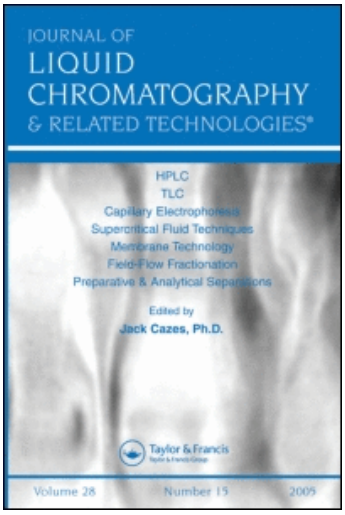

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Estimation of Binding Constants for Diclofenac Sodium and Bovine Serum Albumin by Affinity Capillary Electrophoresis and Fluorescence Spectroscopy

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Abstract: Both affinity capillary electrophoresis (ACE) and fluorescence spectroscopy were used to measure the binding affinities between diclofenac sodium (DFS) and bovine serum albumin (BSA) in this investigation. In ACE, DFS was injected into the borate buffer containing various concentrations of BSA. Mobility ratio (M) was used to deduce the binding constant (K_b), which effectively eliminates the effect of electroosmotic flow (EOF). Both ACE and fluorescence measurements indicated two classes of binding sites between DFS and BSA. The K_b value for high affinity binding sites ($1.9 \pm 0.2 \times 10^5\,M^{-1}$) obtained from ACE is in agreement with that from fluorescence spectroscopy ($2.8 \pm 0.3 \times 10^5\,M^{-1}$). The work demonstrates that ACE and fluorescence spectroscopy are complementary to each other for the determination of binding constants of DFS and BSA.

Keywords: Affinity capillary electrophoresis, Binding constant, Bovine serum albumin, Diclofenac sodium, Fluorescence

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INTRODUCTION

Studying drug/protein interactions is not only important for explaining the relationship between the structures and functions of proteins but crucial for drug discovery within the pharmaceutical industry.[1] Bovine serum albumin (BSA), one of the most abundant proteins in bovine plasma, is the most common model protein in studying drug/protein interactions. The structure of BSA is very analogous to human serum albumin and was well characterized.[2,3] Due to its distinct anti-rheumatic, anti-inflammatory, analgesic, and anti-pyretic activities, diclofenac sodium (2-[(2,6-Dichlorophenyl)amino] benzeneacetic acid sodium, DFS), a non-steroidal anti-inflammatory drug, has been widely used in clinical medicine for the treatment of inflammation.[4] Although several methods were used to investigate the binding characters of diclofenac and serum albumin,[5–11] each of them has one or several inherent limitations. For example, NMR, one of the most powerful techniques for structure characterization, is of low sensitivity and restricted access. In addition, NMR is not suitable for studying the high affinity binding between large proteins and small drug molecules.[10] Equilibrium dialysis suffers from long analysis time,[8,9] which may cause protein denaturation or microbial growth. For electrochemical techniques, the fabrication of special electrodes and the preparation of sample matrices are tough work, and various factors have impacts on the precision of the results.[11]

Recently, capillary electrophoresis (CE) has been extensively used for the exploration of biological interactions.[12–17] Among the existing CE methods for measuring binding constants (K_b),[18] affinity capillary electrophoresis (ACE) is becoming increasingly popular. The well established advantages include, but are not limited to simplicity, versatility, high speed, small quantity of sample, and low running costs.[15,19] The common method used for estimating K_b is to measure the shift in mobility of an injected sample in a running buffer containing different amounts of the additive.[1,20,21] Although K_b can be readily obtained, it is generally more reliable if another independent technique can be used to support the results.[19]

Due to its distinct advantages such as extreme sensitivity and high selectivity, fluorescence spectroscopy played a powerful role in optical techniques for probing the affinity of biomolecules in the last two decades.[22–25] In this work, ACE was used in tandem with fluorescence spectroscopy to study the binding affinities between DFS and BSA. The experimental results showed that K_b values for high affinity binding sites, based on ACE determinations, are in well agreement with those from spectrofluorimetric measurements, providing a reliable approach for measuring binding affinities between drugs and proteins.

