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Characterization of the binding and chiral separation of D- and L-tryptophan on a high-performance immobilized human serum albumin column

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ABSTRACT

High-performance affinity chromatography was used to study the separation and binding of D- and L-tryptophan on an immobilized human serum albumin (HSA) column. Frontal analysis and zonal elution studies indicated that both D- and L-tryptophan were binding to single but distinct sites on HSA. L-Tryptophan bound to the indole site of HSA. D-Tryptophan had indirect interactions with the warfarin site of HSA but no interactions with the indole site. The association constants for the binding of D- and L-tryptophan at pH 7.4 and 25°C were $0.4 \cdot 10^4$ and $2.7 \cdot 10^4$ M^{-1}, respectively. The value of ΔG for these sites ranged from −5.2 to −5.7 kcal/mol (1 cal = 4.184 J) and had a significant entropy component. The effects of varying the pH, phosphate concentration, temperature and polarity of the mobile phase on the binding of D- and L-tryptophan to HSA were examined. The role of sample size in determining peak shape and retention was also considered. From these data, general guidelines were developed for the separation of D- and L-tryptophan on immobilized HSA. Under optimized conditions the enantiomers were separated in less than 2 min with baseline resolution.

INTRODUCTION

The separation of chiral molecules is an area of increasing importance in pharmaceutical and biochemical testing. However, this also represents one of the most difficult challenges in separation science. To meet this challenge, a number of techniques have been developed for the analysis and purification of chiral compounds. Examples include liquid chromatographic methods based on chiral derivatives, complexing agents, ion-pairing agents, and chemically bonded chiral stationary phases [1]. This study will examine the use of high-performance affinity columns using immobilized human serum albumin for the separation of D- and L-tryptophan.

Human serum albumin (HSA) is the most abundant protein in blood [2]. HSA is useful as an affinity ligand since it is known to bind to a variety of biological and pharmaceutical compounds [3]. It is believed that this binding occurs at a number of relatively well-defined sites or regions on HSA. The two most important of these regions are the warfarin–azapropazone and indole–benzodiazepine binding sites [4–6]. These two sites are believed to be involved in the interactions of most compounds with HSA [7]. As its name suggests, the warfarin site is characterized by its binding to warfarin and related substances [4,8–10]. This site is known to show stereoselectivity in binding such compounds as *R*- and *S*-warfarin or *R*- and *S*-phenprocoumon [11]. The indole site binds to a number of indole-

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containing compounds, including L-tryptophan [12,13]. Binding at this site is highly specific and can also be stereoselective in nature [11,14].

L-Tryptophan is one of the essential amino acids and is used as a pharmaceutical antidepressant agent [15]. D-Tryptophan has no known significant biological effects [16,17] but can appear as a contaminant in L-tryptophan samples. In view of recent US Food and Drug Administration (FDA) guidelines regarding chiral drugs [18], this makes the determination of D- and L-tryptophan in pharmaceuticals an area of great potential importance. One way of separating and analyzing D- and L-tryptophan is through the use of immobilized albumin columns [19]. However, baseline resolution of tryptophan enantiomers in a reasonable amount of time (*i.e.*, less than 10 min) has not yet been achieved by this approach.

The aim of this work is to obtain a better understanding of how D- and L-tryptophan bind to immobilized HSA, allowing the development of more rapid separation techniques for these enantiomers. In previous work with HSA immobilized to diol-bonded silica by the Schiff base method, it has been shown that L-tryptophan binds to a single type of site on the immobilized HSA [20]. Data indicated that L-tryptophan was binding to the indole site of HSA, in agreement with solution studies [20]. However, little or no specific information is yet available regarding the binding of D-tryptophan to immobilized HSA.

The first part of this study will use frontal analysis to determine the number of binding sites and the association constants for the interactions of D- and L-tryptophan with the immobilized HSA. Thermodynamic constants for these interactions, such as their changes in enthalpy and entropy, will also be evaluated. The binding of D-tryptophan will be further characterized by examining its competition with *R*-warfarin and L-tryptophan for the warfarin and indole sites of HSA. Changes in the retention and binding of D- and L-tryptophan with pH, phosphate concentration, column temperature, mobile phase polarity and sample size will then be studied. From this data, optimum conditions for the rapid separation of D- and L-tryptophan on the immobilized HSA column will be determined.

