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Studies of phenytoin binding to human serum albumin by high-performance affinity chromatography

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Abstract

High-performance affinity chromatography was used to study the binding of phenytoin to an immobilized human serum albumin (HSA) column. This was accomplished through frontal analysis and competitive binding zonal elution experiments, the latter of which used four probe compounds for the major and minor binding sites of HSA injected into the presence of mobile phases containing known concentrations of phenytoin. It was found that phenytoin can interact with HSA at the warfarin-azapropazone, indole-benzodiazepine, tamoxifen, and digitoxin sites of this protein. The association constants for phenytoin at the indole-benzodiazepine and digitoxin sites were determined to be 1.04 ($\pm$0.05) $\times$ 10^4 M^{-1} and 6.5 ($\pm$0.6) $\times$ 10^3 M^{-1}, respectively, at pH 7.4 and 37 °C. Both allosteric interactions and direct binding for phenytoin appear to take place at the warfarin-azapropazone and tamoxifen sites. This rather complex binding system indicates the importance of identifying the binding regions on HSA for specific drugs as a means for understanding the transport of such substances in blood and in characterizing their potential for drug–drug interactions.

Keywords: Phenytoin; Human serum albumin

1. Introduction

Drug–protein binding in blood affects the transport, distribution, metabolism and elimination of many pharmaceutical agents. These binding processes can also be a source of drug–drug interactions [1–3]. This occurs when there is either direct or indirect competition of two or more drugs for the same binding proteins. As a result, information on the number and types of interaction sites that a particular drug has on a given protein can be valuable in predicting how this agent will be affected by other substances. One protein that is often involved in such interactions is human serum albumin (HSA). HSA has two major binding sites for drugs: the warfarin-azapropazone site (Sudlow site I) and the indole-benzodiazepine site (Sudlow site II) [4]. It is also believed there are various minor binding regions on HSA for drugs, including the tamoxifen and digitoxin sites [5,6].

Phenytoin is an anti-convulsant drug that is widely used for the treatment of seizures. The therapeutic concentration for phenytoin in serum is approximately 40–80 μM [7], with about 90% of this drug being bound to plasma proteins [8–10]. Most of this binding occurs with HSA [11], but a small fraction also binds to α-globulins [12,13] and β-lipoproteins [14]. The results of earlier studies that have examined the interactions of phenytoin with HSA are summarized in Table 1 [11,15–19]. The association constants reported for phenytoin in such studies have ranged from 745 to 2 $\times$ 10^4 M^{-1} and the number of observed binding sites for this drug on HSA have ranged from 0.85 to 8. As this indicates, there is still a great deal of uncertainty in both the number of binding sites for phenytoin on HSA and the strengths of these interactions.

A few papers have considered the additional question of where phenytoin's binding sites are located. For example, some reports have proposed that phenytoin binds to the warfarin-azapropazone site [18,20]. Others have noted that digitoxin increases free phenytoin concentrations in blood, suggesting an interaction of phenytoin with the digitoxin site of HSA [20,21]. However, no systematic examination of phenytoin's binding to these and other sites on HSA has been reported.

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Table 1
Association constants and number of binding sites reported for phenytoin with HSA in previous studies

Association constant (M^{-1})	Number sites on HSA	[Phenytoin] (μM)	[HSA] (μM)	Experimental method and conditions	Ref.
2.0×10^4	0.85	3.7–122.1	536–749	Ultrafiltration, 25 °C	[15]
745	8	0.2–20	75	Equilibrium dialysis, pH 7.4, 37 °C	[16]
9×10^3 (37 °C); 1.6×10^4 (25 °C)	1	4.9–106.6	556–777	Ultrafiltration, 25 and 37 °C	[17]
1.76×10^3 (uremic); 4.10×10^3 (normal)	3.0–3.8; 2.5–2.9	57	304–565	Equilibrium dialysis, 2% ethanol, pH 7.4, 37 °C	[11]
1.7×10^4	1.4	~1–30	18.2	HSA on microparticles, 2% ethanol, pH 7.4, 25 °C	[18]
4.74×10^3 (1%, w/v HSA); 3.92×10^3 (2%, w/v HSA)	Not determined	0.37–739	1–2%, w/v HSA	Equilibrium dialysis, pH 7.4, 37 °C	[19]

The values given in the left column for Ref. [19] represent the product nK, where n is the number of binding sites and K is the apparent association constant.

This study will use high-performance affinity chromatography (HPAC) to examine the binding of phenytoin to specific regions on HSA. HPAC is a liquid chromatographic method that makes use of an immobilized ligand (e.g., HSA) attached to an HPLC support such as silica. In the past it has been shown that immobilized HSA columns can be used as effective models to obtain a variety of information on drug interactions with HSA, including the association constants and binding sites involved in such processes (see review given in Ref. [22]). This current report will use two approaches, frontal analysis and zonal elution, to determine the association constants and binding sites for phenytoin with HSA. Part of this work will involve competition studies between phenytoin and other solutes that have known binding regions on HSA. From these experiments it will be possible to develop a more complete picture of how phenytoin binds to HSA in the circulation.

