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Characterization of thyroxine–albumin binding using high-performance affinity chromatography

I. Interactions at the warfarin and indole sites of albumin

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ABSTRACT

A high-performance affinity column containing immobilized human serum albumin (HSA) was used to study the binding of thyroxine at the warfarin and indole sites of HSA. Frontal analysis, using *R*-warfarin and L-tryptophan as probes for these sites, demonstrated that the immobilized HSA had binding behavior equivalent to that observed for HSA in solution. By injecting *R*-warfarin or L-tryptophan in the presence of excess thyroxine, it was found that thyroxine was binding directly to both types of site. The warfarin and indole sites had relatively strong binding for thyroxine, with association constants at 37°C of $1.4 \cdot 10^5$ and $5.7 \cdot 10^5$ M^{-1}, respectively. The value of ΔG for these sites ranged from -7 to -8 kcal/mol and had a significant entropy component. The techniques used in this study are not limited to thyroxine–HSA interactions, but should also be valuable in examining the site-specific binding of other drugs and hormones to HSA.

INTRODUCTION

Protein binding is an important factor in the transport and release of many drugs and hormones in the body. One compound for which this protein binding is significant is thyroxine (L-3,5,3′,5′-tetraiodothyronine). Thyroxine is the major thyroid hormone in the body. It is important in regulating a number of biological processes, including oxygen consumption, protein synthesis, carbohydrate metabolism, growth and development, and maintenance of body weight. Of the total thyroxine in blood, more than 99.9% is protein-bound. This binding occurs with three serum proteins: thyroxine-binding globulin, thyroxine-binding prealbumin, and human serum albumin (HSA) [1,2].

Of these various thyroxine–protein interactions, the binding of thyroxine to HSA is the least understood. Estimates vary concerning both the number of thyroxine-binding sites on HSA and the association constants for these sites. Most studies agree that HSA has one or two strong binding sites for thyroxine plus a number of weaker binding sites. The association constants assigned to the strong binding sites are typically between 10^5 and 10^6 M^{-1} [3–7], but the exact values are still unclear. In addition, the location of these sites on HSA is not known and little work has been done to determine the energetics or temperature dependence of thyroxine binding at these sites [7].

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Many other compounds that interact with HSA appear to bind at a series of relatively well defined regions on the protein's surface. The two most important of these regions are the warfarin–azapropazone site and the indole–benzodiazepin site [8–10]. As their names imply, these sites are characterized by their ability to bind certain solutes, such as warfarin or indole-containing compounds. Together, these two sites are believed to be involved in the interactions of most compounds with HSA [11].

We have studied the binding of thyroxine at the warfarin and indole sites by using a high-performance affinity column containing immobilized HSA. Thyroxine was applied to this column in the presence of two different probe molecules, *R*-warfarin and L-tryptophan, which were used because they are believed to have 1:1 binding at the warfarin and indole sites of HSA, respectively, and have known association constants for these interactions [12–14]. Such properties make these compounds potentially useful in examining the binding of thyroxine or other compounds to HSA.

In the first part of this study frontal analysis was used to determine the association constants for the binding of *R*-warfarin and L-tryptophan to the immobilized HSA column. These constants were compared with solution values, in order to determine how well the immobilized HSA models the binding of compounds to HSA in solution. The same column was then used in zonal elution experiments to examine the competition of thyroxine with *R*-warfarin and L-tryptophan for the warfarin and indole sites of HSA because if competition occurs, this approach will allow the association constants for thyroxine at each site to be measured. By performing similar experiments at different temperatures, information on the energetics of these interactions could also be obtained.

THEORY

Frontal analysis

The theory for the determination of equilibrium constants by affinity chromatography has been well described [15–18]. One method by which such constants can be measured is frontal analysis, in which a solution with a known concentration of pure solute is continuously applied to a column containing a fixed amount of immobilized ligand. As the ligand becomes saturated, the amount of analyte eluting from the column gradually increases, forming a characteristic breakthrough curve. The mean position of this curve is related to the concentration of applied analyte, the amount of ligand present, and the association constants for the system.

