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Molecular Basis of Indomethacin–Human Serum Albumin Interaction

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Abstract

Studies on the strength and extent of binding of the non-steroidal anti-inflammatory drug indomethacin to human serum albumin (HSA) have provided conflicting results. In the present work, the serum-binding of indomethacin was studied in 55 mM sodium phosphate buffer (pH 7·0) at 28°C, by using a fluorescence quench titration technique.

The interaction of indomethacin with human serum albumin has been studied as a function of temperature, ionic strength and pH. The results suggest that electrostatic interaction plays a major role in the binding. The possible role of lysine residues in this interaction was studied by modifying exposed and buried lysine residues of HSA with potassium cyanate and studying indomethacin binding with the modified HSA. The data suggest that the interaction takes place via a salt bridge formation between the carboxylate group of indomethacin and a buried lysine residue of HSA.

A technique involving fluorescence enhancement of bilirubin upon its interaction with HSA was used to study its displacement by indomethacin. The displacement, although apparently competitive in nature, was not strong suggesting that the primary sites of interaction of bilirubin and indomethacin are different.

As a major soluble protein, human serum albumin (HSA) is the principal transport protein in plasma (Peters 1996). Besides contributing to the osmotic pressure, the outstanding property of HSA is its ability to bind reversibly to an incredible variety of ligands (Kragh-Hansen 1981). Many drugs which enter the blood circulation bind with several molecules to the HSA molecule, which presumably possesses an infinite capacity to bind quite different types of ligands (Kragh-Hansen 1981; Brodersen et al 1984; Carter & Ho 1994; Peters 1996). Some of these ligands compete to varying degrees with each other for binding to the protein, if present at the same time in the blood stream. The distribution of different drugs is therefore primarily determined by the strength of their binding to HSA and their concentration in the blood. A better understanding of the molecular events in the drug–albumin interaction would be provided by knowledge of binding isotherms of different drugs to HSA together with progress made in HSA structure–function studies (He & Carter 1992; Peters 1996) and efforts to obtain diffraction patterns.

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Out of the hundreds of drugs and ligands which have been studied for binding to albumin only a few have been examined in detail to provide knowledge about the molecular basis of interaction. Besides the displacement of one drug by another, the drug-induced bilirubin displacement from its complex with albumin is of biological importance (Brodersen 1979b). Kernicterus is a condition obtained by the precipitation of insoluble bilirubin in brain cells. The risk of kernicterus is increased when the non-protein-bound bilirubin concentration is increased, as may happen when a part of the bilirubin-binding function of HSA is occupied by a drug (Brodersen 1979b; Cashore & Oh 1982; Bratlid et al 1984; Brodersen et al 1995). Silverman et al (1956) reported an outbreak of fatal kernicterus in icteric infants upon treatment with sulfisoxazole. Since then a number of drugs have been found to bind to HSA and cause bilirubin displacement (Brodersen 1979b; Robertson et al 1991). However, generally the molecular basis of their interaction with HSA and the mechanism by which they displace bilirubin, is still poorly understood.

HSA interaction with indomethacin, an anti-inflammatory and antipyretic agent commonly used for closing patent ductus arteriosus in premature

infants, has been studied by several workers using various techniques (Mason & McQueen 1974; Ekman et al 1980; Honoré et al 1983; Honoré & Brodersen 1984; Taira & Terada 1985; Russeva & Mihailova 1996). The results obtained from these studies regarding the binding strength and extent of binding are to some extent conflicting. The only point of agreement is that indomethacin binds to HSA with a primary association constant between 10^5 and $10^6\ \text{M}^{-1}$ and that several binding sites exist.

In the present study, we have used a fluorescence-quenching technique to investigate the indomethacin-HSA interaction in detail, and the possible role of lysine residues in the indomethacin–HSA interaction has been elucidated.

Materials and Methods

Chemicals

HSA, indomethacin, Sephacryl S-200 HR, citraconic anhydride, trinitrobenzenesulphonic acid (TNBS), bilirubin and molecular weight markers were obtained from Sigma Chemical Company, USA. Potassium cyanate was a product of Koch-Light Laboratories Ltd, England. All other chemicals used in this study were high-quality analytical-grade reagents.

Spectral measurements

Light absorption measurements were performed on a Shimadzu double-beam spectrophotometer, model UV-150-02, using matched silica cells of 1-cm path length. Fluorescence emission spectral measurements were performed on a Shimadzu spectrofluorophotometer, model RF-540, equipped with a data recorder, model DR-3. All fluorescence measurements were performed at constant temperature with continuous stirring by using a temperature-controlled cell holder equipped with a mini magnetic stirrer (Hellma cuv-o-stir, model 333) and a constant temperature water circulator, TB-85.

Determination of protein concentration

HSA monomers were prepared from commercial HSA by size-exclusion chromatography on a Sephacryl S-200 HR column. The concentration was routinely obtained from its absorbance value at 279 nm by using the extinction coefficient of $0{\cdot}531\ \text{g}^{-1}\,\text{L}\,\text{cm}^{-1}$ (Peters 1996). Concentrations of modified HSA preparations were determined by the method of Lowry et al (1951) using native HSA as standard.

Indomethacin–HSA interaction

Stock indomethacin solution was prepared by dissolving 100 mg of drug in 1 mL methanol. Subsequently the final volume was made up to 100 mL with 55 mM phosphate buffer, pH 7·0 and an ionic strength of 0·11. Indomethacin concentration was determined spectrophotometrically by using the molar absorption coefficient of $6{\cdot}290\ \text{mol}^{-1}\,\text{cm}^{-1}$ at 319 nm. The solution was stored at 4°C. The interaction of indomethacin with HSA was studied by monitoring the quenching of the fluorescence emission spectra of the protein as a function of indomethacin concentration. A discontinuous titration method was used to obtain the binding isotherms. A fixed amount of HSA (10·1 μM) was taken in a series of test tubes and increasing amounts of indomethacin solution were added. The final volume was made up to 5 mL with the appropriate amount of suitable buffer. The incubation time was not critical but was kept constant at 10 min. The temperature was maintained at 28°C except when temperature dependence was studied. Fluorescence emission spectra for all the solutions were recorded from 300 to 480 nm by exciting the solution at 284 nm. A series of blank emission spectra of solutions containing only indomethacin (without protein) were also recorded. The contribution of drug emission at 340 nm (the emission maximum of HSA) was negligible and was not taken into consideration.