THEORY

Frontal analysis

For the continuous application of solute (E) to a column containing a single type of immobilized ligand site (L), the apparent moles of E required to reach the mean point of the resulting breakthrough curve (m_{Lapp}) is given by the following equation [20,21]:

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

where K_a is the association constant for the binding of E to L, [E] is the concentration of solute applied to the column and m_L is the true number of moles of binding sites on the column. Eqn. 1 predicts that a plot of $1/m_{Lapp}$ *vs.* $1/[E]$ for this type of system will give a straight line with a slope of $1/K_a m_L$ and an intercept of $1/m_L$. The association constant K_a can be determined directly from this plot by calculating the ratio of the intercept to the slope. The true number of binding sites for solute on the column (m_L) can be determined from the inverse of the intercept [20].

Temperature studies

In the injection of a small amount of solute onto a column with a single type of binding site, the capacity factor of the solute (k') can be related to the solute's association constant by the equation [21]:

$$k' = K_a m_L / V_m \quad (2)$$

where V_m is the column void volume and all other terms are the same as defined previously. The association constant for this system can also be related to the absolute temperature (T) by:

$$\ln K_a = -\Delta H/RT + \Delta S/R \quad (3)$$

where ΔH and ΔS are the changes in the enthalpy and entropy of the reaction and R is the ideal gas law constant. By combining eqns. 2 and 3, the following expression can be derived:

$$\ln k' = -\Delta H/RT + \Delta S/R + \ln (m_L/V_m) \quad (4)$$

Eqn. 4 predicts that a plot of ln k' *vs.* $1/T$ will yield a linear relationship with a slope of $-\Delta H/R$ and an intercept of $[\Delta S/R + \ln (m_L/V_m)]$ for a system with single site binding. Using the values of ΔH and ΔS obtained from this plot, the change in the total free energy of the system (ΔG) can also be calculated [20,22].

EXPERIMENTAL

Reagents

The D- and L-tryptophan, HSA (Cohn fraction V, 99% globulin-free), bovine serum albumin (BSA) and *R*-/*S*-warfarin racemic mixture were purchased from Sigma (St. Louis, MO, USA). The Nucleosil Si-300 (4 μm particle diameter, 300 Å pore size) was from Alltech (Deerfield, IL, USA). The reagents for the bicinchoninic acid (BCA) protein assay were purchased from Pierce (Rockford, IL, USA). The HPLC-grade 1-propanol and uracil were from Aldrich (Milwaukee, WI, USA). All other chemicals used were of purest grade available. All solutions were prepared with water from a NANOpure water system (Barnstead, Dubuque, IA, USA).

Apparatus

The chromatographic system consisted of one CM3000 isocratic pump, one CM4000 gradient pump, and one SM3100 UV–Vis variable-wavelength absorbance detector (Milton Roy, Riviera Beach, FL, USA). Samples were injected using a Rheodyne 7012 valve (Cotati, CA, USA) equipped with a PhaseSep event marker (Phase Separations, Queensferry, UK) and a 20-μl injection loop. A Milton Roy Chromlink interface and LCAdvantage software were used for data collection. Chromatographic data were processed using programs written in QuickBASIC (Redmond, WA, USA) with double-precision logic. An Isotemp 9100 circulating water bath (Fisher Scientific, Pittsburgh, PA, USA) was used for temperature control of both the column and mobile phases. All columns were packed using an Alltech HPLC column slurry packer (Deerfield, IL, USA).

Methods

The Nucleosil Si-300 silica was converted to a diol-bonded form using a previously-published method [23]. The diol coverage of the Nucleosil prior to activation was 25 ± 4 μmol (± 1 standard error of the mean, S.E.M.) per gram of silica, as determined in duplicate by the periodate oxidation method [24,25]. HSA was immobilized onto the diol-bonded Nucleosil using the Schiff base method [23]. After the immobilization step, the silica was centrifuged, washed three times with 0.10 *M* phosphate buffer (pH 7.0) containing 2.0 *M* NaCl, and rinsed three times with 0.10 *M* phosphate buffer (pH 7.0). The silica was then stored at 4°C prior to column packing. A small portion of the HSA–silica was further washed with deionized water and dried under reduced pressure for use in the BCA protein assay [26]. The BCA assay was performed using BSA as the standard and diol-bonded silica as the blank.

Chromatography

The HSA-silica and diol-bonded silica were downward slurry-packed into two separate 100.0 mm × 4.1 mm I.D. stainless steel columns at a pressure of 400 bar using 0.10 *M* phosphate buffer (pH 7.0) as the packing solvent. Both columns were enclosed in water jackets for temperature control. All studies, except those examining the temperature dependence of tryptophan–HSA binding, were performed at 25 ± 0.1°C. The mobile phases were filtered prior to use by passing them through 0.45-μm cellulose acetate or 0.45-μm nylon filters. All mobile phases were degassed under vacuum for at least 10 min before use on the HPLC system. Elution of the D- and L-tryptophan was monitored at 290 nm.

