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Role of binding capacity versus binding strength in the separation of chiral compounds on protein-based high-performance liquid chromatography columns
Interactions of D- and L-tryptophan with human serum albumin

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Abstract

Frontal analysis was used to examine changes in the association constant (K_a) and moles of binding sites (m_L) for D- and L-tryptophan on an immobilized HSA column under various elution conditions. Both enantiomers had single-site interactions under all conditions tested. At pH 7.0 and 25°C, the strength of L-tryptophan/HSA binding was determined mostly by the change in enthalpy of the system, while D-tryptophan/HSA binding was dominated by the change in entropy. The interactions of L-tryptophan with HSA showed a large change when varying the temperature, pH, ionic strength or 1-propanol content of the mobile phase. In each case, changes in K_a accounted for most of the shifts in retention that were seen for L-tryptophan during zonal elution studies. However, m_L for this compound was also affected when varying the pH and 1-propanol levels. Changes in K_a were responsible for most of the shifts in D-tryptophan retention that were seen when adjusting the mobile phase pH or ionic strength. In addition, the value of m_L for D-tryptophan was affected by pH, temperature and 1-propanol levels. It was concluded that varying such chromatographic conditions can alter either the binding strength or number of binding sites for solutes injected onto immobilized protein columns.

Keywords: Binding capacity; Binding strength; Enantiomer separation; Association constants; Mobile-phase composition; Frontal analysis; Thermodynamic parameters; Tryptophan; Albumin

1. Introduction

The separation of chiral molecules is an area of increasing importance in pharmaceutical testing and in the separation of biologically active compounds [1–3]. One approach for separating such compounds is to use an HPLC column that contains an immobilized protein capable of binding the solutes of interest. Examples of proteins that have been used for this purpose include bovine serum albumin [4], human serum albumin [5], α_1-acid glycoprotein [6], ovomucoid [7] and α-chymotrypsin [8]. Columns that employ these proteins as stationary phases have been used in the separation of a wide range of different chiral molecules, including drugs, amino acids, and other organic agents [3–8].

Various reports have examined the effects of changing different chromatographic conditions on the retention and selectivity of protein-based columns [3–6,9–11]. However, relatively few studies

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have examined the mechanisms that lead to chiral separations when using immobilized protein supports. One exception is recent work that has been performed with *R*- and *S*-warfarin on a human serum albumin (HSA) column [12]. In this work, it was found that both the binding strength (or association equilibrium constants) and the moles of available binding sites were important in determining the effect of temperature on the retention and resolution of these enantiomers [12]. The aim of this present study is to determine whether or not both of these parameters are important for other chiral molecules and when varying additional chromatographic conditions (e.g., the pH or organic content of the mobile phase). The system to be examined in this work will be based on the binding of D- and L-tryptophan to an immobilized HSA column.

HSA (M_r 66 500) is a protein found in human blood and plasma [13,14]. One function of HSA is to bind and transport throughout the body many organic and inorganic substances, including fatty acids and various hormones or drugs [15]. It is believed that there are several regions on HSA that are involved in these interactions, the two most important of which are the warfarin-azapropazone and indole-benzodiazepine binding sites [15–17].

The compounds D- and L-tryptophan will be used as model analytes in this study since both enantiomers are retained by HSA and have a well-characterized change in retention under various chromatographic conditions [11,18,19]. L-Tryptophan has a single binding region on HSA that is known to be located at the indole-benzodiazepine site [15,17]. Recent crystallographic studies have identified this site as being in the IIIA subdomain of HSA [20] and the amino acid residues that make up this site are relatively well-characterized [15,20–22]. D-Tryptophan is also believed to bind at a single site on HSA, but has a much weaker affinity than L-tryptophan [11,23,24] and interacts at a separate binding region [11]. Although the exact location of the D-tryptophan site is not known, D-tryptophan does have some indirect interactions with the warfarin-azapropazone site of HSA [11].

This work will examine the binding of D- and L-tryptophan to an immobilized HSA column by using the method of frontal analysis. The data obtained by this method will be used to determine the amount of active binding sites on the HSA column and the association equilibrium constants for D- and L-tryptophan at these sites under a wide range of chromatographic conditions. The conditions to be examined will include a variety of temperatures and mobile phases made up at different pHs, ionic strengths and solvent polarities. The frontal analysis data will then be compared with the results of zonal elution studies performed under similar conditions. Frontal analysis experiments will also be used to determine the changes in entropy, enthalpy and total free energy that are associated with the binding of D- and L-tryptophan to HSA. This work should help provide insight into the general mechanisms that are involved in the retention of chiral molecules by HSA and other immobilized proteins.

2. Theory

If an analyte (A) has a single type of binding site on an immobilized ligand (L) and no other interactions with the support to which the ligand is attached, then retention of the analyte on a column that contains this ligand can be described as follows [25]:

$$k' = (K_a m_L)/V_m \quad (1)$$

In Eq. 1, k' is the capacity factor for the injected analyte, as given by the expression $k'=(t_r - t_m)/t_m$, where t_r is the mean elution time of the analyte and t_m is the column void time. The term K_a represents the association equilibrium constant for the binding of A with L, m_L is the total moles of active binding sites in the column, and V_m is the void volume of the column. Eq. 1 assumes that no significant interactions occur between the analyte and any other sites on the ligand or the chromatographic support. It also assumes that the amount of injected solute is small compared to the total column binding capacity (i.e., linear elution conditions are present) and that the mean position of the analyte peak represents the establishment of a local equilibrium between the analyte and ligand in the column. Eq. 1 indicates that k' can be affected by changes in either K_a or m_L. Since both of these factors are directly proportional to k', any decrease in these items should result in a proportional decrease in analyte retention.