2. Theory

The theory for the determination of equilibrium constants by affinity chromatography has been previously reviewed [22]. As a general model, if a solute (A) binds to a single type of immobilized ligand (L), the equations that describe the binding of A to this ligand are as follows,

$$\mathrm{A} + \mathrm{L} \overset{K_\mathrm{a}}{\rightleftharpoons} \mathrm{A} - \mathrm{L} \tag{1}$$

$$K_\mathrm{a} = \frac{\{\mathrm{A} - \mathrm{L}\}}{[\mathrm{A}]\{\mathrm{L}\}} \tag{2}$$

where K_a is the association equilibrium constant for the binding of A to L. In Eq. (2), [A] is the molar concentration of solute in the mobile phase, and $\{\mathrm{L}\}$ and $\{\mathrm{A} - \mathrm{L}\}$ are the surface concentrations of the ligand and solute-ligand complex at equilibrium.

One method that can be used with an affinity column to examine the binding between A and L is frontal analysis. In this technique a solution with a known concentration of pure solute is continuously applied to a column containing a fixed amount of immobilized ligand. As the column becomes saturated, the amount of analyte eluting from the column gradually increases, forming a characteristic breakthrough curve, as shown in Fig. 1(a). The position of this curve is related to the concentration of applied analyte, the amount of ligand present, and the association constants for the system. For a column containing a single type of immobilized ligand site, the apparent moles of analyte required to reach the mean point of the resulting breakthrough curve (m_{Lapp}) is given by Eq. (3) [22],

$$\frac{1}{m_{\mathrm{Lapp}}} = \frac{1}{(K_\mathrm{a}\, m_\mathrm{L}[\mathrm{A}])} + \frac{1}{m_\mathrm{L}} \tag{3}$$

where [A] is the concentration of solute applied to the column, and m_L is the true moles of binding sites in the column. Eq. (3) predicts that a plot of $1/m_{\mathrm{Lapp}}$ versus 1/[A] for

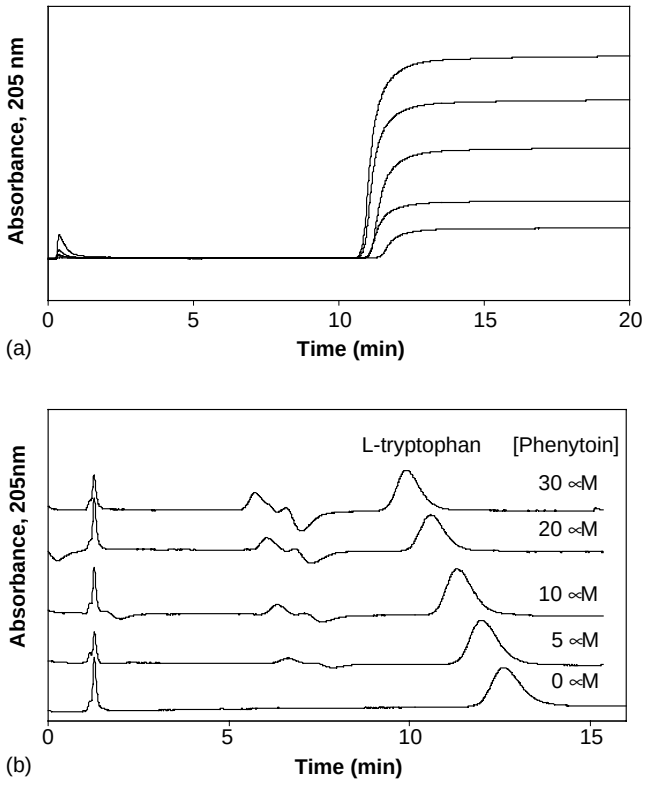

Fig. 1. Examples of chromatograms obtained on HSA columns during (a) frontal analysis studies with phenytoin and (b) competitive zonal elution studies with L-tryptophan as the injected analyte and phenytoin as the mobile phase additive. The experimental conditions are given in Section 3. The concentrations of phenytoin in (a) were 5, 10, 20, 30 and 40 μM.

this system will give a straight line with a slope of $1/(K_a m_L)$ and an intercept of $1/m_L$ for a system with 1:1 interactions. The association constant can be determined directly from this plot by calculating the ratio of the intercept to the slope. For systems with multiple binding sites, plots prepared according to Eq. (3) can also give rise to linear regions at low-to-moderate concentrations of A. This occurs when the concentration of A is sufficiently low to allow binding at its high affinity sites to be much greater than to its low affinity sites. Information on the conditions needed to obtain such behavior can be found in Ref. [23]. Under such conditions, the value of K_a obtained from Eq. (3) can be used as an approximation of the highest association constant in the system being examined [23].

