Frontal Analysis Studies of Indole Compounds and Human Serum Albumin in Chromatographic Measurements of Drug-Protein Binding

Mandi Conrad, Annette Moser, David S. Hage, University of Nebraska, Department of Chemistry, Lincoln, NE 68588

Introduction

The object of this study is to locate a compound with similar binding characteristics compared to L-tryptophan for the protein human serum albumin (HSA) but that is more stable then L-tryptophan. L-Tryptophan is commonly used as a site selective probe for HSA in drug-protein binding studies. However, L-tryptophan is relatively unstable, requiring that fresh solutions of this probe be made on a daily basis. In this study, various related compounds (i.e., indole-3-acetic acid, indole-3-carboxylic acid, indole-3-butyric acid, indole-3-methanol, indole-3-propionic acid, 3-acetylindole, and 3-methylindole) were studied as alternative agents for binding studies using HSA immobilized in HPLC columns. Frontal analysis was used to obtain association equilibrium constants for these compounds with HSA at pH 7.4 and 37°C. These compounds all had greater stability than L-tryptophan, which the time required for sample preparation. Competition studies between these compounds and L-tryptophan were also used to determine if they shared a common binding site on HSA.

Chemical Structures

Figure 1

Method

Column Preparation

HSA was immobilized onto 300 □, 7 □m particle size silica and packed into a 5 cm x 2.1 mm i.d. stainless steel column. The HSA supports were prepared by the Schiff base method, which attaches HSA covalently to the support through amine groups. The protein content of this support was 40 (± 2) μg HSA per mg of silica. The columns were stored in pH 7.4, 67 mM potassium phosphate buffer.

Frontal Analysis

Frontal analysis uses a continuous application of sample to a column. This results in essentially a titration of the active binding sites available for the analyte to interact within the column. The affinity (K_A) and number of binding sites (m_{Lapp}) for analyte binding on the column can be determined using the resulting breakthrough curves. The samples in this study were all made using pH 7.4, 67 mM potassium phosphate buffer and were applied to the column at 37°C using concentrations that ranged from 0.5 μM to 30 μM. The samples were all stored at 4°C when not in use. The analytes that were investigated by this approach were 3-acetylindole, 3-methylindole, indole-3-acetic acid, indole-3-butyric acid, indole-3-carboxylic acid, indole-3-methanol, and indole-3-propionic acid.

Zonal Elution

Zonal elution uses the application of analyte in a small plug injection. This process can be used to determine if compounds are binding to the same site as a competing agent which is included in the mobile phase. A competition study was performed in this project using L-tryptophan as the mobile phase additive and the other indole compounds as the injected solutes. L-Tryptophan was added to the mobile phase at concentrations ranging from 0 μM to 300 μM. The analyte of interest was placed as a 20 μM sample prepared in the mobile phase. The L-typtophan was allowed to saturate the column before a 20 μL of analyte was injected.

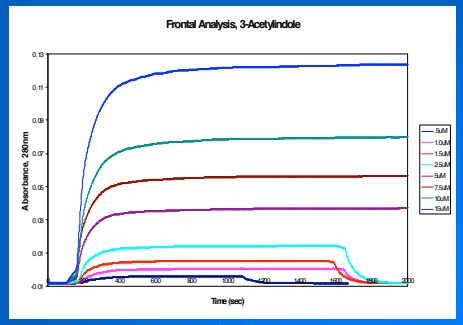

Figure 2

Frontal Analysis Results

All of the compounds were analyzed for their binding to HSA by frontal analysis. Out of the seven compounds tested, only three had K_A values that were in an acceptable range for a replacement compounds for L-tryptophan (Table 1). The breakthrough times were recorded and used to calculate the binding capacity (m_L) of the column for each compound. Plots were then made of $1/m_L$ vs $1/[A]$ (see Figure 2). By using the slope and intercept of the best-fit lines to these plots, the association equilibrium constant (K_A) and the m_{Lapp} were calculated. Indole-3-butyric acid, indole-3-propionic acid were eliminated as candidates early in this work due to their high non-specific binding to a control column. 3-Methylindole was eliminated due to its odor, and indole-3-methanol was eliminated due to high non-specific binding and poor reproducibility from run-to-run. Indole-3-acetic acid, indole-3-carboxylic acid and 3-acetylindole were all used in further competition studies.

$$K_A = \frac{k_a}{k_d} \qquad \frac{1}{m_{Lapp}} = \frac{1}{K_A m_L [A]} + \frac{1}{m_L}$$

$K_{a,A}$= association equilibrium constant
[A]=concentration of your solute
m_{Lapp}=moles of ligand calculated
m_L=actual moles of ligand in the

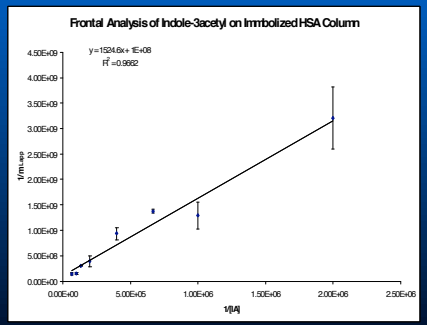

Figure 3

Table 1

Compound	K_A	Stand. Dev.	m_{Lapp}	Stand. Dev
Indole-3-acetic acid	$2.3x10^4M^{-1}$	± $2.1x10^4$	$5.7x10^{-8}$	± $5.1x10^{-8}$
Indole-3-carboxylic acid	$8.5x10^3M^{-1}$	± $4.6x10^3$	$1.2x10^{-7}$	± $6.4x10^{-8}$
3-Acetylindole	$7.3x10^4M^{-1}$	± $5.6x10^4$	$8.9x10^{-9}$	± $6.8x10^{-9}$
3-Methylindole	$5.3x10^4M^{-1}$	± $1.6x10^4$	$2.9x10^{-8}$	± $8.8x10^{-9}$
Indole-3butyric acid	$9.7x10^3M^{-1}$	± $3.2x10^5$	$2.6x10^{-8}$	± $3.1x10^{-9}$
Indole-3propionic acid	$1.1x10^5M^{-1}$	± $3.4x10^4$	$4.5x10^{-8}$	± $9.6x10^{-9}$
Indole-3methanol	$1.2x10^5M^{-1}$	± $5.3x10^4$	$1.2x10^{-9}$	± $5.3x10^{-10}$
L-Tryptophan	$1.1x10^4M^{-1}$	± $0.1x10^4$		

