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Chromatographic analysis of carbamazepine binding to human serum albumin

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Abstract

In this study, high-performance affinity chromatography was used to characterize the binding of carbamazepine to an immobilized human serum albumin (HSA) column. Frontal analysis was first used to determine the association equilibrium constant and binding capacity for carbamazepine on this column at various temperatures. The non-specific binding of carbamazepine within the column was also considered. The results indicated that carbamazepine had a single binding site on HSA with an association equilibrium constant of $5.3 \times 10^3\ M^{-1}$ at pH 7.4 and 37 °C. This was confirmed through zonal elution self-competition studies. The value of ΔG for this reaction was −5.35 kcal/mol at 37 °C, with an associated change in enthalpy (ΔH) of −6.45 kcal/mol and a change in entropy (ΔS) of −3.56 cal/mol K. The location of this binding region was examined by competitive zonal elution experiments using probe compounds with known sites on HSA. It was found that carbamazepine had direct competition with L-tryptophan, a probe for the indole–benzodiazepine site of HSA, but allosteric interactions with probes for the warfarin, tamoxifen and digitoxin sites. Changes in the pH, ionic strength, and organic modifier content of the mobile phase were used to identify the predominant forces in the carbamazepine–HSA interaction.

Keywords: Carbamazepine; HSA; High-performance affinity chromatography; Frontal analysis; Zonal elution

1. Introduction

Protein structure and function are two important areas to consider in the study of biological interactions. An example of one such interaction is the binding of drugs with blood proteins. This process determines the distribution, excretion and activity of many drugs, making it of great interest to the fields of pharmaceutical science, toxicology, and clinical chemistry. One blood protein that interacts with many drugs is human serum albumin (HSA). HSA is the most abundant protein in serum, having a typical concentration of 50 g/L [1,2]. It has a molecular mass of 66,438 Da and consists of a single polypeptide chain of 585 amino acids held together by 17 disulfide bonds [1].

Many small organic compounds show reversible binding to HSA, including both endogenous and exogenous agents such as long-chain fatty acids, steroids, warfarin, tryptophan, ketoprofen, propranolol and diazepam [3–6]. These substances often bind at relatively well-defined regions on HSA. The two most important of these regions are the warfarin–azapropazone site and indole–benzodiazepine site [1,2]. The warfarin–azapropazone site is located in the IIA subdomain of HSA, and the indole–benzodiazepine site is located in the IIIA subdomain [1,2]. In addition, there are other minor binding sites on HSA that have been reported for compounds like digitoxin and tamoxifen; [7,8] however, the exact locations of these other regions has not yet been determined.

Carbamazepine is a drug known to have significant binding to HSA [9,10]. This drug, shown in Fig. 1, is a structural congener of the tricyclic antidepressant imipramine and is used in treating simple partial, complex partial and generalized tonic–clonic seizures. During its use, the total serum concentrations of carbamazepine are often monitored. Along with its binding to HSA, carbamazepine has a small degree

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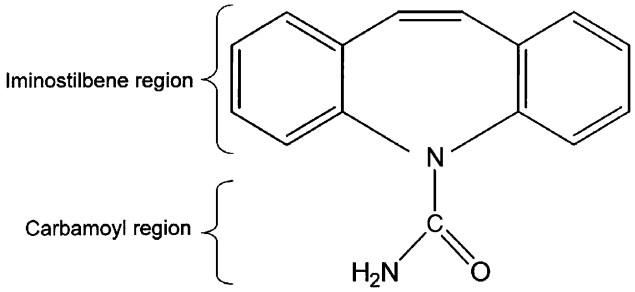

Fig. 1. Structure of carbamazepine.

of binding to another serum protein, α_1-acid glycoprotein (AGP) [9]. The binding of carbamazepine in serum has been studied by equilibrium dialysis, which has given an estimated association equilibrium constant of 10^3–10^4 M^{-1} for the interaction of carbamazepine with HSA [9,10].

There are numerous techniques for examining the binding of solutes to HSA. Examples include spectrofluorometry, ultrafiltration, equilibrium dialysis, crystallography, capillary electrophoresis, and surface plasmon resonance [11–16]. Another technique employed for such work is high-performance affinity chromatography (HPAC) [17–30]. This latter method examines the retention and competition of solutes as they pass through an immobilized HSA column. HSA columns have been used to determine solute binding constants, perform drug–solute competition and displacement studies, generate structure–retention relationships, locate binding regions for solutes, and examine the effects of pH, temperature, ionic strength and organic modifiers on solute–HSA interactions [18–22,24,28–30]. Advantages of this approach include its speed, precision, and good correlation versus solution-based methods [18].

In this study, a combination of several HPAC methods will be used to examine the interactions of carbamazepine with HSA. This will include the use of frontal analysis, self-competition zonal elution studies, zonal elution with known probe compounds as competing agents, and experiments that use changes in the temperature or mobile phase to examine the forces involved in carbamazepine–HSA binding. The purpose of this work is to better characterize the nature of carbemazepine's interactions with HSA and to explore the use of HPAC as a rapid method for investigating solute–ligand binding.