Another approach that can be used with affinity chromatography to obtain equilibrium constants is zonal elution. This method is often performed by placing a known concentration of a competing agent (I) in the mobile phase while small injections of the analyte (A) are made (see Fig. 1(b)). If I and A compete at a single site on L and A has no other interactions with the column, the following equation can be used to describe the observed retention of A [22].

$$\frac{1}{k} = \frac{K_I V_M [I]}{K_a m_L} + \frac{V_M}{K_a m_L} \quad (4)$$

In the above equation, V_M is the void volume of the column (i.e., the elution volume of a non-retained solute), m_L is the moles of binding sites involved in the competition of A with L, [I] is the concentration of competing agent in the mobile phase, K_a is the association constant for the binding of A to L at the site of competition with I, and K_I is the association constant for I at the same site. The term k is the retention factor for the injected solute, or $k = (t_R/t_M) - 1$ where t_R is the retention time of the solute and t_M is the void time of the column. Eq. (4) has been shown to be valuable in studying compounds with multiple binding sites on HSA by using these agents as the competing agent and site-specific probe as the injected solutes [24,25]. This approach will also be used in this report for examining the binding of phenytoin at each of the major and minor binding sites of HSA.

A special situation occurs in zonal elution experiments when the same compound is used as both the competing agent and injected analyte. Under these conditions, Eq. (4) converts to the following form for a system with 1:1 interactions between A and L [22].

$$\frac{1}{k} = \frac{V_M [A]}{m_L} + \frac{V_M}{K_a m_L} \quad (5)$$

This equation indicates that a plot of $1/k$ versus [A] will again give a linear relationship, with the inverse of the slope now giving m_L/V_M and ratio of the slope to the intercept providing K_a. One benefit of using such a plot is it can be used to confirm whether 1:1 interactions really are present between the injected analyte and ligand, since deviations from a linear response should be seen at or near the intercept (i.e., at low analyte concentrations) for systems with multiple binding sites.

3. Experimental

3.1. Reagents

The HSA (99% fatty acid free), phenytoin (99% pure), L-tryptophan (>98% pure), digitoxin (97% pure), and bicinchoninic acid (BCA) reagent were from Sigma (St. Louis, MO). The *R*-warfarin (>99% pure) was from Ultrafine (Manchester, England) and the *cis*-clomiphene was supplied by the Marion Merrell Dow Research Institute (Cincinnati, OH). Nucleosil Si-300 and Si-500 silica (7 μm particle size, 300 or 500 Å pore sizes, respectively) were from P.J. Cobert (St. Louis, MO). All solutions were prepared with water obtained from a Nanopure water system (Barnstead, Dubuque, IA).

3.2. Apparatus

The chromatographic system consisted of two Jasco PU980 pumps (Easton, MD), two Rheodyne six-port valves (Cotati, CA), an Alltech water jacket (Deerfield, IL) and a Milton Roy 3100 UV detector (Riviera Beach, FL). One of the injection valves was used to switch the mobile phase

from a competing agent solution to a pH 7.4 potassium phosphate buffer. The other injection valve was used to inject samples into the desired application buffer. The water jacket held water circulated from an Isotemp 9100 water bath (Fisher Scientific, Pittsburgh, PA). The chromatographic data were collected with programs written using Labview software (National Instruments, Austin, TX).

3.3. Methods

The Nucleosil supports were converted into a diol-bonded form using a previously published method [26]. HSA was immobilized onto the diol-bonded supports using the Schiff base method [27]. After the immobilization step, the silica was centrifuged, and washed three times with pH 7.4, 0.067 M phosphate buffer. This support was stored at 4 °C prior to use. A BCA assay was performed on a dried portion of each support using HSA as the standard and diol-bonded silica as blank [28]. The measured protein content of these supports ranged from 20–54 mg/g silica. The HSA silica and a control support (i.e., diol-bonded silica taken through the immobilization process without HSA being added) were downward slurry-packed into separate 5 cm × 4.6 mm I.D. stainless steel columns at 3500 psi using pH 7.4, 0.067 M phosphate buffer as the packing solution.

Although phenytoin shows significant degradation after 24 h in plasma or blood at 25–37 °C [29,30], this drug has been reported to be stable in an aqueous solution for over 39 weeks at −20 to 25 °C [31]. Experiments in this current study also found no significant change in phenytoin solutions after 6 months of storage in pH 7.4, 0.067 M potassium phosphate buffer at room temperature. In general, all phenytoin solutions used in this report were prepared just before an experiment and used for no more than 1 week.

Although the solubility of phenytoin in aqueous solution at physiological pH has been reported to be 73–105 μM [32–34], it was found that a 50 μM phenytoin solution took up to 12 h to dissolve in pH 7.4, 0.067 M phosphate buffer without the use of an organic additive. To avoid this problem, the phenytoin was instead dissolved in pH 11.0, 0.067 M phosphate buffer and slowly titrated to pH 7.4 by adding pH 6.0, 0.067 M phosphate buffer. This stock solution was then diluted to the desired concentration by adding pH 7.4, 0.067 M phosphate buffer.