Zonal Elution Results

L-Tryptophan was used as the competing agent (I) in the mobile phase at concentrations of 5, 20, 50, 100, 150, 225, and 300 μM. The retention time (t_r) for all the injected compounds were determined using their central moments (Figure 3). By determining t_r, t_m (the column void time) and t_{ex} (the extracolumn void time), the retention factor (k') for the analyte was determined at each given concentration of competing agent.

A plot of $1/k'$ vs [I] was then made for each analyte to examine its competition with L-tryptophan for HSA binding sites. A linear graph for this type of plot is an indication that direct competition at a single site is present. Only 3-acetylindole gave such behavior (see Figure 4). Plots of $k_0/k-k_0$ versus 1/[I] were also made to test for allosteric or multisite interactions, where k_0 is the retention of the analyte with 0 μM L-tryptophan in the mobile phase. These latter plots make it possible to determine the coupling constant ($\alpha_{I\leftrightarrow A}$) for effect of *I* on *A*. When $\alpha_{I\leftrightarrow A} > 1$, *I* has a positive allosteric effect on *A*, while $0 > \alpha_{I\leftrightarrow A} > -1$ indicates that *I* has a negative allosteric effect on *A*. If $\alpha_{I\leftrightarrow A} = 1$ in this plot, then *I* and *A* are binding independently to the immobilized protein. Indole-3-acetic acid and indole-3-carboxylic acid both appeared to bind to multiple sites on HSA, while indole-3-acetic acid and indole-3-carboxylic acid appeared to have negative allosteric interactions with L-tryptophan (see Figure 5).

$$k' = \frac{t_r - t_m}{t_m - t_{ex}} \qquad \frac{1}{k'} = \frac{V_M K_I [I]}{K_A m_L} + \frac{V_M}{K_A m_L}$$

$$\frac{k_0}{k - k_0} = \frac{1}{[I](\beta_{I\leftrightarrow A} - 1)} + \frac{1}{\beta_{I\leftrightarrow A} - 1}$$

$\beta_{I\leftrightarrow A}$=coupling constant
K_I=association equilibrium constant of competing agent
K_A=association equilibrium constant of analyte
V_M=void volume
k'=retention factor

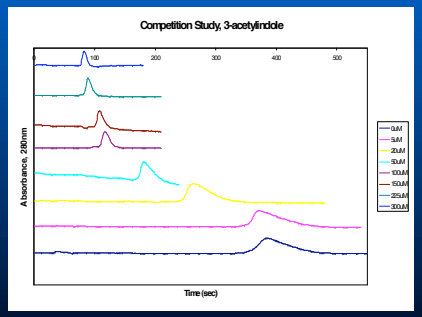

Figure 4

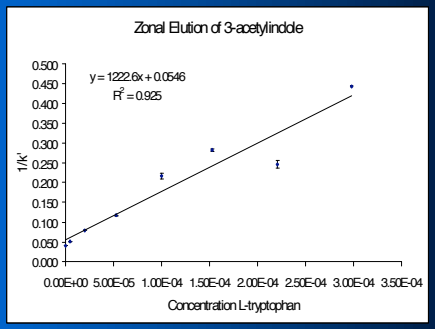

Figure 5

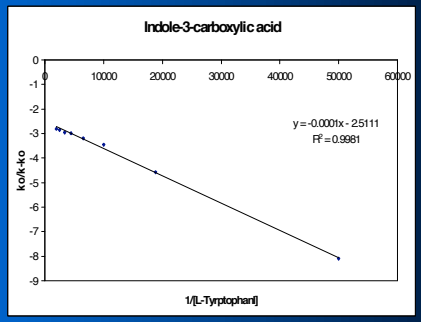

Figure 6

Conclusion

n search for an alternative compound to use for L-tryptophan in drug-protein binding studies involving HSA, compounds with various side chains at the 3 position of the indole ring were investigated. These compounds were looked at for their stability and interactions with HSA. All of the tested solute had greater stability than L-tryptophan based on NMR studies (data not shown). Based on frontal analysis, four compounds were eliminated: indole-3-propionic acid and indole-3-butryic acid had too much non-specific binding; indole-3-methanol had high non-specific binding and non-reproducible results; and 3-methylindole was eliminated due to its odor.

Further competition studies were preformed using 3-acetylindole, indole-3-acetic acid, and indole-3-carboxylic acid. Indole-3-acetic acid and indole-3-carboxylic acid both appeared to bind to multiple sites on HSA, which means they are probably not alternatives for L-tryptophan as a site-selective probes for the indole site of HSA. Indole-3-acetic acid and indole-3-carboxylic acid appeared to have negative allosteric effects from L-tryptophan. 3-Acetylindole was the only analyte that appeared to have direct competition with L-tryptophan at a single site on HSA. This compound also has an affinity for HSA that is comparable to that for L-tryptophan, and yet 3-acetylindole in more stable in aqueous solutions.

Acknowledgements

This research was supported by NIH grant R01GM044931