Analysis of data

The fluorescence data were utilised to calculate the number of moles of indomethacin bound per mole of HSA. The concentration of unbound indomethacin at each concentration of the drug added to albumin was obtained according to the method of Levine (Levine 1977; Tayyab & Trivedi 1995) and was also used for bilirubin–HSA interactions. It was presumed that each bound indomethacin molecule quenched equally. The initial linear part of the fluorescence quenching curve was used to determine the maximal quench as described (Tayyab & Trivedi 1995) and here we only considered the stoichiometrically first bound molecule. In brief, the value of fractional quench, Q, thus obtained was used for calculation of unbound indomethacin [I]:

$$Q = [\text{I–A}]/[\text{A}]_T \qquad (1)$$

where [I−A] is the concentration of indomethacin bound to HSA and $[\text{A}]_T$ is the total HSA concentration. We then obtain the following relations:

$$[\text{I}] = [\text{I}]_T - [\text{I–A}] \qquad (2)$$

$$[I] = [I]_T - Q[A]_T \quad (3)$$

Presuming that the affinity of the stoichiometrically first bound molecule is much stronger than the following we may consider binding of only one indomethacin molecule per HSA molecule:

$$r = [I]K_1/(1 + [I]K_1) \quad (4)$$

where r is the number of bound indomethacin molecules per HSA molecule

The concentration of free HSA, [A], is:

$$[A] = [A]_T - [I\text{–}A] \quad (5)$$

$$[A] = (1 - Q)[A]_T \quad (6)$$

Entering these values into the binding equation yields the following relation:

$$K_1 - K_1Q = Q/[I] \quad (7)$$

Plotting Q/[I] vs Q ideally gives a straight line with the slope $-K_1$.

Effect of temperature, ionic strength and pH on indomethacin binding to HSA

The standard conditions in most experiments were T = 28°C, pH = 7·0 and ionic strength, I = 0·11. The effect of these parameters was studied in the pH range 2·8–10·0 by using glycine–HCl (pH 2·8–3·3), acetate (pH 3·7–5·5), phosphate (pH 6·0–8·0), Tris-HCl (pH 8·0–8·5) and carbonate-bicarbonate (pH 9·35–10·0) buffer systems. The influence of ionic strength was studied in 4 mM phosphate buffer, obtaining different ionic strengths by including the requisite amounts of sodium chloride. The temperature dependence of association was used to compute various thermodynamic parameters. A plot of ln K_1 versus 1/T (van't Hoff plot) gives a straight line with the slope:

$$-\Delta H^\circ/R \quad (8)$$

where ΔH° is the standard enthalpy change and R is the gas constant. By multiplying with R we may calculate the change in standard enthalpy, ΔH°. The change in standard free energy, ΔG° and the change in standard entropy, ΔS°, were calculated at each temperature value by using conventional thermodynamic relationships:

$$\Delta G^\circ = -RT \ln K_1 \quad (9)$$

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T \quad (10)$$

The effect of pH on HSA conformation

The pH-induced conformational changes in the HSA molecule in the pH range 2·8–10·0 were studied by measuring the shift in the fluorescence emission spectra using 284 nm as excitation wavelength.

Carbamylation of HSA

Carbamylation of the lysine side chains of HSA was performed by the procedure of Stark (1972) using potassium cyanate as a modifying reagent. Two approaches to modification were applied to obtain various modified derivatives of HSA.

The first, conventional modification, was performed by treating HSA with 0·5 M potassium cyanate in 0·1 M sodium phosphate buffer, pH 7·7 for different periods of times (2–96 h) with continuous stirring at room temperature. A pilot experiment was performed to achieve the desired extent of modification. This normal protocol allowed only a maximum modification of 80%. Simultaneously a 100%-carbamylated derivative was prepared by performing the reaction in the presence of 6 M guanidine hydrochloride for 96 h.

The second method for preparing a carbamylated preparation was a double-modification procedure as previously described (Mir et al 1992). This method consisted of first masking the surface lysine residues of HSA by using a reversible modifying reagent, citraconic anhydride, followed by carbamylation of the remaining lysine residues in the presence of 6 M guanidine hydrochloride. Finally, the citraconyl groups were removed by dialysing the protein against 50 mM sodium acetate buffer, pH 3·5 for 96 h at 4°C. After decitraconylation, the protein solution was dialysed against 60 mM phosphate buffer, pH 7·0. A detailed protocol of the modifications is given in Table 1. Quantification of the modification was performed with the trinitrobenzenesulphonic acid (TNBS) method (Habeeb 1966) using unmodified protein as standard (Mir et al 1992).

Table 1. Chemical modification of human serum albumin with potassium cyanate.

Reaction conditions	Percent modification	No. of amino groups modified[a]	Stokes radius (nm)	Frictional ratio
Native HSA	0	0	3·39	1·26
2 h	23	14	3·39	1·26
6 h	55	33	3·68	1·37
96 h	77	46	3·98	1·48
96 h[b]	98	59	4·53	1·68
96 h[b,c]	(21)[d]	14	3·39	1·26

All reactions were performed at 25°C, pH 7·7. [a]The number of amino groups was calculated by taking the total number of amino groups to be 60 (one amino terminal + 59 lysine residues). [b]In presence of 6 M guanidine hydrochloride. [c]Citraconylated HSA was prepared according to Mir et al (1992). [d]This preparation has buried lysine residues modified.

Conformational changes in modified HSA

The effect of carbamylation on HSA conformation was studied by hydrodynamic parameters and by circular dichroism (CD). The gel-filtration data, obtained on a Sephacryl column were used according to the method of Ackers (1967) and Laurent & Killander (1964) to calculate the Stokes radii of native and modified albumins. Another parameter, frictional ratio, was calculated from the Stokes radii according to the protocol of Andrews (1970). The secondary structure was determined by CD spectroscopy. CD spectra were recorded on a Jasco J-20 spectropolarimeter. The instrument was calibrated with D-10 camphorsulphonic acid. CD data are expressed as mean residual ellipticity, $[\theta]_{MRW}$ ($^\circ\,cm^2\,dmol^{-1}$).

Interaction of indomethacin with modified HSA derivatives

The interaction of indomethacin with modified derivatives of HSA was studied by the fluorescence quench titration method, as described above.