Frontal analysis was performed by continuously applying to each column a pH 7.4, 0.067 M phosphate buffer containing 5–40 μM phenytoin. The retained phenytoin was eluted by switching to a mobile phase that contained only pH 7.4, 0.067 M phosphate buffer. The amount of phenytoin required to saturate the column was determined by integration of the resulting breakthrough curve. Corrections for the void time and non-specific binding were made by subtracting the breakthrough time needed for the same solution on an identical column containing the control support.

Zonal analysis was performed by injecting small amounts of several probe compounds onto the HSA column or control column. All of these probe solutions were prepared in pH 7.4, 0.067 M phosphate buffer, which was also the mobile phase used in these experiments. Each of these probe solutions was found to be stable for several days except for those containing L-tryptophan, which were prepared fresh daily and stored in the refrigerator when not in use. The injected volumes and concentrations of these injected solutions were as follows: 5 μl, 50 μM phenytoin; 5 μl, 20 μM *R*-warfarin; 20 μl, 5 μM L-tryptophan; 20 μl, 25 μM *cis*-clomiphene (with 0.88 mM β-cyclodextrin in both the sample and mobile phase); and 20 μl, 5 μM digitoxin. No changes in the retention factors for these probes were noted when smaller concentrations of these compounds were injected, indicating that linear elution conditions were present during these studies.

The wavelengths used to monitor the elution of phenytoin, *R*-warfarin, *cis*-clomiphene, digitoxin, and L-tryptophan were 205, 308, 205, 205.5, and 214 nm, respectively. Two or more injections were made under each set of experimental conditions. The column void time was determined by injecting 20 μl of 0.27 mM sodium nitrate onto the chromatographic system throughout this study. The void volume was found to be reproducible throughout this work and did not show any significant changes when using either potassium phosphate buffer as the mobile phase or this buffer plus 50 μM phenytoin as the mobile phase. All retention times were determined by using moment analysis or the $B/A_{0.1}$ and $B/A_{0.5}$ methods [35]. Unless stated otherwise, all experiments described in this report were performed at 37 °C. The zonal elution studies with warfarin were performed at 25 °C as well. A flow rate of 0.2 ml/min was used in the frontal analysis and self-competition zonal elution studies. The flow rate in the zonal elution experiments with *cis*-clomiphene as the probe was 2.0 ml/min. All other experiments used a flow rate of 0.5–1.0 ml/min. No significant differences in the retention factors of the probes (i.e., random variations of only a few percent) were noted over this flow rate range, indicating that sufficiently fast association and dissociation rates were present for use of the HSA columns in equilibrium constant measurements [25]. The pressure across the columns at these flow rates was less than 33 kg/cm^2 (460 psi).

The types of HSA columns used in this study have been shown in previous work to be stable for 6–9 months and over 500 injections with only a gradual loss of activity [24,25]. In this particular study, the HSA columns were used over a much shorter period of time, with the majority of experiments being conducted within three days. No significant shifts in retention for any of the injected probe compounds were noted over this period of time when measured under constant mobile phase conditions. Furthermore, the use of slope and intercept ratios with Eqs. (3)–(5) is known to minimize the effects of any changes in column capacity over time, since this approach provides association constants that are independent of m_L [23–25].

4. Results and discussion

4.1. Frontal analysis and self-competition zonal elution studies

The overall binding properties of phenytoin on the HSA column were first evaluated by frontal analysis [22]. Fig. 2(a) shows the results obtained when these data were plotted according to Eq. (3). When applying phenytoin at concentrations of 5–40 μM to the HSA column, the result was a linear relationship for a plot of $1/m_{Lapp}$ versus 1/[Phenytoin] at 37 °C. The correlation coefficient for this plot was 0.9995 over the five points shown in Fig. 2(a). Normally such a relationship would suggest that a single type of binding site is present for this interaction, but it has been shown that apparently linear behavior can also be obtained for such plots with systems that have multiple binding sites when working at moderate-to-high analyte concentrations (see Ref. [23] for a detailed discussion of the theory behind this effect).

According to Eq. (3), it was possible to determine both the apparent association constant for phenytoin with HSA and binding capacity of the column by using the intercept and slope of the plot in Fig. 2(a). This gave a K_a value for phenytoin and HSA of 8.8 (±2.0) × 10^3 M^{-1} and a binding capacity of 99 (±22) nmol. When this number was combined with a measured column void volume of 0.71 mL, this gave an effective concentration of 140 (±30) μM for the binding sites in the column, or 1.6 times the known amount of immobilized HSA (87 μM). This indicated that more than one mole of phenytoin was binding per mole of HSA. In this type of situation, the value of K_a obtained from Fig. 2(a) can be viewed as an estimate of the affinity for the strongest binding site of phenytoin on HSA, as discussed in Ref. [23].

