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THE INFLUENCE OF SALTS AND DIFFERENCES IN PROTEIN ISOLATION PROCEDURE ON THE BINDING OF QUINIDINE TO HUMAN SERUM ALBUMIN

ODD G. NILSEN and STEN JACOBSEN
Institute of Pharmacology, University of Oslo, Norway

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Abstract—The influence of buffer and two different albumin preparations on the albumin–quinidine interaction was investigated. Human serum albumin was prepared by either alcohol fractionation or ultracentrifugation with subsequent gel filtration. The interaction between albumin and quinidine was determined by equilibrium dialysis. It was inhibited by halide ions and consequently different binding parameters were found to be valid for the complex in sodium phosphate and in Krebs–Ringer phosphate buffer. The influence of Ca^{2+}, Mg^{2+} and SO_4^{2-} in physiological amounts was negligible. The albumin obtained by alcohol fractionation possessed one binding site for quinidine, while the albumin isolated by ultracentrifugation with subsequent gel filtration possessed two binding sites when tested in a Krebs–Ringer phosphate buffer. In sodium phosphate buffer both albumin preparations had two independent binding sites, and showed essentially identical binding parameters.

When investigating the problem of serum protein binding by *in vitro* methods, the relevance of the results to the *in vivo* situation is questionable. The conditions used in the *in vitro* systems should be as close as possible to those in serum concerning electrolytes and the native proteins. However, the *in vitro* methods necessitate the use of artificial buffer systems and isolated protein preparations. The influence of these factors on the binding needs to be clarified.

Drug–protein interactions are known to be affected by the salt concentration of the aqueous phase [1, 2]. The effect of $[H^+]$, $[Ca^{2+}]$ and $[Cl^-]$ ions beyond the physiological range, have been reported to reduce the binding of quinidine to human serum albumin [3]. At the concentrations obtainable in living organisms only chloride ions demonstrated a minimal effect [3]. The *in vitro* interaction between quinidine and human serum albumin has been investigated [4] in a 0.2 M phosphate buffer, pH 7.4. However, an *in vitro* investigation of quinidine–albumin binding in a more 'physiological' buffer than sodium phosphate, containing chloride, calcium and other ions was consequently considered of biological interest.

Human serum albumin is readily available in relatively pure form [5], but the presence of other proteins and components adsorbed to the albumin may vary depending on the isolation procedure and these may interfere differently with the drug–protein interaction. The binding characteristics such as dissociation constant and number of binding sites per protein molecule may not be comparable therefore when the serum albumin is obtained by different isolation procedures. For this reason the binding characteristics for quinidine was determined for two different preparations of albumin.

MATERIALS AND METHODS

Human serum albumin prepared by ultracentrifugation and gel filtration. Preparative ultracentrifugation was performed in a Spinco ultracentrifuge Model L2-65B with a Ti-50 rotor. Fresh sera were obtained from fasting healthy individuals of both sexes, of 23–35 years of age. Serum samples were pooled and potassium bromide added (324.7 g/l) to produce a density of 1.210 g/ml. Serum was then ultracentrifuged for 45 hr at 105,000 *g* and 4°. The floating lipoproteins were withdrawn, and the remaining serum proteins (50 ml) were dialyzed against three changes of 1000 ml Krebs–Ringer phosphate buffer, pH 7.35. 25 ml of the serum proteins devoid of lipoproteins were applied to a Sephadex G-200 column (100 × 5 cm, A.B. Pharmacia, Uppsala, Sweden) equilibrated with the same buffer at 4°. The elution rate was 1 ml/min and serum albumin was eluted in the last protein peak [6]. The albumin fraction used was obtained from the last part of the last eluted protein peak to avoid interference with proteins from the previous peak. The albumin containing fractions were then concentrated to one fifth of their volume 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 3 changes of 1000 ml Krebs–Ringer phosphate buffer, pH 7.35 for 24 hr. This solution containing about 2% (w/v) albumin, was stored at −21°.