Indomethacin-induced bilirubin displacement

Since both bilirubin and indomethacin cause quenching of the intrinsic fluorescence of HSA, it was not possible to study the displacement of bilirubin from its complex with HSA by indomethacin using fluorescence quenching. Instead, we made use of the fact that bilirubin which is nonfluorescent in its unbound form becomes fluorescent upon binding to HSA with excitation and emission maxima at 487 and 528 nm, respectively. On the other hand, indomethacin (bound or free) does not show any fluorescence when excited at 487 nm. Hence, bilirubin–HSA complexes at various concentrations of bilirubin (at bilirubin/HSA ratios of 0–5) were prepared and the displacement of bilirubin by indomethacin was monitored by recording the induced fluorescence spectra at varying indomethacin concentrations (0–100 μM). The bilirubin solutions used were freshly prepared and kept in the dark at 4°C. The concentration was determined by absorption spectroscopy (Jacobsen & Wennberg 1974).

Analysis of displacement data

The fluorescence intensity at 528 nm was plotted against the bilirubin/HSA molar ratio, in the presence or in the absence of indomethacin. The fractional enhancement, E was calculated in the same manner as was used for maximal quench, Q, described above. Since E was linearly related to the extent of binding:

$$E = [B-A]/[A]_T \qquad (11)$$

where [B−A] is the concentration of bilirubin bound to albumin and $[A]_T$ is the total albumin concentration.

Entering equation 11 into the binding equation and considering only the stoichiometrically first-bound molecule, yields the following relationship:

$$K_1 - K_1E = E/[B] \qquad (12)$$

$$K_1 - K_1E = E/\{(R - E)[A]_T\} \qquad (13)$$

where [B] is the concentration of unbound bilirubin and R is the molar ratio of bilirubin to HSA. The value of the association constant, K_1, is determined from a plot of E vs E/[B] which is analysed by the method of least squares (Vorum et al 1993).

Results and Discussion

Binding of ligands to receptor proteins often produces a decrease in protein fluorescence. This quenching phenomenon has previously been used by several workers to study bilirubin–albumin (Levine 1977; Berde et al 1984; Mir et al 1992; Minchiotti et al 1993; Tayyab & Trivedi 1995) and other ligand–protein interactions (Eftink & Ghiron 1981; Gonzalez-Jimenez et al 1995). In the present study we used the same technique to study the interaction of indomethacin with HSA.

Figure 1 shows the fluorescence emission spectra in the presence of increasing concentrations of indomethacin (top to bottom). It can be seen that the contribution of drug fluorescence emission, at the maximum of protein fluorescence emission (at 340 nm) is negligible. The inset shows the relative fluorescence of indomethacin–albumin after appropriate corrections as a function of the indomethacin/albumin ratio. The basic assumption in this method is that fluorescence quenching is linearly related to the extent of binding and the deviation from linearity, as seen in Figure 1, indicates the degree of dissociation.

In Figure 2, we give the complete binding isotherm for indomethacin to HSA. Under the conditions used, indomethacin was bound with a first stoichiometric binding constant, K_1 at $4{\cdot}01 \times 10^5\,M^{-1}$ (range $3{\cdot}63 \times 10^5\,M^{-1}$–$4{\cdot}46 \times 10^5\,M^{-1}$) as obtained by computer fitting of the data (Vorum et al 1993). Although several molecules of indomethacin may bind to HSA (Ekman et al 1980; Honoré et al 1983; Honoré & Brodersen 1984), only binding of the first molecule was considered in the present study. The affinity obtained was similar to findings from previous studies (Honoré et al 1983; Taira & Terada 1985) which reported affinities of $\sim 10^5\,M^{-1}$ while the study of Ekman et al

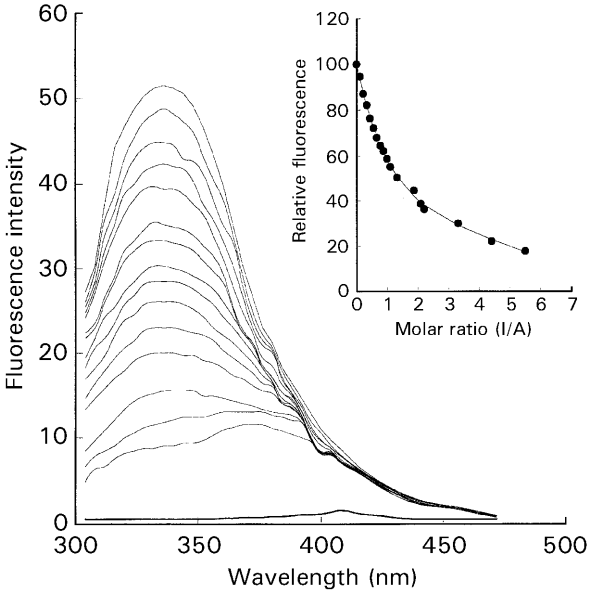

Figure 1. Fluorescence emission spectrum of HSA in the absence and the presence of indomethacin. Molar ratios of indomethacin to HSA were 0·0, 0·11, 0·22, 0·33, 0·44, 0·55, 0·77, 0·88, 1·10, 1·32, 1·98, 2·20, 4·40 and 5·50 (top to bottom). Spectrum of indomethacin alone at 50 μM concentration is represented by the thick line (lowest). Concentration of albumin: 10·1 μM in 55 mM sodium phosphate buffer (pH 7·0), T = 28°C. Inset shows the relation between relative fluorescence and molar ratios of indomethacin to HSA for this interaction.

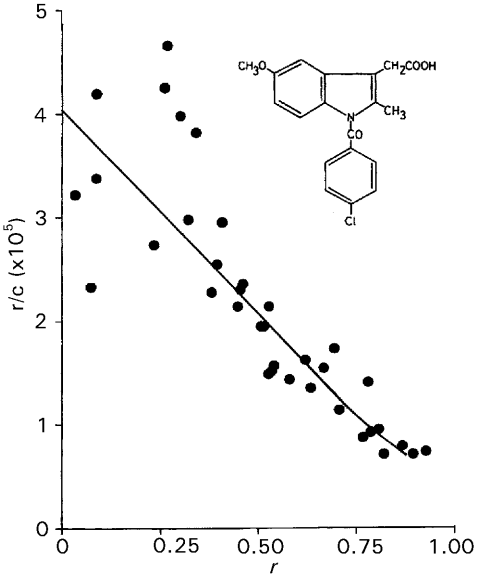

Figure 2. Scatchard plot of the binding of indomethacin to HSA in 55 mM phosphate buffer (pH 7·0) at 28°C. Inset shows the structure of indomethacin.

