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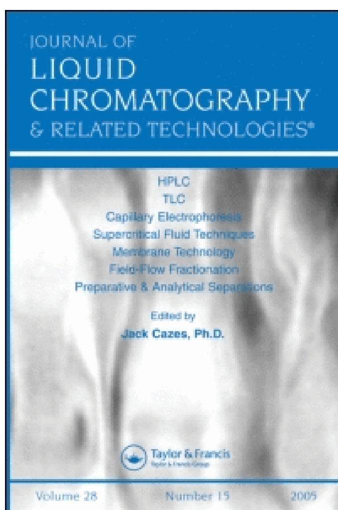

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A MODEL SYSTEM FOR PROTEIN-PORPHYRIN BINDING CONSTANT MEASUREMENT USING CAPILLARY ELECTROPHORESIS

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A MODEL SYSTEM FOR PROTEIN-PORPHYRIN BINDING CONSTANT MEASUREMENT USING CAPILLARY ELECTROPHORESIS

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ABSTRACT

Understanding the process of biological molecule-protein binding in the body is very important. A model system for the application of capillary electrophoresis in the measurement of the binding constants of porphyrin-protein is discussed. The results are described, demonstrating that capillary electrophoresis can be employed as a rapid screening tool for investigating the binding of different forms of porphyrin to a protein.

INTRODUCTION

Porphyrins have been used as photosensitizers in photodynamic therapy of tumors (PDT) and diseases such as the porphyrias, leading to an increased level of porphyrins in the blood. Therefore, investigating how porphyrins are transported in blood is very important. For a long time, albumin was thought to be the main porphyrin binding protein, the carrier, in serum.[1-6] However, it has recently been shown that serum lipoprotein also has high affinity for porphyrins.[7-10] The affinity of serum albumin and lipoprotein for porphyrins indicates a role for these particular classes of proteins as endogenous carriers of porphyrins administered for PDT.[11-14] As a carrier, a protein may aid in the selective delivery of porphyrins to a tumor region and, on the other hand, the same carrier may cause a decrease in the amount of porphyrins available for PDT by its rapid removal from circulation.

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The balance between these two activities might differ from one protein to another and from one porphyrin species to another. The nature of the porphyrin-albumin binding equilibria should be a significant factor in the balance of the porphyrin-carrying activities of albumin. To gain some understanding into the factors involved, the study of the albumin-porphyrin binding constant is very useful.

A number of methods have been used to study porphyrin-protein binding. These methods include: dialysis and ultrafiltration, ultracentrifugation, gel permeation chromatography, electrophoresis, absorption, and fluorescence spectroscopy.[15-19] However, porphyrins possess unique properties that introduce errors into the results obtained by practically all the methods. The experimental errors may be caused by one or more factors such as porphyrin aggregation, interaction with dialysis bags, columns and glassware, chelation of porphyrins with certain metals, leading to altered absorption, and fluorescence spectra.

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Capillary electrophoresis (CE), a powerful separation technique, has developed rapidly in past years.[20-23] CE is a simple system with a relatively low surface-to-volume ratio, an extremely large separation power, reasonable speed and good automation prospects. From the point of view of systematic errors and wide applicability, the use of CE for studying protein-drug binding is attractive. Recent literature in this area of research include the use of affinity capillary electrophoresis to estimate the binding constants (K_b) between carbonic anhydrase B and charged benzenesulfonamides;[24, 25] a comparison between CE and vacancy peak measurement or frontal analysis for studying protein-drug binding; [26] the use of CE to evaluate the binding of anionic carbohydrates to synthetic peptide derived from human serum amyloid P component;[27] and investigation of the enantioselective ligand-protein binding and displacement interactions.[28]

In this study, selected porphyrins were used to determine the feasibility using CE to measure the binding constants with human serum albumin (HSA) and bovine serum albumin (BSA).

EXPERIMENTAL

Chemicals

Hematoporphyrin (HP), Uroporphyrin (UP) I and Coproporphyrin (CP) III were from Porphyrin Products (Logan, UT, USA). Human serum albumin (HSA) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade and were obtained from Fisher Scientific (Fair Lawn, NJ, USA) or Aldrich Chemical Co. (Milwaukee, WI, USA). The run buffer solutions were prepared in doubly deionized water and filtered through a 0.45 µm membrane.