EXPERIMENTAL

Reagents and Chemicals

BSA and DFS were purchased from AMRESCO (Solon, OH, USA) and Sigma (St. Louis, MO, USA), respectively. Boric acid, disodium tetraborate decahydrate, and other reagents were of analytical grade and used as received. All solutions were prepared with deionized water treated with a water purification system (Simplicity 185, Millipore Co., Bedford, MA, USA).

Equipment

CE experiments were performed with a Beckman–Coulter P/ACE MDQ system (Fullerton, CA, USA). Data were collected and analyzed with the Beckman 32 Karat Software. The uncoated fused silica capillary was 60 cm in length with I.D. of 75 μm and O.D. of 375 μm, purchased from Ruipu Chromatography Apparatus Co. (Hebei, China). The effective length of the capillary from the inlet to the detector is 50 cm. Operational voltage of 24 kV was performed in normal polarity and the detection wavelength was set to 276 nm. The temperature of the capillary was maintained at 25°C.

Fluorescence spectroscopy measurements were carried out by using a F-2500 spectrophotometer equipped with 1 cm path cells (Hitachi, Ltd., Japan). The spectral bandwidths of both the excitation slit and the emission slit were set to 5.0 nm. Data were collected and analyzed with the FL Solutions software. Appropriate blanks corresponding to the buffer were subtracted to correct the background of the fluorescence. All the experiments were repeated at least three times and carried out at room temperature (23 ± 2°C).

Sample and Solution Preparations

The borate buffer containing 15 mM sodium tetraborate was adjusted to pH 8.5 with 60 mM boric acid. Samples were prepared by diluting a 25 mM DFS stock solution to 0.4 mM using borate buffer. All the solutions were filtered through 0.22 μm membrane filters (Shanghai Xingya Purification Materials Inc., Shanghai, China) prior to use.

Procedures

In the CE experiments, the capillary was rinsed with NaOH (1 M) for 2 min, and deionized water for 1 min, then buffer for 2 min at 20 psi

successively after each run. Different amounts of BSA were then added into the borate buffers, and the 0–20 μM BSA solutions, which were used as the ACE running buffers, were obtained. The volume and the length of the BSA solutions in capillary were ca. 18 nL and 4 mm (calculated by Poiseulle equation), respectively. The DFS samples were then injected into the capillary with a pressure of 0.5 psi at the anode for 5 s. A 2% (v/v) acetone solution was used as the neutral EOF marker in all the CE experiments.

In fluorescence experiments, the concentration of BSA solution was kept at 5 μM, while the concentration of DFS varied from 0 to 10 μM at an increment of 0.5 μM. Fluorescence spectra of BSA solutions in the absence and presence of DFS were then measured by setting the excitation wavelength to 290 nm and the emission spectra were acquired at the range of 280 to 500 nm.

RESULTS AND DISCUSSION

Determination of ACE Experimental Conditions

In order to obtain an accurate K_b value based on ACE determinations, the following parameters were consecutively optimized: type of additive in the running buffer, concentration and pH of the buffer, concentrations of analyte and additive, and other electrophoretic parameters such as operating voltage, detection wavelength.

For drug/protein interaction studies, either the drug or the protein can be added into the ACE running buffer (termed as the additive).[15,16] Both of the approaches were attempted. Figure 1a depicts the electropherograms of DFS injected into a running buffer in the presence and absence of BSA. The mobility shift of DFS ($\mu_D - \mu_{EOF}$) in the presence of BSA ($-2.21 \times 10^{-4}\,cm^2 \cdot V^{-1} \cdot s^{-1}$ in the top electropherogram of Figure 1a) is apparently different from that in the absence of BSA ($-2.15 \times 10^{-4}\,cm^2 \cdot V^{-1} \cdot s^{-1}$ in the bottom electropherogram of Figure 1a). Similar mobility shift was found between BSA and DFS/BSA complex (Figure 1b). Therefore, BSA was used as an additive in the running buffer for the experiments.