(1980) reported values that were in the order of 10^5 and $10^6\,M^{-1}$.

To understand the molecular basis of the binding of indomethacin to HSA, this interaction was further studied as a function of temperature, ionic strength and pH. Indomethacin fluorescence quenching profiles obtained under various conditions are shown in Figure 3. The association constants obtained by computer fitting (Vorum et al 1993) and by linear regression of the Scatchard plots are listed in Table 2. The methods largely gave similar results. An increase in temperature resulted in a decrease in the association constant. The association constants given in Table 2 were plotted in a van't Hoff plot (inset of Figure 3A). The enthalpy change, ΔH°, was $-29\,kJ\,mol^{-1}$. The values of the other parameters ΔG° and ΔS° at various temperatures were found to be approximately $-33\,kJ\,mol^{-1}$ and approximately $+3\,J\,K^{-1}\,mol^{-1}$, respectively. Previously reported thermodynamic parameters for the bilirubin–albumin interaction have revealed a high negative enthalpy change, $-57\,kJ\,mol^{-1}$, and a free energy change of $-46\,kJ\,mol^{-1}$ with a small entropy change (Jacobsen 1977), resembling the present results. Our results show that the negative free energy for indomethacin–albumin interaction is mainly due to a negative enthalpy change. Since the entropy change is very small, the contribution of hydrophobic interaction seems to be negligible. It is therefore suggested that a hydrophilic interaction is important for binding of the first molecule of indomethacin to albumin.

The ionic strength of the indomethacin–albumin interaction is shown in the inset of Figure 3B. An increase in the ionic strength decreases the K_1, supporting the idea that electrostatic interactions are crucial in this binding mechanism. Thus, it seems that the single carboxylate group of indomethacin (Figure 2) participates in a salt bridge formation with a positively charged group at its binding site in the protein, a situation analogous to that seen for the binding of bilirubin to albumin (Jacobsen 1977). This observation conflicts with a model presented by Russeva & Mihailova (1996) wherein hydrophobic forces are supposed to be the major forces involved in indomethacin–albumin interaction.

Figure 3C shows the pH dependency of the indomethacin–albumin interaction. The association constant of indomethacin to albumin was shown to be constant between pH 6·5 and 8·0 but decreased on either side of this pH region. The decrease in binding constant at higher pH values is difficult to attribute to any ionizable group either in indomethacin (Figure 2, inset) or in the protein, since indomethacin would remain negatively charged in this pH range and it is unlikely that a positively charged group on the protein would undergo any significant deprotonation between pH 8·0 and 9·4. However, it is possible that this decrease is related to the well known N-B transition in albumin (Peters 1996). A very reasonable explanation for the

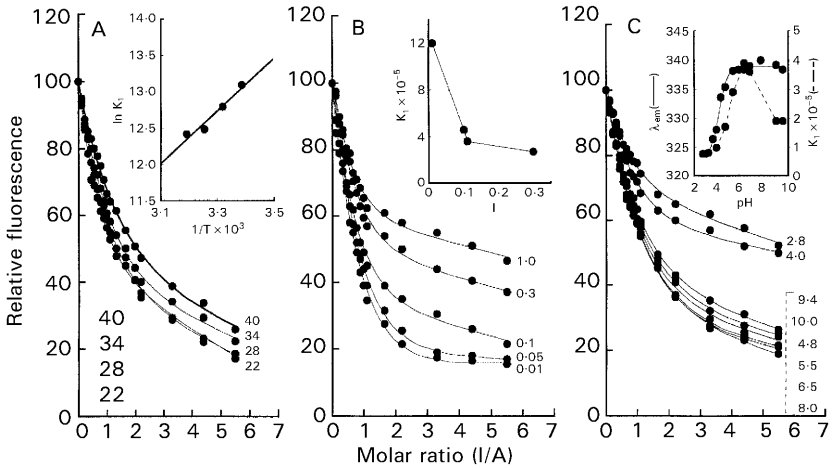

Figure 3. A. The effect of different temperatures on indomethacin binding to HSA: 22°C, 28°C, 34°C and 40°C in 55 mM phosphate buffer (pH 7·0). The inset shows a van't Hoff plot. B. The effect of ionic strength on indomethacin binding to HSA. Ionic strength: 0·01, 0·05, 0·10, 0·30 and 1·0. The inset shows the influence of ionic strength on K_1. C. The effect of pH on indomethacin binding to albumin. pH values: 2·8, 4·0, 4·8, 5·5, 6·5, 8·0, 9·4 and 10·0. The inset shows the plot of pH against K_1 and of pH against emission maxima of HSA.

decrease in K_1 that occurs from pH 6 to pH 4 would be protonation of the sole carboxylate group (pK 4·5) of indomethacin. The plot of pH versus K_1 (inset of Figure 3C) resembles a simple titration curve with a pK of approximately 4·7 strongly supporting this conclusion. The position of the emission maximum of the protein vs pH changes is in parallel with the change in K_1 with pH. In this pH range the well known N-F transition of albumin takes place (Peters 1996). Hence the decrease in K_1 may equally well be explained by the pH-induced N-F conformational change in albumin (Peters 1996).

Table 2. First stoichiometric association constants for binding of indomethacin to HSA at different temperatures, ionic strengths and pH.