Human serum albumin prepared by alcohol fractionation. The commercially available albumin (AB Kabi, Stockholm, Sweden) was prepared by a modified Cohn method [5] from human plasma. This lyophilized albumin preparation was stored in solid form at 4°.

Equilibrium dialysis. The binding of quinidine was determined by equilibrium dialysis in Perspex® cells with 2 chambers of 1 ml separated by a semipermeable membrane [7]. The cells were shaken at 20° for 18 hr. The concentration of quinidine was determined in both chambers after completion of dialysis. Recovery of radioactivity was close to 100 per cent. pH

and concentration of albumin were determined before and after dialysis.

Protein determination. Protein content was determined by the method of Lowry *et al.* [8], using bovine serum albumin, Cohn fraction V (Sigma, St. Louis, U.S.A.) as standard.

Calculation of protein binding. Protein binding was determined as the binding ratio B/F where B and F represent the molar concentration of bound and unbound quinidine respectively as described previously [12].

The number of binding sites and the dissociation constants for the albumin–quinidine complexes were obtained by plotting the data according to Scatchard [9]. The binding parameters 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 [10]. 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 [11]. Corrections were made on the asymptotic lines until the fit with the experimental curve was satisfactory. Each asymptotic line presented one binding site on the protein molecule. The number of binding sites (n) and the dissociation constant (K) were given by the intercept with the abscissa (n) and the ordinate (n/K).

Quinidine determination. The distribution of quinidine after equilibrium dialysis was determined by using a quinidine ^{3}H-labelled isotope with a specific activity of 500 mCi/m-mole [12]. The β-emmitance of the tritiated quinidine was determined by liquid scintillation spectrometry [12].

Analytical polyacrylamide gel electrophoresis. Gel electrophoresis was carried out at pH 9.3 with a Shandon analytical polyacrylamide electrophoresis apparatus with minor modifications of the original method [13] as described in 'Instructions for the Analytical Temperature Regulated Disc Electrophoresis Apparatus' published by Buchler Instrument Inc., New Jersey, U.S.A. Staining was performed with Coomassie Brilliant Blue R obtained from Sigma Chemical Company, St. Louis, U.S.A.

Buffers. 0.15 M sodium phosphate buffer [14], Krebs–Ringer phosphate and bicarbonate buffer [15] was used. Krebs–Ringer bicarbonate buffer was equilibrated with 5% (v/v) carbon dioxide in air during the whole procedure.

Free fatty acids. Free fatty acids were measured according to the method of Lauwerys [28].

RESULTS

The effect of inorganic salts on the quinidine–albumin interaction. The effect of some inorganic salts on the binding of quinidine to human serum albumin (Kabi) was investigated in 0.05 M sodium phosphate buffer, pH 7.35 by adding the sodium and potassium salts of chloride, bromide, iodide and fluoride. The results are presented in Table 1, and demonstrate that the anions $I^- > Br^- Cl^- > F^-$ inhibit the binding of quinidine to serum albumin. No difference was observed between the sodium and potassium salts.

Table 1. The effect of sodium and potassium salts of some halides on the binding of quinidine* to human serum albumin†

Salt (0.1 M) in sodium phosphate buffer (0.05 M) pH 7.35	Binding ratio‡ (B/F)
None	1.46 (1.44–1.49)
NaCl	0.59 (0.57–0.60)
KCl	0.59 (0.57–0.60)
NaF	1.10 (1.09–1.12)
KF	1.12 (1.09–1.14)
NaBr	0.48 (0.46–0.50)
KBr	0.46 (0.46–0.47)
NaI	0.42 (0.41–0.43)
KI	0.43 (0.42–0.43)

* Concentration of quinidine 1×10^{-5} M.

† Concentration of Kabi albumin 1.25% (w/v).

‡ Mean values and range of four experiments expressed as the binding ratio B/F; B and F represent the molar concentration of bound and unbound drug respectively.

