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THE BINDING OF QUINIDINE TO PROTEIN FRACTIONS OF NORMAL HUMAN SERA

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Abstract—In contrast to previous assumptions, albumin is not the only protein in normal human serum responsible for binding quinidine. Human serum proteins were fractionated by gel filtration and floatation. Quinidine binding was determined by equilibrium dialysis. The binding to low (LDL) and high (HDL) density lipoproteins exhibited two binding sites on each protein, and the dissociation constants K and number of binding sites n were calculated. LDL: $K_1 = 2 \times 10^{-5}$, $n_1 = 1$ and $K_2 = 5{\cdot}2 \times 10^{-4}$, $n_2 = 97$. HDL: $K_1 = 1{\cdot}9 \times 10^{-5}$, $n_1 = 0{\cdot}1$ and $K_2 = 1{\cdot}1 \times 10^{-3}$, $n_2 = 14{\cdot}7$.

Serum protein binding of a drug may affect its distribution, elimination and pharmacodynamic effects. To predict changes in protein binding of a specific drug in serum of an individual patient, the extent of binding to the different protein fractions of plasma should be known because of the variations in the serum protein pattern. While albumin is the dominant drug-binding protein in serum, the other serum proteins are often subject to greater changes than albumin. Consequently, if proteins other than albumin contribute to the binding of drugs, more frequent variations in binding are to be expected. Most previous observations on protein binding are based on experiments with whole serum with normal or low concentrations of albumin, or serum albumin preparations.

The binding of quinidine to serum and serum albumin has been evaluated by others [1, 2]. This paper establishes, however, that human serum lipoproteins bind quinidine in normal serum to an extent almost equal to that of the serum albumin. Few other serum proteins exhibit greater individual and interindividual variations in serum concentration than the lipoproteins. Recent observations [3, 4] indicate, however, that some drugs are dissolved in the lipid phase of serum lipoproteins. The present results indicate that quinidine is bound to the lipoproteins, and the interaction is governed by the law of mass action.

The complexes between quinidine, and high density (HDL) and low density (LDL) lipoproteins have been characterized by dissociation constants and number of binding sites.

MATERIALS AND METHODS

Serum. Serum was obtained from fasting healthy individuals of both sexes, 23–35 yr of age.

Gel filtration. This was carried out on a Sephadex G-200 column (A.B. Pharmacia, Uppsala, Sweden) which was equilibrated with 0·1 M sodium phosphate buffer containing 0·1 M sodium chloride at pH 7·30 and 4°. Twenty ml of human serum was applied to a 100 × 5 cm column and eluted with the equilibrating buffer at a constant rate of 0·6 ml per min in fractions of 5 ml. Optical density of the fractions was determined at 280 nm.

Equilibrium dialysis. Dialysis was performed in Perspex® cells with 2 chambers (1 ml) separated by semipermeable membranes (Visking dialysis membrane 20/32). 0·5 ml of the protein solution and the drug was introduced to one of the chambers and 0·5 ml of the buffer to the other. The dialysis was usually run for 18 hr at 20–22° under standardized rotary shaking. The pH of the solutions was determined before and after dialysis. The concentration of quinidine was measured in the solutions of both compartments after dialysis. Recovery of quinidine was 97–101 per cent. Protein concentration was determined before and after dialysis. When serum and Krebs–Ringer bicarbonate buffer was used, dialysis was performed in an atmosphere of carbon dioxide.

Protein binding calculation. Binding was determined at equilibrium; the concentration of quinidine in the protein solution being protein-bound and free unbound drug, while the concentration in the buffer solution is the free drug. The quantitative expression for quinidine binding is presented as the portion of total quinidine which is associated with the protein in per cent ([bound] × 100/[bound] + [free]), or by the ratio ([bound]/[free] × [protein]).

Protein determination. Protein content was determined by the method of Lowry *et al.* [5], using bovine serum albumin as standard.