Frontal analysis was performed by continuously applying 0.05 *M* phosphate buffer (pH 7.4) containing D- or L-tryptophan to the HSA column at a flow-rate of 0.20 ml/min. The analyte solutions were applied using the same valve configuration as reported in ref. 20. The concentrations of D- and L-tryptophan used in this experiment ranged from $6.0 \cdot 10^{-6}$ to $1.0 \cdot 10^{-4}$ *M*. The retained D- and L-tryptophan were eluted by applying 0.05 *M* phosphate buffer (pH

7.4) to the column. The amount of D- or L-tryptophan required to saturate the HSA column was determined by integration of the resulting breakthrough curves [27]. Corrections for the system void volume and non-specific binding were made by subtracting the amount of D- or L-tryptophan needed to saturate the diol-bonded column under identical chromatographic conditions. This correction was within 20% of the system void volume, as measured by injecting uracil or sodium nitrate onto the column.

Zonal elution studies with the D- and L-tryptophan were performed at flow-rates ranging from 0.50 to 1.50 ml/min. The D- and L-tryptophan solutions injected were freshly prepared for each experiment by dissolving up to $1 \cdot 10^{-5}$ *M* of the appropriate enantiomer into the buffer being used as the mobile phase. Three injections of sample were made under each set of mobile phase conditions. The retention times and peak widths of the D- and L-tryptophan peaks were calculated by moments analysis and the $B/A_{0.1}$ method [28]. The void volume was determined by injecting uracil or sodium nitrate under the same chromatographic conditions. The retention times and column void time were corrected for the extra-column volume of the system by making injections of D- or L-tryptophan onto the system with no HSA column present.

Competition between D- and L-tryptophan for HSA binding sites was studied at a flow-rate of 1.00 ml/min by making 20-μl injections of $1 \cdot 10^{-5}$ *M* D-tryptophan in the presence of 0.05 *M* phosphate buffer (pH 7.0) containing $1 \cdot 10^{-4}$ to $1 \cdot 10^{-6}$ *M* L-tryptophan. Competition between D-tryptophan and *R*-warfarin for HSA binding sites was similarly examined by making 20-μl injections of $1 \cdot 10^{-5}$ *M* racemic warfarin in the presence of 0.05 *M* phosphate buffer (pH 7.0) containing $1 \cdot 10^{-4}$ to $1 \cdot 10^{-6}$ *M* D-tryptophan and determining the retention time of the first eluting peak (*i.e.*, *R*-warfarin).

RESULTS AND DISCUSSION

Binding of D- and L-tryptophan to immobilized HSA

The initial properties of the immobilized HSA matrix used in this study are listed in Table I.

TABLE I

INITIAL PROPERTIES OF THE IMMOBILIZED HSA MATRIX

Property	Value (±1 S.D.)
HSA immobilized (nmol/g silica)	500 (±7)
Binding capacity (nmol/g silica)	
L-Tryptophan	170 (±10)
D-Tryptophan	80 (±10)
Specific activity (mol/mol HSA)	
L-Tryptophan	0.34 (±0.02)
D-Tryptophan	0.16 (±0.02)

The coverage of HSA on the matrix, as determined by protein assay, was approximately 0.2 monolayers. This coverage is similar to that obtained in previous work using the same immobilization method for HSA on diol-bonded Nucleosil Si-1000 silica [20]. By assuming 1:1 binding for both the D-tryptophan and L-tryptophan with the immobilized HSA, 16% of the D-tryptophan binding sites and 34% of L-tryptophan binding sites (or indole sites) were determined to be active on this matrix. This relatively low activity has been reported previously for immobilized HSA [20] and is probably the result of such factors as steric hindrance, denaturation, or improper orientation of protein attached to the matrix [29]. The lower specific activity for D-tryptophan may reflect a greater sensitivity of its binding sites to these immobilization effects.

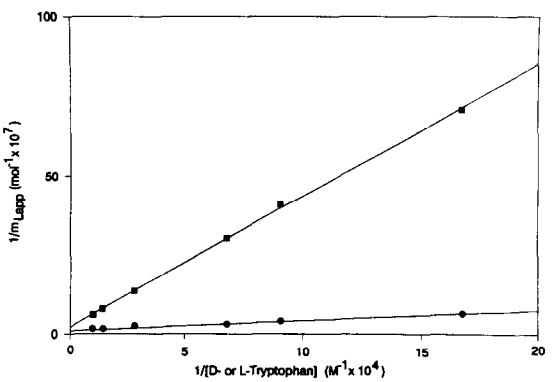

Fig. 1. Frontal analysis results for D- (■) and L- (●) tryptophan on the immobilized HSA column. The chromatographic conditions are given in the text.