The effect of chloride and bromide on the quinidine albumin binding. The inhibitory effect on binding of chloride and bromide was examined with a 1.25% (w/v) solution of human albumin (Kabi) and a 0.05 M phosphate buffer, pH 7.35 with different concentrations of sodium chloride and bromide. Figures 1 and 2 give the double reciprocal plot of the binding data obtained by equilibrium dialysis at different concentrations of quinidine and demonstrate increased

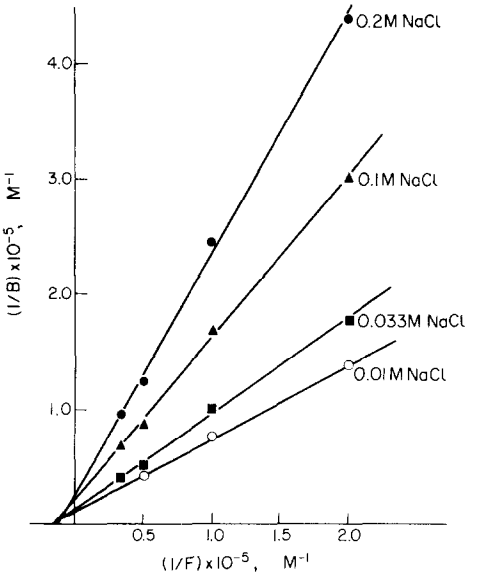

Fig. 1. Inhibitory effect of sodium chloride on the binding of quinidine to human Kabi serum albumin (1.25% (w/v)) in 0.05 M sodium phosphate buffer, pH 7.35 presented as a double reciprocal plot. $1/F$ and $1/B$ represent the reciprocal values of free and bound quinidine respectively. Each point represents the mean value from two experiments and all lines are computerized by least square regression lines with equal weight on each point.

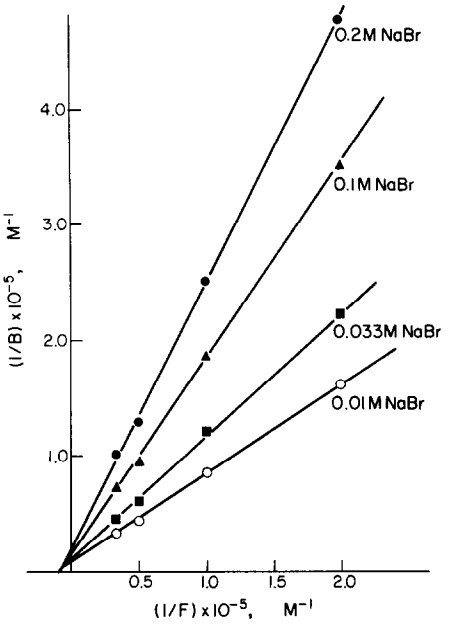

Fig. 2. Inhibitory effect of sodium bromide on the binding of quinidine to human serum Kabi albumin. Conditions and designation are the same as in Fig. 1.

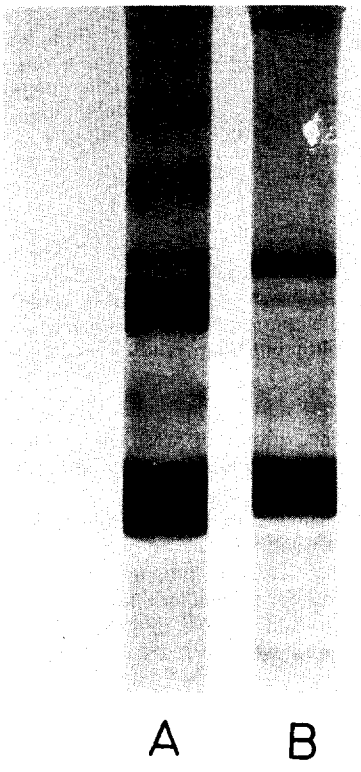

Fig. 3. The albumins isolated by alcohol fractionation and by ultracentrifugation with subsequent gel filtration are designated A and B respectively.

inhibition of the quinidine–albumin interaction by higher concentrations of the salts.

