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QUANTITATIVE STUDIES OF ALLOSTERIC EFFECTS BY BIOINTERACTION CHROMATOGRAPHY:

ANALYSIS OF PROTEIN BINDING FOR LOW SOLUBILITY DRUGS

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Abstract

A new chromatographic method was developed for characterizing allosteric interactions between an immobilized binding agent and low solubility compounds. This approach was illustrated by using it to characterize the interactions between tamoxifen and warfarin during their binding to the protein human serum albumin (HSA), with β -cyclodextrin being employed as a solubilizing agent for these drugs. It was confirmed in this work through several experiments that warfarin had a single binding site on HSA with an association equilibrium constant of $2\text{--}5 \times 10^5 \text{ M}^{-1}$ (average, $3.9 \times 10^5 \text{ M}^{-1}$) at 37°C, in agreement with previous reports. It was also found that tamoxifen had a single major binding site on HSA, with an association equilibrium constant of $3\text{--}4 \times 10^7 \text{ M}^{-1}$ (average, $3.5 \times 10^7 \text{ M}^{-1}$) at 37°C. When warfarin was used as a mobile phase additive in competition studies with tamoxifen, this had a positive allosteric effect on tamoxifen/HSA binding, giving a coupling constant of $2.3 (\pm 0.3)$. Competitive studies using tamoxifen as a mobile phase additive indicated that tamoxifen had a negative allosteric effect on warfarin/HSA binding, providing a coupling constant of $0.79 (\pm 0.03)$. A unique feature of the technique described in this report was its ability to independently examine both directions of the warfarin/tamoxifen allosteric interaction. This approach is not limited to warfarin, tamoxifen and HSA but can also be used to study other solutes and binding agents.

INTRODUCTION

An allosteric effect occurs when the interactions of one compound with a binding agent changes the interactions of a second solute with the same agent. This happens quite often in biological systems, including both drug-protein and ligand-receptor systems.^{1,2} A common example is the presence of allosteric effects during the binding of drugs or other small solutes to the protein human serum albumin (HSA).^{3–6} Most previous studies of allosteric interactions with HSA and other proteins have involved only qualitative observations of increased or decreased binding. Exceptions include work by Stockton et al.⁷ and Ehlert,⁸ in which apparent association equilibrium constants were measured for a receptor with one solute in the presence of several fixed concentrations of a second solute. Another exception is work by Kragh-Hansen,⁹ in which the non-bound fraction of a solute was used to measure its coupling constant to a binding agent in the presence or absence of a second solute.

One disadvantage of these previous reports is that they have used either equilibrium dialysis or ultrafiltration as the basis for their measurements.^{7–9} Such techniques are generally considered to be the reference methods for drug-protein binding studies and can, when combined with other analytical methods, be used for multianalyte systems. However, for some

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analyses these methods can be time-consuming to perform, especially when using equilibrium dialysis, and often require a labeled analog of the analyte for detection. A number of alternative, instrumental methods based on HPLC, affinity capillary electrophoresis and biosensors have been described to provide more rapid and convenient tools for drug-protein binding studies.¹⁰⁻¹⁶ However, these techniques have been used mainly for the analysis of individual drugs or to study direct drug-drug competition and have previously provided only qualitative information on allosteric effects between drugs.

Recently a new chromatographic approach has been reported for the quantitative analysis of allosteric effects.^{6,17} This method, referred to here as biointeraction chromatography, is a variation of zonal elution affinity chromatography in which a trace amount of one solute is injected in the presence of a second solute on a column that contains an immobilized binding agent. The coupling constant between the two solutes is determined by examining the change in retention for the injected solute while various concentrations of the second solute are applied in the mobile phase. Such an approach has already been successfully utilized in examining a number of drug-drug interactions on HSA, including positive and negative allosteric effects as well as direct competition.^{6,17}

One current limitation of this method is it can only be applied to compounds that have at least a moderate solubility in aqueous buffers (i.e., solubilities of at least μM levels). This feature is needed to place enough of the injected compound in solution for detection and to have a sufficiently high concentration of the second solute in the mobile phase to produce a measurable shift in retention. It is possible, in theory, to extend this method to lower solubility substances by adding an organic modifier to the mobile phase or by changing the temperature, pH or ionic strength of the mobile phase. However, these changes might also alter the interactions being studied. An alternative approach that has been used in past work with affinity chromatography is to add a solubilizing agent like β -cyclodextrin to the mobile phase.¹⁸⁻²¹ For instance, this has been used in zonal elution studies with immobilized HSA to determine association equilibrium constants for low solubility compounds like clomiphene and digitoxin.^{18,19}

In this report, the theory and applications of biointeraction chromatography will be expanded from those presented in prior reports to also include low solubility drugs; this will be accomplished by modifying this approach to consider the use of solubilizing agents such as β -cyclodextrin as mobile phase additives. The first goal will be to develop equations for the study of allosteric effects by biointeraction chromatography in the presence of a solubilizing agent. These equations will then be used to study the interactions on HSA between warfarin and tamoxifen (see Figure 1), two drugs with known allosteric effects.^{1,22} Warfarin binds to one of the major binding sites of HSA, referred to as the "warfarin site" or Sudlow site I,^{23, 24} while tamoxifen is believed to bind at a minor binding region on HSA (i.e., the "tamoxifen site").^{1,21} Although it is known that there is an allosteric interaction during the binding of warfarin and tamoxifen to HSA,^{1,22} no quantitative description of this process has been reported. In this work, such information will be obtained for both the effects of warfarin on tamoxifen and for tamoxifen on warfarin. These same analytes will also be used in self-competition studies to illustrate the use of biointeraction chromatography for low solubility compounds that have direct competition on a binding agent.

It has been shown in many previous studies with HSA columns that warfarin has sufficient solubility in aqueous buffers (i.e., approximately 60 μM in pH 7.4, 0.067 M phosphate buffer) to allow this drug to be examined without the use of solubilizing agents (e.g., see Refs. 10 and 25). However, the observed solubility of tamoxifen under the same conditions is in the upper nM to low μM range and does require such agents.¹⁹ These properties make these model drugs useful in this present study for comparing binding parameters that are measured for drugs with HSA in the presence or absence of β -cyclodextrin as a solubilizing agent (i.e., when working

with warfarin), as well as for exploring the extension of biointeraction chromatography to low solubility drugs (i.e., through work with tamoxifen). Based on this information it should then be possible to extend this approach to other drugs and/or low solubility agents.