This work will use frontal analysis to examine the individual changes in K_a and m_L for a ligand (HSA) that has been exposed to a variety of mobile-phase and column conditions. In frontal analysis, a known concentration of analyte is continuously applied to a column that contains a fixed amount of the immobilized ligand. As the ligand becomes saturated with analyte, the amount of analyte that exits from the column will increase until it equals the amount that is entering the column. The result is a breakthrough curve that is characteristic of the column and analyte/ligand system. If it is assumed that the analyte binds at only a single site on L and that the association/dissociation kinetics for this process are fairly rapid on the time scale of the experiment (i.e., a local equilibrium is established), then the following equations can be used to relate the mean position of the breakthrough curve to the concentration of applied analyte and the values of K_a or m_L [26,27]:

$$m_{L,app} = \frac{K_a m_L [A]}{K_a [A] + 1} \tag{2}$$

or

$$\frac{1}{m_{L,app}} = \frac{1}{K_a m_L [A]} + \frac{1}{m_L} \tag{3}$$

In Eqs. 2 and 3, $m_{L,app}$ represents the moles of analyte that is required to reach the mean position of the breakthrough curve at a given concentration of applied analyte ([A]). All other terms are the same as defined earlier. One similarity between Eqs. 2 and 3 and Eq. 1 is that the experimentally measured value ($m_{L,app}$ or k') depends on both K_a and m_L. However, with frontal analysis it is possible to independently determine each of these factors. For example, Eq. 3 predicts that a system with single-site binding should give a plot for $1/m_{L,app}$ versus $1/[A]$ that is a straight line with a slope of $1/(K_a m_L)$ and an intercept of $1/m_L$. From this plot, the value of K_a can be determined by calculating the ratio of the intercept to the slope. The value of m_L can be obtained by taking the inverse of the intercept.

Further information on the mechanism of analyte–ligand binding can be obtained by performing frontal analysis experiments at various temperatures. For a system that has single-site interactions, the following equation can be used to describe the relationship between K_a and the absolute temperature (T) [26,27]:

$$\ln K_a = -\Delta H/(RT) + \Delta S/R \tag{4}$$

Eq. 4 predicts that a plot of $\ln K_a$ versus $1/T$ will yield a linear relationship with a slope of $-\Delta H/R$ and an intercept of $\Delta S/R$, where R is the ideal gas law constant, ΔH is the change in enthalpy and ΔS is the change in entropy for the analyte–ligand interaction. Thus, from this plot the values of ΔH and ΔS for the reaction can be obtained. By using these parameters, the total change in free energy (ΔG) for the reaction can also be determined by using the equation shown below [28].

$$\Delta G = \Delta H - T\Delta S \tag{5}$$

3. Experimental

3.1. Reagents

The D- and L-tryptophan, HSA (Cohn fraction V, 99% globulin-free) and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). The Nucleosil Si-300 (10 μm particle diameter, 300 Å pore size) was from Alltech (Deerfield, IL, USA). The HPLC-grade 1-propanol was purchased from Aldrich (Milwaukee, WI, USA). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). All other chemicals used were of purest grades available. All solutions were prepared with deionized water obtained from a Nanopure water system (Barnstead, Dubuque, IA, USA).

3.2. Apparatus

The chromatographic system consisted of one CM3000 isocratic pump, one CM4000 gradient pump, and one SM3100 UV–Vis variable-wavelength absorbance detector from LDC/Milton Roy (Riviera Beach, FL, USA). Samples in the zonal elution studies were injected by using a Rheodyne 7010 valve (Cotati, CA, USA) equipped with a

PhaseSep event marker (Phase Separations, Queensferry, UK) and a 20-μl injection loop. An LDC/Milton Roy Chromlink interface and LCAdvantage software were used for data collection. The frontal analysis and zonal elution data were processed using in-house programs written in Microsoft QuickBASIC (Redmond, WA, USA). An Isotemp 9100 circulating water bath (Fisher Scientific, Pittsburgh, PA, USA) was used for controlling the temperature of the column and the mobile phases. All columns were packed using an HPLC column slurry packer from Alltech.

3.3. Methods

The immobilized HSA support was prepared by using diol-bonded Nucleosil Si-300 silica and the Schiff base immobilization method, as described previously [11,26]. After preparation, the HSA support was stored at 4°C in 0.10 *M* potassium phosphate buffer (pH 7.0) until use. A small portion of this HSA support was washed with deionized water and dried under reduced pressure for use in a bicinchoninic acid (BCA) protein assay [29], using bovine serum albumin as the standard and diol-bonded Nucleosil Si-300 silica as the blank. Based on duplicate assays, the amount of immobilized HSA was determined to be 33±1 (1 S.D.) mg HSA per gram of silica.

Chromatography

The immobilized HSA support and diol-bonded Nucleosil Si-300 silica were downward slurry-packed into two separate 100.0×4.1 mm I.D. stainless-steel columns at a pressure of $400 \cdot 10^5$ Pa using 0.10 *M* potassium phosphate buffer (pH 7.0) as the packing solvent. Each column was enclosed in a water jacket for temperature control. All studies, except those examining the temperature dependence of tryptophan–HSA binding, were performed at 25±0.1°C. All mobile phases were filtered prior to use by passing them through 0.45-μm cellulose acetate or nylon filters. The mobile phases were also degassed under vacuum for at least 10 min before use on the HPLC system. Elution of the D- and L-tryptophan was monitored by an on-line UV–Vis absorbance detector set at 280 nm.