Quinidine determination. Quinidine was determined spectrofluorometrically in a Farrand spectrofluorometer at 350 nm activation and emission at 450 nm in 0·1 M sulphuric acid. Known amounts of quinidine were added to appropriate protein and buffer solutions as standards. Samples (50 μl) from dialysis and standard solutions were added directly to 3 ml of the sulphuric acid. Blanks of protein solution and buffer were run simultaneously. The results obtained with this direct method were similar to results obtained after extraction [6].

Ultracentrifugation. Preparative ultracentrifugation was performed in a Spinco ultracentrifuge Model L 2-65 B using a Ti-50 rotor. Lipoproteins were separated from other serum proteins by centrifugation at different densities as described by Havel *et al.* [7]. Lipoproteins were prepared from fresh serum of fasting healthy individuals by preparative floatation at different densities obtained by adding solid

potassium bromide to serum before centrifugation at 105,000 *g*. Low density lipoproteins (LDL) were obtained after centrifugation for 22 hr at density 1·019–1·063 g/ml and high density lipoproteins (HDL) for 45 hr and density 1·063–1·195 g/ml. The lipoprotein fractions obtained were then dialyzed against several changes of 0·15 M sodium phosphate buffer pH 7·30 for 24 hr at 4°. Lipoproteins were stored at 4° until used.

Donnan effect. This was evaluated by the distribution of sodium between the buffer and the protein phase after dialysis. Sodium was determined by atomic absorption. After dialysis the sodium content was 1–2·5 per cent higher in the lipoprotein solution; binding results were not corrected for this.

Immunoelectrophoresis. This was performed with rabbit immunoglobulin against human serum proteins on 1% (w/v) agarose [8]. The precipitates were stained with Amido black and Sudan black.

Buffers. 0·15 M sodium phosphate buffer [9], Krebs–Ringer phosphate buffer or Krebs–Ringer bicarbonate [10] buffer was used as noted in the different experiments.

RESULTS

Gel filtration of human serum. Human serum was subjected to gel filtration as described in Methods, and serum proteins were eluted from the column in three main peaks as illustrated in Fig. 1. Fractions belonging to the different peaks were pooled as noted by numbers in Fig. 1, and examined by immunoelectrophoresis. Proteins in protein pool I were shown to be lipoproteins, α_2 and β_2 macroglobulins, in protein pool II mainly γ-globulins and ceruloplasmin, and in protein pool III albumin, α_1-antitrypsin and α_2-globulin as observed by others [11].

The binding of quinidine to the pooled proteins samples was then evaluated by adding 1×10^{-8} moles of quinidine to each dialysis cell, and the results are presented in Table 1. There was minimal binding of quinidine to proteins in pool II, but considerable binding to proteins in pools I and III. The extent of binding relative to the amount of protein was much greater in pool I than in pool III. Fractions of pool III contained mainly serum albumin and of pool I serum lipoproteins and macroglobulins. Quinidine binding to serum albumin has been

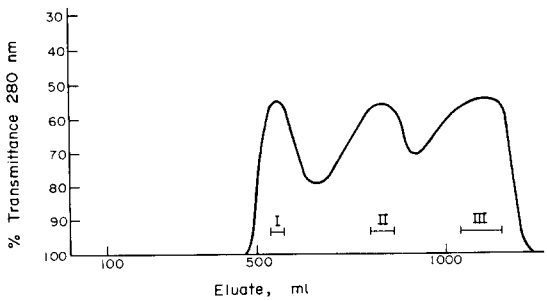

Fig. 1. Content of proteins in consecutive fractions of normal human serum eluted from the Sephadex G-200 column. Protein content is expressed as per cent transmittance at 280 nm. Serum from three healthy individuals was pooled. Fractions from each major peak were pooled and the extension of these fractions is marked (——) with the number I, II and III.

