Biochemical Pharmacology, Vol. 28, pp. 905–911.

0006-2952/79/0315-0905 502.00/0

VARIABLE BINDING OF PROPRANOLOL IN HUMAN SERUM

GEORG SAGER, ODD G. NILSEN and STEN JACOBSEN
Institute of Pharmacology, University of Oslo, Box 1057, Oslo 3, Norway

(*Received* 3 *April* 1978; *Accepted* 23 *July* 1978)

Abstract—Human serum proteins were fractionated by ultracentrifugation and gel filtration. Binding of propranolol was determined by equilibrium dialysis. Propranolol was distributed to lipoproteins independent of drug concentration. Two groups of propranolol binding sites were found to be present in the protein preparation containing albumin, α_1-acid glycoprotein, transferrin and prealbumin. The first binding site with a dissociation constant of 7.5×10^{-7} was present in number equivalent to concentration of α_1-acid glycoprotein. The propranolol binding to serum samples from 21 healthy males expressed as binding ratio B/F and per cent binding ranged from 7.5 to 19.2 and 88.2 to 95.0 respectively. The binding ratio was correlated to concentration of α_1-acid glycoprotein ($r = 0.85$, $P < 0.001$), but not to concentrations of albumin and lipoproteins. The results indicate that α_1-acid glycoprotein is the main propranolol binding protein in human serum.

Although propranolol is the most widely used adrenergic betareceptor blocking agent, and the absorption and elimination of the drug have been carefully investigated [1–3], limited information on the serum protein binding has been available. Propranolol is extensively metabolized in the body [4], and several observations indicate that serum protein binding is of significance for disposition and biological effects of propranolol under steady state conditions [5, 6]. The need for more knowledge about serum protein binding of propranolol is further demonstrated by the observation that correlation exists between total serum concentration of propranolol and biological effects in the individual, but a great variation in effectiveness is found among subjects [7]. On the other hand, a good correlation is present between biological efficacy and concentrations of unbound serum concentration of propranolol between subjects [8]. These findings indicate an interindividual variation in serum protein binding.

The purpose of this study has been to investigate variation in serum protein binding among normal subjects and the influence of the serum proteins as albumin, lipoproteins and α_1-acid glycoprotein on the extent of binding.

MATERIALS AND METHODS

Chemicals. D, L-[4-^{3}H]-Propranolol hydrochloride with a specific activity of 1.18 mCi/mg (radiochemical purity of 99.4 per cent) and racemic propranolol hydrochloride were supplied by Radiochemical and Pharmaceutical Division of Imperial Chemical Industries Ltd., Cheshire, U.K. Purity was established by TLC in 3 separate solvent systems, and the R_f values for the labelled and unlabelled compounds were identical. Other chemicals used were of analytical grade.

Serum, serum proteins of lower molecular weights and lipoproteins. Serum was obtained from three fasting, healthy males, 23–35 years of age, and these sera were pooled. This pool of serum was partly used for preparation of serum lipoproteins and of proteins with lower molecular weight by ultracentrifugation and gel filtration. Potassium bromide was added (293.9 g/l) to serum to produce a density of 1.195 g/ml and then ultracentrifugated for 45 hr at 105,000 *g* at 4°, and the floating lipoproteins were then withdrawn. The remaining serum proteins and the separated lipoproteins were dialyzed for 24 hr against three changes of 1,000 ml Krebs–Ringer phosphate buffer, pH 7.35. The mixture of lipoproteins was then used for binding experiments. Serum proteins (20 ml) devoid of lipoproteins were applied to a Sephadex G-200 column (100 × 5 cm, A.B. Pharmacia, Uppsala, Sweden) and equilibrated with the same buffer at 4°. The elution rate was 1 ml/min and serum proteins as albumin and other proteins of lower molecular weight were eluted in the last protein peak [9]. The protein fraction used was obtained from the middle and terminal part of the last eluted protein peak to avoid interference with proteins from the previous peak. The fractions were then concentrated to one fifth of their initial volumes under nitrogen pressure (1.3 Kp/cm^2) using a Diaflo ultrafiltration PM-10 membrane (Amicon Corp., Lexington, Mass., U.S.A.) and then dialyzed against three changes of 100 ml Krebs–Ringer phosphate buffer of pH 7.35 for 24 hr at 4°. The final protein solution containing about 2% (w/v) albumin, was stored at −21°.