Apparatus

The CE experiments were performed with a laboratory constructed instrument which included an acrylic box, a 30 kV power supply (Glassman High Voltage, Whitehouse Station, NY) and a fully digital controller for the CZE 1000 unit designed by Chamonix Industries (Johnson City, NY). Detection was achieved via a Spectra 200 UV-Vis detector (Spectra Physics, San Jose, CA). The electropherograms were processed on a Perkin-Elmer LCI-100 integrator.

Procedures

The capillaries were coated with linear polyacrylamide according to the method reported by Hjerten.[23] New capillaries were conditioned with 10 mM borate-H_3PO_4 buffer for at least 1 hour before use. The capillary was cleaned frequently during runs by washing with 20 mM phosphate buffer (pH = 7). Sample injection was set for electrokinetic injection (10 KV) at 5s injection time and detection was measured at 230 nm.

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Binding Constant Calculation

Binding constant measurements by affinity capillary electrophoresis (ACE) were first introduced by Whitesides' research group.[24, 25] The following equation was used to estimate the binding constant (K_b):

$$\Delta t_L/[L] = K_b - t_{max} - K_b \Delta t_L \quad (1)$$

where $\Delta t_L = t_L - t_{L=0}$ and $\Delta t_{max} = t_{max} - t_{L=0}$. t_L is the migration time of a protein at ligand concentration [L], $t_{L=0}$ is the migration time at [L] = 0 and t_{max} = the migration time at saturated [L].

Asparagic acid (Asp) was chosen instead of a noninteracting protein as internal reference to monitor the change of EOF since an internal reference protein which does not interact with porphyrins under the electrophoretic conditions can not be found. Therefore asparagic acid was used as the internal reference in all binding constant measurements.

RESULTS AND DISCUSSION

There is a major limitation for measuring binding constants using CE. If the migration time is affected by changes in EOF, Eqn. (1) is not suitable for estimating binding constants. Recently, Comez et al.[25] reported a method to correct for the effect caused by changes in EOF.

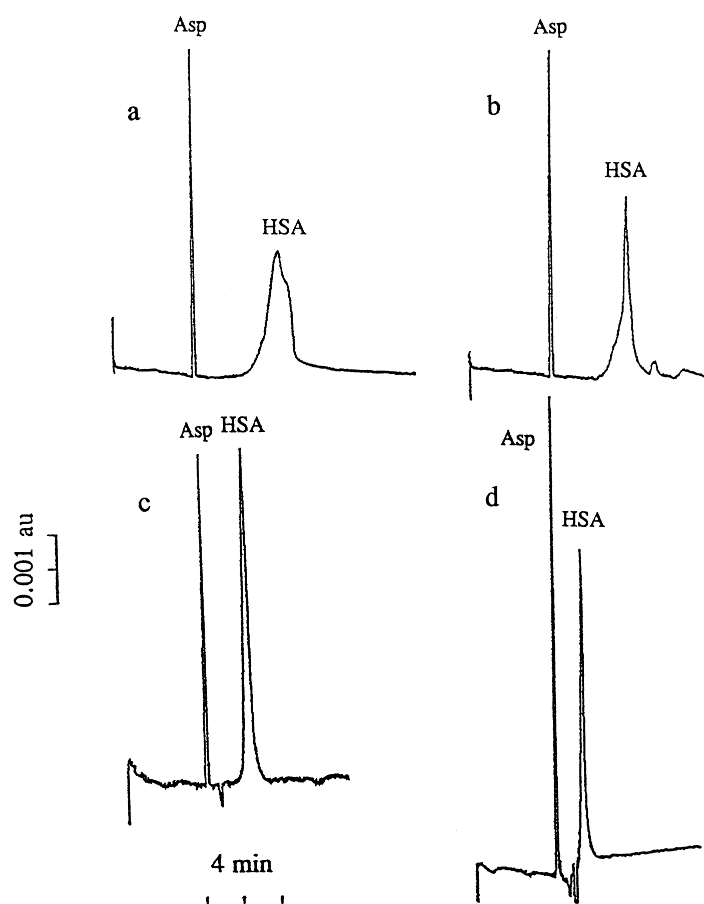

Figure 1. Electropherograms of (a) Asparagic acid (Asp), 10 TM and Human serum albumin (HSA), 15 TM; (b) 5 x 10^{-6} M HP added; (c) 50 x 10^{-6} M HP and (d) 100 x 10^{-6} M HP added. See the text for the detail of CE conditions.