THEORY

Direct Competition in the Presence of a Solubilizing Agent

Zonal elution analysis has been used in many previous reports to study the binding between drugs and immobilized proteins.^{10-12,25} Equations based on the use of solubilizing agents during such experiments have been reported for the case in which an analyte (A) and competing agent (I) have direct competition for an immobilized binding agent or ligand (L) in the presence of a solubilizing agent (S). This reaction scheme is illustrated in Figure 2(a), where K_{AL} and K_{IL} are the association equilibrium constants for the binding of immobilized agent L with A and I, while K_{AS} and K_{IS} are the association equilibrium constants between these compounds and solubilizing agent S. This model assumes there are only 1:1 interactions during these reactions. It is also assumed that L and S have little or no interactions with each other or their respective complexes, as has been shown for HSA and β -cyclodextrin.¹⁹

A typical zonal elution experiment involving direct competition is shown in Figure 3(a), using the self-competition of warfarin as an example. Based on the model in Figure 2(a), the following equation has been previously derived to describe the retention factor (k) for the injected analyte A in the presence of a mobile phase with known total concentrations of competing agent I (C_I) and the solubilizing agent S (C_S), which are passing through a column with a fixed total concentration of binding agent L (C_L).^{19,20}

$$k = \frac{(1 + K_{IS}C_S) K_{AL}C_L}{(1 + K_{AS}C_S) K_{IL}C_I + (1 + K_{IS}C_S)(1 + K_{AS}C_S)} \quad (1)$$

$$\frac{1}{k} = \frac{(1 + K_{AS}C_S) K_{IL}C_I}{(1 + K_{IS}C_S) K_{AL}C_L} + \frac{1 + K_{AS}C_S}{K_{AL}C_L} \quad (2)$$

Eqs 1 and 2 assume that the amount of injected analyte is much smaller than the total amount of binding agent or solubilizing agent in the column (i.e., linear elution conditions are present). They also assume that the concentration of competing agent is much smaller than the concentration of solubilizing agent.^{19,20} Eq 2 has been used in the past to characterize the binding of low solubility compounds with immobilized binding agents such as HSA.¹⁸⁻²¹ This can be performed by injecting a small concentration of A into the presence of various concentrations of I and S in the mobile phase. A plot of $1/k$ versus C_I is then made at each concentration of S, which results in a linear relationship for systems fitting the model in Figure 2(a) (Note: it is also possible to use a non-linear fit to the data according to eq 1; however, the linearized form shown in eq 2 is more convenient for routine analysis and for differentiating between systems with competitive binding versus non-competitive binding).²⁵ The best-fit lines for graphs obtained with eq 2 are then used to make a second graph in which the ratios of the intercepts and slopes are plotted versus the total concentration of solubilizing agent, as described in eq 3,

$$\text{Intercept/Slope} = 1/K_{IL} + (K_{IS}C_S)/K_{IL} \quad (3)$$

where the reciprocal of the intercept is now K_{IL} , and the ratio of the slope and intercept gives K_{IS} .^{19,20}

It is possible to simplify this approach if independent estimates are available for K_{AS} and K_{IS} . Such data can be obtained by spectroscopic methods, electrochemical techniques, calorimetry,

kinetic methods or competition assays.^{26,27} Techniques like the Hummel-Dreyer method,²⁸⁻³¹ reversed-phase chromatography, normal-phase chromatography, cyclodextrin-bonded phase liquid chromatography, thin layer chromatography³²⁻³⁶ and affinity capillary electrophoresis^{37,38} can also be employed for this purpose. Once the values for K_{IS} and K_{AS} are known, eq 2 can then be used in zonal elution experiments that are performed at a single concentration of solubilizing agent. In this situation, the value of K_{IL} is now provided by eq 4,

$$K_{IL} = \frac{\text{Slope}}{\text{Intercept}} \cdot (1 + K_{IS} C_S) \quad (4)$$

where the slope and intercept are the best-fit values obtained using eq 2. If C_L is known, K_{AL} can be determined using eq 5; alternatively, C_L can be calculated from eq 6 by using a known value for K_{AL} .

$$K_{AL} = \frac{1}{\text{Intercept}} \cdot \frac{1 + K_{AS} C_S}{C_L} \quad (5)$$

$$C_L = \frac{1}{\text{Intercept}} \cdot \frac{1 + K_{AS} C_S}{K_{AL}} \quad (6)$$

Self-Competition in the Presence of a Solubilizing Agent

A special situation occurs in zonal elution experiments when A and I are the same compound, as is the case in Figure 3(a). Under these circumstances, self-competition takes place during the binding of the solute to S and L. As a result, the association equilibrium constants K_{IS} and K_{AS} are now identical, as is also true for K_{AL} and K_{IL} . This causes eq 2 to simplify to the following relationship.

$$\frac{1}{k} = \frac{C_A}{C_L} + \frac{1 + K_{AS} C_S}{K_{AL} C_L} \quad (7)$$

In this case, using a fixed total concentration of solubilizing agent (C_S) and various total concentrations of the competing agent (C_A) in the mobile phase will give a linear plot between $1/k$ and C_A . The reciprocal of the slope for this plot will be equal to C_L , which gives the amount of active immobilized binding agent in the column. It is also possible to obtain K_{AL} by using the slope and intercept of this plot along with known values for K_{AS} and C_S , as shown earlier in eq 5.

Allosteric Interactions in the Presence of a Solubilizing Agent

Figure 2(b) shows the model used in this study to examine allosteric interactions between an injected analyte and mobile phase additive for an immobilized binding agent. This was used to examine data obtained from zonal elution experiments like those shown in Figures 3(b) and 3(c). The model in Figure 2(b) that was used to describe these systems is similar to that described in Ref. 6 but includes additional reactions involving a solubilizing agent. In this model, the effect of I on A is described by the association equilibrium constant K'_{AL} , which represents the altered affinity of A when it binds to L that has already formed a complex with I. In addition, the relative change in this association equilibrium constant from its initial value can be described by the ratio $\beta_{I \rightarrow A} = K'_{AL}/K_{AL}$, where $\beta_{I \rightarrow A}$ is the coupling constant for the effect of I and A. In a similar fashion, the allosteric effect of A on I can cause a change in the association equilibrium constant K_{IL} to K'_{IL} , as given by the coupling constant $\beta_{A \rightarrow I} = K'_{IL}/K_{IL}$. If the amount of injected analyte is much smaller than the quantities of I or L, it is possible to ignore the effects of A on the binding of I to L and to obtain information on only the effects of I and A (i.e., $\beta_{I \rightarrow A}$).⁶ However, data on the reverse process (i.e., the effect of A on I, as

described by $\beta_{A \rightarrow I}$) can also be obtained by performing a second experiment in which I is the injected analyte and A is now the mobile phase additive.