Serum samples from healthy volunteers. Serum was obtained from 21 healthy and drug free males (39–53 years old). Venous blood was sampled after fasting overnight. The sample was left for 1 hr at room temperature and then centrifugated (1,100 × *g* in 30 min) at 20°. Determinations of lipid concentrations were carried out the same day, and the serum was then stored at −21°.

Binding experiments. Serum protein binding of propranolol was determined by equilibrium dialysis, using a dialysis membrane 20/32 (Union Carbide Corp., Chicago, Illinois, U.S.A.) clamped between two Perspex® cells. 500μl aliquots of serum or protein prepa-

ration were dialyzed against 500 μl of Krebs–Ringer bicarbonate buffer [10], pH 7.35. Dialysis was performed in an atmosphere of 5% (v/v) carbon dioxide. The dialysis cells were shaken for 18 hr at 20°. Serum, serum fractions and buffer were gassed with carbon dioxide prior to equilibrium dialysis to achieve a pH of 7.35. Propranolol was added to the protein containing solution in concentrations given for the specific experiments. Labelled propranolol was added in tracer amounts.

Propranolol determination. The concentration of propranolol in the buffer and protein solution after equilibrium dialysis was calculated from the distribution of labelled compound, added amount of propranolol and the volumes separated by the dialysis membrane. Changes in volumes during dialysis were determined from changes in protein concentration. Serum and serum protein fractions were diluted 10–18 per cent by the dialysis. Radioactivity of the labelled propranolol was determined in a Packard Tri-Carb liquid scintillation spectrometer, Model 3330. Duplicate samples of 50 μl were obtained and added to 10 ml scintillation fluid [11]. Recovery of labelled propranolol after dialysis was 98 to 103 per cent. Counting efficiency was about 28 per cent in both buffer and protein containing samples. In preliminary experiments, propranolol concentration was also determined by fluorometry [12]. The two different methods gave the same results.

Cholesterol and triglycerides. Isopropanol extracts of serum were analyzed in an Auto Analyzer II, according to instructions given by Technicon Instrument Corporation (work sheet AA II-23 and 24, May, 1971) with the modification that final volume of water was same for standards and serum samples. Two separate determinations were performed on each sample. Coefficient of variation for determinations of cholesterol and triglycerides were ± 3.5 per cent and ± 6 per cent respectively.

Albumin and α_1-acid glycoprotein (orosomucoid). The concentrations of these serum proteins were determined by immunological technique [13] in barbital and veronal buffer respectively, at pH 8.6 using 1% (w/v) agarose. The gels contained either 2% (w/v) antialbumin serum or 2.5% (w/v) antiorosomucoid serum (Dakopatts, Copenhagen, Denmark). Serum standards were obtained from Behringerwerke, Germany. 10 μl serum samples were applied on gel with a thickness of 2 mm.

Total protein. The concentration of total protein in the prepared fractions and in diluted serum was determined by the method of Lowry [14] using bovine serum albumin as standard.

Electrophoresis. Paper electrophoresis of serum was performed at room temperature in barbital buffer containing 1% (w/v) albumin [15]. Electrophoresis strips were dried at 100° for 20 min and then stained with Sudan Schwartz B. This method produced narrow and well defined bands of serum lipoproteins, and chylomicrons would appear as one discrete band at the point of application [15]. Chylomicrons were not detected in the sera from the fasting individuals.