The frontal analysis results obtained for D- and L-tryptophan on the immobilized HSA column are given in Fig. 1. As shown in this figure, plots of $1/m_{L_{app}}$ *versus* both 1/[L-tryptophan] and 1/[D-tryptophan] gave linear relationships over the entire concentration range studied. The correlation coefficients for these plots were 0.9980 and 0.9997, respectively, over the 6 points shown in each graph. According to eqn. 1, this linear behavior indicated that both D- and L-tryptophan were binding to a single type of site on the immobilized HSA matrix. Single-site binding for L-tryptophan to HSA has been previously reported in work with HSA in solution [30] and with HSA immobilized onto high-performance affinity columns [20].

Based on eqn. 1, the plots in Fig. 1 were used to determine the binding capacities (m_L) and association constants (K_a) for D- and L-tryptophan on the immobilized HSA matrix. The resulting values are shown in Tables I and II, respectively. The association constant for L-tryptophan at 25°C was almost an order of magnitude larger than that for the D-tryptophan. This agrees with earlier results reported at 37°C [31]. D- and L-tryptophan also differed in that L-tryptophan had over 2-fold more binding sites on the HSA column. This difference in capacity indicated that D- and L-tryptophan were binding to separate regions on the immobilized HSA.

An experiment examining competitive binding between D- and L-tryptophan was used to confirm that these enantiomers were binding to different regions on HSA. This was done by injecting small amounts of D-tryptophan onto an immobilized HSA column in the presence of mobile phases containing various concentrations of L-tryptophan. No change in the retention of D-tryptophan (*i.e.*, less than 1% variation in k') was noted over the concentration range of 0 to $4 \cdot 10^{-5}$ *M* L-tryptophan. These results indicated that D- and L-tryptophan had no competition (either direct or indirect) in their binding to the HSA. This confirmed that the enantiomers were interacting at separate binding sites.

TABLE II

ASSOCIATION CONSTANTS FOR THE BINDING OF D- AND L-TRYPTOPHAN TO IMMOBILIZED HSA AT 25°C

Compound	Association constant (M^{-1})[a]
L-Tryptophan	$2.7 \cdot 10^4$ ($\pm 0.3 \cdot 10^4$)
D-Tryptophan	$0.4 \cdot 10^4$ ($\pm 0.1 \cdot 10^4$)

[a] Values in parentheses represent ±1 S.D.

A similar competition study was performed between D-tryptophan and *R*-warfarin to determine if D-tryptophan was binding to the warfarin site of HSA. The results are shown in Fig. 2. It was found that the retention of *R*-warfarin changed as different concentrations of D-tryptophan were applied to the column. However, a plot of $1/k'_{R\text{-warfarin}}$ *versus* [D-tryptophan] did not give the linear relationship expected for direct competition at a single site [20]. Instead, the retention of *R*-warfarin initially increased in going from 0 to $0.33 \cdot 10^{-5}$ *M* D-tryptophan, followed by a gradual decrease in retention at higher D-tryptophan concentrations. These results suggest that the binding sites for D-tryptophan and *R*-warfarin are related through allosteric interactions or some other type of indirect competition.

Effect of mobile phase composition on the retention of D- and L-tryptophan

After it was determined that D- and L-tryptophan bound to two distinct sites on the immobilized HSA, work was performed to see how

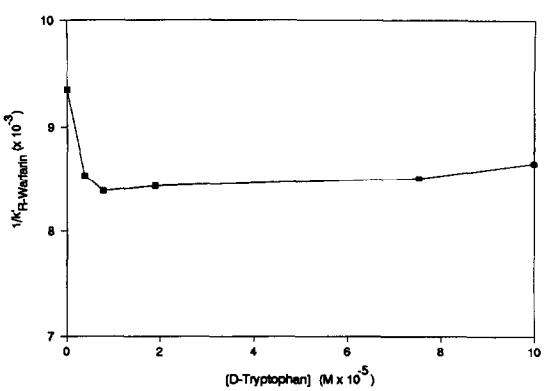

Fig. 2. Competitive binding of *R*-warfarin with D-tryptophan on the immobilized HSA column. The chromatographic conditions are given in the text.

changing the pH, phosphate concentration, temperature, and organic modifier content of the mobile phase would affect the interactions of D- and L-tryptophan at these sites. The effect of varying pH on the retention (k') of both compounds is shown in Fig. 3.