Table 1. Binding of quinidine* to serum protein fractions after gel filtration† and after ultracentrifugation‡ of protein pool I

Protein fraction	Protein concentration (mg/ml)	Concentration of bound quinidine ($M \times 10^{-5}$)	Per cent§ binding ($B \times 100/B + F$)
Pool I	3·2	0·662	49·8 (48·8–50·8)
Pool II	7·2	0·070	6·7 (6·4–7·0)
Pool III	20·0	0·866	60·4 (59·4–61·4)
Density < 1·210 g/ml‡	1·9	0·680	50·8 (49·8–51·8)
Density > 1·210 g/ml‡	3·3	0·032	3·1 (3·0–3·2)

* 1×10^{-8} moles of quinidine added to the cell (1 ml).

† Pools I, II and III from gel filtration of serum (see Fig. 1).

‡ Potassium bromide added to pool I to a density of 1·210 g per ml.

§ Mean values and range of four experiments.

established previously [1, 2], but binding to the fractions of peak I containing lipoproteins and globulins has not been shown before.

Binding of quinidine to separated proteins of peak I from gel filtration. Twenty ml of proteins from pool I obtained by gel filtration of human serum were dialyzed against 0·15 M sodium chloride; 6·494 g of potassium bromide per 20 ml was then added to produce a density of 1·210 g per ml. This solution was centrifuged at 105,000 *g* for 45 hr at 4°. By this procedure the proteins were separated into a supernatant containing proteins of density lower than 1·21 g per ml and a bottom layer containing proteins with density greater than 1·21 g per ml. Immunoelectrophoresis showed that the supernatant contained different lipoproteins, and the bottom layer contained several serum globulins. The immunoelectrophoretic technique, however, does not distinguish between β- and preβ-lipoproteins. The binding of quinidine to these two different protein fractions was evaluated by adding 1×10^{-8} moles of quinidine to the dialysis cell and the results are presented in Table 1. The binding of quinidine to the lipoproteins was much more extensive than to the globulins.

Binding of quinidine to human low density and high density lipoproteins. Low density and high density human serum lipoproteins from four normal individuals were prepared as described in Methods by ultracentrifugation and floatation in the density ranges 1·019 to 1·063 and 1·063 to 1·195 g per ml, respectively.

The binding of quinidine to the two separate classes of lipoproteins was determined by equilibrium dialysis at different concentrations of quinidine. The results, plotted according to the method of Schatchard [12], are shown in Figs. 2 and 3. From these plots the dissociation constants and number of binding site were calculated. Asymtotic straight lines were drawn as the least square regression lines, at both extremities of the Schatchard plot and moved parallel so that the sum of ordinate intercepts equals the total ordinate intercept. The asymtotic lines obtained were then corrected by drawing straight lines from the origin to different points on the curve. The distance from the origin to the curve must equal the sum of the distance from the origin to the two asymtotic lines, according to Rosenthal

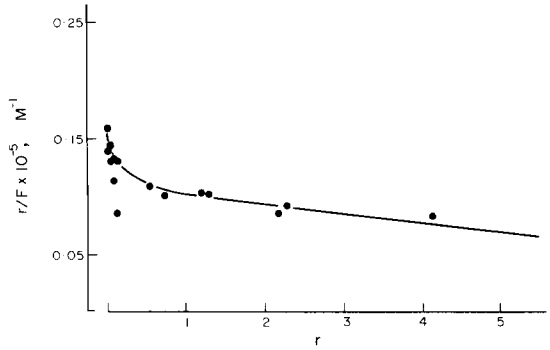

Fig. 2. Binding of quinidine to human α_1-lipoprotein (HDL), plotted according to the method of Schatchard. Points determined experimentally (●). r = moles of quinidine per mole of α_1-lipoprotein; F = free concentration of quinidine in 0·15 M sodium phosphate buffer pH 7·30.

[13]. Only minor adjustments were necessary. The results are presented in Table 2. The number of binding sites is based on average molecular weights of $2{\cdot}5 \times 10^5$ and $2{\cdot}3 \times 10^6$ for the high and the low density lipoproteins, respectively [14], assuming that proteins within each class of lipoproteins possess similar binding characteristics.