2. Theory

2.1. Frontal analysis

The first technique used in this report was frontal analysis. In this method, a known concentration of pure solute is continuously applied to a column containing a fixed amount of an immobilized ligand (see Fig. 2). If an applied analyte (A) binds to only a single type of ligand site (L) and

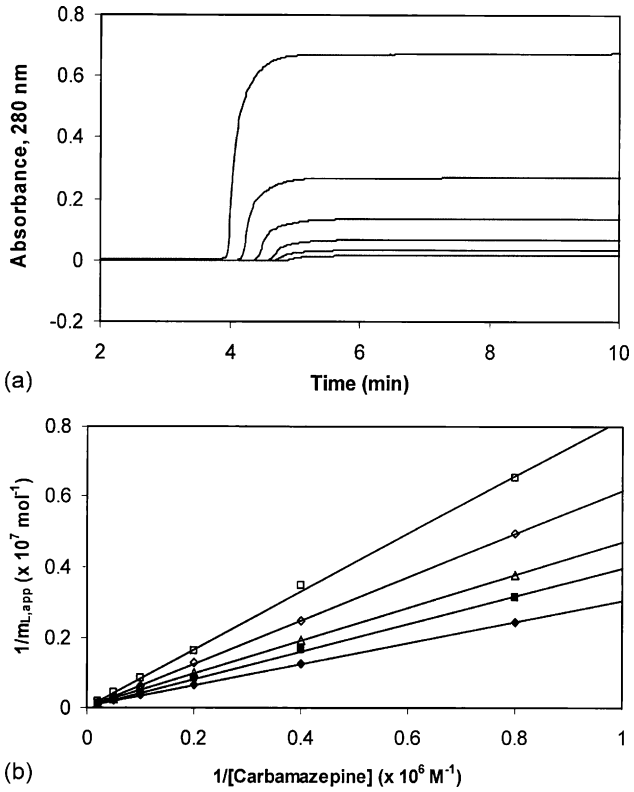

Fig. 2. (a) Frontal analysis curves for carbamazepine at pH 7.4 and 37 °C, where the concentrations of applied carbamazepine (from right to left) are 1.25, 2.5, 5, 10, 20, and 50 μM. (b) Double-reciprocal plots for frontal analysis studies obtained with carbamazepine applied to an HSA column at pH 7.4 and 4 (♦), 15 (■), 27 (△), 37 (◊), or 45 °C (□). Other conditions are given in the text.

this process has fast association/dissociation kinetics, Eq. (1) can be used to relate the true number of binding sites on the column (m_L) to the apparent moles of solute (m_{Lapp}) required to reach the mean point of the breakthrough curve [24].

$$\frac{1}{m_{Lapp}} = \frac{1}{(K_a\, m_L[A])} + \frac{1}{m_L} \quad (1)$$

In this relationship, K_a is the association equilibrium constant for the binding of A to L, and [A] is the concentration of solute applied to the column. This equation predicts that a plot of $1/m_{Lapp}$ versus 1/[A] will give a straight line with a slope equal to $1/(K_a m_L)$ and an intercept of $1/m_L$. This makes it possible to obtain K_a from the ratio of the intercept to the slope and m_L from the inverse of intercept.

If multiple binding sites for A are detected, expanded versions of Eq. (1) can be used. For instance, if a column contains two classes of binding sites, L_1 and L_2, the relationship between $1/m_{Lapp}$ and [A] takes the following form [25].

$$\frac{1}{m_{Lapp}} = \frac{1 + K_{a1}[A] + \beta_2 K_{a1}[A] + \beta_2 K_{a1}^2[A]^2}{m_{Ltot}\{(\alpha_1 + \beta_2 - \alpha_1\beta_2)K_{a1}[A] + \beta_2 K_{a1}^2[A]^2\}} \quad (2)$$

In Eq. (2), K_{a1} is the association equilibrium constant for the highest affinity site (L_1), and α_1 is the fraction of all binding sites that belong to this group (i.e., $\alpha_1 = m_{L1,tot}/m_{Ltot}$, where $m_{L1,tot}$ is the total moles of site L_1 in the column). The term β_2 is the ratio of association equilibrium constants for the low versus high affinity sites, where $\beta_2 = K_{a2}/K_{a1}$ and $0 < K_{a2} < K_{a1}$.

Unlike Eq. (1), the expression in Eq. (2) does not predict a linear relationship between $1/m_{Lapp}$ and [A]. However, this equation does approximate a straight line at low analyte concentrations, as shown in Eq. (2).

$$\lim_{[A]\to 0} \frac{1}{m_{Lapp}} = \frac{1}{m_{Ltot}(\alpha_1 + \beta_2 - \alpha_1\beta_2)K_{a1}[A]} + \frac{(\alpha_1 + \beta_2^2 - \alpha_1\beta_2^2)}{m_{Ltot}(\alpha_1 + \beta_2 - \alpha_1\beta_2)^2} \quad (3)$$

By using Eqs. (2) and (3) it is thus possible to examine frontal analysis data for a two-site system and obtain quantitative information on the relative amount of each site (as represented by α_1) and their affinities (as represented by K_{a1} and β_2) [25].

2.2. Zonal elution

The second technique used in this report was zonal elution. In this case, a known concentration of a competing agent (I) is continuously applied in the mobile phase to a column containing L while small amounts of A are injected. If A and I have direct competition at a single binding site on L and these interactions have fast association/dissociation kinetics, the following equation can be used to describe the retention of A [18].

$$\frac{1}{k} = \left(\frac{K_{aI}V_M\,[I]}{K_{aA}m_L}\right) + \frac{V_M}{K_{aA}m_L} \quad (4)$$

In this equation, k is the retention factor of the injected analyte, where $k = (t_R/t_M - 1)$, t_R is the mean retention time for the analyte, and t_M is the void time of the column. K_{aI} and K_{aA} are the association equilibrium constants for the binding of I and A at their site of competition, and [I] is the concentration of competing agent in the mobile phase. Eq. (4) predicts that a system with single-site competition will give a linear plot for $1/k$ versus [I], where K_{aI} can be obtained from the ratio of the intercept to the slope.

A special case occurs when the competing agent and injected analyte are the same substance. In this situation, Eq. (4) reduces to the following form when A has a single type of binding site in the column [18].

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

In this relationship [A] refers to the concentration of analyte that is in the mobile phase as a competing agent. Like Eq. (4), this new equation predicts a linear relationship between $1/k$ and [A] for a system with 1:1 interactions.

If more than one type of binding site for A is in the column, deviations from the linearity predicted by Eq. (5) will be observed. In this case, Eq. (6) can be used to describe the binding at one of two sites [18].

$$\frac{1}{k_A - k_2} = \frac{V_M[A]}{m_{LI}} + \frac{V_M}{K_{a1}m_{L1}} \quad (6)$$

In this equation, k_2 is the retention factor for A due to site 2 and k_A is the overall retention factor for A due to sites 1 and 2. This equation assumes the retention factors due to the individual sites are additive and that an independent estimate of k_2 can be obtained, allowing the retention factor due to site 1 (k_1) to be determined by using $k_1 = (k_A - k_2)$.