The elimination of the adsorption of proteins onto the capillary inner wall is crucial for the ACE detection because the adsorption will result in poor resolution and loss of separation efficiency.[26–28] In our recent work,[29] it was shown that adsorption of BSA onto the capillary inner wall was negligible when the BSA concentration was lower than 20 μM, and a 60 mM borate buffer at pH 8.5 was employed. Hereby, the borate buffer was applied and the running buffer concentrations of BSA were controlled in the range of 0 to 20 μM.

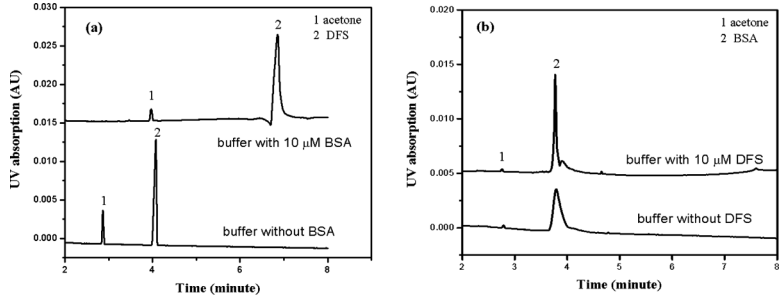

Figure 1. Typical electropherograms of (a) DFS (0.4 mM) in a borate running buffer without and with 10 μM BSA, and (b) 10 μM BSA in a borate running buffer in the absence and presence of 0.1 mM DFS. ACE conditions: 24 kV, 25°C, 5 s injection at 0.5 psi, and detection at 276 nm for DFS and 214 nm for BSA.

The concentration of injected analyte is also of importance for ACE investigations.[30,31] Figure 2 shows that the mobility shift of DFS decreases greatly with the increases of the concentration of DFS initially. While its concentration reaches 0.4 mM, this trend turns to a gradual decrease. In this work, a solution of 0.4 mM DFS was used since such a DFS concentration can be readily detected and the data precision can be improved.

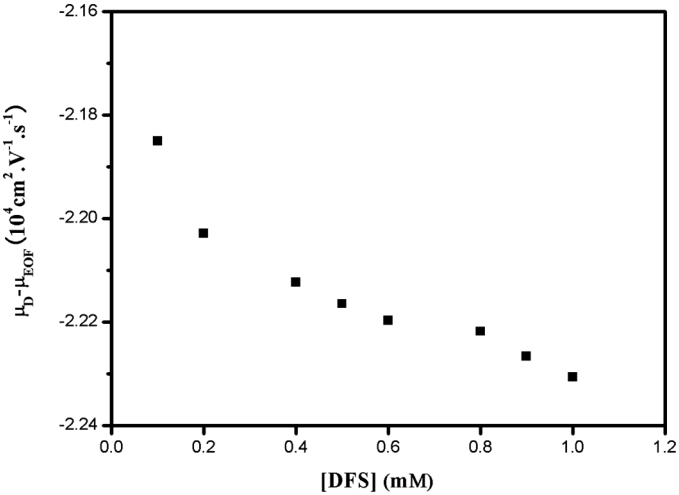

Figure 2. Mobility shift of DFS as a function of DFS concentration. The constant concentration of BSA in the running buffer was 10 μM and the DFS concentrations studied were 0.1, 0.2, 0.4, 0.6, 0.8, 0.9, and 1.0 mM. The ACE conditions were the same as those used for Figure 1.