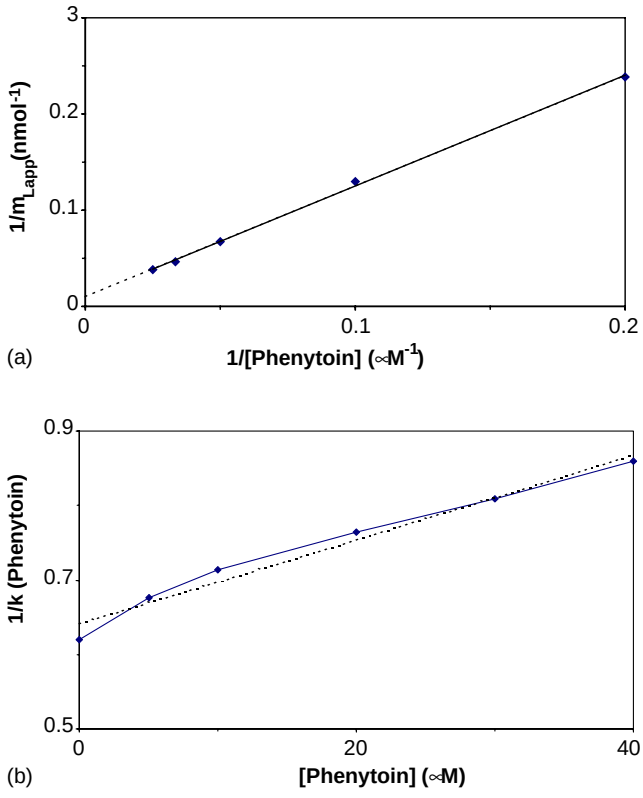

Fig. 2. (a) Plot of $1/m_{L,app}$ vs. 1/[Phenytoin] for frontal analysis experiments of phenytoin binding to immobilized HSA and (b)1/*k* vs. [Phenytoin] for self-competition zonal elution studies. The equation for the best fit line in (a) is $y = 1.15\ (\pm 0.02) \times + 0.010\ (\pm 0.002)$, where the numbers in parentheses represent a range of ±1 S.D. The best fit line in (b) was $y = 0.0057\ (\pm 0.0004) \times + 0.64\ (\pm 0.01)$, with a correlation coefficient of 0.988 ($n = 6$).

The binding of phenytoin to HSA was also examined by performing self-competition zonal elution studies. These were conducted by using 5 μl of 50 μM phenytoin as the injected probe and 0–40 μM phenytoin as the competing agent in the mobile phase. A correction for non-specific binding of phenytoin to the support was made by performing the same experiment on a control column. This involved subtracting phenytoin's retention factor on the control column from that measured on the HSA column under each set of experimental conditions, where the retention on the control column was typically 45–50% of that seen on the HSA column.

One possible problem with this correction is it assumes the same number of non-specific sites are present in both the HSA and control columns. A more sophisticated approach makes an adjustment for the secondary sites covered by HSA based on the known surface area and packing density of the support (35 m^2/g and 0.45 g/ml), the measured protein content of the HSA support (20 mg/g silica) and the approximate area of HSA (40 Å × 140 Å) [36]. From these values, it was determined that HSA covered approximately 65% of the total surface area of the support. This value was then used to adjust the retention factors measured on the control column (i.e., a decrease of 65% in these values) before they were used to correct the HSA data. A similar method could be used to correct the frontal analysis data in Fig. 2(a), but was not required in this study since these particular results were used only to give a preliminary estimate for the association constant of phenytoin at its high affinity sites on HSA.

The results obtained with this latter approach are shown in Fig. 2(b). A similar plot was obtained without the surface area correction for HSA but with slightly higher 1/*k* values (i.e., lower adjusted retention factors). According to Eq. (5), if there is only one type of binding site for phenytoin on HSA, then a plot of 1/*k* versus [Phenytoin] should give a linear relationship. However, the plot in Fig. 2(b) gave a curved relationship even when the possible presence of non-specific binding was considered. Thus, this again indicated that more than one binding site was present on HSA for phenytoin.

4.2. Zonal elution studies with R-warfarin as the injected probe

The next set of zonal elution studies used *R*-warfarin as an injected probe. These experiments were performed to see if there were any interactions for phenytoin at the