Calculation of protein binding. Protein binding was expressed as the binding ratio B/F, B and F representing concentrations of bound and unbound propranolol. Binding capacity and dissociation constants were obtained by plotting the binding at different concentrations of propranolol according to Scatchard [16]. The binding characteristics for two independent binding sites were obtained by drawing asymptotic straight lines to the curve close to the abscissa and ordinate and then moving these lines parallel so that the sum of the distances to the ordinate intercepts equalled the initial distance to the ordinate intercept. From these asymptotic lines the curve was regenerated by drawing arbitrary straight lines through the origin of the coordinates. Along these the distance from the origin to the regenerated curve was made equal to the sum of the distances from the origin to the intercepts of the two asymptotic lines as described by Rosenthal [17]. Corrections were made on the asymptotic lines until the fit with the experimental curve was satisfactory. Each of the asymptotic lines represented one binding site on the protein molecule. The binding capacity (n) and the dissociation constant (K) were given by the intercept with the abscissa (n) and the ordinate n/K. The binding ratio could be calculated from the equation,

$$\frac{B}{F} = \frac{n \times P}{K + F},$$

P being the concentration of binding proteins.

RESULTS

Binding to lower molecular weight serum proteins. The protein preparation containing serum albumin and other proteins of lower molecular weight was separated from one pool of human sera. Electrophoresis of the fractions used, show the presence of prealbumin, albumin, transferrin and α_1-acid glycoprotein. Binding to these proteins was determined by equilibrium dialysis at different concentrations of propranolol and the results are plotted in Fig. 1 according to Scatchard [16]. The experimental values are distributed along a curve, indicating the presence of more than one binding site for these proteins. Assuming two separate and independent groups of binding sites, binding characteristics (Table 1) were calculated according to Rosenthal [17]. The molar concentrations of albumin and α_1-acid glycoprotein in the prepared serum fraction after dialysis are also given.

The results indicate the existence of one binding site with low binding capacity, characterized by high affinity for propranolol. The other site has greater binding capacity with lower affinity for propranolol.

Binding to serum lipoproteins. The fraction of serum lipoproteins was prepared from the pool of sera as described in Methods, and contained a mixture of very low, low and high density lipoproteins, while chylomicrons were absent. The concentration of lipoproteins in this fraction was adjusted by the protein content to be equal to that of lipoproteins in the diluted serum used later. The interaction of these proteins with propranolol was determined by equilibrium dialysis against Krebs–Ringer buffer. The results in Fig. 2 show that the interaction is not caused by binding obeying the law of mass action, but by a concentration independent distribution between the aqueous phase and the lipoproteins.

Binding in serum. Propranolol binding was determined in whole serum diluted with buffer to an albumin concentration equal to that of the protein preparation containing albumin and other lower molecular weight

