/α_1-AG	Capacity n_2P_2 (μM)	Dissoc. const. Kd_2 (μM)	n_2P_2/Kd_2
Serum	9·2	3·7	0·70	230	150	1·5
DEAE-cellulose-treated	0·2	0·4	—	—	—	1·3[b]
Sulphosalicylic acid-treated	7·2	6·7	0·58	—[c]	—[c]	—[c]
Albumin	0·1	0·8	—	—	—	1·1[b]
α_1-AG	4·0	5·5	0·34	—[c]	—[c]	—[c]

[a] Protein concentrations are shown in Table 3.
[b] Only the n_2P_2/Kd_2 value is shown, since the saturable binding was not detected.
[c] Low affinity binding was not detected.

DEAE-cellulose-treated serum and purified albumin also seem to have high affinity binding components (Kd = 0·4–0·8 μM), although the binding capacity of albumin is small (approximately 1·5–3·0 $\times 10^{-4}$). Hence its contribution to the total binding can be considered minor.

The binding ratio (C_b/C_f) of various drugs, including imipramine, desipramine, propranolol and salicyclic acid, to the differently treated sera is shown in Table 5. For each drug, the binding ratio for sulphosalicylic acid-treated serum is comparable to that for purified α_1-AG. The binding ratios of imipramine, desipramine and propanolol for DEAE-cellulose-treated serum were greater than those for purified albumin. On the other hand, the binding ratios of salicylic acid to DEAE-cellulose-treated serum and purified albumin were approximately the same. From the binding data of each drug to

Table 5. Binding ratio (C_b/C_f) of propranolol, imipramine, desipramine and salicylic acid.[a]

	Pooled I[b]		Pooled II[b]		
Sample	Prop-ranolol	Desip-ramine	Imip-ramine	Desip-ramine	Salicylic acid
Serum	3·6	4·3	2·4	2·7	7·1
DEAE cellulose-treated	1·7	3·9	1·8	2·3	7·1
Sulphosalicylic acid-treated	1·0	1·0	0·3	0·4	0·0
Albumin	1·0	1·6	1·1	—	8·8
α_1-AG	0·8	0·3	0·4	—	0·0
Contribution of α_1-AG[c]	0·37	0·20	0·15	0·15	0·00

[a] Each data set is the average value of two or three experiments.
[b] Protein concentrations are shown in Table 3.
[c] (Binding ratio for sulphosalicylic acid-treated serum)/(Binding ratio for sulphosalicyli acid-treated serum + Binding ratio for DEAE-cellulose-treated serum.)

sulphosalicylic acid-treated seurm and DEAE-cellulose-treated serum, the contribution of α_1-AG to the serum binding of each drug was estimated to be approximately 40, 15 and 15–20% for propranolol, imipramine and desipramine, respectively (Table 5). On the other hand, no contribution of α_1-AG was observed in the serum binding of salicylic acid.

DISCUSSION

In the present binding studies we first sought an appropriate buffer for equilibrium dialysis and comparison of the binding ratio (C_b/C_f) of propranolol obtained by an ultrafiltration technique, Sörensen buffer or phosphate buffered saline was found to be suitable (Table 1). To make a valid comparison of the C_b/C_f obtained by different methods and buffers, both pH and temperature dependencies of the binding of propranolol were examined (Fig. 1, Table 2). Although the changes in pH and temperature affected the binding, the extent was at most 30%, suggesting that the comparison of the C_b/C_f shown in Table 1 might be reasonable.

The contribution of α_1-AG to the serum binding of basic drugs was estimated using a DEAE-cellulose-treated serum in which α_1-AG is deficient and sulphosalicylic acid-treated serum in which almost all the proteins except α_1-AG in serum are deficient.

Treatment of human serum by DEAE-cellulose at pH 4·1 removed almost all the α_1-AG, while, treatment of serum by sulphosalicylic acid removed most of the proteins with little change in α_1-AG concentration. This is consistent with our previous finding that a serum sample treated with sulphosalicylic acid showed a major single band on SDS-polyacrylamide gel electrophoresis which corresponded to human α_1-AG (Sugiyama et al 1985). Since the change in the affinity for propranolol caused by the sulphosalicylic acid-treatment seemed to be minor, if any (Table 4), such treatment might be useful in purifying α_1-AG from human serum.

As shown in Table 4, the binding capacity for the high affinity site (expressed as μM of α_1-AG) has a tendency to decrease, as the purification of α_1-AG proceeded, although the mechanism is not known.

The binding index for the low affinity sites (n_2P_2/Kd_2), which is probably the result of binding to proteins other than α_1-AG differs among untreated and DEAE-cellulose-treated serum and albumin. The difference between the untreated and DEAE-cellulose-treated serum might be explained by DEAE-cellulose-treatment removing β-lipoprotein (5%) and albumin (20%) from serum. The difference between the DEAE-cellulose-treated serum and albumin could be due to the presence of β-lipoprotein in the former, since it contributes to the plasma binding of some basic drugs (Vallner & Chen 1977; Pike et al 1982).

The binding site with the high affinity and very low capacity was observed in both DEAE-cellulose treated serum and commercial albumin and is possibly due to minor contamination by α_1-AG (Lima & Salzer 1981). The sensitivity of our method for α_1-AG was approximately 0·05 mg ml^{-1}, and therefore contamination by α_1-AG below that level cannot be excluded.

By the use of method I (see introduction), both the dissociation constant (Kd) and the contribution of α_1-AG to the serum binding of propranolol were estimated from published data (Sager et al 1979; Glasson et al 1980; Abramson et al 1982). If it is assumed that drug binding is linear and that the inter-individual difference in serum levels of other binding proteins such as albumin and β-lipoprotein is small, the inter-individual difference in the serum binding of the drug is mainly due to the α_1-AG concentration. Thus, the following equation can be derived:

$$C_b/C_f = (n/Kd) \times (\text{concentration of } \alpha_1\text{-AG}) + \text{constant} \quad (4)$$

Therefore, the n/Kd value can be calculated from the slope of the plot. In addition, the contribution of α_1-AG at the average serum α_1-AG concentration of 0·55 mg ml^{-1} (13 μM) to the serum binding of propranolol can be estimated from the following equation,

$$\text{Contribution} = \frac{(n/Kd) \times 13\,\mu M}{(n/Kd) \times 13\,\mu M + \text{constant}} \quad (5)$$

Our calculations based on the literature data gave the Kd/n values of 3·5–5 μM and 40–80% contribution to the binding. The Kd/n values and the extent of the contribution obtained in the present studies are 4–7 μM (Table 4) and 37% (Table 5), respectively, which are comparable with the above calculated values.

Pike et al (1983) estimated the contribution of α_1-AG to serum binding of basic drugs by an immunoadsorption method using antibody against human α_1-AG. This method is superior in specificity but it required large amounts of antibody and cannot be performed readily. On the other hand, the methods that are described can be carried out relatively easily.

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