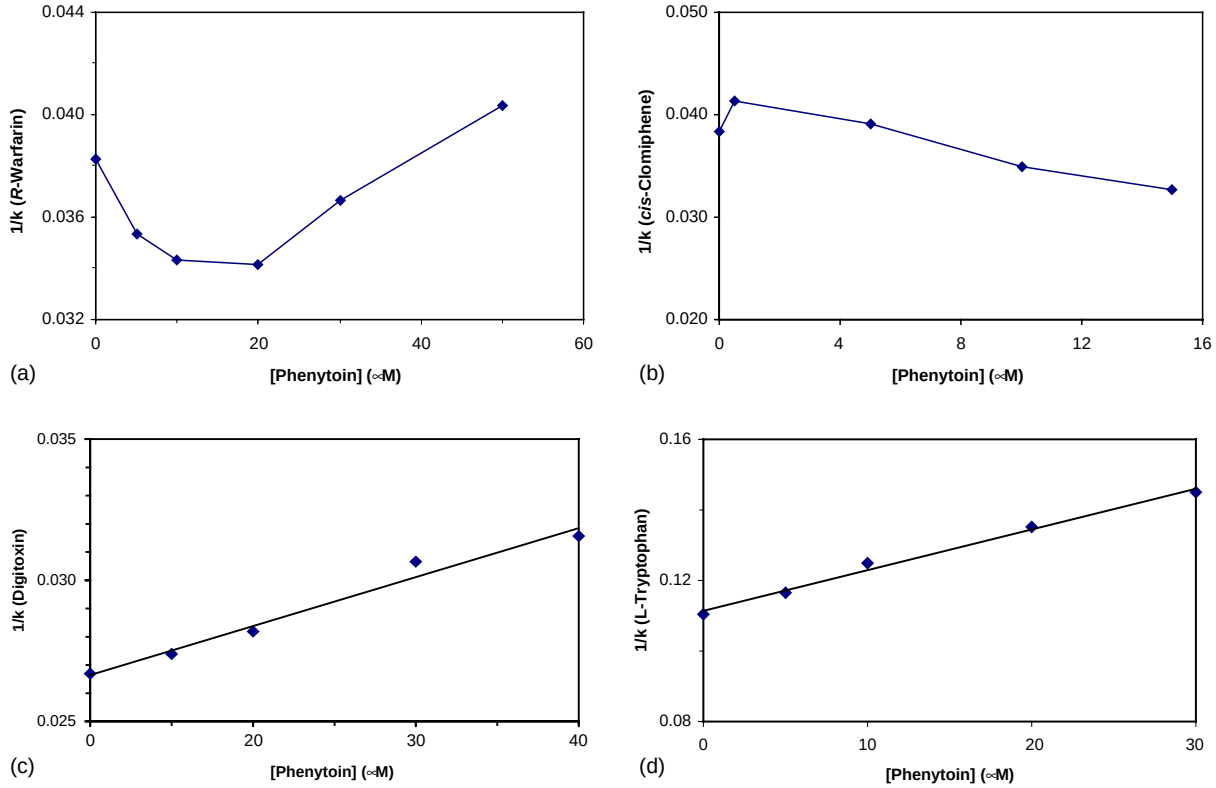

Fig. 3. Zonal elution plot of $1/k$ vs. [Phenytoin] for injections of (a) *R*-warfarin at 0–40 μM phenytoin, (b) *cis*-clomiphene at 0–15 μM phenytoin (with a correction for secondary binding based on results obtain with a control column), (c) digitoxin at 0–30 μM phenytoin, and (d) L-tryptophan at 0–30 μM phenytoin. The best fit line in (c) was $y = 1.73\ (\pm 0.16) \times 10^{-4} \times + 0.0266\ (\pm 0.0003)$, with a correlation coefficient of 0.988 ($n = 5$). The best fit line in (d) was $y = 1.13\ (\pm 0.05) \times 10^{-3} \times + 0.109\ (\pm 0.001)$, with a correlation coefficient of 0.997 ($n = 5$).

warfarin-azapropazone site of HSA, to which *R*-warfarin is known to bind. In this work, the retention of *R*-warfarin to the control column was less than 3% of its retention on the HSA column (as noted in earlier studies) [24,27], so no corrections for non-specific binding by this probe to the support had to be made in these experiments.

Fig. 3(a) shows how the retention of *R*-warfarin changed at 37 °C as the concentration of phenytoin was varied in the mobile phase. This gave an increase in retention for *R*-warfarin as 0–20 μM phenytoin was added to the mobile phase (i.e., a decrease in $1/k$), followed by a decrease in retention (i.e., increase in $1/k$) at higher phenytoin concentrations. A similar trend was noted at room temperature when using a different HSA column. These results indicated that multiple interactions are taking place between phenytoin and *R*-warfarin. Previous work with phenytoin and warfarin has noted direct competition between these two compounds on HSA [18,20]. This fits the behavior observed at high phenytoin concentrations to the right of Fig. 3(a); however, this does not fit the behavior seen at lower phenytoin concentrations. The failure to note this pattern in earlier studies may have been due to their emphasis on relatively high phenytoin concentrations [18,20]. However, further information on the affinity for the direct competition site in Fig. 3(a) is needed before any conclusive comparison can be made between these results and those in the literature.

The curve obtained in Fig. 3(a) again indicates that phenytoin has at least two sites of interaction with HSA. In this case, one site might allow for the direct competition or negative allosteric effects between phenytoin and *R*-warfarin. The linear region in the right-hand portion of Fig. 3(a) indicates that direct competition is the more likely of these two possibilities. However, the curvature seen at low phenytoin concentrations can be explained by the presence of a second binding site that involves positive allosteric interactions between *R*-warfarin and phenytoin. The presence of direct competition between these two agents suggests that phenytoin binds to the warfarin-azapropazone site of HSA, which is the major binding region for *R*-warfarin under the conditions used in this study. The location of the other region, which gives rise to the positive allosteric effects between *R*-warfarin and phenytoin, will be examined later through the use of probes for the other sites on HSA.