The results in Fig. 3 were obtained by injecting tryptophan samples onto the HSA column using a 0.10 *M* phosphate buffer at pH values ranging from 4.0 to 7.4. As the pH decreased, the capacity factors for both D- and L-tryptophan decreased. The retention of both compounds on the diol-bonded column was negligible (*i.e.*, $k' \leq 0.05$) throughout this entire pH range. In going from pH 7.4 to 4.0, the capacity factor for D-tryptophan dropped from 0.52 to 0.25 (52%), while the capacity factor for L-tryptophan decreased from 1.43 to 0.23 (84%). The fact that these changes were not of the same relative size indicates that the binding sites of the L- and D-tryptophan were subjected to different local ionic interactions. This supports the model proposed earlier in which the two enantiomers bind to two distinct sites. As the pH was decreased from 7.4 to 4.0, the separation factor for D- and L-tryptophan (α, where $\alpha = k'_{\text{L-Trp}}/k'_{\text{D-Trp}}$) decreased from 2.75 to 1. The best separation of the enantiomers was obtained at pH 7.4. This was the pH used in the remainder of the study.

The ionic strength of the mobile phase is another parameter which can affect the stereoselectivity of an immobilized protein. In this study, this parameter was examined by maintaining the mobile phase at pH 7.4 while varying the concentration of the phosphate buffer from 0.010 to 0.250 *M*. As shown in Fig. 4, the capacity factors of both D- and L-tryptophan on the HSA column decreased with increasing phosphate buffer concentration. Binding to the diol–silica column was again negligible under these conditions (*i.e.*, $k' < 0.05$). The changes observed on the HSA column are consistent with previous work performed with oxazepam hemisuccinate on immobilized HSA–silica [32] and chiral separations performed on immobilized BSA–silica [33,34].

In this study, the capacity factor for L-tryptophan decreased much faster with increasing phosphate concentration than the capacity factor for D-tryptophan (*i.e.*, 72% *versus* 12% change, respectively). This suggested that the binding site for L-tryptophan was more sensitive to changes in the mobile phase ionic strength. This agrees with the results noted earlier in the pH studies. One result of this difference in sensitivity to changes in the ionic strength was that the value of α for D- and L-tryptophan dropped from 7.62 to 2.44 over the range of phosphate concentrations studied. Optimum separation of the two enantiomers was obtained at phosphate concentrations of 0.05 *M* or less. This concentration range was used in all later work.

Temperature is a third parameter which can be varied to adjust the resolution of an enantio-

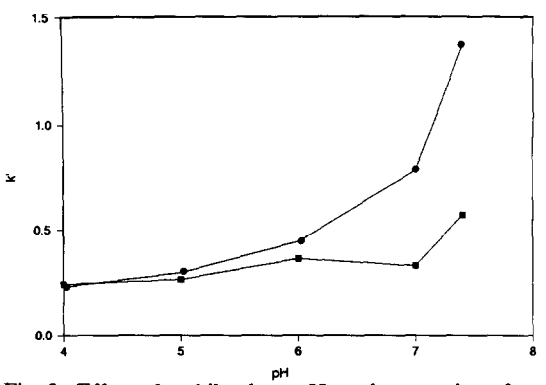

Fig. 3. Effect of mobile phase pH on the retention of D- (■) and L- (●) tryptophan on the immobilized HSA column. The chromatographic conditions are given in the text.

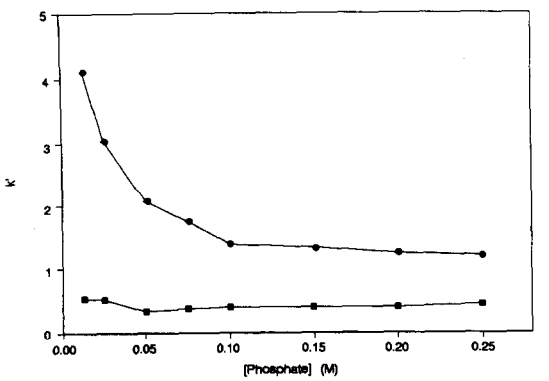

Fig. 4. Effect of phosphate concentration on the retention of D- (■) and L- (●) tryptophan on the immobilized HSA column. The chromatographic conditions are given in the text.