Effects of operating voltage and detection wavelength were also investigated. Electropherograms of DFS in borate buffer containing 10 μM BSA at various voltages (from 6 to 24 kV), indicated that within this range the applied voltage had no influences on the DFS peak shape, and the current increased linearly with the applied voltage. Thus, 24 kV was chosen as the operating voltage. There are two absorption peaks at 199 and 276 nm in the DFS absorption spectrum. The absorbance values are 0.21 (199 nm) and 0.05 (276 nm). Given the fact that BSA also displayed large absorption at 199 nm but small at 276 nm, the detection wavelength was set at 276 nm.

Measurement of the DFS/BSA Binding Constant by ACE

Affinity interactions between protein and drug molecules are commonly investigated by measuring the mobility shift of a sample as a function of additive concentrations in the running buffer. Figure 3 is a typical ACE electropherogram of DFS binding by various concentrations of BSA. The complexation of negatively charged BSA with DFS leads to a decrease of the charge to mass ratio lowering the mobility. Then the retention time of DFS was extended. The mobility ratio M was

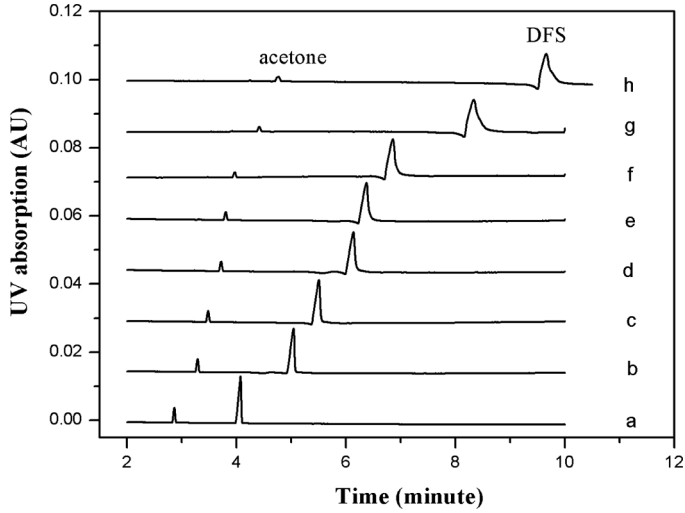

Figure 3. Representative electropherograms of DFS in the presence of different BSA concentrations in the running buffer; 0.4 mM DFS was injected. From curve a–h, BSA concentrations: 0, 2, 4, 6, 8, 10, 15 μM, respectively. The ACE conditions were the same as those given in the caption of Figure 1.

inducted to eliminate the effects of the variation of electroosmotic flow (EOF),[16,32] as defined in the following equation:[16]

$$M = [l_c l_d/(Vt)]/[l_c l_d/(Vt_{\mathrm{EOF}})] + 1 = t_{\mathrm{EOF}}/t + 1 \quad (1)$$

where l_c (cm) is the total length of the capillary, l_d (cm) is the effective length of the capillary, t (s) is the measured analyte migration time, t_{EOF} (s) is the migration time of the neutral marker, and V is the operating voltage. Clearly, M is independent of CE experimental parameters, such as the voltage, capillary length, and viscosity of the running buffer.

Typical Scatchard analysis of ACE yields a continuous plot with two regions (Figure 4). Linear regression analysis of both regions implies K_b values of $2.4 \pm 0.2 \times 10^4$ and $1.9 \pm 0.2 \times 10^5\,\mathrm{M}^{-1}$ with correlation coefficients R^2 greater than 0.98. The reproducibility of mobility ratio M was determined in the presence of different BSA concentrations (0–20 μM). The relative standard deviations (RSD) for six replicate injections ranged from 2.1% to 7.6%. Furthermore, the RSD for day to day M_D values tested over a period of 5 days ranged from 3.2 to 9.7%.