Frontal analysis was performed by continuously applying various concentrations of D- or L-tryptophan to the HSA column. The initial experiments were performed at 25°C and using a mobile phase that consisted of 0.05 *M* potassium phosphate buffer (pH 7.0) that contained no organic modifier. In later experiments, either the system temperature, mobile-phase phosphate concentration, pH, or organic modifier content was systematically varied while all other operating conditions were held constant. The typical flow-rates used in these studies were 0.20 to 0.25 ml/min; however, equivalent results were obtained at 0.10 to 0.40 ml/min, thus indicating the presence of a local equilibrium at the mean position of the breakthrough curves. The analyte solutions were made fresh daily and were applied by using the same valve configuration as described in Ref. [26]. The concentration of applied D- or L-tryptophan ranged from 6.0 to 100 μM. Retained tryptophan was eluted by washing the HSA column with application buffer that contained no added analyte. The amount of D- or L-tryptophan that was needed to saturate the HSA column was determined by integration of the resulting breakthrough curves [30]. Corrections for the system void volume and for non-specific binding to the support were made by performing identical studies on the diol-bonded silica column. The breakthrough volume for D- and L-tryptophan on this latter column was within 20% of the estimated void volume, as determined by making injections of sodium nitrate onto the chromatographic system. Zonal elution studies were performed in the same fashion as described earlier [11]. These studies were carried out at flow-rates of 0.50 to 1.50 ml/min by injecting fresh solutions of 10 μM D- or L-tryptophan made up in the desired mobile phase. Consistent results (i.e., less than 5% variation in k') were obtained throughout this flow-rate range, indicating that a local equilibrium was present in the column during these experiments. In addition, it was found that the given amount of injected tryptophan was small enough to avoid the presence of any significant non-linear elution effects (i.e., changes in k' with analyte concentration).

Three separate injections of D- and L-trypto-

phan were made under all sets of conditions. The retention times for these compounds were calculated by using the first statistical moment of each analyte's peak [31]. The column void time was determined by injecting sodium nitrate onto the HSA column under the same conditions. The retention times and column void time were corrected for the extra-column void time by making injections of D- or L-tryptophan onto the system when no HSA column was present. The corrected values for t_r and t_m were then used to calculate k', as described in Section 2.

Throughout the course of this study, the stability of the HSA column was periodically examined (e.g., at the beginning and end of each study) by making injections of 10 μM D- and L-tryptophan under a standard set of zonal elution conditions. This was performed with a mobile phase that contained 0.05 M potassium phosphate buffer (pH 7.0) and that was applied to the HSA column at 25°C and 1.0 ml/min. No significant change (i.e., less than 8% variation) was noted in the separation factor (α) and capacity factors for both D- and L-tryptophan under these conditions during the entire course of this work (a period of 30 months or about one thousand sample injections). This indicated that there were no appreciable changes in the column's binding characteristics during this study.

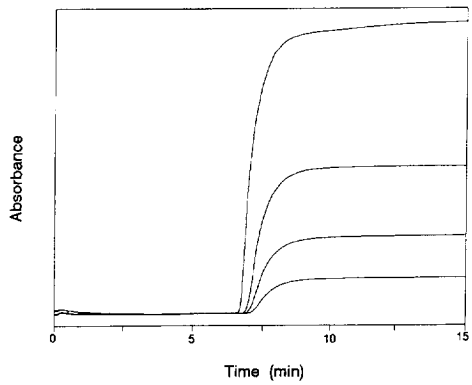

Fig. 1. Typical frontal analysis curves for L-tryptophan obtained at 25°C on an immobilized HSA column. The concentrations of L-tryptophan (from left to right) were 100, 50, 25 and 12.5 μM. The flow-rate was 0.25 ml/min and the application buffer was 0.05 M potassium phosphate buffer (pH 7.0). The void time under these conditions was approximately 3.6 min.

4. Results and discussion

4.1. General behavior of frontal analysis results

Frontal analysis was used throughout this work to examine the effects of various chromatographic conditions on the binding properties of an immobilized HSA column. Fig. 1 shows a typical set of breakthrough curves that were obtained for this column when applying L-tryptophan at several concentrations. The frontal analysis results for D-tryptophan, and L-tryptophan under other conditions, showed similar behavior to those in Fig. 1 but had different mean positions for their breakthrough curves. As discussed in Section 2, the mean positions of these curves are related not only to the concentration of the applied analyte, but also to the moles of active binding sites in the column and the equilibrium constants for these sites. It is this type of behavior that makes breakthrough curves useful in examining the changes in column binding that occur under various operating conditions.

The data obtained from the breakthrough curves were analyzed by plotting the results according to Eq. 3. Some typical results for L-tryptophan are shown in Fig. 2 for data collected between 4 and 45°C at pH 7.0 in 0.05 M phosphate buffer. Each plot in this figure gave a linear relationship over the entire range of concentrations tested. In this particular figure, the correlation coefficients varied from 0.9950 to 0.9997 for the five data points shown in each plot, with only random variations being noted in the distribution of data about the best-fit lines. According to Eq. 3, this type of linear behavior suggests that L-tryptophan was binding to a single type of site on the immobilized HSA column. This is in agreement with previous studies that have examined the binding of L-tryptophan with HSA in solution [15,23,24] and with HSA immobilized to solid-phase supports [11,19,26].

Work with D-tryptophan under the same conditions as used in Fig. 2 gave similar, linear graphs when the results were plotted according to Eq. 3. A random

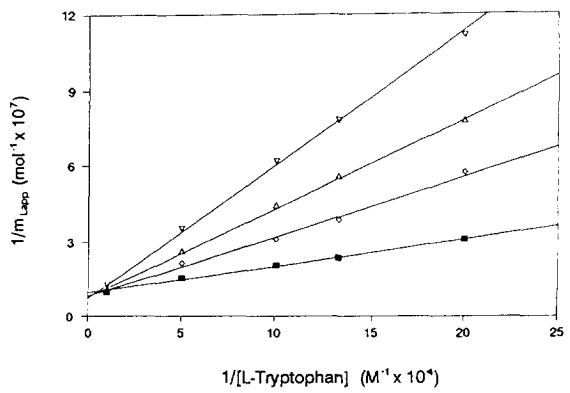

Fig. 2. Frontal analysis results obtained for L-tryptophan on an immobilized HSA column at temperatures of 4°C (■), 25°C (◇), 37°C (△) and 45°C (▽), as plotted according to Eq. 3.

distribution of the results about the best-fit lines was again seen, with correlation coefficients of 0.9850 to 0.9999 being obtained over the 5 to 6 data points in each plot. This agrees with earlier studies that have noted a single binding site for D-tryptophan on immobilized HSA [11].