As noted previously, it is possible with this model to describe a system with direct competition ($\beta = 0$) or no competition ($\beta = 1$), as well as positive allosteric effects ($\beta > 1$) or negative allosteric effects ($0 < \beta < 1$).⁶ If the same conformation is obtained for the final complex regardless of the binding order, this will be represented by the situation where $\beta_{I \rightarrow A} = \beta_{A \rightarrow I}$. However, it is also possible with this model to describe a system with two states for the final conformation of the complex, depending on the order in which A and I interact with the binding agent. In this latter case, $\beta_{I \rightarrow A}$ and $\beta_{A \rightarrow I}$ will now have different values.

Based on the model in Figure 2(b), the following equation was obtained in this report to describe the retention of A in the presence of both I and S (see Appendix for derivation).

$$k = \frac{(1 + K_{IS}C_S)K_{AL}C_L}{(1 + K_{AS}C_S)(1 + K_{IS}C_S + K_{IL}C_I)} + \frac{K_{IL}K'_{AL}C_I C_L}{(1 + K_{AS}C_S)(1 + K_{IS}C_S + K_{IL}C_I)} \quad (8)$$

This expression can be converted to the following linear form,

$$\frac{k_0}{k - k_0} = \frac{1}{(\beta_{I \rightarrow A} - 1)} \cdot \left(\frac{1 + K_{IS}C_S}{K_{IL}C_I} + 1 \right) \quad (9)$$

in which k_0 is the retention factor for A when no competing agent is present (i.e., $C_I = 0$). Eq 9 indicates that a plot $k_0/(k - k_0)$ versus $1/C_I$ should give a linear relationship for systems with either direct competition ($\beta_{I \rightarrow A} = 0$) or allosteric competition for an immobilized binding agent and in the presence of a solubilizing agent (Note: for solutes with no competition there will be no observed change in k with the C_I). From the slope and intercept of this plot, the association equilibrium constant for the binding of I to L (K_{IL}) and the coupling constant for the allosteric effect of I on A can be determined, as shown in eqs 10 and 11.

$$K_{IL} = \frac{\text{Intercept}}{\text{Slope}} (1 + K_{IS}C_S) \quad (10)$$

$$\beta_{I \rightarrow A} = \frac{K'_{AL}}{K_{AL}} = \frac{1}{\text{Intercept}} + 1 \quad (11)$$

If the same compound is used as both the injected analyte and competing agent (i.e., $K_{AL} = K_{IL}$ and $K_{AS} = K_{IS}$), eq 9 reduces to eq 12.

$$\frac{k_0}{k - k_0} = \frac{1}{(\beta_{I \rightarrow A} - 1)} \cdot \left(\frac{1 + K_{AS}C_S}{K_{AL}C_A} + 1 \right) \quad (12)$$

In this case, the expression giving $\beta_{I \rightarrow A}$ is the same as eq 11 but the slope and intercept ratio now provides K_{AL} through the use of eq 13, as shown below.

$$K_{AL} = \frac{\text{Intercept}}{\text{Slope}} \cdot (1 + K_{AS}C_S) \quad (13)$$

Corrections for Secondary Interactions

One assumption made in the previous equations is that the same number of sites are involved in the binding of A and I. However, this may not always be the case. For instance, it is possible that an immobilized binding agent like HSA will have different degrees of activity at different regions due to variations in local steric hindrance or denaturation effects.^{39,40} It is also possible

that one solute may have binding regions that do not affect the interaction of the second solute with L. As suggested in Ref. 6, it is possible to correct for these effects by using the following equations in place of eq 9 or 2, respectively,

$$\frac{k_0 - x}{k - k_0} = \frac{1}{\beta_{I \rightarrow A} - 1} \cdot \left(\frac{1 + K_{IS} C_S}{K_{IL} C_L} + 1 \right) \quad (14)$$

$$\frac{1}{k - x} = \frac{(1 + K_{AS} C_S) K_{IL} C_I}{(1 + K_{IS} C_S) K_{AL} C_L} + \frac{1 + K_{AS} C_S}{K_{AL} C_L} \quad (15)$$

where x is the retention factor for A due to its additional secondary interactions.^{6,25}

Note in the case of eq 14 that a correction is only required in the numerator on the left, since the value of x is constant and will cancel out in the denominator when the difference is taken between k and k_0 . As demonstrated with similar equations in Ref. 6, eq 14 can also be used when the number of binding sites for A and I are not identical. Eq 14 indicates that a plot of $(k_0 - x)/(k - k_0)$ versus $1/C_I$ should again give a straight line. Eqs 10 and 11 can then be used with the slope and intercept of this plot to determine the association equilibrium constant and coupling constant for I, as described in the previous section.

For interactions involving the support material, the value of x in eqs 14 and 15 can be estimated using a control column of the same size and made in the same way as the affinity column, except with no immobilized binding agent now being present. For secondary interactions involving the binding agent, an estimate of x can be obtained if independent estimates are available for the association equilibrium constant and amount of the given binding region within the column. As an alternative, the value for x in eqs 14 and 15 can also be estimated through non-linear regression.

EXPERIMENTAL SECTION

Reagents

The HSA (99% pure, fatty acid free), racemic warfarin (> 98% pure), tamoxifen (i.e., [Z]-1-[*p*-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene) and β -cyclodextrin (> 98% pure) were from Sigma (St. Louis, MO). Nucleosil Si-1000 silica (5 μ m particle size, 1000 Å pore size) was purchased from P.J. Cobert (St. Louis, MO). The reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). All solutions were prepared with water obtained from a Nanopure water system (Barnstead, Dubuque, IA).

Apparatus

The chromatographic system consisted of a Jasco PU980 pump (Easton, MD), a Thermoseparations AS3000 autosampler (Riviera Beach, FL) equipped with a 20 μ l sample loop, an Alltech water jacket (Deerfield, IL), and a Milton Roy SM3100 UV detector (Riviera Beach, FL). The temperature of the column was controlled by an Isotemp 9100 circulating water bath from Fisher Scientific (Pittsburgh, PA). The same circulating water bath was used to pre-equilibrate the mobile phase at the column temperature. Chromatographic data were collected using programs written with Labview software from National Instruments (Austin, TX).

Methods

The Nucleosil-1000 was converted into a diol-bonded form according to a previous method.⁴¹ The diol content of this support was 37 ($\pm$ 1) μ mol/g silica ($\pm$ 1 SD) as determined in triplicate by an iodometric capillary electrophoresis assay.⁴² HSA was immobilized onto the diol-

bonded Nucleosil using the Schiff base method.³⁹ A control support was prepared by taking a portion of the same batch of diol-bonded silica through the Schiff base method but with no HSA being added during the immobilization step.