3. Experimental

3.1. Reagents

The carbamazepine, digitoxin and tamoxifen were from Sigma (St. Louis, MO, USA); all of these agents were more than 98% pure according to the supplier. *R*-Warfarin was purchased from Gentest (Woburn, MA, USA). The HSA (Cohn fraction V, essentially fatty acid and globulin free) was obtained from Fluka (Milwaukee, WI, USA). The Nucleosil Si-300 (7 μm particle diameter, 300 Å pore size) was from Macherey Nagel (Düren, Germany). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). All other chemicals were of the highest grades available. All buffers and aqueous solutions were prepared using water from a NANOpure system (Barnstead, Dubuque, IA, USA) and filtered using Osmonics 0.22 μm nylon filters from Fisher (Pittsburgh, PA, USA).

3.2. Apparatus

The chromatographic system consisted of one PU-980i isocratic pump (Jasco, Tokyo, Japan), one P4000 gradient pump, and one UV100 absorbance detector (ThermoSeparation Products, Riviera Beach, FL, USA). Samples were injected using a Rheodyne Lab Pro valve (Cotati, CA, USA) and 20 μL loop. The BCA protein assay was performed using a UV-160A spectrophotometer (Shimadzu, Kyoto, Japan). An Isotemp 9100 circulating water bath from Fisher was used for temperature control of the columns and mobile phases. The diol coverage of the silica was determined with an MDQ capillary electrophoresis system (Beckman, Fullerton, CA, USA). All columns were packed using an Alltech column slurry packer (Deerfield, IL, USA). Chromatographic data were collected and processed using programs written in LabView 5.1 (National Instruments, Austin, TX, USA).

3.3. Methods

Nucleosil Si-300 silica was converted into a diol-bonded form according to a previous procedure [31]. The final diol coverage of this material was measured in triplicate by an iodometric capillary electrophoresis assay [32] and found to be 336 μmol/g (±4) silica (±1 S.D.). This diol-bonded silica was then used in a Schiff base method for the immobilization of HSA [33]. This was accomplished by converting the diol-bonded silica into an aldehyde form through oxidation with periodic acid [31]. Next, 5 g of the aldehyde silica was combined with 150 mg HSA and 70 mg sodium cyanoborohydride in 10 mL of pH 6.0, 0.10 M potassium phosphate buffer. The immobilization reaction was allowed to proceed for 5 days at 4 °C. The HSA silica was washed with pH 8.0, 0.10 M phosphate buffer and treated with three portions of 10 mg sodium borohydride to convert the excess aldehyde groups on the support into alcohols. The support was then washed several times with pH 7.4, 0.067 M potassium phosphate buffer and stored in this buffer at 4 °C until use.

A control support was prepared by performing the Schiff base method on a separate portion of the diol-bonded silica, with no HSA being added during the immobilization step. This control material was washed and stored in the same manner as the immobilized HSA support. Small portions of both the HSA silica and control support were washed several times with deionized water and dried under vacuum at room temperature. These dried samples were analyzed in triplicate using a BCA protein assay in which HSA was the standard and the control silica was the blank. With this procedure, the final protein content of the HSA silica was found to be 410 nmol HSA/g (±7) silica.

The HSA silica and control support were downward slurry packed at 3500 p.s.i. (214 bar) into separate 50 mm × 4.6 mm i.d. or 2.1 mm i.d. stainless steel columns using pH 7.4, 0.067 M potassium phosphate buffer as the packing solution. Each column was placed into a water jacket for temperature control.

All mobile phases for the chromatographic studies were degassed at least 15 min prior to use. The elution of carbamazepine was monitored at 280 nm. Other injected compounds were detected at the following wavelengths: L-tryptophan, 214 nm; digitoxin, 221 nm; tamoxifen, 260 nm; and *R*-warfarin, 310 nm. Column pressures less than 300 p.s.i. were present during all of the chromatographic studies, with no variations in retention with pressure being observed under these conditions.

The samples and mobile phases containing carbamazepine were prepared in pH 7.4, 0.067 M potassium phosphate buffer at concentrations of 0–50 μM and were stored at 4 °C until use. These solutions were stable for several months under such conditions [34]. Solutions containing *R*-warfarin or L-tryptophan were prepared by adding 0–50 μM of these agents to pH 7.4, 0.067 M potassium phosphate buffer. In the zonal elution studies with digitoxin and tamoxifen, 2.5 mM β-cyclodextrin was also added to the pH 7.4, 0.067 M potassium phosphate buffer as a solubilizing agent [7]; the concentration of digitoxin or tamoxifen in these solutions was varied from 0 to 50 μM. Solutions containing L-tryptophan were prepared daily. All other solutions were used over the course of a few weeks and were stored at 4 °C between studies.

Frontal analysis was performed using pH 7.4, 0.067 M potassium phosphate buffer that contained 0–50 μM carbamazepine. This solution was applied at a flow rate of 0.1 mL/min. This flow rate was found to be well within the range needed to establish a local equilibrium in the HSA column, in agreement with earlier results reported for other solutes [24,35]. All experiments were performed in triplicate under each set of tested conditions. The retained carbamazepine was eluted and the column regenerated between studies by passing pH 7.4, 0.067 M potassium phosphate buffer through the column. The amount of carbamazepine required to saturate a column was determined from the mean position of the resulting breakthrough curve [36]. The results obtained for the control column were subtracted from those obtained for an HSA column of identical size to correct for the column void time and to correct for secondary interactions between carbamazepine and the support. A correction for the system void time was made by performing similar experiments using sodium nitrate as a non-retained solute.