The use of pH 7.0, 0.05 *M* phosphate buffer as the mobile phase was not the only case that gave linear plots like those in Fig. 2. The graphs in Fig. 2 are also representative of the other column conditions that were examined in this work. For these other conditions, the correlation coefficients obtained for plots of $1/m_{L,app}$ versus 1/[L-tryptophan] ranged from 0.9870 to 0.9997 over 5 to 6 data points. The correlation coefficients of similar graphs made for D-tryptophan ranged from 0.9814 to 0.9999 for 5 to 6 points. In all of these plots, the data showed a random distribution about each best-fit line. These results indicate that single-site interactions with HSA continued to be present for D- and L-tryptophan under all of the experimental conditions that were examined in this study.

4.2. Effect of temperature on the binding of D- and L-tryptophan to HSA

The association equilibrium constants for D- and L-tryptophan on the immobilized HSA column were determined from the slopes and intercepts of the plots in Fig. 2 and similar graphs obtained at other temperatures. The results are given in Table 1. The relative precision of the association constants measured in this work was ±10–22% (1 R.S.D.) for the L-tryptophan results (mean, ±13%) and ±8–35% for the D-tryptophan values (mean, ±29%). The values measured for K_a show good agreement (i.e., less than 8–15% difference) with previous estimates made for the binding of D- and L-tryptophan to the same type of immobilized HSA column at 25°C and under similar mobile-phase conditions [11]. These results are also similar to those noted by Lagercrantz et al. at pH 7.4 and 37°C for D- and L-tryptophan applied to an HSA-agarose column [19]. In addition, the L-tryptophan values in Table 1 are of the same order of magnitude as the intrinsic association constants that have been reported by McMenamy and coworkers between 2 and 37°C [23,24].

In general, the equilibrium constants given in Table 1 for L-tryptophan are much greater than those for D-tryptophan and show a larger change with temperature. For example, in going from 4 to 45°C the total change in K_a for L-tryptophan is −76%, while for D-tryptophan the change in K_a is −21%. In going from 4 to 37°C, the change in K_a for L-

Table 1
Association equilibrium constants (K_a) for the binding of D- and L-tryptophan to immobilized HSA at 4 to 45°C

Compound	Temperature (°C)	Association constant, K_a (M^{-1})[a]
L-Tryptophan	4	$8.2\ (\pm 0.9)\cdot 10^4$
	10	$7.8\ (\pm 0.8)\cdot 10^4$
	18	$4.7\ (\pm 0.7)\cdot 10^4$
	25	$3.2\ (\pm 0.7)\cdot 10^4$
	37	$2.4\ (\pm 0.3)\cdot 10^4$
	45	$2.0\ (\pm 0.2)\cdot 10^4$
D-Tryptophan	4	$3.8\ (\pm 1.1)\cdot 10^3$
	10	$3.7\ (\pm 1.2)\cdot 10^3$
	18	$3.7\ (\pm 0.3)\cdot 10^3$
	25	$3.7\ (\pm 1.3)\cdot 10^3$
	37	$3.6\ (\pm 1.2)\cdot 10^3$
	45	$3.0\ (\pm 1.0)\cdot 10^3$

All of the above results were obtained using 0.05 *M* potassium phosphate buffer (pH 7.0) as the mobile phase.
[a] The values in parentheses represent ±1 S.D.

tryptophan is −71% and the change in K_a for D-tryptophan is only −5%. This different dependence on temperature for the binding of D- and L-tryptophan is consistent with a model in which these two enantiomers are binding to two distinct regions on HSA [11].

Fig. 3 shows a comparison of the association equilibrium constants measured by frontal analysis and the values of k' that were observed for D- and L-tryptophan during zonal elution studies carried out under the same mobile phase and column conditions. Fig. 3 also includes the total moles of binding sites that were measured by the frontal analysis experiments. As shown in the figure, the decrease noted in the capacity factor for L-tryptophan with increasing temperature was similar to the decrease seen in L-tryptophan's K_a value. At the same temperatures, only small changes were detected in the value of m_L for L-tryptophan. These results indicate that variations in the binding strength of L-tryptophan (i.e., K_a) were responsible for the changes seen in L-tryptophan's retention over the temperatures that were tested.

The data in Fig. 3 show that a similar, but smaller decrease in retention occurred for D-tryptophan as the temperature was raised. In this case, the decrease in k' (i.e., −31% between 4 and 45°C) was much larger than what would be predicted based on just the observed changes in K_a. A close examination of Fig. 3c indicates that the moles of binding sites for D-tryptophan became lower at higher temperatures (i.e., a decrease of 48% in m_L between 4 and 45°C). Thus, the change in retention for D-tryptophan over temperature appeared to be the result of variations in both m_L and K_a. As mentioned earlier, a similar conclusion has been reached in studies that have examined the temperature dependence of *R*- and *S*-warfarin binding to an immobilized HSA column [12].

In earlier work with *R*- and *S*-warfarin, it has been noted that there is a change in the amount of some active sites on HSA as the column temperature is varied [12]. Although the exact reason for this phenomenon is not known, it probably reflects conformational changes in the protein at or near the regions that are responsible for analyte binding [12,33,34]. In this study, the fact that only D-tryptophan showed any significant temperature dependence for m_L again supports the hypothesis that D- and L-tryptophan are binding to different regions on HSA.