Evaluation of the quinidine binding to serum proteins. The binding of quinidine to other preparations of serum protein was evaluated both from equilibrium dialysis experiments and calculations based on the dissociation constants, number of binding sites and protein concentrations. The equation used is based on the law of mass action [15]:

$$B = N \times P \times F/K + F$$

where B is the amount of ligand bound, N is the number of binding sites per protein molecule, P is the concentration of protein, F is the amount of free ligand and K is the dissociation constant of the ligand–protein complex.

The proteins were isolated from a pool of three different sera. The experiments with LDL and HDL were carried out with 0·15 M sodium phosphate buffer pH 7·30. The binding parameters for albumin (human lyophilized, grade A, supplied by A. B. Kabi) and experimental values for albumin and the protein mixture were achieved by using Krebs–Ringer phosphate buffer. Krebs–Ringer bicarbonate buffer was used to determine the binding of quinidine in serum. All experiments were performed at pH 7·30. In the

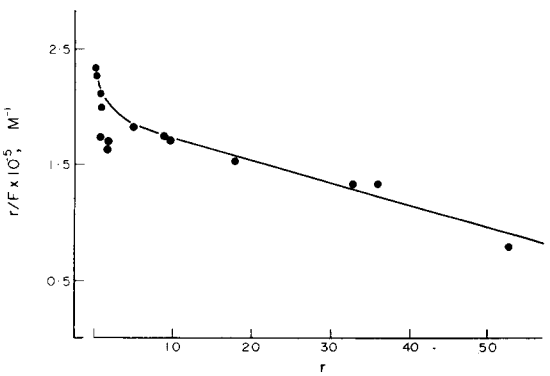

Fig. 3. Binding of quinidine to human β-lipoprotein (LDL), plotted according to the method of Schatchard. The symbols and conditions are as described in Fig. 2.

Table 2. Some characteristics of the quinidine–lipoprotein interaction*

	Dissociation constant	Number of binding sites per molecule
α_1-lipoprotein quinidine complex [5]		
First binding site	$(1{\cdot}9 \pm 0{\cdot}8) \times 10^{-5}$ M	0·10 ± 0·04
Second binding site	$(1{\cdot}1 \pm 0{\cdot}3) \times 10^{-3}$ M	14·7 ± 3·4
β-lipoprotein quinidine complex [5]		
First binding site	$(2{\cdot}0 \pm 0{\cdot}4) \times 10^{-5}$ M	1·0 ± 0·2
Second binding site	$(5{\cdot}2 \pm 0{\cdot}4) \times 10^{-4}$ M	97·0 ± 9·4

* The results are given ± standard error, S.E.M. The numbers in brackets [ ] give number of experiments.

protein mixture of LDL + HDL + albumin the proteins are added to the cell in concentrations roughly corresponding to their serum concentration. As can be seen in Table 3, the binding of quinidine by equilibrium dialysis and from theoretical calculations is roughly in agreement both with respect to the isolated fractions and to the mixture of proteins. The three proteins in the mixture, however, do not explain the total binding in serum.

DISCUSSION

The binding of quinidine to some serum proteins was thoroughly investigated by Conn and Luchi [1, 2]. Their results indicated that serum albumin bound most of the quinidine in human serum. However, the characteristics of the quinidine–serum albumin complex were calculated from experimental data obtained with preparations of purified human serum albumin in sodium phosphate buffer at pH 7·30. Recent observations by Jacobsen *et al.* (to be published) demonstrate that the binding of quinidine to human serum albumin is influenced by both the albumin preparation and the composition of buffer used. Sodium phosphate buffer produces a greater binding than Krebs–Ringer phosphate at pH 7·30, the latter buffer being more equal to the aqueous phase of serum. The results obtained with this buffer should then be more appropriate to the binding taking place in serum. Under their conditions the binding of quinidine to albumin would only partially account for the total binding in serum. This paper demonstrates that other serum protein fractions contribute to the binding of quinidine in serum. Equilibrium dialysis was used to determine the extent of protein binding. The ultrafiltration method was used initially, but was not satisfactory, because an equilibrium between the aqueous phase of the protein solution and ultrafiltrate was not achieved. In the dialysis experiments equilibrium was reached within 5 hr, but dialysis was usually continued for 18 hr.