Influence of other salts. The binding of quinidine to human serum albumin (Kabi) was performed in a 0.15 M sodium phosphate buffer and a Krebs–Ringer phosphate buffer with and without $MgSO_4$ and $CaCl_2$ at three different concentrations of quinidine. Table 2 demonstrates that in the absence of $MgSO_4$ and $CaCl_2$ the binding ratio is not changed from that obtained in Krebs–Ringer phosphate buffer, while sodium phosphate buffer produces a much higher binding ratio. All albumin fractions were dialyzed against their respective buffer prior to equilibrium dialysis. This was performed at pH 7.35 and 20°.

The binding of quinidine to two different preparations of human serum albumin. Analytical polyacrylamide gel electrophoresis was carried out with albumin obtained by alcohol fractionation and by ultracentrifugation with subsequent gel filtration. As can be seen from Fig. 3, both preparations contained in addition to serum albumin, traces of transferrin. The commercial serum albumin preparation prepared by Cohn fractionation was also shown to contain a large quantity of dimer and polymer aggregates of albumin, while the albumin prepared in the laboratory by ultracentrifugation with subsequent gel filtration also contained traces of prealbumin, but no aggregates of albumin. The binding of quinidine to both human serum albumin preparations was evaluated in a 0.15 M sodium phosphate buffer, pH 7.35 and in a Krebs–Ringer phosphate buffer, pH 7.35, with concentrations of quinidine ranging from 1.25×10^{-6} to 4.00×10^{-4} M. The results were plotted according to the method of Scatchard [9] and are shown in Figs. 4 and 5. All the experimental values are distributed along a curved line except those for the commercially available Kabi albumin in Krebs–Ringer phosphate buffer, where the experimental values represent a straight line. This demonstrates that in sodium phosphate buffer albumin of both preparations possess more than one group of binding sites while in Krebs–Ringer phosphate buffer the Kabi albumin possesses

Table 2. Binding* of quinidine to albumin† in different buffers at pH 7.35

Concentration of quinidine (M)	0.15 M Sodium phosphate buffer	Krebs–Ringer phosphate buffer	Krebs–Ringer phosphate buffer without $MgSO_4$‡	Krebs–Ringer phosphate buffer without $CaCl_2$§
4.00×10^{-4}	1.21 (1.20–1.22)	0.49 (0.48–0.50)	0.48 (0.47–0.49)	0.49 (0.48–0.50)
1.00×10^{-5}	3.41 (3.37–3.45)	0.68 (0.67–0.69)	0.64 (0.62–0.66)	0.66 (0.64–0.68)
1.25×10^{-6}	3.55 (3.51–3.59)	0.70 (0.69–0.71)	0.70 (0.69–0.71)	0.70 (0.68–0.72)

* Mean values and range of two experiments expressed as the binding ratio B/F.
† Concentration of Kabi albumin 2% (w/v).
‡ Original concentration in the buffer 1.23 mM.
§ Original concentration in the buffer 1.30 mM.

Table 3. Influence of buffers and protein preparations on the binding of quinidine to human serum albumin

Protein	Sodium phosphate buffer Number of binding sites	Sodium phosphate buffer Dissociation constant (M)	Krebs–Ringer phosphate buffer Number of binding sites	Krebs–Ringer phosphate buffer Dissociation constant (M)
Albumin A*	0.1	2.8×10^{-5}	2.0	7.7×10^{-4}
	1.5	1.4×10^{-4}		
Albumin B	0.06	2.1×10^{-5}	0.01	6.9×10^{-6}
	1.4	1.1×10^{-4}	1.3	6.8×10^{-4}

* Albumin A refers to the protein fraction isolated by alcohol fractionation (Kabi albumin) and B to the albumin prepared by ultracentrifugation with subsequent gel filtration. Mean values from 3 experiments are given.

one and the other albumin preparation possesses more than one group of binding sites per albumin molecule.

The binding parameters evaluated from these plots are shown in Table 3. As can be seen from Table 3, the two different albumin preparations possessed almost equal binding parameters for quinidine in sodium phosphate buffer while different parameters for the two preparations were obtained in Krebs–Ringer phosphate buffer.