After immobilization, each support was centrifuged and washed three times with pH 7.4, 0.067 M potassium phosphate buffer. These were then stored in a pH 7.4, 0.067 M potassium phosphate buffer at 4°C prior to use. A small portion of the HSA support was further washed with water, dried under vacuum and analyzed for its protein content by a BCA assay.⁴³ The HSA content of the supports used in this study ranged from 7.0-11 mg HSA/g silica, as obtained for two separate batches of immobilized HSA. The HSA support and control support were downward slurry-packed into separate 5 cm × 2.1 mm I.D. stainless steel columns. These columns were packed at 4000 psi using pH 7.4, 0.067 M potassium phosphate buffer as the packing solution.

All mobile phases were prepared using pH 7.4, 0.067 M potassium phosphate buffer. Zonal analysis was performed by injecting 20 µl samples of warfarin or tamoxifen dissolved in the corresponding mobile phase. These injections were made on both the HSA and control columns after the columns had been equilibrated with the mobile phase. When warfarin was used as both the competing agent and injected analyte, the samples contained a warfarin level that was 0.06 µM higher than the mobile phase's warfarin concentration. When warfarin was used as the competing agent and tamoxifen was the analyte, the samples contained tamoxifen concentrations below 0.1 µM. The mobile phase concentration of β-cyclodextrin in these studies was 2.2 mM, except where otherwise noted. Under these conditions, there was no shift observed in analyte retention with a decrease in sample concentration, indicating that linear elution conditions were present.

In the first set of experiments that used tamoxifen as competing agent, the mobile phase contained 2.2 mM β-cyclodextrin and 0-2.75 µM tamoxifen. The tamoxifen samples in these studies contained tamoxifen that was 0.46 µM higher in concentration than the level of this drug in the mobile phase, in addition to 2.2 mM β-cyclodextrin. The warfarin samples used in these experiments contained 0.25 µM warfarin. In a related set of studies, the mobile phase contained 0.88 mM β-cyclodextrin and 0-2.4 µM tamoxifen, with the samples containing 3.0 µM tamoxifen or 0.50 µM warfarin, and 0.88 mM β-cyclodextrin. Tamoxifen was found to be completely dissolved under these conditions, as was confirmed by absorbance measurements at 205 nm. No observable shift was seen in the retention of the analytes during these studies when lower sample concentrations were used, again indicating linear conditions were present.

The temperature in all these studies was 37°C. The elution of warfarin and tamoxifen was monitored at 204-205 nm. All experiments were performed in at least duplicate. The column and system void times were determined by making 20 µl injections of 0.27 mM sodium nitrate, which was also monitored at 204 nm. All experiments were performed at flow rates of 0.1-0.3 ml/min. No significant change in the retention factors were observed when these flow rates were further decreased (i.e., random variations of less than 5%), indicating that the observed peak means were not susceptible to kinetic effects and could be used in the determination of binding constants. The pressure across the columns was less than 500 psi during these studies. The retention time for each peak was determined by moment analysis⁴⁴ after baseline correction (i.e., based on linear fits to the background or background subtraction).

Non-specific binding of warfarin to the support in the affinity columns was negligible in these studies, accounting for less than 3% of the total measured retention for warfarin on the HSA column. The non-specific binding for tamoxifen was larger and varied with the experimental conditions. Work performed with the control columns indicated that this non-specific binding accounted for 11-49% of tamoxifen's total retention on the HSA column in the presence of 2.2

mM β -cyclodextrin and 14-56% of tamoxifen's total retention in the presence of 0.88 mM β -cyclodextrin. This information was later used along with eqs 14 and 15 to correct for these secondary interactions in studies of tamoxifen binding to HSA.

RESULTS AND DISCUSSION

Self-Competition Studies with Warfarin

The first series of studies examined the self-competition of warfarin on an immobilized HSA column in the presence of β -cyclodextrin. This made it possible to examine the use of eqs 7 and 12 in the analysis of a drug-protein system with direct competition before moving on to the more complex case of allosteric interactions. Warfarin is a common anticoagulant with two enantiomers, *R*- and *S*-warfarin, that have a single major binding site on HSA with well-defined association equilibrium constants.^{24,40} In this work, racemic warfarin was used since only one peak was generally observed for these enantiomers on the HSA column in the presence of β -cyclodextrin, as shown in Figure 3(a). Furthermore, no correction was needed for non-specific binding between warfarin and the support, since no significant retention was seen for this solute on the control column.⁴⁰

These initial studies were conducted by making injections of warfarin into mobile phases that had known concentrations of warfarin as a competing agent and β -cyclodextrin as a solubilizing agent. As shown in Figure 3(a), this gave rise to a shift to lower retention times for the injected warfarin as more warfarin was placed into the mobile phase as a competing agent. The retention factors measured in these experiments are summarized in Table 1 and had precisions of ± 0.3 to 0.9% (1 relative standard deviation). When these self-competition data were plotted according to eq 7, the resulting graph of $1/k$ versus C_{Warfarin} gave a linear relationship with a correlation coefficient of 0.997 ($n = 5$), as shown in Figure 4(a). According to eq 7, this linearity indicated that direct competition was occurring between the injected warfarin and warfarin in the mobile phase at a single type of site on HSA. This agrees with previous studies reporting that both warfarin enantiomers have a single binding region on HSA under the conditions used in this study.^{39,40}

More detailed information could be obtained from Figure 4(a) by combining these results with previous measurements examining the binding of warfarin with β -cyclodextrin. For instance, the total concentration of β -cyclodextrin in Figure 4(a) was 2.2 mM, giving the value for C_S in eq 7. In addition, a previous report has found that K_{AS} for warfarin and β -cyclodextrin is $5.2 (\pm 0.3) \times 10^2 \text{ M}^{-1}$ under the conditions used in this study.²⁶ By combining these results with the best-fit line in Figure 4(a), the association equilibrium constant for warfarin with HSA (i.e., K_{AL} in this experiment) was estimated from eq 5 to be $5.4 (\pm 0.3) \times 10^5 \text{ M}^{-1}$. This result is similar to previous values of $2.4\text{--}4.1 \times 10^5 \text{ M}^{-1}$ that have been reported for this interaction in the absence of any solubilizing agent.^{40,45,46}

By using the reciprocal of the slope, the binding capacity of the immobilized HSA column for warfarin could also be obtained from Figure 4(a). This gave an effective concentration of $33 (\pm 1) \mu\text{M}$ for the warfarin binding sites in the column. The total protein content for this particular HSA column was $11 (\pm 1) \text{ mg/g}$, giving an effective concentration of $74 \mu\text{M}$ HSA in the column. When this value was compared with the estimated amount of warfarin binding sites, the activity of the immobilized HSA for warfarin was found to be $45 (\pm 4)\%$. This result agrees with earlier work performed with other HSA columns prepared under similar conditions,⁴⁰ in which the relative activity of immobilized HSA for *R*-warfarin was found to be $53 (\pm 3)\%$.