Zonal elution was typically performed at 0.1 mL/min. In general, less than a 1% variation in the retention of all injected compounds was noted as the flow rate was varied from 0.1 to 0.5 ml/min, confirming that a local equilibrium had been established on the HSA and control columns under these conditions. At each concentration of competing agent, triplicate injections of the analyte or desired probe compound were made. The concentrations of injected compounds were as follows: 1 μM carbamazepine, 2 μM L-tryptophan, 10 μM *R*-warfarin, 8 μM digitoxin, and 10 μM tamoxifen. These levels were sufficiently low to avoid any significant changes in the retention factor due to overloading effects (i.e., variations less than 1–2%), thus indicating that linear elution conditions were present. The mean retention time for a peak was obtained by calculating its first statistical moment. The column void time was found by injecting sodium nitrate as a non-retained solute. In some experiments, the system temperature, buffer concentration, pH, or organic modifier content of the mobile phase was varied while similar measurements were made of solute retention.

4. Results and discussion

4.1. Frontal analysis and initial characterization of HSA column

The initial properties of the HSA column used in this study are listed in Table 1. The amount of immobilized HSA was determined by a protein assay to be approximately 27 mg/g (±1) silica, or 410 nmol HSA/g (±7) silica. This corresponds

Table 1
Initial properties of the immobilized HSA column

Property	Result[a]
Amount of immobilized HSA (nmol/g silica)	410 (±7)
Binding capacity for carbamazepine (nmol/g silica)	306 (±5)
Specific activity for carbamazepine (% mol/mol HSA)	74 (±3)

[a] The values in parentheses represent a range of ±1 S.D. The numbers given for the binding capacity and specific activity were obtained at 37 °C in pH 7.4, 0.067 M potassium phosphate buffer.

to a coverage of roughly 0.15 monolayers for HSA on the silica's surface, based on a size for HSA of 140 × 40 Å [1]. This is consistent with previous values reported for HSA on the same type of silica and under similar immobilization conditions [19,24].

Some typical frontal analysis curves obtained for carbamazepine on the immobilized HSA column are shown in Fig. 2(a). As indicated by this example, the mean position of these curves shifted to the left (i.e., to shorter breakthrough times) as the concentration of applied carbamazepine increased. This shift was related to the moles of binding sites in the column and the concentration of carbamazepine by analyzing the results according to Eq. (1), as shown in Fig. 2(b). The plots obtained for $1/m_{Lapp}$ versus 1/[carbamazepine] gave linear relationships at all temperatures studied, with correlation coefficients ranging from 0.997 to 0.999 ($n = 6$). According to Eq. (1), this suggested that only a single type of binding site was present for carbamazepine on the immobilized HSA. This was in agreement with observations made in previous solution-phase studies [24].

The number of binding sites and association constants obtained for carbamazepine and HSA during these studies are listed in Table 2. In general, the association constants for this interaction were found to be in the range of $0.4 \times 10^4\ M^{-1}$ to $2 \times 10^4\ M^{-1}$ over the temperatures examined in this study, with a value of $0.53 \times 10^4\ M^{-1}$ being noted at 37 °C. This agrees with the range of 10^3–$10^4\ M^{-1}$ previously observed for this interaction in solution [9,37,38]. A statistically identical value was obtained in this study through self-competition zonal elution studies, which gave an association constant of $0.51\ (\pm 1.1) \times 10^4\ M^{-1}$ at 37 °C.

Based on the binding capacities in Table 2 and the known amount of HSA in the column, it was determined that 74% (±3) of the immobilized HSA had active binding regions for carbamazepine. This is higher than the specific activities reported for similar HSA columns in the binding of L-tryptophan (34%) and *R*-warfarin (31%) [19,39]. The fact that this activity is less than 100% is caused by such factors as steric hindrance, denaturation, or improper orientation of HSA during the immobilization process [18].

Another interesting item seen in Table 2 is that there was an increase in HSA's binding capacity for carbamazepine with temperature. In this case, an increase from 4 to 45 °C gave almost a two-fold increase in this value. A previous study with *R*- and *S*-warfarin on a similar HSA column gave a similar but smaller increase in binding capacity over the same temperature range (i.e., a 30% increase between 4 and 45 °C) [24]. The reason for this effect is not currently known; however, it might be caused by the greater flexibility in HSA at higher temperatures, thus decreasing steric hindrance and creating more accessible binding regions.

4.2. Corrections for secondary interactions

It was noted during the frontal analysis studies that carbamazepine had significant binding to the control column. This was indicated by the presence of breakthrough curves for this column that were well beyond the expected void time of the system. The results in Fig. 2 and Table 2 were corrected for this by subtracting the breakthrough times for carbamazepine on the control column from those observed on the HSA column under equivalent conditions. To confirm the validity of this correction, the frontal analysis data for the control column were examined in more detail to determine the binding constant for carbamazepine to this column.

The frontal analysis results generated with the control support are shown in Fig. 3(a). Like the corrected data for the HSA column in Fig. 2, this plot gave linear behavior when prepared according to Eq. (1), with correlation coefficients of 0.997–0.999 ($n = 6$) at the various temperatures examined. From the slopes and intercepts of these graphs, the association constants and binding capacities for carbamazepine on the control column were determined at each temperature used in this study. These values are included in Table 2.

This table shows that carbamazepine had lower association constants on the control column than the HSA column. However, the control column also had a higher binding capacity. This meant that both the secondary interactions of carbamazepine with the support and its more specific interactions with HSA played significant roles in determining this

Table 2
Binding capacities and association equilibrium constants for carbamazepine on the HSA and control columns[a]

Temperature (°C)	Binding capacity ($\times 10^{-7}$ mol)		Association equilibrium constant ($\times 10^4\ M^{-1}$)	
	HSA column	Control column	HSA column	Control column
4	1.60 (±0.05)	2.7 (±0.1)	2.1 (±0.1)	1.01 (±0.05)
15	2.09 (±0.07)	3.0 (±0.4)	1.2 (±0.1)	0.66 (±0.09)
25	2.30 (±0.12)	3.8 (±0.4)	1.1 (±0.2)	0.59 (±0.07)
37	3.06 (±0.05)	7.0 (±2.4)	0.53 (±0.08)	0.23 (±0.07)
45	3.17 (±0.09)	10.4 (±4.3)	0.46 (±0.12)	0.05 (±0.02)

[a] All results were obtained at pH 7.4 in 0.067 M potassium phosphate buffer. The values in parentheses represent a range of ±1 S.D.

drug's overall retention on the HSA column. This was later confirmed in zonal elution experiments, in which the retention factor for carbamazepine on the HSA column was 5.1 under physiological conditions and 3.3 on the control column under the same conditions.