If a solute (E) binds to a single type of immobilized ligand site (L), the reactions that describe binding of E to the column are as follows:

$$E + L \overset{K_3}{\rightleftharpoons} E\text{–}L \tag{1}$$

$$K_3 = \frac{\{E\text{–}L\}}{[E]\,\{L\}} \tag{2}$$

where K_3 is the association constant for the binding of E to L, and [E] is the concentration of solute applied to the column. {L} and {E–L} represent the surface concentrations of the ligand and ligand–solute complex, respectively. For this system, the following equation can be used to relate the true number of binding sites on the column (m_L) to the apparent moles of solute required to reach the mean position of the breakthrough curve (m_{Lapp}):

$$\frac{1}{m_{Lapp}} = \frac{1}{K_3 m_L\,[E]} + \frac{1}{m_L} \tag{3}$$

Equivalent expressions have been derived previously by others [15]. Eqn. 3 predicts that a plot of $1/m_{Lapp}$ *versus* 1/[E] for a system with single-site binding will give a straight line with a slope of $1/K_3 m_L$ and an intercept of $1/m_L$. The value of K_3 can be determined directly from this plot by calculating the ratio of the intercept to the slope. The value of m_L can be obtained from the inverse of the intercept. In this study, the values of m_L and K_3 will be used to determine the number of warfarin and indole binding sites on the HSA column and the association constants of these sites for the probe molecules.

Zonal elution

In the technique of zonal elution, a known concentration of an inhibitor (I) is continuously applied to a column containing an immobilized ligand (L) while injections of a solute (E) are made. If I and E compete at a single site on L and E binds to no other sites on the matrix, then the following reactions take place in the column:

$$E + L \overset{K_3}{\rightleftharpoons} E\text{–}L \quad (4)$$

$$I + L \overset{K_2}{\rightleftharpoons} I\text{–}L \quad (5)$$

$$K_2 = \frac{\{I\text{–}L\}}{[I]\,\{L\}} \quad (6)$$

where K_2 is the association constant for the binding of I to L at the site of competition, [I] is the concentration of inhibitor applied to the column and {I–L} is the surface concentration of the inhibitor–ligand complex at the analyte's binding site. All other terms are the same as defined previously. For this set of reactions, the following relationship has been derived to describe the retention of E as [I] is varied [19,20]:

$$\frac{1}{k'_E} = \frac{K_2 V_m [I]}{K_3 m_L} + \frac{V_m}{K_3 m_L} \quad (7)$$

In eqn. 7, k'_E is the capacity factor for solute E, V_m is the void volume of the column, and [I] is the concentration of inhibitor applied to the column. For a system with single-site competition, this equation predicts that a plot of $1/k'_E$ *versus* [I] will yield a linear relationship with a slope ($K_2 V_m / K_3 m_L$) and an intercept of ($V_m / K_3 m_L$). By calculating the ratio of the slope to the intercept for such a plot, the value of K_2 an be directly obtained. One advantage of eqn. 7 is that it allows the association constant for I to be measured only at the site at which I and E compete. This is useful in studying a system with multisite interactions, such as the binding of thyroxine to HSA, because it allows individual sites to be monitored by injecting appropriate probe molecules (*i.e.* *R*-warfarin or L-tryptophan) into the presence of a solution containing the inhibitor of interest (*i.e.* thyroxine).

Temperature studies

Using association constants measured at several temperatures by either zonal elution or frontal analysis, the free energies can be obtained as follows:

$$\ln K = -\Delta H/R\,T + \Delta S/R \quad (8)$$

$$\Delta G = -R\,T \ln K \quad (9)$$

where K is the association constant of interest, T is the absolute temperature, R is the ideal gas law constant, ΔG is the total free energy change of the reaction, ΔH is the change in enthalpy and ΔS is the change in entropy. Eqn. 8 predicts that a plot of ln K *versus* $1/T$ will yield a linear relationship with a slope of $-\Delta H/R$ and an intercept of $\Delta S/R$. This provides the values of ΔH and ΔS for the reaction. By using eqn. 9 and the values of K and T, ΔG for the reaction may also be obtained [21].

EXPERIMENTAL

Reagents

HSA (Cohn fraction V, 99% globulin-free) and L-tryptophan were from Sigma (St. Louis, MO, USA). L-Thyroxine was from Eastman Kodak (Rochester, NY, USA). The *R*-warfarin was generously provided by DuPont Pharmaceuticals (Wilmington, DE, USA). The Nucleosil Si-1000 (7 μm particle diameter, 1000 Å pore size) was obtained from Alltech (Deerfield, IL, USA). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). All other chemicals and biochemicals used were of the purest grades available. All solutions were prepared using water from a Nanopure water system (Barnstead, Dubuque, IA, USA).