Conditions	Association constant, K_1 (M^{-1})
Temperature (°C) (pH = 7·0, I = 0·11 M)	
22	$4{\cdot}87 \times 10^5$ ($6{\cdot}12 \times 10^5$)
28	$3{\cdot}61 \times 10^5$ ($4{\cdot}16 \times 10^5$)
34	$2{\cdot}65 \times 10^5$ ($1{\cdot}68 \times 10^5$)
40	$2{\cdot}48 \times 10^5$ ($0{\cdot}82 \times 10^5$)
Ionic strength (M) (pH = 7·0, T = 28°C)	
0·01	$1{\cdot}20 \times 10^6$ ($2{\cdot}50 \times 10^6$)
0·05	nd ($8{\cdot}49 \times 10^5$)
0·10	$4{\cdot}60 \times 10^5$ ($4{\cdot}45 \times 10^5$)
0·11	$3{\cdot}61 \times 10^5$ ($4{\cdot}16 \times 10^5$)
0·30	$2{\cdot}70 \times 10^5$ ($3{\cdot}18 \times 10^5$)
1·00	nd ($1{\cdot}48 \times 10^5$)
pH (I = 0·11 M, T = 28°C)	
2·8	nd ($1{\cdot}88 \times 10^4$)
4·0	$9{\cdot}80 \times 10^4$ ($6{\cdot}64 \times 10^4$)
4·8	$1{\cdot}70 \times 10^5$ ($2{\cdot}49 \times 10^5$)
5·5	$2{\cdot}90 \times 10^5$ ($2{\cdot}61 \times 10^5$)
6·5	$3{\cdot}90 \times 10^5$ ($3{\cdot}35 \times 10^5$)
7·0	$3{\cdot}61 \times 10^5$ ($4{\cdot}16 \times 10^5$)
8·0	$3{\cdot}90 \times 10^5$ ($3{\cdot}57 \times 10^5$)
9·4	$1{\cdot}90 \times 10^5$ ($2{\cdot}03 \times 10^5$)
10·0	$1{\cdot}90 \times 10^5$ ($2{\cdot}16 \times 10^5$)

The values of association constant were obtained by computer fitting (Vorum et al 1993). Those in parentheses are obtained by Scatchard analysis using the method of Levine (1977).

In earlier studies by others (Roosdorp et al 1977), as well as by some of us (Tayyab & Qasim 1986; Mir et al 1992), modification proved to be very useful in establishing the role of lysine residues in interaction with different ligands. We resorted to chemical modification of lysine residues in order to reveal their role in the interaction. Previous results based on modification of bovine serum albumin have indicated that approximately 80% of lysine residues are exposed in the native protein (Tayyab & Qasim 1986). Carbamylation of albumin attempted in this study is fairly specific for amino groups under the experimental condition used (Nakagawa et al 1972; Stark 1972) and produces no, or little, conformational changes in the protein molecule (Mir et al 1992). Two different procedures were adopted to obtain different carbamylated albumin preparations. In the conventional modification method, albumin was treated with 0·5 M potassium cyanate for varying lengths of time in pilot experiments (data not shown). In another attempt the buried lysine residues were subjected to carbamylation according to a double modification procedure previously described (Mir et al 1992). The strategies used to modify the albumin are summarized in Table 1. The extent of modification determined by the TNBS method (Habeeb 1966) is depicted and it can be seen that after decitraconylation the overall modification was found to be 21%. Thus in this way carbamylation could be

restricted to approximately 20% lysine residues which are not readily accessible in the native protein. All the modified albumin preparations were found to be pure as judged by polyacrylamide gel electrophoresis and size-exclusion chromatography (data not shown). The size-exclusion chromatographic profiles were used to compute the Stokes radii of unmodified and modified albumin derivatives by the method of Ackers (1967) and Laurent & Killander (1964) using various molecular-weight markers. The average of Stokes radii thus obtained by both treatments is summarised in Table 1. The relationship between the percent of modification and the changes in Stokes radii is shown in Figure 4A. The values of frictional ratios obtained according to the method of Andrews (1970) are also tabulated in Table 1. The increase in Stokes radius from 3·39 nm to 4·50 nm and of frictional ratio from 1·26 to 1·68 for native to maximally modified albumin suggests that the protein molecule undergoes considerable conformational changes upon modification. The changes in both parameters are more pronounced in the two highly modified derivatives (77%- and 98%-carbamylated albumin), but remain unaffected up to 23% modification. Moreover, insignificant conformational changes were observed when 21% modification of albumin (having modification at buried lysine residues) was performed in two steps. This suggests that removal of citraconyl groups, modified during the first step of modification (after the second step of carbamylation) was successful. The CD spectra in the far ultraviolet region of native and modified albumin derivatives are shown in Figure 4B. These CD spectra provide further support for the above mentioned results, that some of the ordered structure is lost especially in the two highly carbamylated albumin preparations. However, the loss in ellipticity observed during the citraconylation of albumin (in the first step of the double-modification procedure) is restored after the removal of citraconyl groups in the second step of the carbamylation procedure. The final product of this double-modified albumin (21% carbamylated), being modified only at buried lysine residues, has only insignificant loss of CD signal, thus showing the usefulness of this procedure. This preparation has an almost identical secondary structure to that of native albumin or 23%-carbamylated albumin as obtained by the conventional procedure.

The interaction of indomethacin with these carbamylated albumin preparations is depicted in Figure 5. A significant decrease in relative fluorescence can be seen which depends upon the extent of modification. Interestingly, the 21%-carbamylated albumin preparation shows a high degree of change in relative fluorescence as compared with its counterpart, 23%-carbamylated albumin. This is in close agreement with the Scatchard analysis shown in the inset to Figure 5. The slope decreases with the increasing degree of modification and it was found to be minimum for the double-modified (21% carbamylated) albumin. The K_1 values are summarized in Table 3. A 52-fold decrease in association constant with the 21%-carbamylated albumin derivative strongly indicates the participation of buried lysine residues in the indomethacin–albumin interaction. A 9-fold decrease in K_1 seen with the maximally modified albumin derivative seems to be due to conformational changes rather than the modification of any critical lysine residues. Similarly, the 2- to 7-fold decrease in K_1 with the other modified albumin derivatives is also likely to be due to the varying extent of conformational changes in the albumin molecule upon modification.