The correction for secondary binding used in preparing Fig. 2 assumed that the HSA and control columns had a similar number of secondary binding sites. If this were not the case, it would have resulted in an error in the HSA results given in Table 2. To test this, the frontal analysis data for the HSA column were also examined without subtracting away the control column results. In this case, the only correction made was for the void time of the system, as measured by using sodium nitrate as a non-retained solute. This gave rise to the plots shown in Fig. 3(b). These plots were examined according to a two-site model based on Eq. (3).

In fitting Eq. (3) to the data in Fig. 3(b), it was possible to use the association equilibrium constant that had been determined independently for secondary interactions of carbamazepine on the control column. This gave values of K_{a1} for the carbamazepine–HSA interaction that differed by only 7–18% from the association equilibrium constants given in Table 2 after simple subtraction of the HSA and control column breakthrough times. Thus, it was concluded that the earlier approach used to correct for secondary interactions was valid in this present study.

Although the nature of these secondary sites is not currently known, it has been determined that this is not due to residual silanol groups. This was determined by performing injections of carbamazepine under the same conditions as used in this study onto a column that contained only the original bare silica as a support. The result was a retention factor of only 0.2, which is much smaller than that observed on the control column. Another possibility is that carbamazepine was interacting with the propyl groups or some other portion in the backbone of the modified supports.

4.3. Thermodynamic studies

It can be seen from Table 2, that the association equilibrium constant for carbamazepine with HSA decreased as the temperature increased from 4 to 45 °C. This same trend has been seen for many other compounds in their binding to HSA, including L-tryptophan, *R*-warfarin, *S*-warfarin and L-thyroxine [19,24]. The effect of temperature on the binding strength of carbamazepine to HSA was characterized by plotting the data in Table 2 according to Eq. (7).

$$\ln K_a = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \tag{7}$$

where ΔS and ΔH are the changes in entropy and enthalpy for the observed interaction, T the absolute temperature, and R is the ideal gas law constant [24]. The result, shown in Fig. 4, was a linear relationship between $\ln K_a$ and $1/T$. This gave a correlation coefficient of 0.971 ($n = 5$) over the temperature range examined in this report (4–45 °C). This linearity again confirmed that the interactions of carbamazepine with HSA could be described by a single site model. Furthermore, this behavior indicated that the underlying assumption in Eq. (7) that the change of enthalpy (ΔH) was essentially constant was valid under these conditions.

Based on the slope and intercept of Fig. 4, the changes in enthalpy and entropy for the carbamazepine/HSA interaction were calculated by using Eq. (7). Also, the total change in free

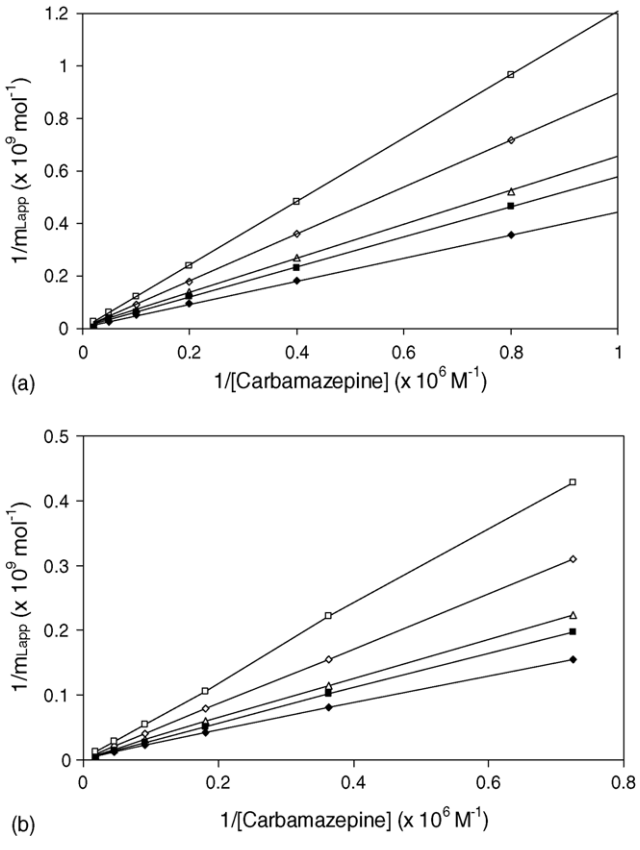

Fig. 3. Double-reciprocal frontal analysis plots for carbamazepine on (a) the control column at temperatures of 4 (♦), 15 (■), 27 (△), 37 (◊), or 45 °C (□) and (b) the HSA column without corrections for binding to the control column.

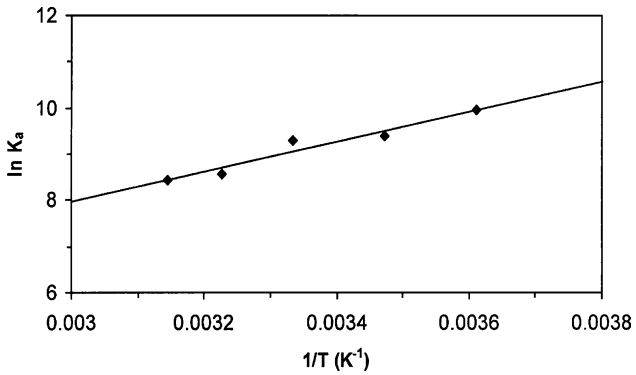

Fig. 4. Van't Hoff plot for the interactions of carbamazepine with HSA. The best-fit slope was 3.24 (±0.02) × 10^3 M^{-1} and the best-fit intercept was −1.76 (±0.01). The correlation coefficient was 0.971 ($n = 5$).