Apparatus

The chromatographic system consisted of one CM3000 isocratic pump, one CM4000 gradient pump, and one SM3100 UV–VIS variable-wavelength detector from Milton Roy (Riviera Beach, FL, USA). Samples were injected using a Rheodyne 7012 injection valve (Cotati, CA, USA) equipped with a PhaseSep event marker (Phase Separations, Queensferry, UK). Data were collected using a Milton Roy Chromlink interface

and LCadvantage software. Chromatograms were processed by programs written in Microsoft QuickBASIC (Redmond, WA, USA) using double-precision logic. Columns and mobile phases were temperature-controlled using an Isotemp 9100 circulating water bath (Fisher Scientific, Pittsburgh, PA, USA). Columns were prepared using an HPLC column slurry packer from Alltech (Deerfield, IL, USA).

Methods

HSA column. Diol-bonded silica was prepared from Nucleosil Si-1000 using previously published procedures [22]. The diol coverage of the Nucleosil prior to activation was 20 ± 3 μmol (± 1 S.D.) per gram of silica, as determined in duplicate by the periodate oxidation method [23,24]. HSA was immobilized onto the diol-bonded Nucleosil using the Schiff base method [22], with the silica being sonicated under vacuum for 15 min at the beginning of the activation and immobilization steps [25]. After sonication in the immobilization step, 55 mg of HSA and 100 mg of sodium cyanoborohydride per gram of silica were added to the silica suspension. The resulting mixture was then shaken at 4°C for five days [22].

After the immobilization step, any aldehyde groups remaining on the matrix were reduced by adding 90 mg of sodium borohydride per gram of silica [22] and shaking the mixture for 2 h at room temperature. The silica was then centrifuged, washed with 2 *M* sodium chloride and 0.067 *M* phosphate buffer (pH 7.4), and stored at 4°C until further use. A portion of the silica was washed three times with deionized water, vacuum-dried at room temperature, and assayed in duplicate for protein content by the BCA method [26], using HSA as the standard and diol-bonded silica as the blank.

Chromatography. The HSA and diol-bonded silica matrices were downward slurry-packed at 240 bar into two separate 45 mm × 4.1 mm I.D. columns of a previously published design [27]. Both columns were enclosed in a water jacket for temperature control. All studies, except those examining the temperature dependence of thyroxine–HSA binding, were performed at 37 ± 0.1°C. All mobile phases and packing solvents used in this work were prepared using 0.067 *M* potassium phosphate buffer (pH 7.4). Prior to use, the mobile phases were filtered using a 0.45-μm cellulose acetate filter and degassed under vacuum for 10 min. Elution of the L-tryptophan and *R*-warfarin was detected by monitoring absorbance at 290 and 310 nm, respectively.

Frontal analysis was performed by continuously applying solutions containing L-tryptophan or *R*-warfarin to the HSA column at flow-rates of 0.1–0.5 ml/min. Concentrations of $5 \cdot 10^{-6}$ to $1 \cdot 10^{-4}$ *M* of L-tryptophan were used and concentrations of $2 \cdot 10^{-6}$ to $9 \cdot 10^{-6}$ *M* of *R*-warfarin were used. Retained L-tryptophan and *R*-warfarin were eluted by later applying phosphate buffer (pH 7.4) to the column. Application of these solutions to the column was controlled using a Rheodyne 7012 switching valve. The amount of L-tryptophan or *R*-warfarin needed to saturate the column was determined by integration of the resulting breakthrough curves [28]. Contributions due to the system void volume and non-specific binding were corrected by performing similar measurements on the diol-bonded silica column. The measured breakthrough volumes of the *R*-warfarin and L-tryptophan on the diol-bonded silica column were within 2 and 9%, respectively, of the calculated system void volume.