Free fatty acids. The concentration of free fatty acids in the Kabi albumin and in the albumin separated by ultracentrifugation and gel filtration was 0.030 and 0.013 meq/g protein, respectively. Free fatty acids were determined after equilibrium dialysis and the concentration of both albumin preparations was 2% (w/v) after dialysis.

DISCUSSION

The binding of quinidine to human serum albumin was inhibited to the same extent by the sodium and potassium salts of several halide ions. Consequently the inhibitory effect seems to be related to the anions which is in accordance with earlier observations establishing that more than 11 chloride ions were bound per albumin molecule in a 0.15 M sodium chloride solution, while only a small amount of sodium ions was bound [16].

The increased concentration of chloride and bromide in Figs. 1 and 2 will increase the negative charge on the albumin molecule because of the binding of these anions. The observed inhibition of quinidine binding to albumin may therefore be explained by some electrostatic interaction between the negatively charged albumin molecule and the positively charged quinidine molecule at pH 7.4; ($pKa \approx 8.6$). The inhibition cannot be explained by an electrostatic attraction between these molecules because an increased negative charge on the albumin molecule would have increased the binding of quinidine. The inhibitory effect can, however, be explained by a shielding effect or an indirect inhibition of binding sites by the halide ions. An indirect inhibition could be caused by the additional charged halide ions on the albumin molecule making an interaction possible between protons and negatively charged binding sites usually available for quinidine. The resulting neutrality could result in the loss of this binding site for quinidine.

Salts are also known to influence enzyme activity [18] and protein denaturation [17] by affecting protein structure. It has been shown that anions can be ranked by their capacity to alter the organized structure of several macromolecules [19]. Other work established that the effect on protein conformation was in the order $Cl^- < Br^- < I^-$ [18, 20]. As the binding of quinidine to albumin was also inhibited in the order $F^- < Cl^- < Br^- < I^-$, an additional explanation for the inhibition may be conformational changes produced in the albumin molecule.

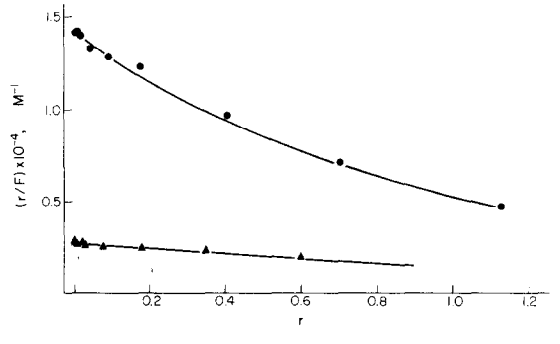

Fig. 4. Binding of quinidine to human albumin isolated by alcohol fractionation plotted according to the method of Scatchard. ●, binding in sodium phosphate buffer pH 7.35; ▲, binding in Krebs–Ringer phosphate buffer pH 7.35. Concentration of albumin is 2% (w/v).

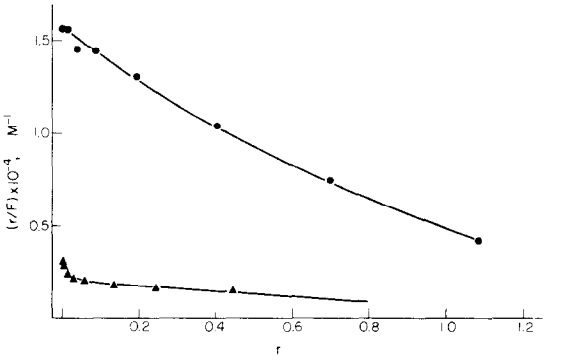

Fig. 5. Binding of quinidine to human albumin isolated by ultracentrifugation with subsequent gel filtration plotted according to the method of Scatchard. The symbols and conditions are as in Fig. 4.