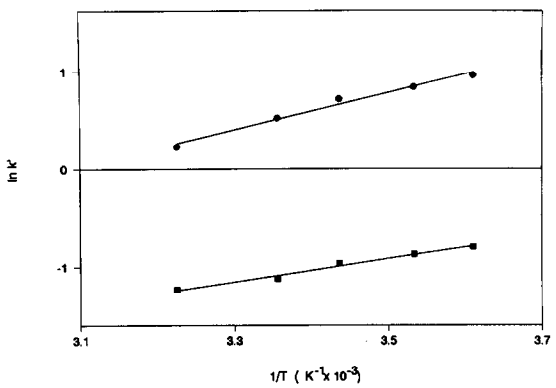

Fig. 5. Temperature dependence of D- (■) and L- (●) tryptophan binding to the immobilized HSA column. The chromatographic conditions are given in the text.

meric separation. The effect of temperature on the binding of D- and L-tryptophan to the immobilized HSA column was examined using a mobile phase containing 0.05 *M* phosphate buffer (pH 7.4). The resulting plots of ln *k' versus* $1/T$ are shown in Fig. 5. Both D- and L-tryptophan gave linear relationships over the temperature range of 4 to 37°C. The correlation coefficients for these plots were 0.9922 and 0.9925, respectively, over the 5 points shown in each graph. According to eqn. 4, these linear relationships confirmed that both the D- and L-tryptophan had single-site interactions with the immobilized HSA.

From the slopes and intercepts of these plots and the data in Table I, it was possible to calculate the change in enthalpy, entropy, and total free energy for the binding of each of these enantiomers. The results are given in Table III. The change in total free energy (ΔG) for these sites ranged from −5.2 to −5.7 kcal/mol (1 cal = 4.184 J) at 25°C. These values agree with those calculated using the association constants from Table II. For both the D- and L-tryptophan, a significant portion of the total free energy change (*i.e.*, 33–54%) was due to the entropy term ($-T\,\Delta S$). This significant entropy contribution has been previously observed in the binding of L-thyroxine to a similar immobilized HSA column [20].

In Fig. 5 it was found that the capacity factors for both the D- and L-tryptophan decreased proportionately as the temperature increased. For example, the value of k' for L-tryptophan decreased from 2.62 to 1.26 (48%) in going from 4 to 37°C while the k' value for D-tryptophan decreased from 0.45 to 0.29 (64%). The result was that the separation factor α changed only slightly over this temperature range. This indicated that adjusting the temperature was not particularly useful as a means for obtaining optimum resolution in this separation. In further work, a temperature of 25°C was used for the sake of convenience.

The use of small amounts of mobile phase modifiers, such as 1-propanol, is yet another technique which has commonly been reported as a way of decreasing retention on protein columns while maintaining or improving stereoselectivity. The effect of using 1-propanol as an organic modifier in the separation of D- and L-tryptophan was examined by using a mobile phase containing 0.05 *M* phosphate buffer (pH 7.4) with 1-propanol levels of 0 to 5% (v/v). The effects of adding 1-propanol on the k' values for the D- and L-tryptophan are shown in Fig. 6. In this figure, increasing the concentration of 1-propanol from 0 to 5% caused the capacity factor for L-trypto-

TABLE III

THERMODYNAMICS FOR THE BINDING OF D- AND L-TRYPTOPHAN TO IMMOBILIZED HSA

Values in parentheses represent ±1 S.D.

Compound	ΔG at 25°C (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol K)
L-Tryptophan	−5.7 (±0.4)	−3.8 (±0.3)	6.3 (±0.7)
D-Tryptophan	−5.2 (±0.4)	−2.4 (±0.2)	9.4 (±1.3)

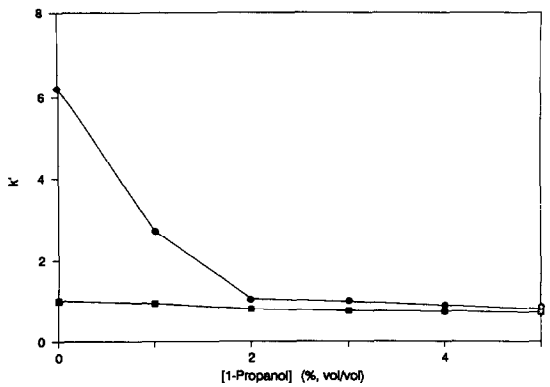

Fig. 6. Effect of mobile phase 1-propanol content on the retention of D- (■) and L- (●) tryptophan on the immobilized HSA column. The chromatographic conditions are given in the text.