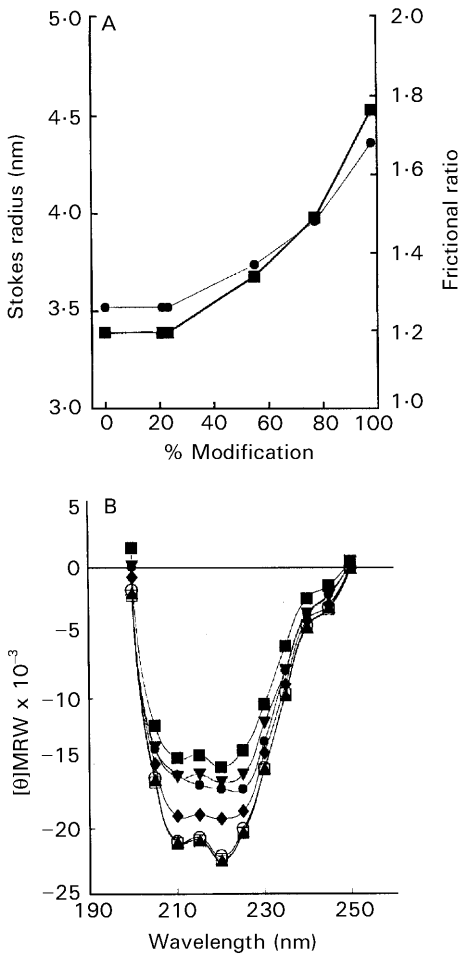

Figure 4. A. Effect of modification of HSA on the Stokes radius (■) and frictional ratio (●). B. Far UV-CD spectra of native and various modified HSAs. ○ Native, ▲21%-carbamylated, □ 23%-carbamylated, ◆ 55%-carbamylated, ● 77%-carbamylated, ■ 98%-carbamylated and ▼ ~80%-citraconylated (before decitraconylation).

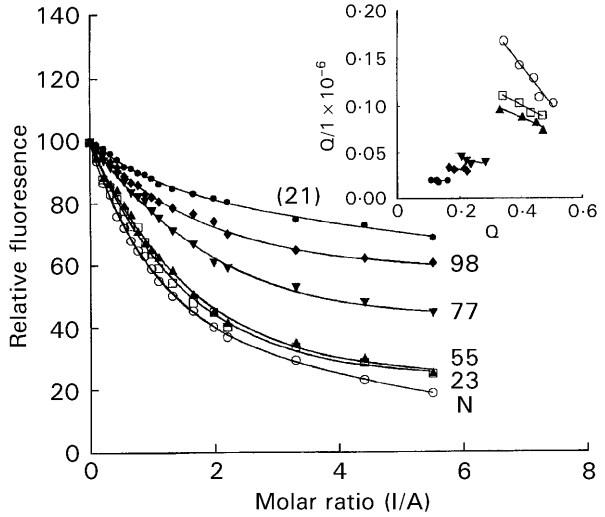

Figure 5. Interaction isotherms of indomethacin to native (○), ● 21%-, □ 23%-, ▲ 55%-, ▼ 77%- and ◆ 98%-carbamylated HSAs. Inset shows a Scatchard plot analysis of the isotherms.

Table 3. Association constants for the binding of indomethacin to modified HSA.

HSA derivative	Association constant, K_1 (M^{-1})
Native HSA	$4{\cdot}16 \times 10^5$
23%-carbamylated HSA	$1{\cdot}99 \times 10^5$
55%-carbamylated HSA	$1{\cdot}51 \times 10^5$
77%-carbamylated HSA	$0{\cdot}58 \times 10^5$
98%-carbamylated HSA	$0{\cdot}45 \times 10^5$
(21%)-carbamylated HSA	$0{\cdot}08 \times 10^5$

In previous reports (Tayyab & Qasim 1987; Mir et al 1992) we have shown, in agreement with others (Jacobsen 1978; Reed & Mackay 1985), that lysine residues of albumin participate in bilirubin–albumin interaction. The possible involvement of lysine-240, proposed by Jacobsen (1978) and lysine-195, proposed by Reed & Mackay (1985) has been discussed in a recent study by Minchiotti et al (1993), based on a Herborn variant of human serum albumin. Several studies on the mode and mechanism of bilirubin–albumin interaction are of interest in the context of the present study (Jacobsen & Brodersen 1983; Lightner et al 1986, 1988). These studies have emerged with the concept of a salt-bridge formation between a carboxylate group of bilirubin and a positively charged (lysine) residue of albumin (Jacobsen 1977; Mir et al 1992). Previous results from our group (Tayyab & Qasim 1987) have shown a critical role of buried lysine residues in the formation of the bilirubin–bovine serum albumin complex. Due to these similarities, we suggest that indomethacin and bilirubin may strongly displace each other if the lysine residues involved are the same.

To explore the possibility of a common binding nature for bilirubin and indomethacin with albumin, we studied the fluorescence enhancement pattern of this complex in the absence and presence of indomethacin. The fluorescence enhancement spectra of the bilirubin–albumin complex, obtained at various ligand–protein molar ratios in the absence and presence of 100 μM indomethacin concentration are shown in Figure 6. The spectrum

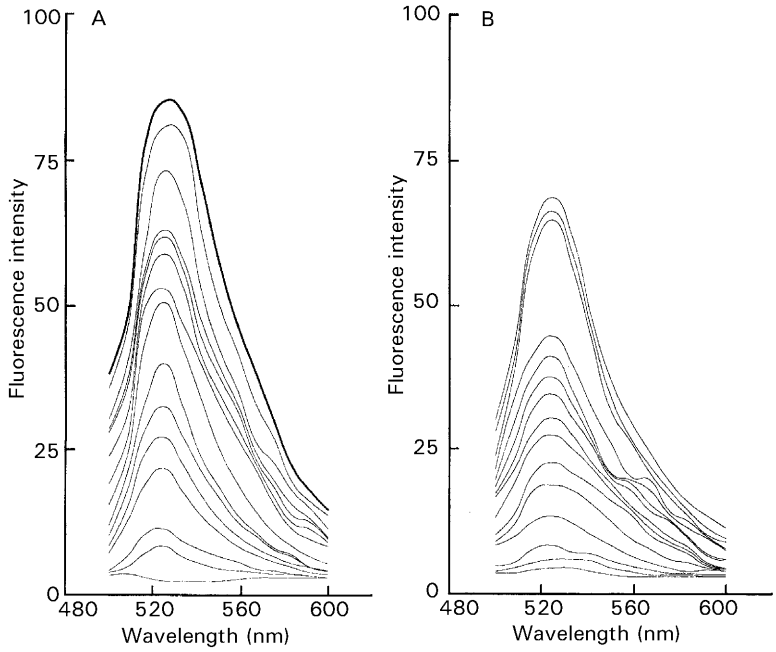

Figure 6. Fluorescence emission spectra of bilirubin–HSA complex in the absence (A) and presence (B) of indomethacin. Molar ratios of bilirubin-HSA (from bottom to top): 0, 0·05, 0·10, 0·15, 0·30, 0·40, 0·50, 0·60, 0·70, 0·80, 0·90, 1·0, 4·0, 1·5 and 2·5. The albumin concentration was 5 μM. All experiments were carried out in 50 mM phosphate buffer (pH 8·0) at 28°C.

is characterized by an emission maximum of 528 nm at an excitation wavelength of 487 nm. Increasing concentrations of bilirubin produce an increase in fluorescence intensity at 528 nm. The contribution of bilirubin or albumin is negligible in the wavelength range 500–600 nm. As can be seen from Figure 6B, the addition of indomethacin to the bilirubin–albumin complex leads to a decreased enhancement of fluorescence at 528 nm due to the release of bilirubin from its complex with albumin. The incubation time for maximum displacement by drug was calculated to be 10 min (data not shown).