The data in Figure 4(a) were next analyzed by eq 12 to determine the types of interactions that were present between the injected warfarin and warfarin in the mobile phase. The result is shown in Figure 4(b), where a plot of $k_0/(k - k_0)$ versus $1/C_{\text{Warfarin}}$ gave a linear relationship,

with a correlation coefficient of 0.997 ($n = 4$). By employing the slope and intercept of this plot along with the previously given values for C_S and K_{IS} , it was possible to use eq 13 to obtain the association equilibrium constant between warfarin and HSA. This gave a value of $4.2 (\pm 0.7) \times 10^5 \text{ M}^{-1}$, which was statistically identical to the value found earlier by more traditional zonal elution analysis.

From the best-fit line Figure 4(b), it was possible to calculate the coupling constant between the injected warfarin and warfarin in the mobile phase. This was accomplished by using the intercept of this plot along with eq 11. This gave a coupling constant of $-0.16 (\pm 0.19)$, which was statistically equal to zero (i.e., zero was within 1 SD of this value). This result is what would be expected for a system with direct competition and confirms that warfarin had a single site of interaction on HSA. It was concluded from this analysis that eq 11 could be used to analyze and detect direct competition for such a system in the presence of a solubilizing agent.

Self-Competition Studies with Tamoxifen

The next series of experiments used self-competition studies to characterize the binding of tamoxifen with immobilized HSA. This was necessary to obtain more detailed information on the affinity of tamoxifen for HSA, as well as the number of binding sites for tamoxifen with this protein. The chromatograms and shifts in retention seen in these studies were similar to those given in Figure 3(a) for the self-competition of warfarin. The retention factors obtained in these studies are summarized in Table 1 and had precisions of ± 0.4 to 1.7%. During these experiments, some non-specific binding was noted between tamoxifen and the control support, as discussed in the Experimental Section. A correction for these secondary interactions was made by subtracting the retention factor measured for tamoxifen on the control columns (as represented by the term x) from the overall retention seen on the HSA column at each given concentration of the competing agent. This was performed by employing eq 15 and approaches similar to those described in previous studies.^{6,10}

When using 0- 2.75 μM tamoxifen as a competing agent along with 2.2 mM β -cyclodextrin as a solubilizing agent, a plot of $1/(k - x)$ versus $C_{\text{Tamoxifen}}$, for injections of tamoxifen gave a linear relationship, as shown in Figure 5(a). The best-fit line for this plot had a correlation coefficient of 0.98 ($n = 5$). According to eq 15, this indicated tamoxifen had 1:1 competition with itself on the immobilized HSA. In previous studies it has been determined that the association equilibrium constant for tamoxifen with β -cyclodextrin is $1.1 (\pm 0.1) \times 10^4 \text{ M}^{-1}$ under the conditions used in this study.²⁶ Based on the same approach as described earlier for warfarin, this information was used along with the known concentration of β -cyclodextrin and the best-fit slope and intercept from Figure 5(a) to estimate the association equilibrium constant for tamoxifen with HSA, giving a value of $4.6 (\pm 1.7) \times 10^7 \text{ M}^{-1}$.

Figure 5(a) was also used to determine concentration of binding sites for tamoxifen in the HSA column. By using the reciprocal of the best-fit slope, this gave an effective binding site concentration of $13 (\pm 1) \mu\text{M}$. The measured protein content for the support in this case was $7.0 (\pm 0.8) \text{ mg HSA/g silica}$, which lead to an effective HSA concentration of $47 (\pm 5) \mu\text{M}$ in the column. This resulted in an estimated activity of $28 (\pm 4)\%$ for HSA in its binding to tamoxifen. This value is lower than the activity determined in the last section for warfarin, as could be caused by different immobilization effects at the warfarin and tamoxifen sites of HSA. Similar differences in activity between binding regions have been previously reported between the warfarin and indole-benzodiazepine sites of immobilized HSA.^{10,39} Using this activity with data from Ref. 26 gave an association equilibrium constant from this earlier report of $3.2 (\pm 0.5) \times 10^7 \text{ M}^{-1}$, which is statistically equal to the value obtained in this current study for tamoxifen in the presence of β -cyclodextrin.

The tamoxifen data from these experiments were next analyzed by preparing a plot of $(k_0 - x)/(k - k_0)$ versus $1/C_{\text{Tamoxifen}}$ according to eq 14. The graph obtained gave a linear relationship, as shown in Figure 5(b), which again suggested that tamoxifen had a single-site of interaction with the immobilized HSA. The correlation coefficient of this line was 0.94 ($n = 4$) and the association equilibrium constant determined for tamoxifen with HSA from this graph was $1.1 (\pm 0.3) \times 10^8 \text{ M}^{-1}$, a value about two-fold higher than that obtained in Figure 5(a) (Note: this difference may be due to the greater sensitivity of eq 14 versus eq 15 to deviations in the measured retention factors and corrections for secondary interactions). The coupling constant determined from the intercept of this plot was $0.08 (\pm 0.04)$, which was statistically equal to zero (i.e., zero was within 2 SD of this value) and confirmed that tamoxifen was undergoing direct competition with itself for sites on HSA.

Similar studies were conducted at higher mobile phase concentrations of tamoxifen (0.3–2.4 μM) and using a lower level of β -cyclodextrin (0.88 mM), a combination expected to give more free tamoxifen in the mobile phase for binding to HSA. In this situation, the plot of $1/(k - x)$ versus $C_{\text{Tamoxifen}}$ again gave a linear relationship (data not shown), with a correlation coefficient of 0.988 ($n = 6$). The association equilibrium constant determined for tamoxifen with HSA in this case was $2.9 (\pm 0.9) \times 10^7 \text{ M}^{-1}$, which was close to the value determined when using 2.2 mM β -cyclodextrin as a solubilizing agent. A plot of $(k_0 - x)/(k - k_0)$ versus $1/C_{\text{Tamoxifen}}$ for the same data gave a linear relationship with a correlation coefficient of 0.976. This latter plot provided an association equilibrium constant of $2.9 (\pm 0.5) \times 10^7 \text{ M}^{-1}$ and a coupling constant of $0.16 (\pm 0.6)$.