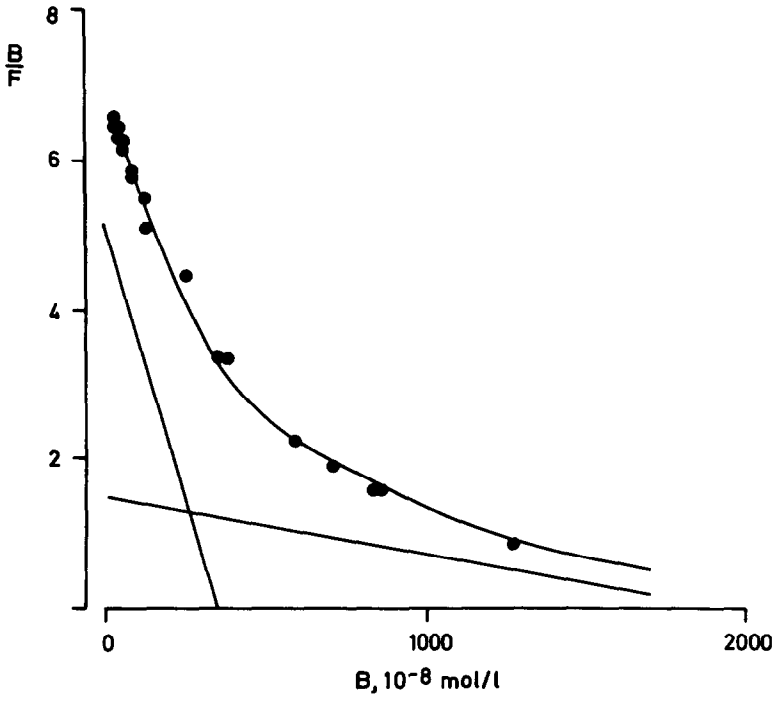

Fig. 1. Binding of propranolol to the protein preparation containing albumin, α_1-acid glycoprotein (see Table 1), prealbumin and transferrin plotted according to the method of Scatchard [16]. *B* and *F* represent concentrations of bound and unbound propranolol respectively. Each point represents mean of four separate experiments. The curve is regenerated from the two straight lines which were derived according to the method of Rosenthal [17].

proteins. Serum from the same pool was used for separation of serum proteins. Binding was determined by equilibrium dialysis at 20°, and the results are given in Fig. 3 as a Scatchard plot, with no correction for protein content. The experimental binding values indicate the presence of more than one binding site for propranolol.

The binding lines obtained in the Scatchard plots (Figs. 1 and 2) for lower molecular weight serum proteins and lipoproteins respectively, are inserted in Fig. 3, but without correction for the protein content since the concentrations of proteins in the prepared fractions were similar to those in diluted serum. The resultant curve from these separate lines was constructed according to the method of Rosenthal [17]. The experimental binding values obtained in diluted serum follow the constructed curve and indicate that total serum binding is explained by the observed propranolol binding to lower molecular serum proteins and to lipoproteins.

Variation in serum binding. Sera from 21 healthy and fasting males were examined separately for propranolol binding by equilibrium dialysis at 20°. Labelled and unlabelled propranolol was added to all serum samples, producing a serum concentration of 3×10^{-7} mol/l. The concentration of albumin, α_1-acid glycoprotein, triglycerides and cholesterol were determined in all sera. The lipid concentrations would reflect the levels of serum lipoproteins [18].

Results are given in Table 2. The serum binding expressed as per cent binding and the binding ratio *B/F* were in the range 88.2–95.0 and 7.5–19.2 per cent

Table 1. Binding and protein characteristics* of serum proteins of lower molecular weight

Total protein concentration (g/l)	21.5 ± 0.3
Albumin concentration (mol/l)	$(3.1 \pm 0.2) \times 10^{-4}$
α_1-AGP-concentration (mol/l)	$(3.4 \pm 0.3) \times 10^{-6}$
Binding capacity for propranolol (mol/l)	
1. SITE	$(3.3 \pm 0.2) \times 10^{-6}$
2. SITE	$(18.7 \pm 0.4) \times 10^{-6}$
Dissociation constant for propranolol (mol/l)	
1. SITE	$(7.5 \pm 1.5) \times 10^{-7}$
2. SITE	$(1.2 \pm 0.2) \times 10^{-5}$

* Mean values with S.D. of 4 separate experiments after equilibrium dialysis.