The various isotherms of bilirubin–albumin interactions with 0, 20, 50 and 100 μM indomethacin (in the form of fluorescence intensity at 528 nm and molar ratio of bilirubin to albumin) is shown in Figure 7A. In all cases, the decrease in fluorescence intensity at 528 nm at higher bilirubin–albumin molar ratios may be due to precipitation of bilirubin, since the solubility of bilirubin is very low, around 7 nM (Brodersen 1979a). Figure 7B shows a Scatchard plot of the interaction. The value of the association constant for binding of the first molecule of bilirubin to albumin ($5{\cdot}71 \times 10^7\,mol^{-1}$) was found to be in close agreement with previous reports (Berde et al 1984; Minchiotti et al 1993). Upon addition of higher concentrations of indomethacin (i.e. 20, 50 and 100 μM) compared with the albumin concentration at 5 μM, we found that indomethacin was not a strong bilirubin displacer. The value of association constants for the bilirubin–albumin interaction in the presence of 20, 50 and 100 μM indomethacin was $1{\cdot}47 \times 10^7\,M^{-1}$, $6{\cdot}8 \times 10^6\,M^{-1}$ and $5{\cdot}1 \times 10^6\,M^{-1}$, respectively. A decrease in K_1 by a factor of 11 was observed with the addition of 100 μM indomethacin (indomethacin/albumin ratio of 20). This result was in agreement with previous studies by us (Honoré et al 1983) and by Lam et al (1990) who have shown that a concentration of indomethacin up to 12·6 μM is not capable of displacing bilirubin from binding to albumin in serum. Thus, even though the molecular mechanisms of binding of indomethacin and bilirubin to albumin are similar, both involving salt-bridge formation to lysine residues, clearly their primary sites of interaction are different.

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References

Ackers, G. K. (1967) Molecular sieve studies of interacting protein systems. I. Equations for transport of associating systems. J. Biol. Chem. 242: 3026–3034

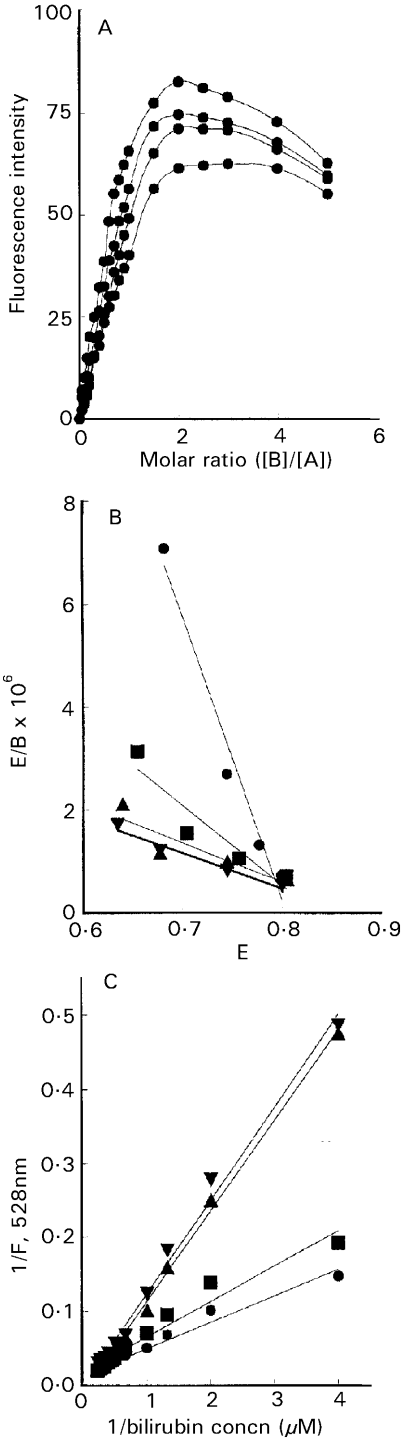

Figure 7. A. Bilirubin displacement isotherms in the presence of 0, 20, 50 and 100 μM (from top to bottom) indomethacin. B. Scatchard plot analysis of the data shown in A for ● 0, ■ 20, ▲ 50 and ▼ 100 μM indomethacin. C. Double reciprocal plot for indomethacin-induced bilirubin displacement for ● 0, ■ 20, ▲ 50 and ▼ 100 μM indomethacin. The plots were transformed from the data given in A (in bilirubin–HSA molar ratio range 0·05–1·0). The HSA concentration was 5 μM. All experiments were performed in 50 mM phosphate buffer (pH 8·0) at 28°C.

Andrews, P. (1970) Estimation of molecular size and molecular weights of biological compounds by gel filtration. Methods Biochem. Anal. 18: 1–53

Berde, C. B., Benitz, W. E., Rasmussen, L. F., Kerner, J. A., Johnson, J. D., Wennberg, R. P. (1984) Bilirubin binding in the plasma of newborns: critical evaluation of a fluorescence quenching method and comparison to the peroxidase method. Pediatr. Res. 18: 349–354

Bratlid, D., Cashore, W. J., Oh, W. (1984) Effect of acidosis on bilirubin deposition in rat brain. Pediatrics 73: 431–434

Brodersen, R. (1979a) Bilirubin. Solubility and interaction with albumin and phospholipid. J. Biol. Chem. 254: 2364–2369

Brodersen, R. (1979b) Binding of bilirubin to albumin. CRC Crit. Rev. Clin. Lab. Sci. 11: 305–399

Brodersen, R., Honoré, B., Larsen, F. G. (1984) Serum albumin – a non-saturable carrier. Acta Pharmacol. Toxicol. 54: 129–133