Injections of Tamoxifen using Warfarin as a Mobile Phase Additive

Once the binding of tamoxifen and warfarin to HSA had been studied separately, experiments were performed to examine the interactions between these compounds. This was first performed by injecting tamoxifen as the analyte and using warfarin as the competing agent in the mobile phase. This was conducted in the presence of various concentrations of warfarin in a pH 7.4, 0.067 M phosphate buffer that contained 2.2 mM β -cyclodextrin as a solubilizing agent. The result, as illustrated in Figure 3(b), was a shift in tamoxifen retention to longer retention times as more warfarin was placed into the mobile phase. The resulting retention factors are summarized in Table 1 and had precisions of ± 0.3 to 2.9%. In this case eqs 14 and 15 were used to correct for the non-specific binding seen for tamoxifen on the support.

Figure 3(b) and the plot of $1/(k - x)$ versus C_{Warfarin} in Figure 6(a) shows there was an increase in tamoxifen retention, or a decrease in $1/(k - x)$, as the mobile phase concentration of warfarin was increased. This relationship is contrary to what would be expected for direct competition (as modeled by eq 15) but does fit a model in which the binding of warfarin with HSA has a positive allosteric effect on the binding of tamoxifen with HSA. When the same data were plotted according to eq 14, as illustrated in Figure 6(b), this gave a linear relationship with a correlation coefficient of 0.999 ($n = 4$). From this plot the association equilibrium constant for warfarin at its binding site was determined to be $2.2 (\pm 0.5) \times 10^5 \text{ M}^{-1}$ and the coupling constant for its effect on tamoxifen was found to be $2.3 (\pm 0.3)$. This coupling constant is consistent with previous reports noting a positive allosteric interaction between warfarin and tamoxifen during their binding to HSA.¹ In addition, the association equilibrium constant measured here for warfarin agrees with earlier values obtained in the literature and this current study for the interactions of this drug with HSA.^{44,45,46}

Injections of Warfarin using Tamoxifen as a Mobile Phase Additive

The last set of studies used tamoxifen as a competing agent in the mobile phase and warfarin as the injected analyte. This was the reverse of the experiments in the last section. Although the retention for injected tamoxifen was found in the previous section to increase with an

increase in warfarin concentration in the mobile phase, when the reverse experiment was performed completely different behavior was noted. This is illustrated in Figures 3(c) and 7 (a), where the retention of warfarin decreased (or $1/k$ increased) with an increase in the mobile phase concentration of tamoxifen. The retention factors obtained this study are summarized in Table 1 and gave precisions of ± 0.1 to 1.7%.

In further examining these data, the values for C_S (2.2 mM β -cyclodextrin) and K_{IS} ($1.1 \times 10^4 \text{ M}^{-1}$ for tamoxifen with β -cyclodextrin)²⁶ were known, as was C_I (the total concentration of tamoxifen in the mobile phase). With this information, it was possible to use eq 9 to further examine the retention data for warfarin in the presence of tamoxifen. The resulting plot of $k_0/(k - k_0)$ versus $1/C_{\text{Tamoxifen}}$ is shown in Figure 7(b). This gave a straight line with a correlation coefficient of 0.91 ($n = 4$). From the best-fit line, the association equilibrium constant for tamoxifen with HSA was determined to be $3.7 (\pm 1.4) \times 10^7 \text{ M}^{-1}$ which was in good agreement with previous values determined in the tamoxifen self-competition study and reported in the literature.²⁴

From the intercept of Figure 7(b), the coupling constant for the effect of tamoxifen on warfarin during their binding to HSA was found to be $0.79 (\pm 0.03)$. This meant there was a negative allosteric effect for tamoxifen on warfarin, weakening the binding of warfarin with HSA and increasing the free concentration of warfarin in solution. Such an effect is consistent with the observation that concomitant administration of warfarin and tamoxifen increases the effect of warfarin in the body.⁴⁷⁻⁴⁹ This result is quite different from the positive allosteric effect seen in the last section when using warfarin as a competing agent, which gave a coupling constant of $2.3 (\pm 0.3)$. This suggests that the order of binding of tamoxifen versus warfarin to HSA effects the final conformation of this protein in its complex with these drugs. Similar differences in coupling constants have been noted for other solutes and HSA when analyzed by biointeraction chromatography (e.g., phenytoin and L-tryptophan).⁶ This demonstrates the value of being able to examine an allosteric interaction in both directions (i.e., the effect of I on A and A on I), as can be accomplished by the method described in this report.

CONCLUSIONS

In this report a method was described for the quantitative analysis of allosteric drug-drug interactions on a protein when using a solubilizing agent. This approach was tested by using it to examine the interactions between warfarin and tamoxifen on HSA. Self-competition studies with warfarin and tamoxifen gave similar association equilibrium constants to those in previous studies examining the interactions of these agents with HSA in the absence of β -cyclodextrin; this indicated that this solubilizing agent did not have any measurable affect on binding parameters measured in this current study between these drugs and HSA. In both cases, the coupling constants obtained during self-competition studies were statistically equal to zero, indicating that direct competition was occurring for each drug with itself. In addition, it was determined that both warfarin and tamoxifen had 1:1 interactions with HSA.

It was confirmed in further competition studies between tamoxifen and warfarin that these drugs had allosteric effects during their binding to HSA. Figure 8 summarizes the corresponding association equilibrium constants and coupling constants that were determined in this study. This model gave good qualitative agreement with previous studies of warfarin/tamoxifen interactions. This same model explains why an earlier report noted that *trans*-clomiphene (i.e., a compound similar to tamoxifen in structure) decreased the binding of immobilized HSA to phenol red (i.e., a compound that binds at the warfarin site of HSA) but gave positive allosteric interactions when phenol red was used as a competing agent.²¹

The tools developed in this report are not limited to the study of HSA or warfarin and tamoxifen, but can be employed with other systems. As illustrated in this report, such an approach can be used to examine both direct competition and allosteric effects. The use of a solubilizing agent like β -cyclodextrin also allows this method to be employed with solutes that have either high or low solubility in aqueous buffers. In addition, it was shown how biointeraction chromatography can be used to obtain equilibrium constants and coupling constants for relatively complex solute-protein interactions. As noted earlier, other advantages of this method when it is performed as part of an HPLC system include its high precision, speed, and ability to work with the same affinity column for hundreds of experiments.^{5,6,10} Such features should make this technique valuable as a tool for the further study of drug-drug interactions and in obtaining a better description of drug behavior within the body.