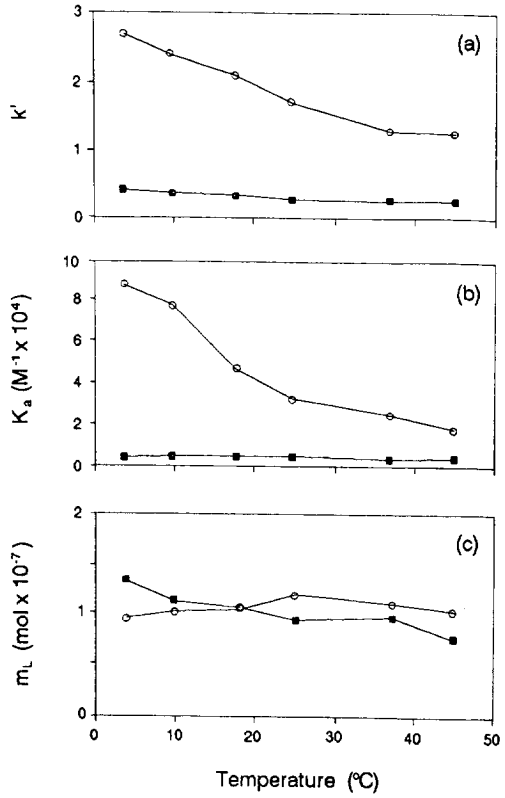

Fig. 3. Effect of temperature on the capacity factor (a), association equilibrium constant (b) and total moles of active binding sites (c) for D-tryptophan (■) and L-tryptophan (○) on an immobilized HSA column. The application buffer was 0.05 *M* potassium phosphate (pH 7.0). All other conditions were the same as described in Section 3.

In previous zonal elution experiments, shifts in k' with temperature have been used to estimate the changes in enthalpy and entropy that are associated with the binding of D- and L-tryptophan to immobilized HSA [11]. One assumption made in this approach is that the amount of binding sites remains constant over the range of conditions that are being examined. However, the data in Fig. 3c indicate that this assumption is not necessarily valid for the tryptophan/HSA system. In this present work, the

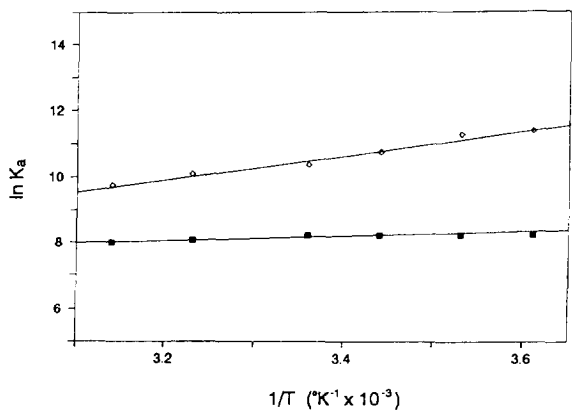

Fig. 4. Change in the association equilibrium constant (K_a) with absolute temperature (T) for D-tryptophan (■) and L-tryptophan (◇), as plotted according to Eq. 4. The experimental conditions were the same as in Fig. 3.

changes in enthalpy and entropy were instead determined by using the K_a values that were measured from the frontal analysis studies. A plot of these values was then made according to Eq. 4, as shown in Fig. 4.

The results in Fig. 4 for both D- and L-tryptophan gave linear behavior over the entire temperature range studied. The correlation coefficients of the plots for D- and L-tryptophan were 0.9932 and 0.9996, respectively, over the six points in each graph. According to Eq. 4, the linearity of these graphs supports a model in which each of these enantiomers is binding to a single type of site on HSA. This behavior is consistent with that obtained with HSA in the earlier zonal elution studies [11] and for D-tryptophan in similar work performed with bovine and sheep serum albumin columns [18,33]. However, in the studies with the bovine and sheep serum albumin, significant curvature was noted in temperature plots made for L-tryptophan [18,33]. This difference from the behavior seen with HSA probably reflects the slightly different amino acid sequences of these albumins in the vicinity of the indole binding site [18].

The changes in enthalpy and entropy that were determined from Fig. 4 are summarized in Table 2. The values of ΔG that were obtained by applying Eq. 5 to this data are also included. The results for ΔG and ΔH show reasonable agreement with those determined in previous zonal elution measurements [11]. However, the values obtained for ΔS are significantly different from those reported earlier. This was expected since this parameter would have been the most affected by the assumption that a constant amount of binding sites were present. Since this assumption was not made with the values in Table 2, these results should provide a more accurate representation of these thermodynamic parameters.

An inspection of the changes in enthalpy and entropy that are given in Table 2 shows that there are a number of important differences in the interactions of D- and L-tryptophan with HSA. For example, at room temperature the total change in energy for the binding of L-tryptophan with HSA is controlled mainly by the change in enthalpy (ΔH), which makes up over 90% of ΔG under these conditions. In contrast, the binding of D-tryptophan is dominated by the change in energy due to entropy ($-T\Delta S$), which makes up 65% of ΔG for this compound. The larger change in enthalpy seen for L-tryptophan indicates that this enantiomer has more or stronger bond formation during its interactions with HSA, while the larger value of $-T\Delta S$ for D-tryptophan indicates that the increase in entropy which occurs during the binding of this enantiomer is an important process in

Table 2
Thermodynamic parameters for the binding of D- and L-tryptophan to immobilized HSA[a]

Compound	ΔG at 25°C (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol °K)	$-T\Delta S$ at 25°C (kcal/mol)
L-Tryptophan	−6.2 (±0.6)	−5.7 (±0.4)	1.7 (±1.3)	−0.5 (±0.4)
D-Tryptophan	−4.8 (±0.3)	−1.7 (±0.2)	10.5 (±0.6)	−3.1 (±0.2)

All of the above results were obtained using 0.05 M potassium phosphate buffer (pH 7.0) as the mobile phase.
[a] The values in parentheses represent ±1 S.D.

promoting D-tryptophan–HSA interactions. An increase in entropy as the result of solute adsorption to HSA has been observed for other compounds and is probably due to the release of solvent molecules from the solute and/or HSA as a result of the binding process [12,24,26,27,35].