Zonal elution in the thyroxine studies was performed by applying mobile phases containing $7 \cdot 10^{-7}$ to $9 \cdot 10^{-6}$ *M* L-thyroxine in phosphate buffer (pH 7.4) to the HSA column while 20-μl injections of *R*-warfarin or L-tryptophan were made. All mobile phases used in this study were prepared from a stock solution containing $2.5 \cdot 10^{-5}$ *M* thyroxine. This stock solution was prepared in 0.067 *M* potassium phosphate buffer (pH 11.0) and slowly adjusted to pH 7.4 by the addition of 0.067 *M* potassium phosphate buffer (pH 2.5) while being held at 40–50°C. This solution was then diluted to the desired concentration using phosphate buffer (pH 7.4). Thyroxine solutions prepared in this manner were found to be stable for at least one week when stored at room temperature. The injected samples were prepared

by dissolving $4 \cdot 10^{-5}$ *M* *R*-warfarin or $3 \cdot 10^{-4}$ *M* L-tryptophan in the L-thyroxine solution being used as the mobile phase. The retention times of the warfarin and tryptophan peaks were calculated from their central moments [29]. The column void volume was determined by making similar injections onto the diol-bonded silica column. The retention times and column void time were corrected for the extra-column volume of the system, as determined by making injections of *R*-warfarin or L-tryptophan with no column present in the system.

Zonal elution experiments examining the competition of *R*-warfarin and L-tryptophan for the immobilized HSA were performed at a flow-rate of 0.5 ml/min, using 20-μl injections of $1.5 \cdot 10^{-4}$ *M* *R*-warfarin dissolved in the appropriate mobile phase. The mobile phases consisted of the phosphate buffer (pH 7.4) containing 0 to $3.0 \cdot 10^{-5}$ *M* L-tryptophan. The retention of *R*-warfarin was determined as in the thyroxine zonal elution studies.

RESULTS AND DISCUSSION

Warfarin and tryptophan binding to immobilized HSA

The initial properties of the HSA matrix used in this study are summarized in Table I. The coverage of HSA on the matrix was *ca.* 0.3 monolayers. The specific activity of this matrix, as determined by frontal analysis, indicated that 53% of the warfarin sites and 12% of the indole sites on the matrix were active, assuming 1:1 binding of *R*-warfarin and L-tryptophan with HSA. This relatively low activity is common with immobilized proteins and is the result of such factors as steric hindrance, denaturation, or improper orientation of protein attached to the matrix [18]. The lower specific activity obtained at the indole site may reflect a greater sensitivity of this site to immobilization effects. However, it should be noted that full recovery of HSA activity was not required for this study since the values of K_3 and K_2 could be obtained from eqns. 3 and 7, respectively, without knowledge of the actual number of binding sites on the HSA column.

TABLE I

INITIAL PROPERTIES OF THE IMMOBILIZED HSA MATRIX

Property	Value (± 1 S.D.)
HSA immobilized (nmol/g silica)	196 (± 1)
Binding capacity (nmol/g silica)	
L-Tryptophan	24.0 (± 0.9)
R-Warfarin	104 (± 6)
Specific activity (mol/mol HSA)	
L-Tryptophan	0.12 (± 0.01)
R-Warfarin	0.53 (± 0.03)

The first part of this work examined the effectiveness of the immobilized HSA matrix in modeling the binding behavior of HSA in solution. This was studied by measuring the association constants on a column containing the immobilized HSA matrix using compounds having well characterized interactions with HSA in solution. As already mentioned, *R*-warfarin and L-tryptophan were chosen for this work since each binds to a single, distinct site on HSA, with no allosteric interactions between their respective binding regions [12–14,30]. The results obtained by frontal analysis for *R*-warfarin on the immobilized HSA column at 37°C are given in Fig. 1. As shown, a plot of $1/m_{L_{app}}$ *versus* 1/[*R*-warfarin] gave a linear relationship with a correlation coefficient of 0.9998 over the four concentrations studied. According to eqn. 3, this linear behavior

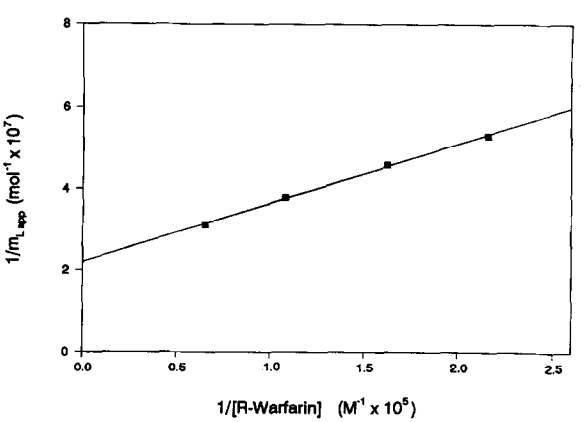

Fig. 1. Frontal analysis results for *R*-warfarin on the immobilized HSA column.

indicated that the *R*-warfarin was binding to a single type of site on the immobilized HSA. This same model has been proposed to describe the binding of *R*-warfarin to HSA in solution [12].