Table 3
Thermodynamic parameters for the binding of carbamazepine to HSA[a]

ΔG at 37 °C (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol K)	$-T\Delta S$ at 37 °C (kcal/mol)
−5.35 (± 0.13)	−6.45 (± 0.05)	−3.56 (± 0.26)	1.10 (± 0.08)

[a] All results were obtained at pH 7.4 in 0.067 M potassium phosphate buffer. The values in parentheses represent a range of ±1 S.D.

energy (ΔG) was determined by using the data in Table 2 and Eq. (8),

$$\Delta G = -RT\ln(K_a) \quad (8)$$

The results are shown in Table 3. This indicated that the binding of carbamazepine to HSA had a negative change in energy due to enthalpy ($\Delta H = -6.45$ kcal/mol) but a positive change in energy due to entropy under physiological conditions ($-T\Delta S = 1.1$ kcal/mol at 37 °C). Of these two components, the greatest contribution to the total change in free energy at 37 °C was the change in enthalpy. The decrease in entropy seen upon the binding of carbamazepine to HSA is somewhat unusual in that most drugs and small solutes show an increase in entropy when they bind to this protein [18,24,40–43]. However, a decrease in entropy has been noted in some cases, such as in the binding of benzodiazepines or heptacarboxyl porphyrin to HSA [41].

4.4. Zonal elution studies

The next set of experiments used zonal elution to examine the competition of carbamazepine with several agents that have known binding sites on HSA. All of these studies were performed on both the HSA and control columns. The first compounds used in this work were *R*-warfarin and L-tryptophan. *R*-Warfarin at normal therapeutic levels is known to have single site interactions with the warfarin–azapropazone site of HSA and has a well-characterized association equilibrium constant for this binding [1,2]. Similarly, L-tryptophan binds in a 1:1 stoichiometry with HSA at the indole–benzodiazepine region and has a known equilibrium constant for this interaction [1,2]. In addition, neither of these agents has any appreciable secondary binding to the control support used in this study [24,35].

Fig. 5 shows how the retention factor for carbamazepine changed as it was injected onto the HSA column in the presence of various concentrations of *R*-warfarin or L-tryptophan in the mobile phase. Fig. 6 shows the results of the reverse experiment, in which carbamazepine was used as the competing agent. Studies on the control column gave no measurable change in retention for either carbamazepine or L-tryptophan when the other agent was used as a competing agent. But this was not what was observed on the HSA column. For instance, in Fig. 6(a) a plot of $1/k$ for L-tryptophan versus [carbamazepine] gave a straight line with a correlation coefficient of 0.991 ($n = 6$). According to Eq. (5), this indicated L-tryptophan and carbamazepine had direct competition for a single common binding region. This was not surprising since many compounds similar in structure to carbamazepine also interact at this site (e.g., amitriptyline or opipramol) [2]. However, the reverse experiment in Fig. 5 indicated these sites were easily saturated or that the some additional allosteric interactions occurred between carbamazepine and L-tryptophan in their binding to HSA.

The presence of direct competition between carbamazepine and L-tryptophan was confirmed by using Eq. (4) with the best-fit line in Fig. 6(a) and the results at low tryptophan concentrations in Fig. 5(a) to estimate the association constants for L-tryptophan and carbamazepine at their common binding site. The association equilibrium constant obtained for L-tryptophan at this site was 1.01 (± 0.01) $\times 10^4\ M^{-1}$, which is equivalent to previous values reported for this interaction using either soluble or immobilized HSA [19]. The association equilibrium constant estimated for carbamazepine at this site, after correction for retention on the control column, was 5.2 (± 0.1) $\times 10^3\ M^{-1}$ which agreed with previous values given in this current report. Thus, these experiments confirmed that carbamazepine and L-tryptophan had direct competition on HSA and that the location of this competition was the indole–benzodiazepine site.

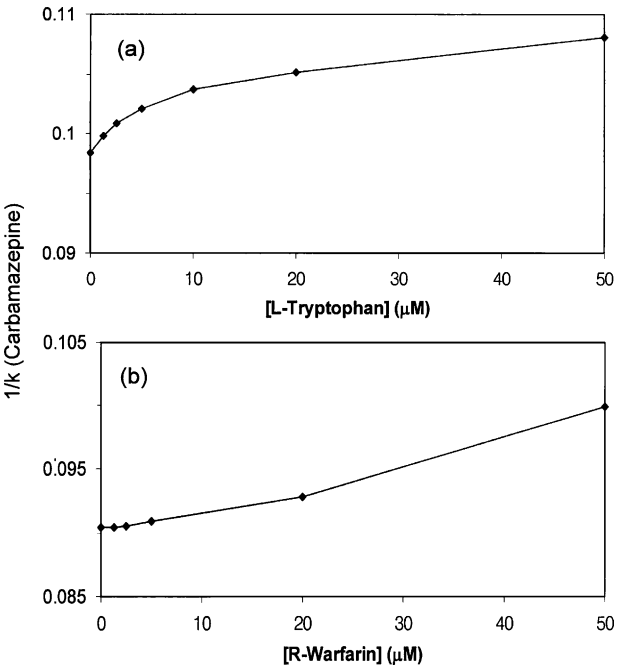

Fig. 5. Competition of carbamazepine with (a) L-tryptophan and (b) *R*-warfarin as mobile phase additives at pH 7.4 and 37 °C. The experimental conditions are given in the text. The best-fit slope for the first three data points in (a) was 1.001 (± 0.002) $\times 10^3\ M^{-1}$ and the best-fit intercept was 0.0985 (± 0.003). The correlation coefficient for this fit was 0.999.