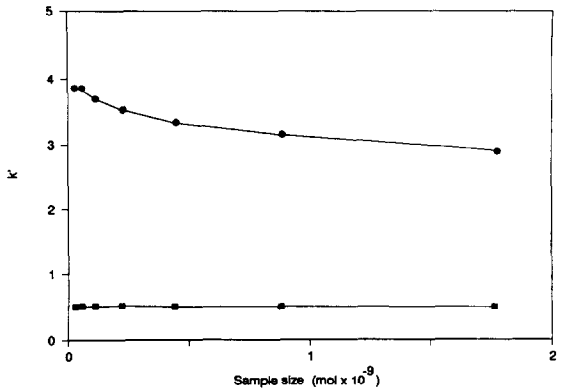

Fig. 7. Effect of sample size on the retention of D- (■) and L- (●) tryptophan on the immobilized HSA column. Chromatographic conditions: mobile phase, 0.015 *M* phosphate buffer (pH 7.4); 20-μl injection loop; flow-rate, 1.00 ml/min.

phan to decrease from 1.98 to 0.24 (88%) while the capacity factor for D-tryptophan changed only from 0.32 to 0.24 (25%) under the same conditions. The binding of both compounds to the diol-silica column was negligible ($k' < 0.05$) under these conditions.

The effect of 1-propanol noted in Fig. 6 agrees with that observed by Allenmark and Bomgren [33,34] on a BSA-silica column and that seen by Dominici *et al.* [32] for the separation of hemisuccinate enantiomers on an HSA-silica column. In our study, the separation factor for D- and L-tryptophan fell from 6.2 to 1.16 in going from 0 to 5% 1-propanol. This decrease reflects the greater sensitivity of the L-tryptophan site to the organic modifier. Since the separation factor decreased by adding 1-propanol, and both D- and L-tryptophan were already separated in a reasonable amount of time when no 1-propanol was added, 1-propanol was not used in the remainder of this study.

Effect of sample size on peak retention and symmetry

The effect of sample size on the separation of D- and L-tryptophan was investigated by determining the values of k' for D- and L-tryptophan as different amounts of each solute were loaded onto the HSA column. As shown in Fig. 7, the capacity factor for D-tryptophan remained relatively stable (*i.e.*, less than 2% change) throughout the entire range of solute concentrations studied while the capacity factor for L-tryptophan decreased by over 32% over the same concentration range. These results are consistent with effects observed by Gilpin *et al.* [19] for the separation of D- and L-tryptophan on a BSA column. This supports the model in which the D- and L-tryptophan are binding to two distinct regions on the immobilized HSA. The greater sensitivity of the L-tryptophan site to sample size is somewhat surprising since there were almost two-fold more binding sites for L-tryptophan than for D-tryptophan present on the HSA column. However, these results may reflect differences in the kinetics or binding mechanisms of D- and L-tryptophan with the immobilized HSA.

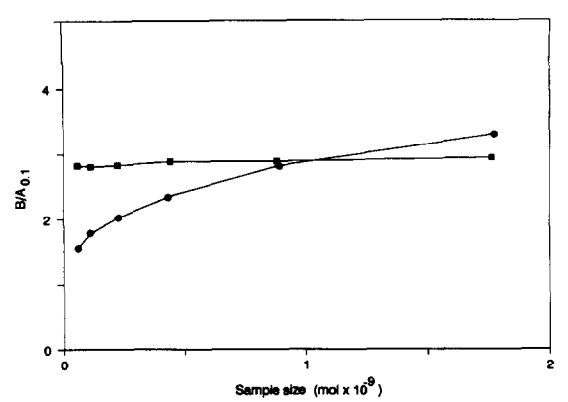

Fig. 8. Effect of sample size on the peak symmetry ($B/A_{0.1}$) of D- (■) and L- (●) tryptophan on the immobilized HSA column. The chromatographic conditions were the same as in Fig. 7.

The effect of sample size on peak shape was examined by using the tenth height B/A ratio ($B/A_{0.1}$) as a measure of peak symmetry. Changes observed in the $B/A_{0.1}$ ratio with sample size are shown in Fig. 8. For D-tryptophan, the $B/A_{0.1}$ ratio varied by only 20% over the range of sample loads studied. But for L-tryptophan, this ratio varied by 54% over the same sample load range and became stable only at very small sample sizes (*i.e.*, $<10^{-10}$ mol per injection). These results are consistent with the observed changes in the capacity factor with sample size. From the data in Fig. 8 it was determined that relatively small sample sizes (*i.e.*, 10^{-11} to 10^{-10} mol per injection) are needed for the optimum resolution of D- and L-tryptophan on this type of immobilized HSA column.