During these studies, the use of phenytoin as the injected analyte and *R*-warfarin or other probes as the mobile phase additive was also considered. However, the analysis of data in such experiments was complicated by the fact that phenytoin had multiple binding sites on HSA, with only a few of these being affected by any given competing agent. This gave rise to mixed retention behavior for phenytoin even when using a competing agent with only one binding site on HSA. In addition, the slope and intercept ratio obtained

from Eq. (4) gives the association constant for the mobile phase additive and not for the injected analyte in this approach. Since the association constants were already known for the various probes used in this study, these additional experiments did not provide any additional information beyond that shown in this current report.

The biphasic behavior noted in Fig. 3(a) indicates the site of interaction involving allosteric effects with *R*-warfarin might become saturated at relatively low concentrations of phenytoin. If true, this suggests this site has a high affinity for phenytoin and/or is present in a small amount versus phenytoin's second binding region. However, whether or not this first site is really saturated is difficult to determine since direct competition becomes dominant at only slightly higher concentrations. A more detailed analysis of this situation and its causes will be considered in future work.

4.3. Zonal elution studies with cis-clomiphene as the injected probe

The interactions of phenytoin with the tamoxifen site of HSA were examined by using *cis*-clomiphene as the injected probe. Due to the low solubility of *cis*-clomiphene, 0.88 mM β-cyclodextrin (β-CD) was used as a solubilizing agent to prepare the *cis*-clomiphene solution. β-CD has been shown in previous work with HSA columns to make it easier to examine the protein binding of non-polar substances like *cis*-clomiphene without interfering in the interactions of such substances with HSA [37,38]. To avoid any mismatch in detector response between the mobile phase and probe solutions, the same concentration of β-CD was also added to the mobile phase. Identical solutions were used in experiments with the control column. Although Eq. (4) no longer applies to a system that has additional interactions with a binding agent in the mobile phase, an expanded version of this equation can be used under these conditions. Like Eq. (4), this alternative expression predicts a linear relationship between $1/k$ and the concentration of mobile phase additive for an analyte and additive with single site competition on an immobilized ligand, provided the experiment is performed under linear elution conditions and in the presence of a fixed excess of solubilizing agent. Further details on this approach can be found in Refs. [37,38].

Due to the strong retention of *cis*-clomiphene on the HSA column, a flow rate of 2.0 ml/min was used to help reduce the analysis time. Under the given solvent conditions, the retention of *cis*-clomiphene on the control column was about half of its observed retention on the HSA column, so an adjustment for these secondary interactions was required. This correction was made in the same fashion as described in Section 4.2 for the phenytoin self-competition studies. In this case, an adjustment for the area covered by HSA was calculated by using a surface area for the support of 100 m^2/g, a packing density of 0.45 g/ml, and a protein content of 35.5 mg/g. The result indicated that 21.7% of the support's total surface area was covered by HSA. Thus, the retention factors measured on the control column were decreased by this amount before they were used to correct the retention data for *cis*-clomiphene on the HSA column.

Fig. 3(b) shows the results obtained when a plot was made of $1/k$ for *cis*-clomiphene versus [Phenytoin] in the mobile phase after correcting for secondary binding of the probe. Similar results, but with higher $1/k$ values, were seen when no correction was made for the surface area covered by HSA. As shown in Fig. 3(b), a slight decrease in *cis*-clomiphene retention was noted as the phenytoin concentration was increased from 0 to 0.5 μM (giving a statistically significant increase in $1/k$ for multiple injections of the analyte), followed by an increase in retention (a decrease in $1/k$) at higher phenytoin concentrations. The first part of this plot indicated there was either direct competition or a negative allosteric effect between phenytoin and *cis*-clomiphene. If this is due to direct competition, then it would be taking place at the tamoxifen site of HSA. In addition, the behavior seen at higher phenytoin concentrations indicates that positive allosteric effects were occurring under these conditions. This latter effect explains a previous observation that tamoxifen given to phenytoin-treated patients gives higher plasma concentrations for tamoxifen than when tamoxifen is given alone [39]. This behavior is also consistent with the results obtained in Section 4.2 for *R*-warfarin and phenytoin, since a positive allosteric effect is known to be present between the warfarin-azapropazone and tamoxifen sites of HSA [20,40]. Thus, the presence of such effects for phenytoin in both the warfarin and tamoxifen studies suggests that this drug has binding at both these regions.

4.4. Zonal elution studies with digitoxin as the injected probe

The next site examined on HSA was the digitoxin site. This was accomplished by using digitoxin as the injected probe. Previous studies with digitoxin on HSA columns have used mobile phases that contained β-CD as a solubilizing agent for this drug [6,37,38]. However, it was found in this work that solutions with acceptable levels of digitoxin could also be prepared in pH 7.4, 0.067 M phosphate buffer by sonicating this solution for 2 h after adding digitoxin. This made it possible to prepare a 5 μM solution of this probe. When using 0–40 μM phenytoin in the mobile phase, digitoxin gave retention on the control column that was less than 3% of that observed on the HSA column. Thus, secondary binding of this probe to the support was minimal and no corrections for such binding were required.