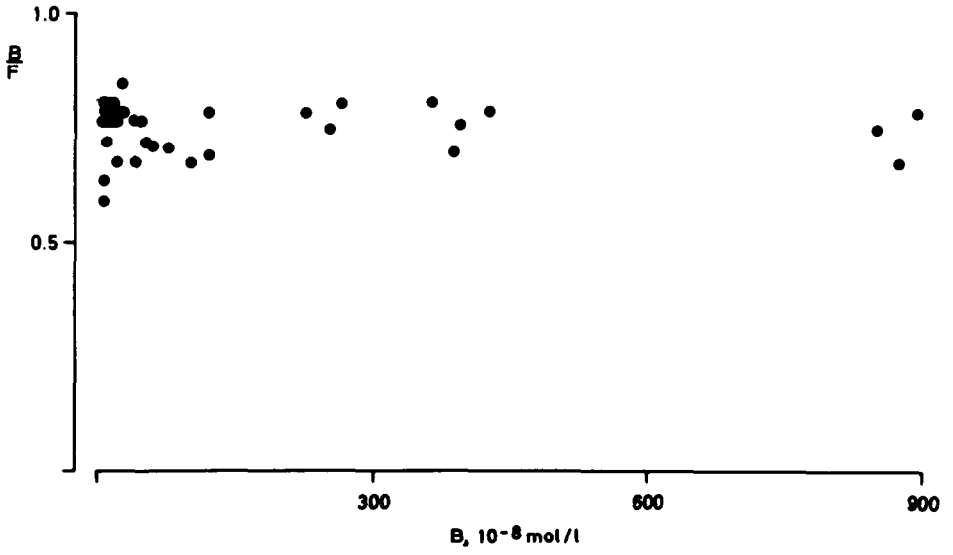

Fig. 2. Binding of propranolol to the preparation containing very low, low and high density lipoproteins plotted according to the method of Scatchard [16]. *B* and *F* represent bound and unbound propranolol respectively. A regression line was determined by the least square method. The slope of the curve was not significantly different from zero and intercepted with the ordinate at 0.75.

respectively. This variation in serum binding results in free concentrations of propranolol ranging from 5.0 to 11.8 per cent of total concentration in serum.

The binding of propranolol in the individual serum, expressed as the binding ratio B/F, was plotted against the respective serum concentrations of serum albumin, triglycerides, cholesterol and α_1-acid glycoprotein. A linear and significant correlation was found between binding ratios and concentrations of α_1-acid glycoprotein ($r = 0.85$, $P < 0.001$) shown in Fig. 4. No significant correlation was found between binding ratio and cholesterol ($r = -0.167$, $P > 0.1$), triglycerides ($r = -0.078$, $P > 0.1$) and albumin concentrations ($r = 0.01$, $P > 0.1$) (Table 3).

DISCUSSION

A previous study on the serum binding of propranolol showed that 91.0 to 95.8 per cent was bound [5], and the results indicated that propranolol was mainly bound to serum albumin, but also to other proteins. The present results reveal that propranolol is bound mainly

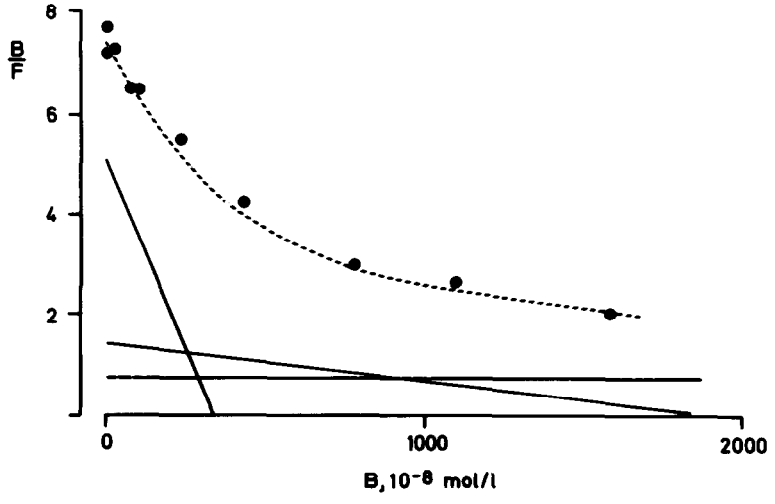

Fig. 3. Binding of propranolol to diluted serum is plotted according to the method of Scatchard [16]. *B* and *F* represent bound and unbound concentrations of propranolol respectively. Each point represents mean of four separate experiments. The straight lines with slope different from zero represent binding capacity of prealbumin, albumin, transferrin and α_1-acid glycoprotein (Fig. 1). The straight line with a slope not different from zero represents the binding capacity of lipoproteins (Fig. 2). The curve (presented as the dotted line) is constructed from the straight lines according to the method of Rosenthal [17].