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APPENDIX

It has been shown in previous work with zonal elution that the moles of an immobilized binding agent that are occupied in the presence of a given concentration of competing agent (m_{LI}) can be related to the total moles of binding agent (m_L) through the following equation,³⁹

$$m_{LI} = \frac{K_{IL} [I]}{1 + K_{IL} [I]} \cdot m_L \quad (\text{A1})$$

where eq A2 gives the moles of binding agent L that remain unoccupied (m_{L2}).

$$m_{L2} = m_L - m_{LI} = \frac{1}{1 + K_{IL} [I]} \cdot m_L \quad (\text{A2})$$

Other terms in these equations include K_{IL} , the association equilibrium constant for I with L, and $[I]$, the molar concentration of I in the mobile phase.

For this system, the retention factor for A due its binding to L with no I present (k_1) can be written as follows,¹⁹

$$k_1 = \frac{K_{AL} [L]_1}{1 + K_{AS} C_S} \quad (\text{A3})$$

where K_{AL} is the association equilibrium constant for A with L, K_{AS} is the association equilibrium constant between A and solubilizing agent S, $[L]_1$ is the molar concentration of unoccupied binding agent, and C_S is the total concentration of S. Similarly, the retention factor for A due to its binding to L that has already bound to I (k_2) is given by,

$$k_2 = \frac{K'_{AL} [L]_2}{1 + K_{AS} C_S} \quad (\text{A4})$$

where K'_{AL} is the association equilibrium constant for A with L after I has bound to L, and $[L]_2$ is the concentration of binding agent occupied by I. The total retention factor for A (k) will be equal to the sum of the retention factors for A at these two classes of sites, or $k = k_1 + k_2$. Using this fact with eqs A3 and A4 gives eq A5.

$$k = \frac{K_{AL} [L]_1}{1 + K_{AS} C_S} + \frac{K'_{AL} [L]_2}{1 + K_{AS} C_S} \quad (\text{A5})$$

Eqs A1 and A2 can be rewritten to relate the total concentration of binding agent (C_L) to the concentrations of the individual forms of this binding agent. This is shown in eqs A6-A7, where $C_L = m_L/V_M$, $[L]_1 = m_{L1}/V_M$, and $[L]_2 = m_{L2}/V_M$, and V_M is the column void volume.

$$[L]_1 = \frac{C_L}{1 + K_{IL} [I]} \quad (\text{A6})$$

$$[L]_2 = \frac{K_{IL} [I]}{1 + K_{IL} [I]} \cdot C_L \quad (\text{A7})$$

When eqs A6 and A7 are substituted into eq A5, this results in eq A8.

$$k = \frac{K_{AL} \cdot \frac{C_L}{1 + K_{IL} [I]}}{1 + K_{AS} C_S} + \frac{K'_{AL} \cdot \frac{K_{IL} [I]}{1 + K_{IL} [I]} \cdot C_L}{1 + K_{AS} C_S} \quad (\text{A8})$$

Furthermore, the non-bound concentration of competing agent I in the mobile phase can be described as shown below,¹⁹

$$[I] = C_I / (1 + K_{IS} C_S) \quad (\text{A9})$$

where C_I is the total concentration of I, and K_{IS} is the association equilibrium constant between I and S. Eq A9 assumes the concentrations of I and A are much smaller than C_S , allowing the amount of bound solubilizing agent to be negligible versus its total concentration. Placing eq A9 into A8 gives,

$$k = \frac{K_{AL} \cdot \frac{C_L}{1 + K_{IL} C_I / (1 + K_{IS} C_S)}}{1 + K_{AS} C_S} + \frac{K'_{AL} \cdot \frac{K_{IL} C_I / (1 + K_{IS} C_S)}{1 + K_{IL} C_I / (1 + K_{IS} C_S)} \cdot C_L}{1 + K_{AS} C_S} \quad (\text{A10})$$

which leads to eq 8 when common terms in this relationship are combined.

If no competing agent is present in the mobile phase (i.e., $C_I = 0$), eq A10 reduce to eq A11.

$$k_0 = \frac{K_{AL} \cdot C_L}{1 + K_{AS} C_S} \quad (\text{A11})$$

By subtracting eq A10 from A11, eq A12 is then obtained.

$$k - k_0 = \frac{(K'_{AL} - K_{AL}) \cdot \frac{K_{IL} C_I / (1 + K_{IS} C_S)}{1 + K_{IL} C_I / (1 + K_{IS} C_S)} \cdot C_L}{1 + K_{AS} C_S} \quad (\text{A12})$$

Furthermore, if eq A12 is divided by eq A10 and $\beta_{I \rightarrow A}$ is substituted in for K'_{AL}/K_{AL} , the form shown in eq 9 is acquired. A similar process is used to derive eqs 14 and 15 when dealing with systems where A has non-specific binding or secondary interactions that are independent of I.^{6,10,50}

NOMENCLATURE

$\beta_{A \rightarrow I}$, Coupling constant for the effect of A on the binding of I to L, where $\beta_{A \rightarrow I} = K'_{IL}/K_{IL}$
 $\beta_{I \rightarrow A}$, Coupling constant for the effect of I on the binding of A to L, where $\beta_{I \rightarrow A} = K'_{AL}/K_{AL}$
 A, Injected analyte
 C_S , Total mobile phase concentration of solubilizing agent S

C_I , Total mobile phase concentration of competing agent I
 C_L , Total concentration of immobilized binding agent L
 I , Competing agent present in the mobile phase
 $[I]$, Molar concentration for non-bound I in the mobile phase
 k , Retention factor for injected analyte A
 k_0 , Retention factor for A when there is no competing agent in the mobile phase
 k_1 , Retention factor for A due to binding with L
 k_2 , Retention factor for A due to binding with L in the presence of bound I
 K_{AL} , Association equilibrium constant for A with L
 K_{AL}' , Association equilibrium constant for A with L in the presence of bound I
 K_{IL} , Association equilibrium constant for I with L
 K_{IL}' , Association equilibrium constant for I with L in the presence of bound A
 K_{IS} , Association equilibrium constant for I with S
 K_{AS} , Association equilibrium constant for A with S
 L , Immobilized binding agent
 $[L]$, Effective total concentration of immobilized binding agent
 $[L]_1$, Concentration of binding agent that is not bound to I
 $[L]_2$, Concentration of binding agent that is bound to I
 m_L , Total moles of immobilized binding agent
 m_{L1} , Moles of binding agent that have formed a complex with I
 m_{L2} , Moles of binding agent that have not formed a complex with I
 S , Solubilizing agent
 x , Retention factor for A due to secondary interactions that are independent of I

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Warfarin

Tamoxifen

Figure 1.
Structures of warfarin and tamoxifen.