Fig. 3(c) shows the plot of $1/k$ versus [Phenytoin] that was obtained for digitoxin on the HSA column. This gave a straight line with a correlation coefficient of 0.988 over five points. According to Eq. (4), this indicated that direct competition was present between digitoxin and phenytoin at the digitoxin site of HSA. From the slope and intercept of this plot, the association constant for phenytoin at this site was determined to be 6.5 ($\pm$0.6) $\times$ 10^3 M^{-1}, which is consistent

with the range of values listed in Table 1. In addition, the results in Fig. 3(c) agree with observations made in other studies that have reported a decrease in phenytoin binding to HSA in the presence of digitoxin [20,21].

4.5. Zonal elution studies with L-tryptophan as the injected probe

The last binding region on HSA that was examined was the indole-benzodiazepine site. This was studied by using L-tryptophan as the injected probe. In the presence of 0–30 μM phenytoin, the non-specific retention of L-tryptophan to the control column was less than 20% of the total retention seen on the HSA column. In the same manner as described in Section 4.2, the retention due to this non-specific binding on the HSA column was estimated by using the retention of the probe on the control column, the protein content of the HSA support (54 mg/g silica) and the support's surface area (100 m^2/g). This gave an estimated surface coverage of 33% for HSA in its column. A proportional correction to the retention measured on the control column was then made when subtracting this from the measured retention on the HSA column.

When a plot of 1/*k* versus [Phenytoin] was made for L-tryptophan on the HSA column, as shown in Fig. 3(d), this gave a straight line with a correlation coefficient of 0.994 over five points. This indicated that phenytoin had direct competition with L-tryptophan and was interacting with the indole-benzodiazepine site of HSA. From this data, the association constant for phenytoin at this site was found to be 1.04 (±0.05) × 10^4 M^{-1}, a value consistent with the highest affinities listed in Table 1 and the estimate of K_a provided by Fig. 2(a). This result is also consistent with an earlier observation that the serum level of free L-tryptophan increases in the presence of phenytoin [41].

5. Conclusions

This work studied the binding of phenytoin to HSA by using HPAC. Both frontal analysis and self-competition zonal elution studies suggested that multi-site binding was present between phenytoin and HSA. Zonal elution studies with four probes representing the major and minor sites on HSA were then performed to identify some of these sites. It was found that phenytoin had some interactions with all of the tested sites. Direct competition was seen between phenytoin and probes for the indole-benzodiazepine and digitoxin sites of HSA, while more complex behavior was seen between phenytoin and probes for the warfarin-azapropazone and tamoxifen sites. For these latter sites, the experimental results suggested that both direct competition (or possibly negative allosteric effects) and positive allosteric interactions were occurring.

Fig. 4 summarizes the model for phenytoin–HSA binding that was developed in this study. It was found that

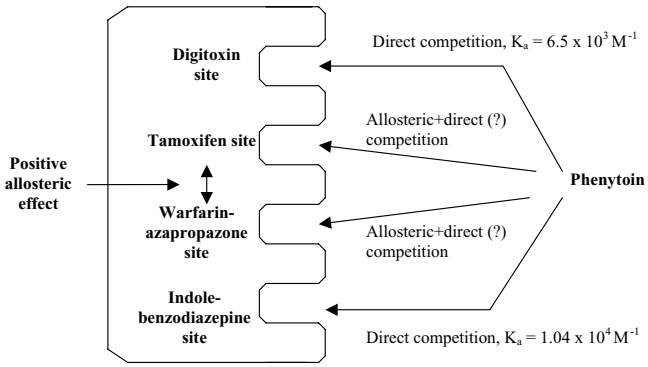

Fig. 4. Proposed model for phenytoin binding to HSA. The data in this report is consistent with a model in which phenytoin has direct binding at both the warfarin-azapropazone and tamoxifen sites, although it is possible that the competition noted in this study could have been due to negative plus positive allosteric effects between this drug and *R*-warfarin or *cis*-clomiphene.

the association constants measured for phenytoin at the indole-benzodiazepine and digitoxin sites agree with the range of values that have been reported in previous studies (see Table 1). The fact that all four of the major and minor sites on HSA appear to interact with phenytoin also agrees with the range of binding sites that have been measured for phenytoin with HSA. In addition, the data obtained in this study fit previous observations of drug–drug interactions between phenytoin and other compounds in serum or plasma. Given the relatively weak binding of phenytoin at these various sites, it is likely that other solutes which bind HSA will have an effect on phenytoin–HSA interactions. When combined with the relatively high degree of phenytoin binding to plasma proteins like HSA and the narrow therapeutical range for this drug [7–14], such interactions could result in clinically relevant changes in phenytoin's non-protein bound fraction and activity. This indicates the value of information like that in Table 1 and Fig. 4 in predicting the effects other drugs may have on phenytoin and in obtaining better models for phenytoin's interactions in blood.

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