Affinity Measurements Using Fluorescence Spectroscopy

At pH 8.5, as can be seen from Figure 5, addition of increasing concentrations of DFS caused gradual decreases of the fluorescence

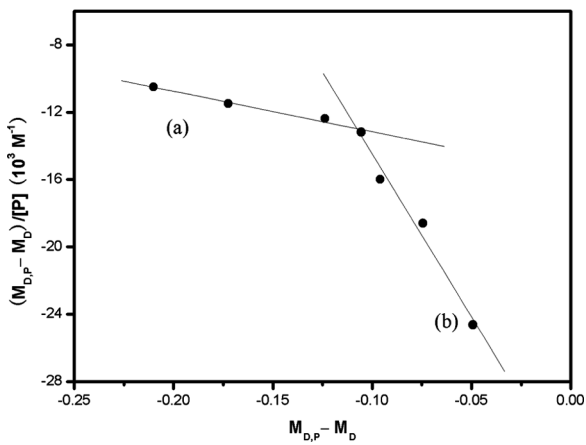

Figure 4. Estimation of binding constants for DFS binding to BSA using Scatchard analysis by ACE. Equations of the linear fitting ($n = 4$): (a) $y = -15568 - 24056x$, $R = 0.99$; (b) $y = -33824 - 193464x$, $R = 0.99$.

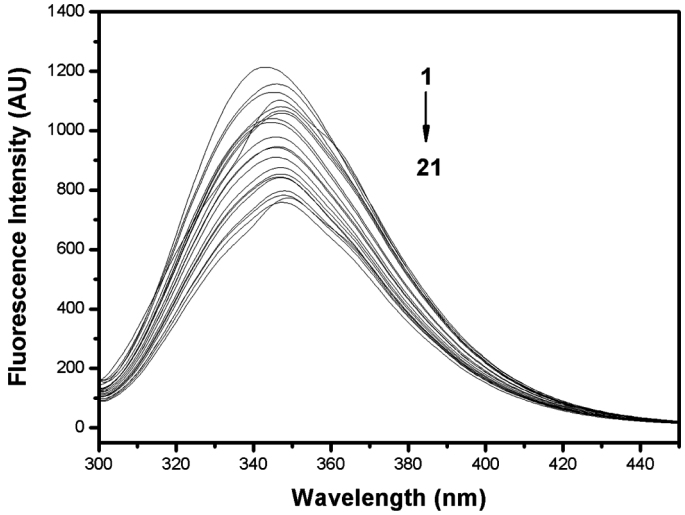

Figure 5. Emission spectra of BSA in the presence of various concentrations of DFS (298 K, pH 8.5 and $\lambda_{ex} = 290$ nm). From curves 1–21, BSA concentration was at 5.0 μM, DFS concentrations: 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9, 9.5, and 10.0 μM, respectively.

intensity in the BSA emission spectra. The apparent binding constant K_b can be obtained from Eq. (2):[33]

$$\log[(F_0 - F)/F] = \log K_b + n \log[D] \quad (2)$$

where F_0 and F are the fluorescence intensities before and after the addition of the drug, $[D]$ is the total drug concentration and n is binding sites.

It can be seen from Figure 6 that the plot of log $[(F_0 - F)/F]$ vs. log $[D]$ has two regression segments intersecting at $[D] = 4.0\,\mu M$. The K_b values were deduced to be $2.8 \pm 0.3 \times 10^5$ and $5.4 \pm 0.5 \times 10^2\,M^{-1}$ in curves a and b, respectively. The equations of the linear fitting showed that when $[D] < 4.0\,\mu M$, the numbers of binding sites are 0.64; while in the case of $[D] > 4.0\,\mu M$, the numbers of binding sites are 1.13. These results demonstrated that other binding sites with higher affinity were involved at higher DFS concentrations. At pH 7.4, there is only one regression segment and the K_b value was deduced to be $1.8 \pm 0.1 \times 10^5\,M^{-1}$ (data not shown).