Table 2. Serum concentrations of lipids, α_1-acid glycoprotein, albumin and binding of propranolol in serum. Concentration of propranolol in serum was 3×10^{-7} mol/l

		Mean*	S.D.	Range
Serum concentrations (mmoles/l)	Triglycerides	2.77	1.12	1.13–5.50
	Cholesterol	7.50	1.31	5.57–10.13
	Albumin	0.54	0.05	0.44–0.62
	α_1-AGP	0.027	0.011	0.012–0.051
Binding of propranolol in serum	B/F†	12.30	3.29	7.46–19.17‡

*Mean value of 21 sera after equilibrium dialysis.
† B and F represent concentrations of bound and free propranolol respectively.
‡ Equals 88.18–95.04, expressed as bound propranolol as per cent of total concentration of propranolol in serum.

Table 3. Correlation between binding ratio (B/F) and concentrations of lipids and proteins in serum

Triglycerides		Cholesterol		Albumin		α_1-Acid glycoprotein	
r-value	P-value*	r-value	P-value	r-value	P-value	r-value	P-value
−0.078	> 0.1	−0.167	> 0.1	0.010	> 0.1	0.849	<0.001

*Significance for r towards zero.

to α_1-acid glycoprotein in serum and less to other serum proteins, and observed variation in serum binding is correlated to concentration of this protein.

Based on the observations that the basic drug quinidine was bound to serum albumin [19,20], lipoproteins [21] and α_1-acid glycoprotein [22], serum proteins were separated into two main fractions for the present binding studies. One fraction contained lower molecular weight serum proteins as albumin, transferrin, prealbumin and α_1-acid glycoprotein and the other was a mixture of serum lipoproteins.