Brodersen, R., Vorum, H., Robertson, A. F. (1995) Calculation of the bilirubin displacing effect of a drug from drug binding data obtained in the presence and absence of bilirubin. J. Pharm. Sci. 84: 148–151

Carter, D. C., Ho, J. X. (1994) Structure of serum albumin. Adv. Protein Chem. 45: 153–203

Cashore, W. J., Oh, W. (1982) Unbound bilirubin and kernicterus in low-birth-weight infants. Pediatrics 69: 481–485

Eftink, M. R., Ghiron, C. A. (1981) Fluorescence quenching studies with proteins. Anal. Biochem. 114: 199–227

Ekman, B., Sjödin, T., Sjöholm, I. (1980) Binding of drugs to human serum albumin-XV. Characterization and identification of the binding sites of indomethacin. Biochem. Pharmacol. 29: 1759–1765

Gonzalez-Jimenez, J., Corrales, S., Moreno, F., Garcia Blanco, F. (1995) Study of the interaction between xylazine and bovine serum albumin by fluorescence quenching measurements. Chem. Pharm. Bull. 43: 1949–1952

Habeeb, A. F. (1966) Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal. Biochem. 14: 328–336

He, X. M., Carter, D. C. (1992) Atomic structure and chemistry of human serum albumin. Nature 358: 209–215

Honoré, B., Brodersen, R. (1984) Albumin binding of anti-inflammatory drugs. Utility of a site-oriented versus a stoichiometric analysis. Mol. Pharmacol. 25: 137–150

Honoré, B., Brodersen, R., Robertson, A. (1983) Interaction of indomethacin with adult human albumin and neonatal serum. Dev. Pharmacol. Ther. 6: 347–355

Jacobsen, J. (1977) Studies of the affinity of human serum albumin for binding of bilirubin at different temperatures and ionic strength. Int. J. Pept. Protein Res. 9: 235–239

Jacobsen, C. (1978) Lysine residue 240 of human serum albumin is involved in high-affinity binding of bilirubin. Biochem. J. 171: 453–459

Jacobsen, J., Brodersen, R. (1983) Albumin-bilirubin binding mechanism. J. Biol. Chem. 258: 6319–6326

Jacobsen, J., Wennberg, R. P. (1974) Determination of unbound bilirubin in the serum of newborns. Clin. Chem. 20: 783

Kragh-Hansen, U. (1981) Molecular aspects of ligand binding to serum albumin. Pharmacol. Rev. 33: 17–53

Lam, B. C., Wong, H. N., Yeung, C. Y. (1990) Effect of indomethacin on binding of bilirubin to albumin. Arch. Dis. Child. 65: 690–691

Laurent, T. C., Killander, J. (1964) A theory for gel filtration and its experimental verification. J. Chromatogr. 14: 317–330

Levine, R. L. (1977) Fluorescence-quenching studies of the binding of bilirubin to albumin. Clin. Chem. 23: 2292–2301

Lightner, D. A., Reisinger, M., Landen, G. L. (1986) On the structure of albumin-bound bilirubin. Selective binding of intramolecularly hydrogen-bonded conformational enantiomers. J. Biol. Chem. 261: 6034–6038

Lightner, D. A., Wijekoon, W. M., Zhang, M. H. (1988) Understanding bilirubin conformation and binding. Circular dichroism of human serum albumin complexes with bilirubin and its esters. J. Biol. Chem. 263: 16669–16676

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275

Mason, R. W., McQueen, E. G. (1974) Protein binding of indomethacin: binding of indomethacin to human plasma albumin and its displacement from binding by ibuprofen, phenylbutazone and salicylate, in vitro. Pharmacology 12: 12–19

Minchiotti, L., Galliano, M., Zapponi, M. C., Tenni, R. (1993) The structural characterization and bilirubin-binding properties of albumin Herborn, a [Lys-240 → Glu] albumin mutant. Eur. J. Biochem. 214: 437–444

Mir, M. M., Fazili, K. M., Abul Qasim, M. (1992) Chemical modification of buried lysine residues of bovine serum albumin and its influence on protein conformation and bilirubin binding. Biochim. Biophys. Acta 1119: 261–267

Nakagawa, Y., Capetillo, S., Jirgensons, B. (1972) Effect of chemical modification of lysine residues on the conformation of human immunoglobulin G. J. Biol. Chem. 247: 5703–5708

Peters, T. (1996) All About Albumin. Biochemistry, Genetics, and Medical Applications. Academic Press, San Diego

Reed, R. G., Mackay, C. M. (1985) Locating the bilirubin binding site of human serum albumin. Fed. Proc. Fed. Am. Soc. Exp. Biol. 44: 1618

Robertson, A., Karp, W., Brodersen, R. (1991) Bilirubin displacing effect of drugs used in neonatology. Acta Paediatr. Scand. 80: 1119–1127

Roosdorp, N., Wann, B., Sjöholm, I. (1977) Correlation between arginyl residue modification and benzodiazepine binding to human serum albumin. J. Biol. Chem. 252: 3876–3880

Russeva, V., Mihailova, D. (1996) Modelling of indometacin and diclofenac sodium binding to the molecule of human serum albumin. Arzneim. Forsch. 46: 288–292

Silverman, W. A., Andersen, D. H., Blanc, W. A., Crozier, D. N. (1956) A difference in mortality rate and incidence of kernicterus among premature infants alloted to two prophylactic antibacterial regimens. Pediatrics 18: 614–625

Stark, G. R. (1972) Use of cyanate for determining NH_2-terminal residues in proteins. Methods Enzymol. 25: 579–584

Taira, Z., Terada, H. (1985) Specific and non-specific ligand binding to serum albumin. Biochem. Pharmacol. 34: 1999–2005

Tayyab, S., Qasim, M. A. (1986) A correlation between changes in conformation and molecular properties of bovine serum albumin upon succinylation. J. Biochem. 100: 1125–1136

Tayyab, S., Qasim, M. A. (1987) Influence of succinylation of bovine serum albumin on its conformation and bilirubin binding. Biochim. Biophys. Acta 913: 359–367

Tayyab, S., Trivedi, V. D. (1995) Binding of bilirubin to goat serum albumin: Determination of binding constants. Biochem. Educ. 23: 98–100

Vorum, H., Bang, D., Fisker, K. (1993) New procedure for analysing drug binding data, exemplified by warfarin binding to human serum albumin. Biochem. Pharmacol. 45: 1715–1720