(a) Direct Competition

(b) Allosteric competition ($C_I \gg C_A$) **Figure 2.**

General model for (a) direct competition or (b) allosteric competition for the binding of analyte A and mobile additive I with immobilized binding agent L in the presence of solubilizing agent S . The other symbols are defined in the text.

Figure 3.

Chromatograms for (a) self-competition zonal elution studies for warfarin, (b) zonal elution studies examining the competition of injected tamoxifen in the presence of various mobile phase concentrations of warfarin, and (c) zonal elution studies examining the competition of injected warfarin in the presence of various mobile phase concentrations of tamoxifen. The dashed lines show the mean retention times measured for the injected analytes in the presence of no competing agent (Note: in (c) the dashed line is shown for the second warfarin peak, which is due to the *S*-enantiomer of warfarin). The total concentration of warfarin (C_{Warfarin}) or tamoxifen ($C_{\text{Tamoxifen}}$) that was used as a competing agent in the mobile phase is indicated to the right of each chromatogram. Other conditions are given in the text.

Figure 4.

Plots of (a) $1/k$ versus the total mobile phase concentration of warfarin (C_{Warfarin}) and (b) $k_0/(k - k_0)$ versus $1/C_{\text{Warfarin}}$ for self-competition zonal elution studies with warfarin in the presence of 2.2 mM β -cyclodextrin as a solubilizing agent. The equations for the best-fit lines were: (a) $y = 0.030 (\pm 0.001) \times + 0.120 (\pm 0.003)$, with a correlation coefficient of 0.997 ($n = 5$); and (b) $y = -4.42 (\pm 0.24) \times - 0.86 (\pm 0.14)$, with a correlation coefficient of 0.997 ($n = 4$). The error bars in (a) and (b) represent a range of ± 2 S.D.

Figure 5.

Plots of (a) $1/(k-x)$ versus the total mobile phase concentration of tamoxifen ($C_{\text{Tamoxifen}}$) and (b) $(k_0-x)/(k-k_0)$ versus $1/C_{\text{Tamoxifen}}$ for self-competition zonal elution studies with tamoxifen in the presence of 2.2 mM β -cyclodextrin. The term x here represents the non-specific binding of tamoxifen to the support, as measured on the control column. The equations for the best-fit lines in this figure were (a) $y = 0.079 (\pm 0.009) \times + 0.043 (\pm 0.015)$, with a correlation coefficient of 0.98 ($n = 5$); (b) $y = -0.24 (\pm 0.06) \times - 1.08 (\pm 0.05)$, with a correlation coefficient of 0.94 ($n = 4$). The error bars in (a) and (b) represent a range of ± 2 S.D.

Figure 6.

Plots of (a) $1/(k-x)$ versus the total mobile phase concentration of warfarin (C_{Warfarin}) and (b) $(k_0-x)/(k-k_0)$ versus $1/C_{\text{Warfarin}}$ for tamoxifen binding to immobilized HSA in the presence of 2.2 mM β -cyclodextrin and various concentrations of warfarin in the mobile phase. In these plots, k and k_0 are the retention factors for tamoxifen in the presence or absence of warfarin in the mobile phase, respectively. The term x is the retention factor due to non-specific binding. The equation for the best-fit line in (b) is $y = 7.6 (\pm 0.3) \times + 0.79 (\pm 0.17)$, with a correlation coefficient of 0.999 ($n = 4$). The error bars in (a) and (b) represent a range of ± 2 S.D.

Figure 7.

Plots of (a) $1/k$ versus the total mobile phase concentration of tamoxifen ($C_{\text{Tamoxifen}}$) and (b) $k_0/(k - k_0)$ versus $1/(C_{\text{Tamoxifen}})$ for injections of warfarin in the presence of 2.2 mM β -cyclodextrin and various concentrations of tamoxifen in the mobile phase. In these plots, k and k_0 are the retention factors for warfarin in the presence or absence of tamoxifen in the mobile phase, respectively. The equations for the best-fit lines are as follows: (a) $y = 0.0064 (\pm 0.0017) \times + 0.093 (\pm 0.003)$, with a correlation coefficient of 0.91 ($n = 5$); (b) $y = -3.3 (\pm 1.1) \times - 4.8 (\pm 0.8)$, with a correlation coefficient of 0.91 ($n = 4$). The error bars in (a) and (b) represent a range of ± 2 S.D.

Figure 8.

General model for the interactions between warfarin and tamoxifen on HSA. The association equilibrium constants (K) given here are the averages of the various values measured in this study. The coupling constants (β) were determined from the competition experiments described in the text between warfarin and tamoxifen. The overall range of association constants found in this report for warfarin with HSA was $2\text{--}5 \times 10^5 \text{ M}^{-1}$ ($n = 3$) at 37°C . The range of association equilibrium constants determined for tamoxifen for HSA was $3\text{--}4 \times 10^7 \text{ M}^{-1}$ ($n = 4$) at 37°C (Note: one additional value of $1.1 \times 10^8 \text{ M}^{-1}$ was determined, but this was not included in the given average since it was a suspected outlier; when this was included, the average association equilibrium constant for tamoxifen with HSA was $5.0 (\pm 3.9) \times 10^7 \text{ M}^{-1}$).

Table 1

Retention factors obtained in zonal elution competition studies on an immobilized HSA column for injections of warfarin or tamoxifen in the presence of various mobile phase concentrations of these solutes^a

C_{Warfarin} (μM)	k_{Warfarin}	$k_{\text{Tamoxifen}}$	$C_{\text{Tamoxifen}}$ (μM)	k_{Warfarin}	$k_{\text{Tamoxifen}}$
0	8.27 (± 0.03)	30.3 (± 0.6)	0	11.11 (± 0.02)	33.54 (± 0.14)
1	6.71 (± 0.02)	33.5 (± 1.2)	0.9	9.75 (± 0.17)	11.15 (± 0.11)
2	5.42 (± 0.02)	36.4 (± 1.5)	1.4	9.70 (± 0.04)	9.72 (± 0.05)
3	4.87 (± 0.03)	38.0 (± 0.1)	1.8	9.31 (± 0.04)	9.67 (± 0.06)
4	4.11 (± 0.04)	40.5 (± 1.2)	2.8	9.26 (± 0.01)	7.36 (± 0.13)

^aThe terms k_{Warfarin} and $k_{\text{Tamoxifen}}$ are the average retention factors measured for injections of warfarin and tamoxifen, respectively. The terms C_{Warfarin} and $C_{\text{Tamoxifen}}$ are the mobile phase concentrations of these agents during the given experiments. The numbers in parenthesis represent a range of 1 S.D. All listed retention factors were measured at pH 7.4 and at 37°C. Other conditions are given in the text.