The frontal analysis results for L-tryptophan on the HSA column at 37°C are given in Fig. 2. A plot of $1/m_{Lapp}$ *versus* 1/L-tryptophan] gave a linear relationship, with a correlation coefficient of 0.9999 over the four concentrations studied. From eqn. 3, this indicated that L-tryptophan also exhibited single-site binding to the immobilized HSA matrix. This behavior agrees with previous work examining the binding of L-tryptophan ot HSA in solution [14] and to HSA immobilized onto a low-performance affinity column [31].

To confirm that *R*-warfarin and L-tryptophan were binding to separate sites on the immobilized HSA, injections of *R*-warfarin were made in the presence of mobile phases containing different concentrations of L-tryptophan. The change in *R*-warfarin retention with [L-tryptophan] was then examined to see if these compounds competed for HSA binding sites. It was found that there was no significant change (*i.e.* less than 1.5%) in the retention of *R*-warfarin over the concentration range from 0 to $3.8 \cdot 10^{-5}$ *M* L-tryptophan. This indicated that the *R*-warfarin and L-tryptophan were binding to two separate sites on the immobilized HSA. This observation is also in agreement with solution studies [11,32].

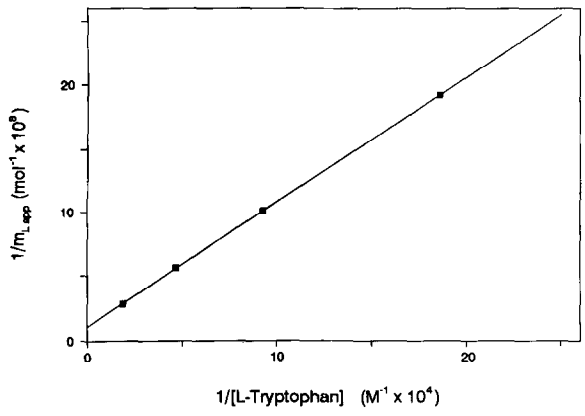

Fig. 2. Frontal analysis results for L-tryptophan on the immobilized HSA column.

From the slopes and intercepts of the best-fit lines in Fig. 1 and 2, the association constants for the binding of *R*-warfarin and L-tryptophan to the immobilized HSA were calculated. The results are given in Table II. The precision of the association constants measured in this experiment ranged from ± 1% for *R*-warfarin to ± 4% for L-tryptophan. The association constants obtained for *R*-warfarin and L-tryptophan differed by less than 1 and 15%, respectively, from those obtained previously by equilibrium dialysis. These results indicated that the HSA column was an effective model in studying the binding of compounds at the warfarin and indole sites of HSA.

TABLE II

ASSOCIATION CONSTANTS FOR THE BINDING OF *R*-WARFARIN AND L-TRYTOPHAN TO HSA AT 37°C

Association constant (M^{-1})[a]	Method	Reference
R-Warfarin		
$2.47 \cdot 10^5$ ($\pm$ $0.03 \cdot 10^5$)	Frontal analysis	Present work
$2.47 \cdot 10^5$	Equilibrium dialysis	Larsen *et al.* [36]
$3.3 \cdot 10^5$	Zonal clution	Lagercrantz and co-workers [31,35]
L-Tryptophan		
$1.10 \cdot 10^4$ ($\pm$ $0.04 \cdot 10^4$)	Frontal analysis	Present work
$1.3 \cdot 10^4$	Equilibrium dialysis	McMenamy and Seder [14]
$1.1 \cdot 10^4$	Zonal elution	Lagercrantz and co-workers [31,35]

[a] Values in parentheses represent ± 1 S.D. All association constants were measured at pH 7.4 except those given in ref. 14 (pH 7.6).

Thyroxine binding to immobilized HSA

Once it had been confirmed that the immobilized HSA column could be used to model the interactions of compounds at the warfarin and indole sites of HSA, this column was next used to examine the binding of thyroxine as these sites. This was done by performing zonal elution experiments in which thyroxine was used as the competing agent and either *R*-warfarin or L-tryptophan were injected onto the system as a probe molecule. The resulting plots of $1/k'$ *versus* [thyroxine] for *R*-warfarin and L-tryptophan at 37°C are shown in Fig. 3. Both plots were linear over the concentration range studied, with correlation coefficients of 0.9964 and 0.9917, respectively. The intercepts of both plots were in agreement with values predicted from the frontal analysis studies. According to eqn. 7, the linearity of these plots indicated that thyroxine had single-site competition with both of these probe molecules. Competition between thyroxine and L-tryptophan for a common site on HSA has also been suggested in previous solution studies [33].