The data obtained in Fig. 5(b) when using *R*-warfarin as a competing agent gave insignificant variations in the retention factor for carbamazepine at low warfarin concentrations (i.e., 0–2.5 μM). However, a decrease in carbamazepine binding was seen at higher warfarin levels. This indicated that some indirect competition was present between carbamazepine and *R*-warfarin as the latter agent was binding at the warfarin–azapropazone site. Much larger changes in retention were noted when the competitive binding studies were reversed and carbamazepine was used as the competing agent, as shown in Fig. 6(b). In this case, a non-linear relationship was again obtained, indicating that allosteric interactions were present between the binding regions for these two compounds. No significant changes in retention for either *R*-warfarin or carbamazepine were noted when similar studies were performed on the control column.

Allosteric effects between the indole–benzodiazepine site and warfarin site have been reported in other studies. For instance, Fitos et al. investigated the allosteric interactions of lorazepam and lorazepam acetate at the indole–benzodiazepine site of immobilized HSA in the presence of warfarin as a competing agent [44]. Domenici et al. also confirmed the existence of an allosteric interaction between the indole–benzodiazepine and warfarin sites in their use of an immobilized HSA column to study the enantioselectivite binding of benzodiazepines [45].

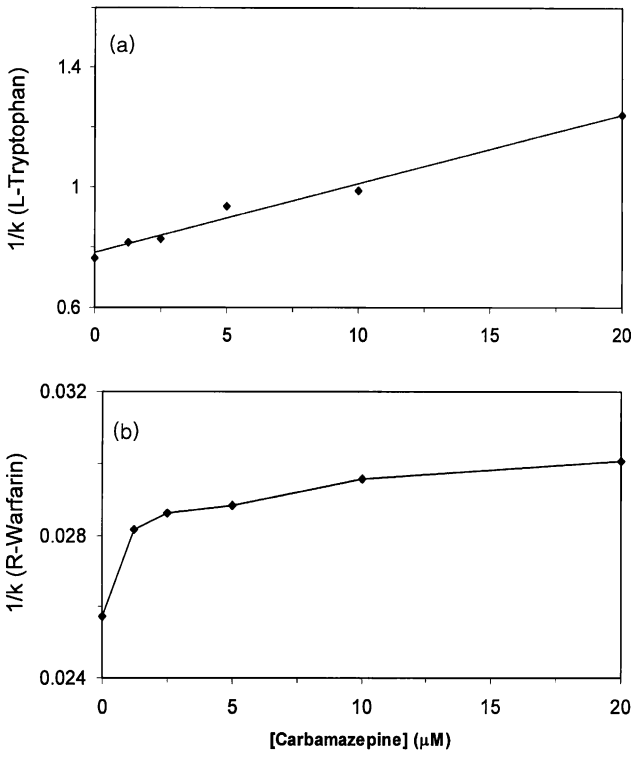

Fig. 6. Competition of (a) L-tryptophan and (b) *R*-warfarin with carbamazepine as a mobile phase additive at pH 7.4 and 37 °C. The best-fit slope in (a) was 1.988 (±0.002) × 10^4 M^{-1} and the best-fit intercept was 0.750 (±0.003). The correlation coefficient for this fit was 0.9861 (n = 6).

The competition of carbamazepine with probes for the minor binding regions of HSA was also considered by using digitoxin and tamoxifen as probes for these sites [7]. When either of these two agents was used as a competing agent, only small random variations were noted in the retention factor for carbamazepine (±0.8–1.9%) at competing agent concentrations up to 10 μM (data not shown). But there was some change in retention for both digitoxin and tamoxifen when the experiment was reversed and carbamazepine was used as mobile phase additive (see Fig. 7). In this case, both probe compounds gave non-linear behavior in plots of 1/k versus [carbamazepine], indicating they had only allosteric or indirect competition with carbamazepine on HSA. No such behavior was seen when using the control column.

4.5. *Effects of mobile phase composition on the binding of carbamazepine to HSA*

Binding of carbamazepine to HSA was also characterized by changing the pH, ionic strength and organic modifier content of the mobile phase (see Fig. 8). All of these studies were performed on both the HSA and control columns. The effect of varying the mobile phase pH was examined by using 0.10 M phosphate buffers with pH values ranging from 2.6 to 7.8. This is illustrated in Fig. 8(a). As the pH increased, no significant trends were noted in the retention of carbamazepine. This was partially due to the fact that carbamazepine has approximately the same charge (+1) throughout this range of conditions (pK_a, 9.2). However, this also suggested that the interactions of carbamazepine with HSA were not sensitive

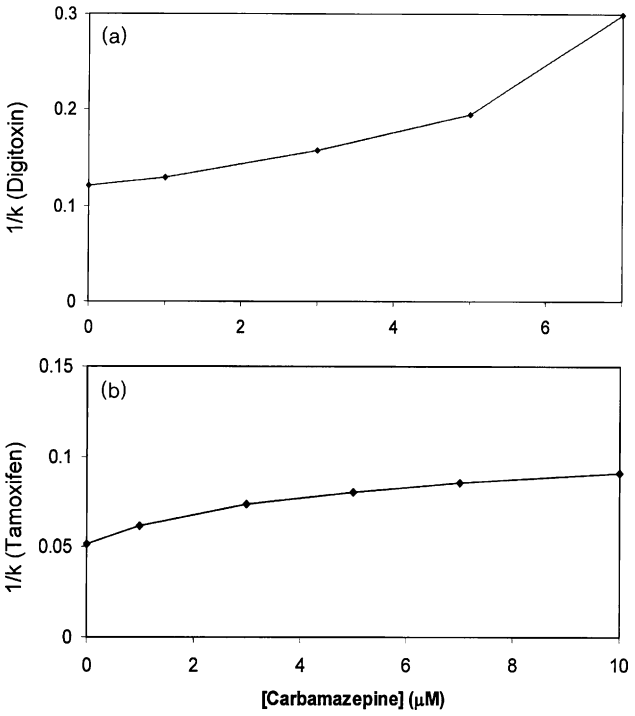

Fig. 7. Competition of (a) digitoxin and (b) tamoxifen with carbamazepine as a mobile phase additive at pH 7.4 and 37 °C.