It is of interest that the inhibitory effect of the halide ions on the quinidine–albumin binding is not according to their affinity for serum albumin, which increases according to $Cl^- < F^- < I^-$ [21, 22]. This indicates that the inhibitory effect of the halide ions is not due to competition with quinidine for the same binding site on the albumin molecule. This is also confirmed by the inhibition pattern in the double reciprocal plots. The binding of phenol red by serum albumin is reported [1] to be similar to that of quinidine with respect to the order of inhibition by small anions.

Table 3 shows essentially equal binding parameters for both albumin preparations in sodium phosphate buffer. The small differences are probably caused by heterogeneity of the two albumin preparations, one containing dimers and other polymers of albumin, the other pre-albumin in addition to monomeric albumin. Table 3 gives an exceptionally low number of binding sites (0.1, 0.06 and 0.01) for quinidine on the albumin molecule. This may be due to endogenous substances being bound to the albumin molecule with a high affinity, thus partly blocking the high affinity binding site for quinidine. The low number may also be caused by heterogeneity of the serum albumin molecules combined with a disappearance of binding sites due to polymerization of albumin molecules.

The binding parameters for both albumin preparations were different when evaluated in sodium phosphate and Krebs–Ringer phosphate buffer. As the SO_4^{2-}, Mg^{2+} and Ca^{2+} ions present in the Krebs–Ringer phosphate buffer did not seem to effect the binding of quinidine to human albumin and the electrolytic strength was equal in the two buffers, the observed differences in binding must be due to the chloride ions altering either the charge on the albumin molecule or its conformation.

The disappearance of the high affinity binding site for quinidine from Kabi albumin but not from the other albumin preparation in Krebs–Ringer phosphate buffer could be due to interference by endogenous substances such as free fatty acids or bilirubin bound to albumin. Both substances are known to affect the binding of many drugs to albumin [23–25]. Comparing the two albumin preparations, Kabi albumin contained double the amount of free fatty acids per gram of protein. Even small variations in the strongly bound free fatty acids [26] are known to produce conformational changes in the albumin molecule. Different amount of free fatty acids in the two albumin preparations could, besides blocking the high affinity binding sites for quinidine to different degrees, also produce different conformations of the two albumin preparations. These changes alone are probably not sufficient to make the whole binding site disappear, but the additional binding of chloride ions may be able to block the high affinity binding site in one albumin preparation completely, but not in the other because of conformation changes or differences in the loss of the high affinity binding site due to endogenous substances. The involvement of chloride ions in the latter problem is due to the high affinity binding site on the Kabi albumin not disappearing when sodium phosphate buffer is used. The mechanism of such a combined effect of endogenous substances and ions is unclear.

The high affinity site observed in Krebs–Ringer phosphate buffer for the albumin fraction isolated by ultracentrifugation may represent binding to contaminants rather than to albumin. However, the identical binding result obtained with the two albumin preparations in sodium phosphate buffer make this explanation improbable.

The few experimental points on the binding curve close to the abscissa in Figs. 4 and 5 resulted in an inaccuracy for the first extrapolation to the intersection point with the abscissa. However, this was corrected for by regenerations of the whole binding curve from the asymptotic lines as described in Methods. In this way each asymptotic line and consequently the total number of binding sites, were not only determined by a few points but also by the whole binding curve.

These results demonstrate that the binding of quinidine to human serum albumin is greatly affected by the electrolytes and the isolation procedure for the albumin. These factors will probably also influence the binding of other drugs to serum proteins. To obtain protein binding representative of the *in vivo* situation, two factors must be considered. First, the use of a buffer containing all of the electrolytes present in normal undialyzed serum. Secondly that the isolation procedure for the protein should be as mild as possible in order that naturally occurring endogenous substances remain on the protein.

To be sure that *in vitro* results are applicable to the *in vivo* situation, the sum of the binding to the isolated proteins of serum, should equal the total binding by whole serum. Results (to be published) indicate that the albumin preparation obtained by ultracentrifugation and gel filtration binds quinidine just like native serum albumin, while the Kabi preparation has other properties.

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