It is interesting to note in Fig. 3 that there is excellent agreement between the experimentally measured intercepts and the best-fit values. In both plots there was no significant difference (*i.e.* less than 1%) obtained in these values. From eqn. 7, this indicates that the equilibrium constants for the probe molecules, and their number of binding sites, were not affected by changes in the thyroxine concentration. This supports a model in which there is direct, rather than allosteric, competition between thyroxine and *R*-warfarin or L-tryptophan. Since thyroxine is known to bind at *ca.* five or six sites on HSA [2], these data also suggest that thyroxine binding at the warfarin and indole sites is not affected allosterically by the binding of thyroxine at other regions on HSA over the concentration range used in this study.

Using eqn. 7 and the best-fit parameters of Fig. 3, the association constants for thyroxine at the warfarin and indole sites were determined. The results are given in Table III. The association constants measured at these sites had precisions of ± 7% and ± 14%, respectively. Both sites had a relatively high affinity for thyroxine, with binding at the indole site being approximately four times stronger than at the warfarin site under the given experimental conditions.

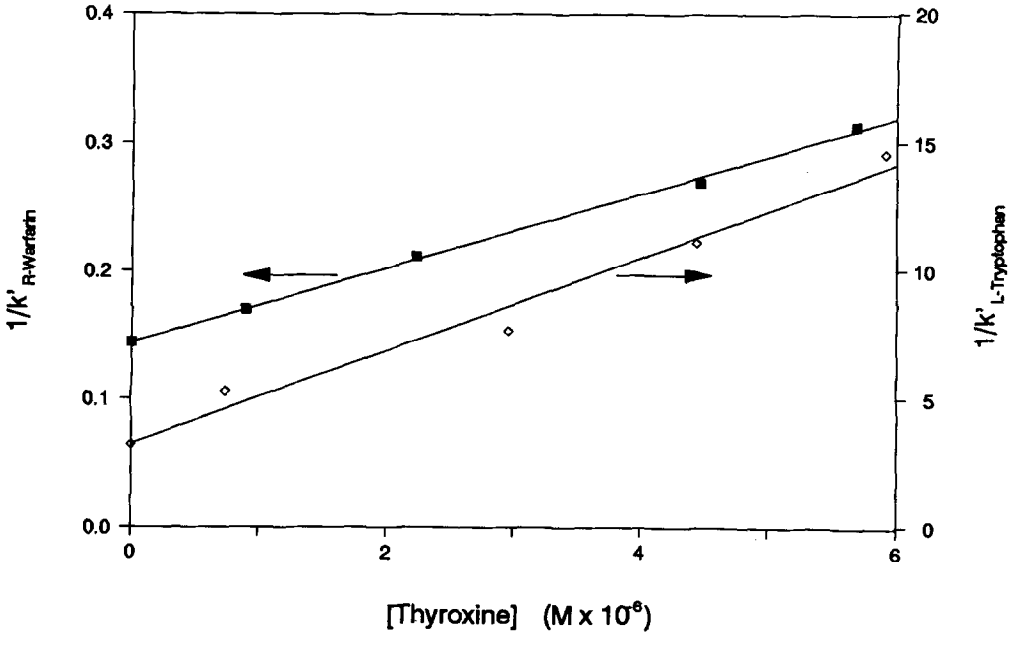

Fig. 3. Competitive binding of thyroxine with *R*-warfarin (■) and L-tryptophan (◇) on the immobilized HSA column.

TABLE III

ASSOCIATION CONSTANTS FOR THE BINDING OF L-THYROXINE TO HSA AT 37°C

Values measured at pH 7.4 in 0.067 M phosphate buffer. Numbers in parentheses represent ± 1 S.D.