These thermodynamic results agree with what is currently known about the binding of D- and L-tryptophan to HSA. For example, in recent NMR studies it has been shown that D- and L-tryptophan have different degrees of attachment to HSA, with L-tryptophan having a tighter fit to its corresponding binding region [36]. This fits with a model in which the interactions of D- and L-tryptophan are driven mainly by the corresponding changes in entropy and enthalpy, respectively. The importance of enthalpy changes during the binding of L-tryptophan to HSA has been noted in previous work [23,24]. Most of this energy arises from non-polar interactions between the indole group of L-tryptophan and hydrophobic amino acids (e.g., tyrosine 411) that occur at one end of the indole-benzodiazepine site [15,21,23,24]. Additional energy comes from the association of the carboxyl group of L-tryptophan with cationic groups at the other end of the indole site [21,23,24]. The third interaction in this binding process is believed to involve the fit of the α-hydrogen on L-tryptophan to the binding site surface [23]. The sensitivity of the binding affinity to substitution at this position [23] helps explain why D-tryptophan does not bind to the same region of HSA.

4.3. Effect of mobile-phase composition on the binding of D- and L-tryptophan to HSA

The different thermodynamic parameters in Table 2 for the interactions of D- and L-tryptophan with HSA suggest that there should also be significant differences in the way that the binding of these compounds is affected by varying such conditions as the pH, ionic strength and polarity of the mobile phase. Previous work with D- and L-tryptophan using zonal elution has shown that this is the case [11,32]. As in the temperature studies, frontal analysis was used to determine whether variations in K_a and/or m_L were responsible for the shifts in solute retention that were observed during the zonal elution studies. The results are summarized in Figs. 5–7.

The data obtained by varying the pH of the mobile phase are shown in Fig. 5. As noted previously, both D- and L-tryptophan showed a decrease in k' with pH, with the L-enantiomer showing the largest change in retention in going from pH 7.4 to 4.0. Some change in the amount of binding sites was observed over this pH range, but the overall variation in m_L (i.e., −13% for L-tryptophan and −30% for D-tryptophan) was relatively small when compared with the change that was observed in k' (i.e., −83% for L-tryptophan and −52% for D-tryptophan). For both enantiomers, most of the variation in k' appeared to come from the corresponding change in K_a. In going from pH 7.4 to 4.0, the association constant for D-tryptophan dropped by 83%, while the association constant for L-tryptophan decreased by 99%. The same type of decrease has been noted by McMenamy and Oncley in work performed with L-tryptophan and HSA between pH 7.4 and 9.0 [23]. Such a decrease in binding strength may reflect either conformational changes at the binding regions or changes in the coulombic interactions and/or hydrogen bonding of D- and L-tryptophan with HSA [3,24].

Altering the ionic strength of the solvent is another factor that can be used to control the interactions between a solute and an immobilized protein [3]. In this work, the ionic strength was varied by changing the mobile-phase phosphate concentration. The results are shown in Fig. 6. As the phosphate concentration was increased from 0.0125 *M* to 0.25 *M*, there appeared to be only random variations in the amount of binding sites that were measured for D- and L-tryptophan (i.e., changes of ±14% and ±9%, respectively). The association equilibrium constant for L-tryptophan showed a large decrease under the same conditions (−74%), while D-tryptophan gave a 10% decrease in its K_a value. The capacity factors measured for these compounds showed behavior that was similar to that seen for the K_a values (i.e., a net decrease in k' of 26% for D-tryptophan and 70% for L-tryptophan). Thus, in this situation it was determined that alterations in the binding strength, and not the amount of binding sites, was responsible for

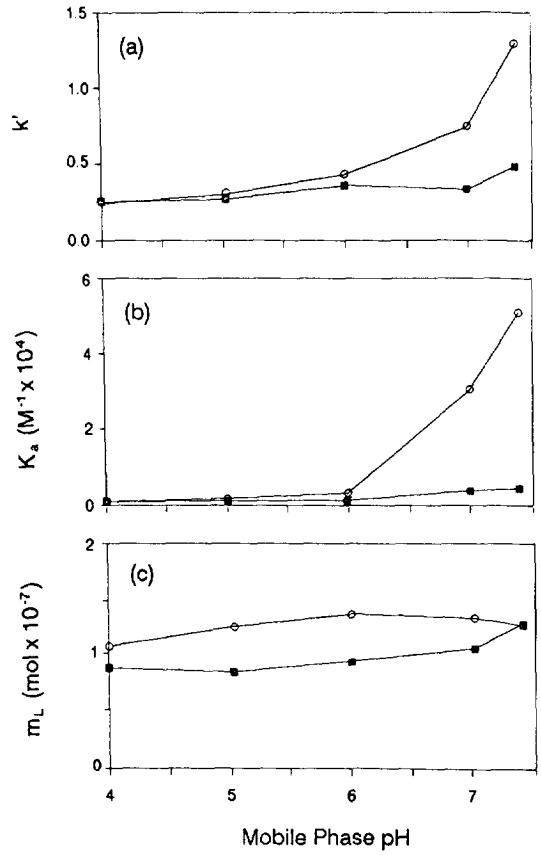

Fig. 5. Effect of mobile-phase pH on the capacity factor (a), association equilibrium constant (b) and total moles of active binding sites (c) for D-tryptophan (■) and L-tryptophan (○) on an immobilized HSA column. The application buffer was 0.05 *M* potassium phosphate buffer adjusted to the given pH values. The temperature was 25°C. All other conditions were the same as described in Section 3.