The procedure used to obtain lipoproteins provides a

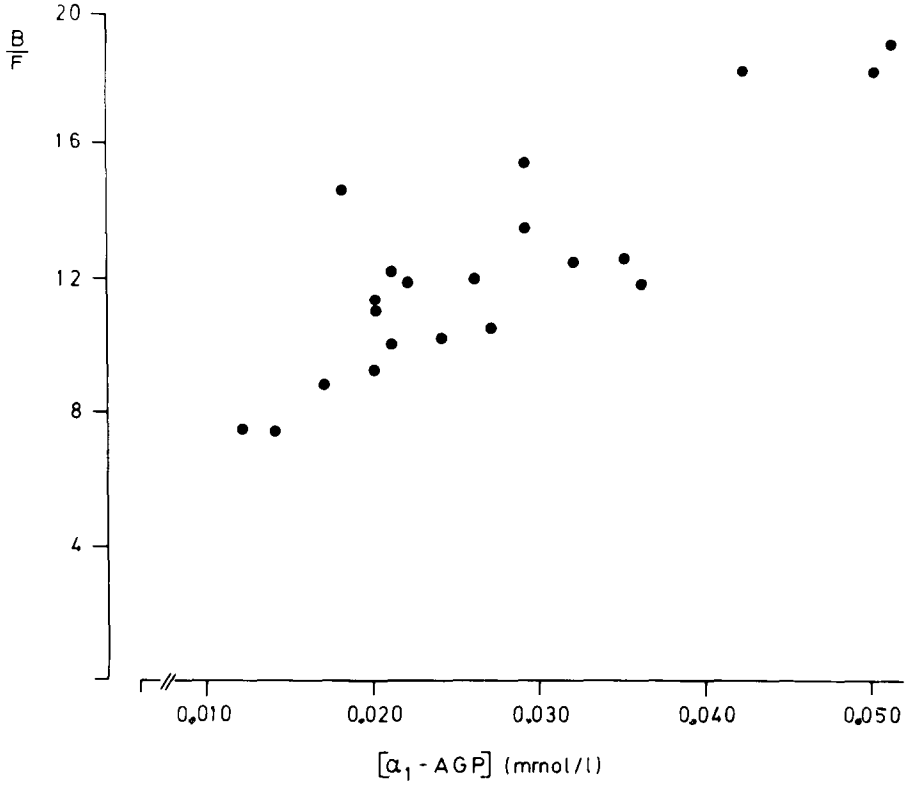

Fig. 4. Correlation between serum levels of α_1-acid glycoprotein and binding ratio bound/unbound (B/F) of propranolol in serum samples from 21 healthy males. The ratios were distributed along the line $Y = 5.33 + 259X$, with a correlation coefficient of 0.849.

mixture of different lipoproteins which did not contain chylomicrons [15]. The present results show that propranolol is not bound, but distributed to lipoproteins independent of propranolol concentration. This differs from the observed binding of quinidine to lipoproteins [21], but is similar to the interaction between tetracyclines and lipoproteins [23]. At therapeutic serum concentrations of propranolol distribution to lipoproteins constitutes a minor fraction of that bound in serum, even in the present hyperlipemic sera.

The other preparation with proteins of lower molecular weights contained albumin, prealbumin, transferrin and α_1-acid glycoprotein. Only the concentrations of α_1-acid glycoprotein and albumin have been determined in the protein preparation, and the presence of the others was confirmed by paper electrophoresis. The propranolol binding characteristics of this preparation, given in Table 1, suggest that two separate binding sites are present in this protein mixture. The first binding site has a low dissociation constant for propranolol and a binding capacity, similar to the molar concentration of α_1-acid glycoprotein (3.3×10^{-6} mol/l), in the protein preparation. The second binding site has a higher dissociation constant for propranolol and a binding capacity equivalent to 18.7×10^{-6} mol/l in the protein preparation, but lower than the albumin concentration (3.1×10^{-4} mol/l).

These binding characteristics explain the total serum binding of propranolol, because the binding curve for serum is quite similar to that constructed from the binding characteristics of lower molecular weight serum proteins and the distribution to lipoproteins. These results show that about 75 per cent of the serum bound propranolol is located to the first binding site with a low dissociation constant, and 25 per cent to the second binding site and lipoproteins in sera.

The sera used to evaluate interindividual variability in binding and the possible correlation with concentrations of albumin, α_1-acid glycoprotein and lipoproteins, were obtained from assumed healthy subjects. The present selection of subjects was chosen because adrenergic beta-blockers to a great extent are administered to males of this age with symptoms of cardiovascular diseases only. Consequently, small variations in serum albumin concentration, but some variability in serum concentrations of other proteins and lipids are expected, as supported by the present results. The results show a variable serum binding and a significant correlation between binding expressed as the binding ratio B/F and α_1-acid glycoprotein concentration in serum, but not with albumin concentration, and not for lipoprotein concentration, as expressed by cholesterol and triglycerides. No conclusive evidence for α_1-acid glycoprotein as the main binding protein for propranolol in serum has been presented. However, the characteristics of the first binding site with a binding capacity similar to the molar concentration of α-$_1$-acid glycoprotein, and the highly significant correlation between binding and concentration of this protein, make the conclusion very probable. The present results do not indicate the localization of the second binding site, and other proteins of lower molecular weight than albumin have to be investigated for this binding.

The serum concentration of the α_1-acid glycoprotein is normally in the range of 0.012–0.042 m mol/l, but the concentration was higher in some of these subjects. However, the observed interindividual variation in binding may be enlarged in groups of patients, and also anticipated individually, because α_1-acid glycoprotein concentration increases by stress [24, 25], inflammation [26] and malignancy [27].

An increased serum binding of propranolol produced by higher concentration of α_1-acid glycoprotein will simultaneously decrease biological half-life and volume of distribution of propranolol [5], and decrease unbound and biological effective concentration of propranolol without any change in total concentration of the drug under steady state conditions [6].