The different lipoprotein preparations were stored at 4° for less than 2–3 weeks to avoid denaturation. Denaturation could be recognized by precipitation in the protein solution and by an increase in fluorescense detected spectrofluorometrically at 350 and 450 nm. When such alterations of the lipoproteins in the controls were present after dialysis, the binding data were excluded.

The binding of quinidine to fractions of serum proteins obtained by gel filtration (Table 1) demonstrates that proteins other than albumin obviously bind quinidine. The protein pools from peaks I and

Table 3. Binding of quinidine to isolated protein fractions, serum and mixtures of protein fractions, evaluated by equilibrium dialysis and theoretical calculations*

Protein	Protein concentration ($M \times 10^{-6}$)	Unbound quinidine ($M \times 10^{-6}$)	Bound quinidine ($M \times 10^{-6}$) Experimental	Calculated
LDL	12·3	4·04	9·90	11·27
HDL	34·5	7·76	4·50	4·55
Albumin	525·0	6·29	6·51	6·47
LDL	1·07			
+ HDL	12·50	5·18	8·03	7·54
+ albumin	500·00			
Human serum		3·57	10·71	

* Theoretical calculations of binding are based on the dissociation constants and number of binding sites.

III from gel filtration demonstrate extensive binding properties, protein pool I containing mainly lipoproteins and macroglobulines, pool III mainly albumin. More quinidine is bound per weight to proteins of pool I than to proteins of pool III.

The lipoproteins of protein pool I with densities lower than 1·210 g/ml were the dominant quinidine binding macromolecules as shown in Table 1. The different lipoproteins classes isolated by ultracentrifugation and used for binding studies were fairly free of other proteins judged by immunoelectrophoresis. The characteristics of quinidine–lipoprotein complexes were estimated on the assumption that low and high density lipoprotein classes are homogeneous. This is valid for the low density but not for the high density lipoproteins, the latter being composed of at least two different lipoprotein entities [14].

The data presented in Table 2 show that both lipoprotein classes have two independent binding sites. The primary binding sites for quinidine are characterized by lower dissociation constants and lower numbers of sites per molecule than the secondary binding sites. The number of primary binding sites for quinidine on high density lipoprotein is less than 1. The partial occupation of this binding site by other ligands of endogenous origin with high affinity, or the possibility that only some proteins within this heterogeneous group of lipoproteins bind quinidine, may explain this finding.

The lipoproteins used in our experiments were obtained from the serum of normal fasting individuals; lipoprotein preparations from individuals with disorders inducing changes of the serum lipids may have different properties.

The experimental observations demonstrate that the interactions between lipoproteins and quinidine binding are governed by the law of mass action. The experiments do not state, however, whether the interaction takes place with the lipid or the protein part of the lipoprotein molecules.

Some other observations [3, 4] indicate that drugs are dissolved within the lipid part of protein. Quinidine being a very lipid soluble molecule at pH 7·30, could be subject to a dissolution phenomenon. The partition ratio, determined in our experiments as bound (B)/free (F), should be constant, when subject to a dissolution, as the concentration of quinidine in the two phases is far from the saturation level. The partition ratio between the lipid and aqueous phase should increase at higher concentrations, in contrast to the actual interaction between quinidine and lipoproteins.

It should be considered, however, that lipoproteins like other proteins can be altered by the different separation procedures used, when isolated from other macromolecules and by the influence by an aqueous phase different for that of serum. With these reservations the observed binding properties of the isolated proteins should be applicable to the binding in mixtures of proteins as well as serum if all protein fractions participating in the binding are recognized. The binding to whole serum cannot be explained by albumin, α_1 and β lipoprotein exclusively, but preliminary results indicate that the very low density lipoproteins are of importance for the major part of the residual binding of quinidine to macromolecules in whole serum.

The calculated parameters of binding should be used with caution, however, because the analytical plotting-methods used depend on an identity of dissociation constants and sufficient separation of the constants to exclude an interference in the graphical analysis. The binding parameters can be used, however, to approximate the extent of binding to the isolated proteins.

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