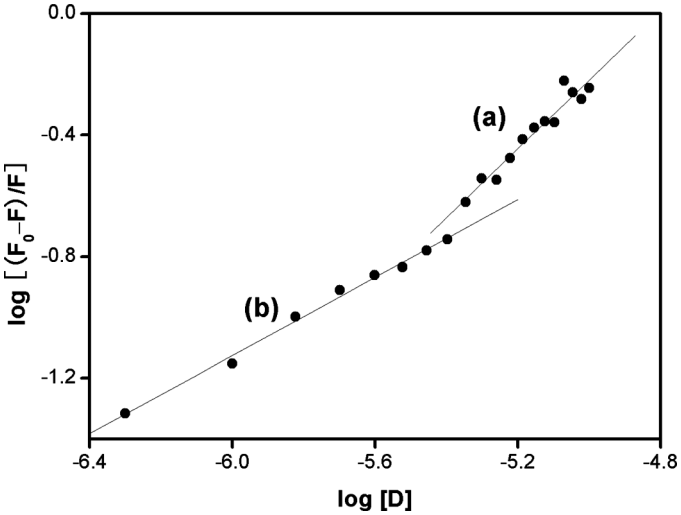

Figure 6. The plots of log $(F_0 - F)/F$ vs. log $[D]$ at pH 8.5. Equations of the linear fitting: (a) $y = 5.44 + 1.13x$, $R = 0.97$; (b) $y = 2.73 + 0.64x$, $R = 0.99$.

Comparison of the Fluorescence Measurements to ACE and Other Assays

Both ACE and fluorescence measurements indicated two classes of binding sites for serum albumin molecule and DFS at pH 8.5: One is the high affinity binding sites and the other is the low affinity binding sites. This is in accordance with most researchers' findings.[34–36] On high affinity binding sites, the K_b value determined from the fluorescence measurements ($2.8 \pm 0.3 \times 10^5\,M^{-1}$, pH 8.5) is in good agreement with that obtained by ACE ($1.9 \pm 0.2 \times 10^5\,M^{-1}$, pH 8.5). Both K_b values on high affinity binding sites are comparable with those obtained by equilibrium dialysis ($0.9 \times 10^5\,M^{-1}$, pH 8.4),[9] further consolidating the validity of our measurements. At lower pH value (pH 7.4), K_b value deduced is $1.8 \pm 0.1 \times 10^5\,M^{-1}$ based on fluorescence measurements, which is comparable with those obtained by other fluorescence determination ($2.2 \times 10^5\,M^{-1}$, pH 7.4).[37] On low affinity binding sites, K_b value determined from the fluorescence measurements ($5.4 \pm 0.5 \times 10^2\,M^{-1}$, pH 8.5) is evidently different from that by ACE ($2.4 \pm 0.2 \times 10^4\,M^{-1}$, pH 8.5). However, the values are not entirely unreasonable, since the constant of $1.7 \pm 0.2 \times 10^2\,M^{-1}$ (pH 7.4) has been measured by NMR[10] whereas another K_b value of $6 \times 10^4\,M^{-1}$ (pH 7.4) has been acquired by equilibrium dialysis.[8]

The agreement between ACE and fluorescence on high affinity binding sites suggests that these two techniques are comparable for binding studies. As a matter of fact, protein adsorption onto the capillary inner wall is a big hindrance for the use of ACE to assess biological binding at various pH values. However, nonspecific adsorption of protein does not affect fluorescence analysis, so the results obtained from fluorescence spectroscopy can be used to validate the value from ACE.

CONCLUSION

In the present work, the interactions of DFS and BSA were investigated with ACE and fluorescence spectroscopy. The mobility ratio *M* was used to correct the EOF. Compared to other techniques (e.g., equilibrium dialysis and NMR), ACE is high performance, rapid, and cost effective. It was also demonstrated that fluorescence spectroscopy can not only serve as a useful technique to validate the ACE results, but can also calibrate the value from ACE when the adsorption of proteins on the wall of capillary can not be neglected.

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