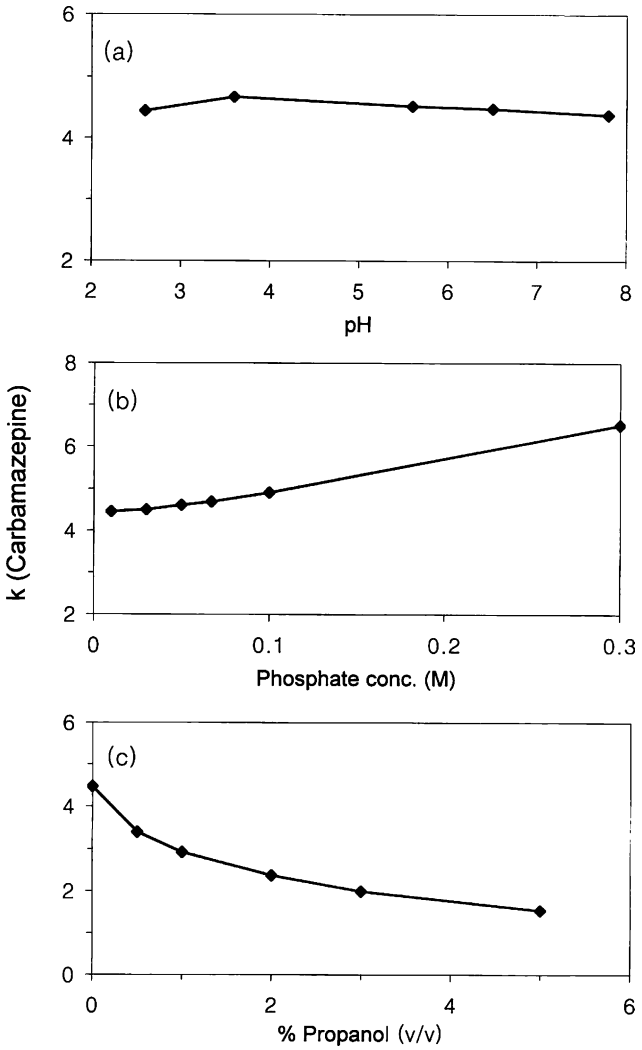

Fig. 8. Effects of (a) pH, (b) ionic strength, and (c) organic modifier content of the mobile phase on the binding of carbamazepine to HSA at 37 °C.

to variations in pH. Similar studies performed on the control column gave only minor variations in retention (less than 0.2%) throughout this pH range.

The ionic strength of the mobile phase was another item varied in this work. This was altered by adjusting the concentration of pH 7.4 phosphate buffer from 0.010 to 0.30 M, giving an ionic strength of 0.0142–0.426 M. As the ionic strength was increased over this range, the retention factor for carbamazepine increased by 47%, as shown in Fig. 8(b). This indicated that the binding of carbamazepine to HSA was much more sensitive to changes in ionic strength than pH. The fact that a decrease in retention at a higher ionic strength was not observed suggests that coulombic interactions did not play a major role in the binding of carbamazepine to HSA. This agrees with the lack of a pH effect in Fig. 8(a). Instead, the increase in retention with ionic strength may reflect an enhancement in non-polar interactions between carbamazepine and HSA as the mobile phase becomes a more polar environment. Similar studies performed on the control column gave

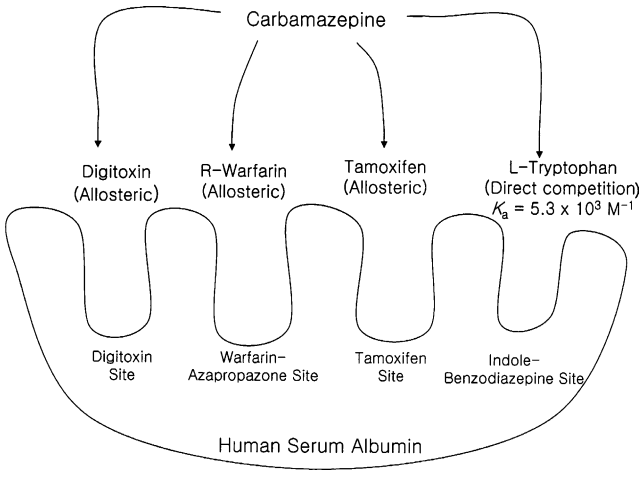

Fig. 9. Proposed model for the binding of carbamazepine to HSA.

only minor variations in retention (less than 0.5%) throughout this concentration range.

The effects of adding small amounts of an organic modifier to mobile phase were also considered. As shown in Fig. 8(c) placing up to 5% 1-propanol in the mobile phase gave rise to a 66% decrease in retention for carbamazepine. This again indicates that non-polar interactions played an important role in the binding between carbamazepine and HSA, as has been suggested for other drugs that show similar solvent effects [18,19,24].

5. Conclusions

This work examined the binding of carbamazepine to HSA through the use of an immobilized form of this protein in an HPLC column. Various approaches for correcting secondary interactions due to the support were also considered. Based on frontal analysis, it was possible to determine the binding capacities and association equilibrium constants of the immobilized HSA for carbamazepine at a number of temperatures. The binding capacities of the HSA column increased when the temperature was raised, but the association equilibrium constants decreased. Competitive binding studies examining the various binding regions of HSA were performed by zonal elution. From these results, it was possible to develop a model to describe the overall binding of carbamazepine to HSA, as shown in Fig. 9. It was found that carbamazepine had direct competition with L-tryptophan at the indole–benzodiazepine site but allosteric interactions with the warfarin–azapropazone site and minor binding regions.

Acknowledgment

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