Several biological effects are related to the unbound concentration of propranolol in serum, and the possible clinical consequences of α_1-acid glycoprotein being the main propranolol binding protein, should be investigated further.

Acknowledgements—The support from the Norwegian Research Council for Science and the Humanities and the Norwegian Council on Cardiovascular Diseases is gratefully acknowledged. We wish to thank Ms. Claire Poulsson for technical assistance, Dr. I. Aakesson for assistance in immunological determination of albumin, α_1-acid glycoprotein and paper electrophoresis of serum proteins and Ms. Kari Haug for the typing of the present manuscript.

REFERENCES

1. J. W. Paterson, M. E. Conolly, C. T. Dollery, A. Hayes and R. G. Cooper, *Pharmac. Clin.* **2**, 127 (1970).
2. D. G. Shand, E. M. Nucolls and J. A. Oates, *Clin. Pharmac. Ther.* **11**, 112 (1970).
3. D. G. Shand and R. E. Rangno, *Pharmacology* **7**, 159 (1972).
4. T. Walle and T. E. Gaffney, *J. Pharmac. exp. Ther.* **182**, 1, 83 (1972).
5. G. H. Evans, A. S. Nies and D. G. Shand, *J. Pharmac. exp. Ther.* **186**, 114 (1973).
6. G. H. Evans and D. G. Shand, *Clin. Pharmac. Ther.* **14**, 494 (1973).
7. R. Zacest and J. Koch-Weser, *Pharmacology* **7**, 178 (1972).
8. D. G. McDevitt, M. Frisk-Holmberg, J. W. Hollifield and D. G. Shand, *Clin. Pharmac. Ther.* **20**, 152 (1976).
9. P. Flodin and J. Killander, *Biochim. biophys. Acta* **63**, 403 (1962).
10. W. W. Umbreit, R. H. Burris and J. F. Stauffer, *Manometric Techniques and Tissue Metabolism.* Burgess, Minneapolis (1951).
11. O. G. Nilsen, D. Fremstad and S. Jacobsen, *Eur. J. Pharmac.* **33**, 131 (1975).
12. I. Offerhaus and J. R. van der Vecht, *Br. J. Clin. Pharmac.* **3**, 1061 (1976).
13. G. Mancini, A. O. Carbonara and J. F. Heremans, *Immunochemistry* **2**, 235 (1965).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. R. S. Lees and F. T. Hatch, *J. Lab. clin. Med.* **61**, 518 (1963).
16. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
17. H. E. Rosenthal, *Analyt. Biochem.* **20**, 525 (1967).
18. L. H. Myers, N. R. Phillips and R. J. Havel, *J. Lab. clin. Med.* **88**, 491 (1976).
19. H. L. Conn and R. J. Luchi, *J. Pharmac. exp. Ther.* **133**, 76 (1961).
20. H. L. Conn and R. J. Luchi, *J. Clin. Invest.* **40**, 509 (1961).
21. O. G. Nilsen and S. Jacobsen, *Biochem. Pharmac.* **24**, 995 (1975).

22. D. Fremstad, K. Bergerud, J. F. W. Haffner and P. K. M. Lunde, *Eur. J. Clin. Pharmac.* **10**, 441 (1976).
23. G. Powis, *J. Pharm. Pharmac.* **26**, 113 (1974).
24. K. F. Aronsen, G. Ekelund, C. O. Kindmark and C. B. Laurell, *Scand. J. clin. Lab. Invest.* **9**, 127 (1972).
25. B. G. Johansson, C. O. Kindmark, E. Y. Trell and F. A. Wollheim, *Scand. J. clin. Lab. Invest.* **9**, 117 (1972).
26. S. Weisman, B. Goldsmith, R. Winzler and M. H. Lepper, *J. Lab. clin. Med.* **57**, 1, 7 (1961).
27. A. Milford Ward, E. H. Cooper, R. Turner, J. A. Anderson and A. M. Neville, *Br. J. Cancer* **35**, 170 (1977).