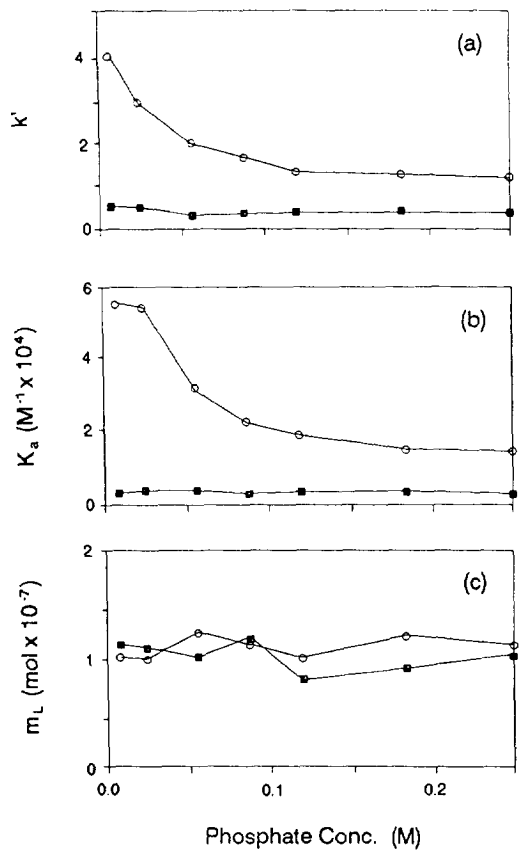

Fig. 6. Effect of mobile-phase phosphate concentration on the capacity factor (a), association equilibrium constant (b) and total moles of active binding sites (c) for D-tryptophan (■) and L-tryptophan (○) on an immobilized HSA column. The application buffer was pH 7.0 potassium phosphate buffer made up at the given concentrations. The temperature was 25°C. All other conditions were the same as described in Section 3.

the observed shifts in solute retention. The decrease in K_a (and k') that was seen at high phosphate concentrations probably reflects an increased shielding of ionic interactions or a decrease in dipole–dipole interactions between these compounds and HSA as the ionic strength of the mobile phase was increased [3].

The solvent polarity was changed in this work by adding different amounts of 1-propanol to the mobile phase. The results are given in Fig. 7. As reported earlier [11], both D- and L-tryptophan gave a steady decrease in k' as the mobile-phase content of 1-propanol was raised. However, the data obtained by frontal analysis indicated that this decrease in k' was actually the result of several different effects. For example, the association equilibrium constant for L-tryptophan showed a large decrease between 0 and 1% 1-propanol (i.e., a change in K_a of −79%). This was followed by much smaller variations in K_a between 1-propanol levels of 1 and 5%, with a possible increase in K_a for L-tryptophan between 3 and 5% 1-propanol. For D-tryptophan, a small but significant increase in K_a was found to occur

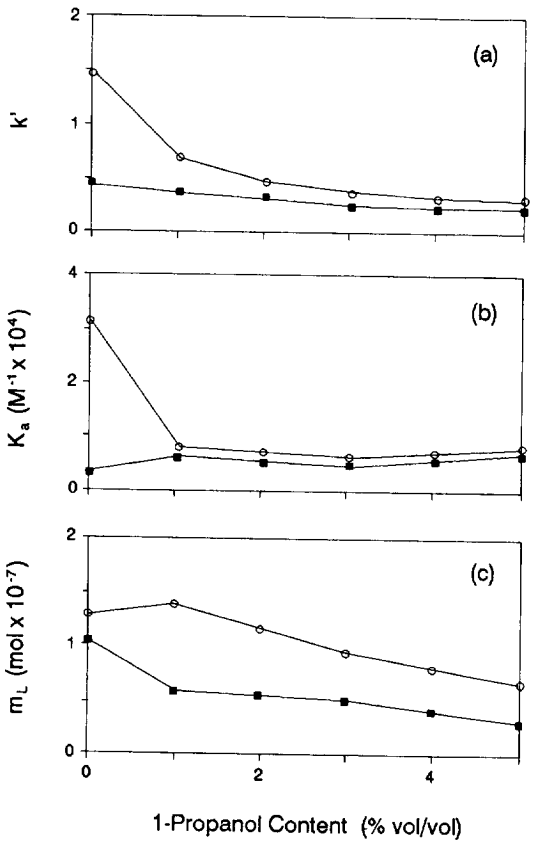

Fig. 7. Effect of adding 1-propanol to the mobile phase on the capacity factor (a), association equilibrium constant (b) and total moles of active binding sites (c) for D-tryptophan (■) and L-tryptophan (○) on an immobilized HSA column. The application buffer was 0.05 *M* potassium phosphate buffer (pH 7.0). The temperature was 25°C. All other conditions were the same as described in Section 3.

throughout the entire range of 0 to 5% 1-propanol. In addition, a decrease in m_L was seen for D- and L-tryptophan as the 1-propanol levels were increased. The result was that both K_a and m_L appeared to be important in determining the overall change in D- and L-tryptophan retention under these conditions.

The decrease in m_L that was seen for D- and L-tryptophan at increasing 1-propanol levels may indicate the presence of some changes in the structure or conformation of the binding regions on HSA. If present, such changes would probably affect K_a as well by either changing the interactions that take place between these analytes and HSA or by altering the changes in entropy that are associated with these binding processes. The parallel behavior seen in the K_a values for D- and L-tryptophan between 3 and 5% 1-propanol suggests that these compounds were exposed to similar changes in entropy under this range of solvent conditions.

5. Conclusion

This work used frontal analysis to examine the changes that occur in the binding of D- and L-tryptophan to an immobilized HSA column under a variety of chromatographic conditions. The response of the frontal analysis curves indicated that each enantiomer was interacting at a single type of site on HSA under all of the conditions that were tested. As noted earlier [11], L-tryptophan was the enantiomer whose retention was most susceptible to changes in temperature and the pH, ionic strength or polarity of the mobile phase. This is consistent with the thermodynamic studies performed in this work, which found that most of the energy released during L-tryptophan–HSA binding at pH 7.0 and 25°C came from the associated change in enthalpy (i.e., bond breaking and formation). At the same pH and temperature, the energy released during D-tryptophan–HSA binding was determined mainly by the change in entropy of the system. This explains why D-tryptophan was less susceptible than L-tryptophan to shifts in retention on the HSA column during changes in the column and mobile-phase conditions.

The association constants (K_a) for D- and L-tryptophan were affected to some degree by all of the chromatographic conditions that were examined. The value of K_a for L-tryptophan showed a large decrease when decreasing the pH, increasing the temperature, or increasing the ionic strength of the mobile phase. The value of K_a for L-tryptophan also showed a large decrease when adding up to 1% (v/v) 1-propanol to the mobile phase. In each of these cases, the change seen in K_a appeared to account for most of the corresponding shifts in retention that were noted for L-tryptophan during zonal elution studies. The value of K_a for D-tryptophan showed a small decrease as the temperature or ionic strength was raised, and as a

lower pH was used in the mobile phase. In addition, a small increase in K_a was seen for D-tryptophan as the amount of 1-propanol in the mobile phase was increased from 0 to 5%. The variations that were observed in K_a accounted for the majority of the shift in D-tryptophan retention in only two of these cases (i.e., the changes produced by pH and ionic strength).