Binding region	Association constant (M^{-1})
Warfarin site	$1.4 \cdot 10^5$ ($\pm$ $0.1 \cdot 10^5$)
Indole site	$5.7 \cdot 10^5$ ($\pm$ $0.8 \cdot 10^5$)

The temperature dependence of thyroxine binding at the warfarin and indole sites was examined by performing several zonal experiments between 4 and 45°C. At each temperature studied, the same linear behaviour as seen in Fig. 3 was noted. The change in the values of ln K_2 with $1/T$ are shown in Fig. 4 for the two sites. For both the indole and warfarin sites, the plots in Fig. 4 gave linear behavior, with correlation coefficients of 0.8900 and 0.9586, respectively. From these plots, it was found that the binding strength of thyroxine at the indole and warfarin sites changed only slightly with temperature. These results are in agreement with earlier solution studies examining the overall temperature dependence of thyroxine–HSA binding [7].

The association constants measured at both 37°C and other temperatures agree well with those determined in previous work examining thyroxine–HSA binding. For example, the association constants obtained for thyroxine at both the indole and warfarin sites fall within the range 10^5–10^6 M^{-1} reported for the high-affinity binding sites of thyroxine on HSA [3–7]. Although the highest affinity site for thyroxine–HSA binding has usually been assigned an association constant of $1 \cdot 10^6$ M^{-1} at 37°C, in work by Sterling [6] the high-affinity site was determined to be only $5 \cdot 10^5$ M^{-1}. This is very close to the value measured in this work for the indole site. An association constant for the high affinity site of less than 10^6 M^{-1} has also been measured by Snyder *et al.* [34]. In studies by Tabachnick [3], a model was proposed in which HSA has two primary binding sites for thyroxine, with an average association

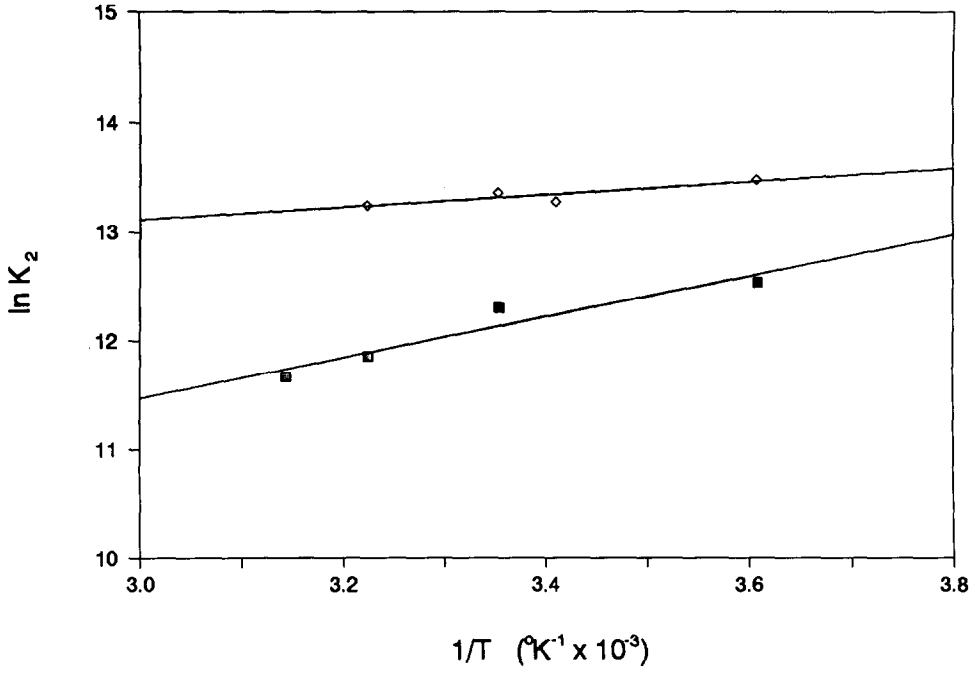

Fig. 4. Effect of temperature on the binding of thyroxine at the indole (◇) and warfarin (■) sites of HSA.

TABLE IV

THERMODYNAMICS OF THYROXINE–HSA BINDING AT THE WARFARIN AND INDOLE SITES

Values measured at pH 7.4 in 0.067 *M* phosphate buffer. Numbers in parentheses represent ± 1 S.D.

Binding region	ΔG at 37°C (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol K)
Warfarin site	− 7.3 (± 1.1)	− 3.7 (± 0.8)	12 (± 3)
Indole site	− 8.2 (± 0.6)	− 1.2 (± 0.4)	23 (± 1)

constant of $3 \cdot 10^5\ M^{-1}$ at 30°C. By comparison, in this work the average association constant at 30°C for thyroxine at the indole and warfarin sites was calculated to be $3.9\ (\pm\ 0.5) \cdot 10^5\ M^{-1}$. These results suggest that the indole and warfarin sites are the same as the first and second high-affinity thyroxine–HSA binding sites identified in these earlier reports.