Optimized separation of D- and L-tryptophan

From the previous studies examining the effects of various mobile phase compositions on the separation of D- and L-tryptophan, the optimum chromatographic conditions for this separation were determined. These conditions included using a neutral mobile phase (pH 7.4) with a low-ionic-strength phosphate buffer (<0.05 *M*), 0% 1-propanol, and a sample size of 10^{-10} to 10^{-11} mol injected at room temperature. An example of a separation obtained under these conditions is shown in Fig. 9. At a flow-rate of 3.0 ml/min, the D- and L-tryptophan were completely resolved ($R_s = 1.72$, $\alpha = 2.56$) in less than 2 min. It is expected that D- and L-tryptophan could be separated in an even shorter period of time if higher flow-rates were used.

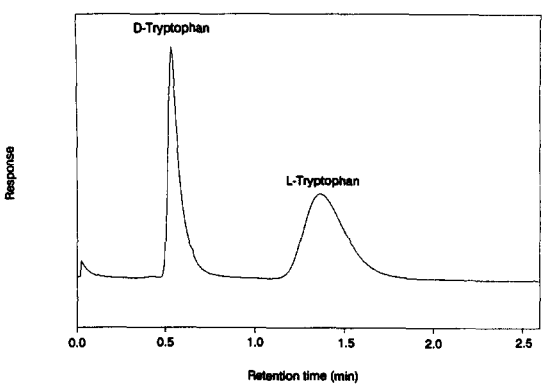

Fig. 9. Separation of D- and L-tryptophan on an immobilized HSA–silica column. Chromatographic conditions: mobile phase, 0.015 *M* phosphate buffer (pH 7.4); sample, $2.75 \cdot 10^{-6}$ *M* D-/L-tryptophan, 20-μl injection loop; flow-rate, 3.00 ml/min. The void time of this separation was at 0.31 min.

The HSA columns used in this study were found to be quite stable under the optimum mobile phase conditions given. No significant change in column behavior was noted over the course of 500 injections and over the range of pH, temperature, and 1-propanol conditions used in this study. The only item noted to give a permanent decrease in HSA activity and tryptophan retention was the use of phosphate buffers with concentrations greater than 0.10 *M*. The mechanism for the loss in tryptophan retention at high phosphate buffer concentrations is currently under investigation.

CONCLUSIONS

In this study high-performance affinity chromatography was used to study the separation and binding of D- and L-tryptophan on an immobilized HSA column. From frontal analysis and zonal elution studies it was found that both D- and L-tryptophan were binding to single but distinct sites on HSA. In earlier work, it has been shown that L-tryptophan binds to the indole site of the immobilized HSA [20]; however, little information has previously been reported on the binding of D-tryptophan to this protein. In competition studies with L-tryptophan and *R*-warfarin, it was determined that the D-tryptophan had indirect interactions with the warfarin site of HSA but no interactions with the indole site. Although the exact location of the D-tryptophan site is still not known, these results are of interest since they definitely show that the two enantiomers are binding to totally different regions on HSA.

More detailed information on the interactions of D- and L-tryptophan with HSA was obtained by measuring the association constants and thermodynamic parameters for these binding processes. The L-tryptophan bound to HSA more strongly than D-tryptophan, with association constants of $2.7 \cdot 10^4$ and $0.4 \cdot 10^4$ M^{-1}, respectively, at pH 7.4 and 25°C. The value of

ΔG for the binding of D- and L-tryptophan to HSA was in the range of −5.2 to −5.7 kcal/mol at 25°C and had a significant contribution due to entropy.

This work also examined the effects of pH, phosphate concentration, temperature and 1-propanol content of the mobile phase on the binding of D- and L-tryptophan to HSA. In each case the retention of L-tryptophan was affected to a greater extent than that of the D-tryptophan. These differences in behavior agree with a model in which the enantiomers are binding to two distinct regions on HSA. Another parameter considered was the role of sample size in determining the retention and peak shape for each enantiomer. It was found that L-tryptophan had a greater sample size dependence in its chromatographic behavior than was noted for D-tryptophan.

Based on these studies, several guidelines were developed for the separation of D- and L-tryptophan on immobilized HSA. The strongest binding and best separation factor for these enantiomers was obtained when working with a neutral pH, low ionic strength phosphate buffer containing no organic modifier (*i.e.*, 1-propanol). Temperature was found to have only a small effect on the separation of D- and L-tryptophan. Under the optimized conditions, D- and L-tryptophan were separated in less than 2 min with baseline resolution. This method is much faster than current separation techniques and should prove useful in the analysis of these compounds in food or pharmaceutical preparations.

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