Due to a strong absorption of porphyrins or both HSA and BSA to the inner fused silica capillary wall, it is impossible to evaluate binding constants using a bare capillary. To overcome this problem, a coated capillary must be used to eliminate the EOF and the adsorption of porphyrins and proteins to the coated capillary wall is negligible. Since porphyrins had no interference in the UV absorption region between 214-230 nm where protein was determined, the range of porphyrin concentrations employed could be large. However, at high porphyrin concentrations (greater than 200 μM), negatively charged porphyrin can be adsorbed onto the coated capillary wall that generated a negative charge

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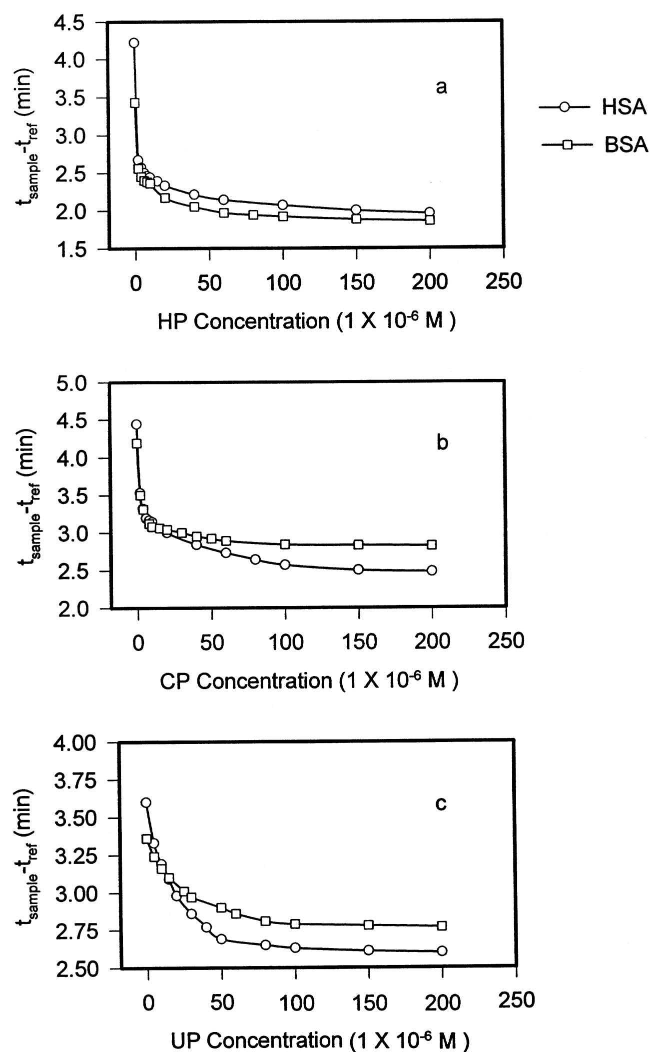

Figure 2. Plots of -t (difference in retention time between the Asp (the internal reference) and interest protein) versus the concentrations of ligand: (a) HP; (b) CP and (c) UP.

layer on the inner surface where EOF increased. No significant increase of EOF was observed at a porphyrin concentration less than 150 μM and the capillary was washed with 20 mM phosphate buffer (pH=7) after each run to clean up any porphyrin or protein residue.

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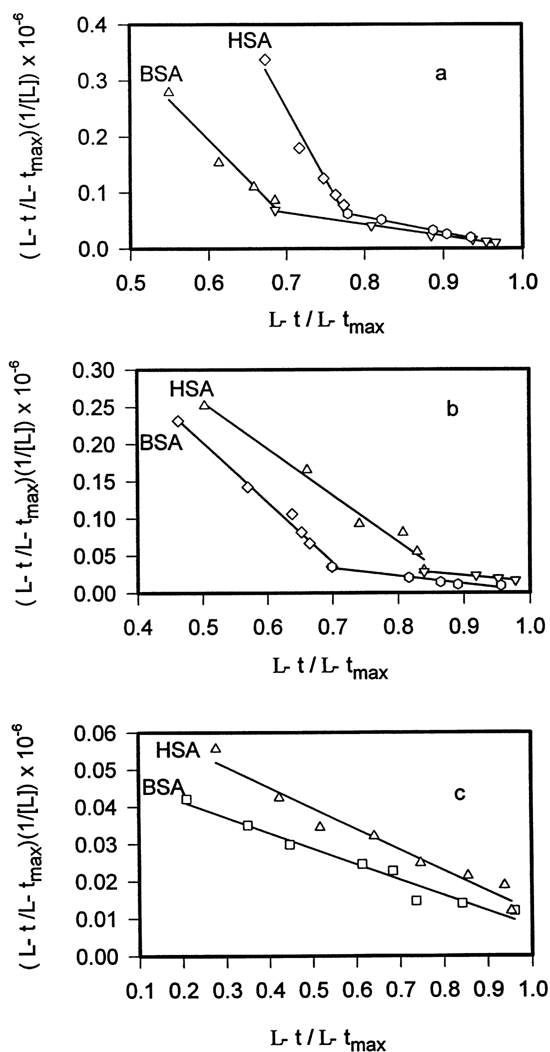