The amount of active binding sites in the HSA column (m_L) was also affected by many of the changes that were made in the chromatographic conditions. For D-tryptophan, a decrease in m_L was seen when increasing the column temperature, decreasing the mobile phase pH, or increasing the amount of 1-propanol in the mobile phase. For L-tryptophan, a decrease in m_L was found to occur with decreasing pH or increasing 1-propanol levels. The only factor that did not appear to significantly affect the amount of binding sites for either D- or L-tryptophan was the mobile-phase ionic strength. This was not surprising, since those factors which did affect m_L (i.e., changes in organic modifier content, pH and temperature) also have the greatest possibility of causing conformational changes to HSA and its binding sites.

The results of this study clearly demonstrate that varying the temperature or mobile-phase composition of an immobilized protein column can result in changes in either the strength or number of binding sites for solutes injected onto that column. This means that altering these conditions can have multiple effects on solute retention and resolution. In addition, it was shown that the exact nature of these changes will depend on the regions of the protein that are involved in solute binding and the physical nature of these interactions. As a result, care must be taken when optimizing the separation of new solutes on protein-based supports and when comparing the elution of solutes that are injected under different temperature or mobile-phase conditions.

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References

[1] D.W. Armstrong, Anal. Chem., 59 (1987) 84A.
[2] T.J. Ward, Anal. Chem., 66 (1994) 633A.
[3] S. Allenmark, Chromatographic Enantioseparation: Methods and Applications, 2nd ed., Ellis-Horwood, New York, NY, 1991.
[4] S. Allenmark, J. Liq. Chromatogr., 9 (1986) 425.
[5] T.A.G. Noctor, G. Felix and I.W. Wainer, Chromatographia, 31 (1991) 55.
[6] J. Hermansson and M. Eriksson, J. Liq. Chromatogr., 9 (1986) 621.
[7] T. Miwa and T. Miyakawa, J. Chromatogr., 408 (1987) 316.
[8] I.W. Wainer, P. Jadaud, G.R. Schombaum, V. Kadodkar and M.P. Henry, Chromatographia, 25 (1988) 903.
[9] I.W. Wainer, in W.J. Lough (Editor), Chiral Liquid Chromatography, Chapman and Hall, New York, NY, 1989, Ch. 7.
[10] R.C. Williams, J.F. Edwards and M.J. Potter, J. Liq. Chromatogr., 16 (1993) 171.
[11] J. Yang and D.S. Hage, J. Chromatogr., 645 (1993) 241.
[12] B. Loun and D.S. Hage, Anal. Chem., 66 (1994) 3814.
[13] A. Dugiaezyk, S.W. Law and O.E. Dennison, Proc. Natl. Acad. Sci. USA, 79 (1982) 71.
[14] A. Kaplan and L.L. Szabo, Clinical Chemistry: Interpretation and Techniques, Lea and Febiger, Philadelphia, PA, 1979, Ch. 5.
[15] W.E. Müller, K.J. Fehske and S.A.C. Schläfer, in M.M. Reidenberg and S. Erill (Editors), Drug–Protein Binding, Praeger Publishers, New York, NY, 1986.
[16] J.-P. Tillement, G. Houin, R. Zini, S. Urien, E. Albengres, J. Barre, M. Lecomte, P. D'Athis and B. Sebille, Adv. Drug Res., 13 (1984) 59.
[17] W.E. Müller and U. Wollert, Nauyn-Schmiedeberg's Arch. Pharmacol., 288 (1975) 17.
[18] V. Tittelbach and R.K. Gilpin, Anal. Chem., 67 (1995) 44.
[19] C. Lagercrantz, T. Larsson and I. Denfors, Comp. Biochem. Physiol., 69C (1981) 375.
[20] X.M. He and D.C. Carter, Nature, 358 (1992) 209.
[21] D.C. Carter and J.X. Ho, Adv. Protein Chem., 45 (1994) 153.
[22] U. Kragh-Hansen, Pharmacol. Rev., 33 (1981) 17.
[23] R.H. McMenamy and J.L. Oncley, J. Biol. Chem., 233 (1958) 1436.
[24] R.H. McMenamy and R.H. Seder, J. Biol. Chem., 238 (1963) 3241.
[25] I.M. Chaiken (Editor), Analytical Affinity Chromatography, CRC Press, Boca Raton, FL, 1987.
[26] B. Loun and D.S. Hage, J. Chromatogr., 579 (1992) 225.
[27] B. Loun and D.S. Hage, J. Chromatogr. B, 665 (1995) 303.
[28] J.G. Kirkwood and I. Oppenheim, Chemical Thermodynamics, McGraw-Hill, New York, NY, 1961.
[29] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson and D.C. Klenk, Anal. Biochem., 150 (1985) 76.
[30] U. Lund, J. Liq. Chromatogr., 4 (1981) 1933.

[31] E. Grushka, M.N. Myers, P.D. Schettler and J.C. Giddings, Anal. Chem., 41 (1969) 889.
[32] S. Allenmark, B. Bomgren and H. Borén, J. Chromatogr., 237 (1982) 473.
[33] R.K. Gilpin, S.E. Ehtesham and R.B. Gregory, Anal. Chem., 63 (1991) 2825.
[34] G.E. Schulz and R.H. Schirmer, Principles of Protein Structure, Springer-Verlag, New York, NY, 1979.
[35] M. Tabachnick, J. Biol. Chem., 242 (1967) 1646.
[36] G. Uccello-Barretta, C. Bertucci, E. Domenici and P. Salvadori, J. Am. Chem. Soc., 113 (1991) 7017.