The thermodynamics of thyroxine–HSA binding were examined in more detail using the data shown in Fig. 4. By applying eqns. 8 and 9 to this data, the changes in free energy for thyroxine interactions at the warfarin and indole sites were determined. The results are shown in Table IV. The values obtained for ΔH and ΔS at the indole site were in good agreement with previous estimates made in solution for the binding of thyroxine at the high-affinity site of HSA [7]. This again supports the hypothesis that the indole site is one of the primary binding sites for thyroxine on HSA. The total change in free energy (ΔG) for thyroxine binding at the two sites ranged from −7 to −8 kcal/mol at 37°C. In both cases, a large portion of this free energy change (*i.e.* 50–85%) was due to a large entropy term ($-T\Delta S$). This significant entropy contribution has also been observed for thyroxine–HSA binding by Tabachnick [7]. It has been suggested that this large change in entropy results from the release of water from HSA and thyroxine during the binding process [7].

CONCLUSION

In this study it was found that thyroxine binds directly to both the warfarin and indole sites on HSA. The association constants of these sites for thyroxine at 37°C are $1.4 \cdot 10^5$ and $5.7 \cdot 10^5\ M^{-1}$, respectively. Based on these values and the temperature dependence of binding at these sites, it was proposed that the indole site of HSA is the same as the high-affinity thyroxine site identified in earlier work. It was also proposed that the warfarin site represents the second high-affinity site noted in previous reports. However, it should be noted that since only the warfarin and indole sites were examined in this study, these results do not rule out the existence of high-affinity thyroxine sites at other regions on HSA. In the future it should be possible to develop a more complete picture of thyroxine–HSA binding by using additional probes to examine binding at these other regions.

High-performance affinity columns containing immobilized HSA were found to be useful as models in studying the interactions of compounds at the major binding sites of HSA. This was demonstrated by frontal analysis using *R*-warfarin and L-tryptophan. These experiments indicated that the warfarin and indole sites on HSA immobilized to diol-bonded silica had behavior equivalent to that observed for HSA in solution. This was indicated both by the type of binding observed for *R*-warfarin and L-tryptophan (*i.e.* single-site) and the association constants measured for these interactions. Similar argeement between the binding behavior of HSA immobilized to agarose and HSA in solution has been noted by Lagercrantz and co-workers [31,35].

The approach of using *R*-warfarin and L-tryptophan as probes to examine the interactions of thyroxine at specific sites on the HSA column has a number of advantages. For example, in studying the overall binding of thyroxine to HSA it is necessary to estimate both the number and binding strength of the low-affinity thyroxine sites. This has been one of the main difficulties in previous thyroxine–HSA studies. A second problem has been the need to ensure that other thyroxine-binding proteins (*i.e.* thyroxine binding globulin) are not contaminating the sample. In this study, both of these problems were minimized or eliminated by examining the binding of thyroxine at specific sites on HSA.

Another advantage of using specific probes injected in the presence of thyroxine was that the association constant for thyroxine at a given site could be determined directly from the concentration of thyroxine used and the resulting retention of the probe molecule. Knowledge of the actual number of binding sites was not required. The reason for this is that eqn. 7 allows the association constants for thyroxine to be calculated based on slope/intercept ratios. The use of such ratios is not only convenient, but also helps to produce stable association constant measurements, because the results are relatively unaffected by long-term changes in the column binding capacity. For example, over the course of 140 injections, less than ± 2% variation was noted in the association constant for thyroxine at the warfarin site, even though the number of these binding sites, as determined by frontal analysis, decreased by 20%.

Advantages of using high-performance affinity chromatography in studying thyroxine–HSA interactions include both the speed and precision of this technique. For example, the chromatographic time required for the frontal analysis of four concentrations of either *R*-warfarin or L-tryptophan was only 3–4 h. A complete zonal elution experiment using five thyroxine concentrations typically took only 5–6 h to perform. These times are much shorter than required by conventional methods for association constant measurements, such as equilibrium dialysis. The precision obtained using high-performance affinity columns was also quite good. This is reflected by the precision of the association constants measured in this work, all of which had relative standard deviations in the range 1–14%. Based on these results, it is expected that high-performance affinity chromatography should continue to be a valuable tool in studying thyroxine–protein binding and other biological interactions.

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