Figure 3. Scatchard plots derived from Figure 2: (a) HP; (b) CP and (c) UP.

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Figure 1 showed a representative series of electropherograms of HSA sample in run buffers containing various concentrations of HP (the ligand). The retention time of HSA decreased with increasing of the HP concentrations. While the interaction with HP increased, the peak of protein became much sharper and the separation efficiency was enhanced. Similar results were observed in the electrolyte solutions containing UP and CP as run buffer additives.

Table 1

Measured Binding Constants by Affinity Capillary Electrophoresis

Ligand (porphyrin)	Interest Protein	K_1 ($1 \times 10^6 M^{-1}$)[a]	K_2 ($1 \times 10^6 M^{-1}$)[b]
Hemeto-	Human Serum Albumin	2.54 ± 0.51	0.28 ± 0.03
	Bovine Serum Albumin	1.42 ± 0.38	0.21 ± 0.02
Copro	Human Serum Albumin	0.81 ± 0.15	0.11 ± 0.02
	Bovine Serium Albumin	0.59 ± 0.05	0.08 ± 0.02
Uro-	Human Serum Albumin	0.056 ± 0.012	
	Bovine Serum Albumin	0.041 ± 0.010	

[a,b] K values were average for three separated runs.

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Figure 2 showed the change in retention time as a function of porphyrin concentrations. It can be seen that Δt values for both HSA and BSA increased dramatically at low porphyrin concentrations, Δt reached maximum values until porphyrin concentrations reached the saturation level. The saturation concentration was approximately 60 to 100 μM for these porphyrins. The binding of hematoporphyrin to the protein had the largest changes, followed by coproporphyrin and uroporphyrin, indicating that the binding of HP or CP was relatively stronger than the uroporphyrin's.

Figure 3 showed Scatchard plots derived from the experimental data. The binding constants were estimated based on the slopes. Two slopes were found from the plots for hematoporphyrin and coproporphyrin, indicating there were two existent binding sites, whereas only a single slope was found for uroporphyrin.

The K_1 values between the porphyrins and HSA were measured to be $2.54 \times 10^6 M^{-1}$, $8.1 \times 10^5 M^{-1}$ and $5.6 \times 10^4 M^{-1}$ for HP, CP and UP, respectively, and $1.42 \times 10^6 M^{-1}$ (HP), $5.1 \times 10^5 M^{-1}$ (CP), $4.1 \times 10^4 M^{-1}$(UP) for BSA (Table 1). These results were close to the previously reported values by conventional methods[15, 16] (hematoporphyrin (K_b) = $1.4 - 2 \times 10^6 M^{-1}$; coproporphyrin ($K_b$) = 1.3 to $1.6 \times 10^5 M^{-1}$). Smaller binding constants were found for uroporphyrin. As suggested by earlier work,[29] the binding between uroporphyrin and albumin is relatively weak and may explain the anomalous in vivo distribution of this porphyrin when compared to other porphyrins in human subjects.[30]

CONCLUSION

Binding constants between the porphyrins and the proteins can be estimated by the affinity capillary electrophoresis under conditions in which the migration time is affected by changing the concentration of ligand in the buffer solution. The advantages of the use of CE for binding studies are: (1) exceedingly small sample volumes are required (10-18nL); (2) it does not require high purity of the protein or an accurate value of its concentration, since values of K_b are based on migration times, not peak areas; (3) as a result of the high resolving power of capillary electrophoresis, measurement of K_b can be applied simultaneously to several proteins in the same solution; and (4) high reproducibility of data can be obtained from fully automated instruments.

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