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Interactions between isoprenaline hydrochloride and bovine serum albumin (BSA) in capillary zone electrophoresis and affinity capillary electrophoresis

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Capillary zone electrophoresis (CZE) and affinity capillary electrophoresis (ACE) were developed to investigate the interactions between isoprenaline hydrochloride (IH) and bovine serum albumin (BSA). In CZE, the binding constant (K_b) was 4.07×10^4 M^{-1} (298 K) and 4.76×10^4 M^{-1} (310 K) using the Scatchard analysis. The number of binding sites (n) in this interaction was approximately one ($n \cong 1$). Furthermore, thermodynamic parameters, such as changes in Gibbs free energy (ΔG), enthalpy (ΔH), and entropy (ΔS) of the binding procedure were also obtained. At 298 K, ΔG, ΔH, and ΔS were -26.30 kJ·mol^{-1}, 10.02 kJ·mol^{-1}, and 0.12 kJ·mol^{-}·K^{-1}, respectively. The ΔG at 310 K was -27.76 kJ·mol^{-1}, whereas the ΔH and ΔS at 310 K were identical with that at 298 K. In ACE, a more reliable parameter, mobility ratio (M), was applied in the calculation of K_b. K_b (310 K) obtained through this method was 9.80×10^4 and 9.24×10^4 M^{-1} when IH and BSA were added to the buffer in varying concentrations, respectively. The obtained K_b may help in gaining some insights on the possible drug/protein interactions and in the early evaluation of the pharmacokinetic profile of the drug during cardiovascular drug screening.

1. Introduction

Cardiovascular diseases remain the biggest cause of death worldwide, although cardiovascular mortality rates have declined in many high-income countries but have increased at an astonishingly fast rate in low- and middle-income countries over the last two decades. Isoprenaline hydrochloride (IH) is an effective cardiovascular drug stimulating $beta_1$ adrenergic receptors in the heart, which causes an increase in rate and strength of heart beats. This phenomenon allows the blood to be pumped effectively around the body in conditions such as heart failure. The basic chemical structure of the drug is beta phenylethylamine, which belongs to the classic $beta_1$ and $beta_2$ agonists. Similar to many biological reactions, its function is triggered by cellular interactions between the protein and the drug. In other words, the pharmacodynamic activity of the drug correlates with its binding effect to protein. Furthermore, screening of natural and synthetic compounds based on the protein-drug interaction is an indispensable strategy in the search for drugs for cardiovascular disease treatment. Unfortunately, studies on the interaction between IH and proteins such as bovine serum albumin (BSA) have not been reported so far. In binding studies, binding constants (K_b) are important parameters. Hence, determination of K_b is very important in describing and understanding the molecular interactions and is essential for drug development (Tabaka and Terabe 2002).

Many methods have been performed to investigate drug-protein interactions, such as nuclear magnetic resonance (NMR), fourier transform infrared spectrometry (FTIR), affinity chromatography (AC), mass spectrometry (MS), X-ray, fluorescence, surface plasmon resonance spectrometry (SPR), polyacrylamide gel electrophoresis (PAGE), equilibrium dialysis, capillary electrophoresis (CE) (Zhang et al. 2009; Østergard et al. 2009) and so on. SPR, AC, PAGE, and equilibrium dialysis are time-consuming procedures and demand a large sample size for analysis. NMR, X-ray, MS, fluorescence, and FTIR require experiments and elaborate procedures for sample purification prior to analysis. By contrast, CE is an ideal tool for this purpose owing to its several advantages, such as short analysis time, low sample size requirement, high efficiency, and flexible applications. In addition, CE can provide physiological or near-physiological buffer conditions in the analysis of biomolecules (He et al. 2002). CE is based on the mobilities of charged and neutral species through a buffer-filled silica capillary upon the application of a high voltage. CE has been used in a wide range of binding studies between many biomolecular systems, such as protein-protein, protein-drug, protein-DNA, peptide-sugar, peptide-peptide, antibody-antigen, and peptide-carbohydrate systems (He et al. 2004; Heegaard 2003; Østergaard and Heegaard 2003; Sladkov 2010; Taga et al. 2010; Liu et al. 2006). Among the various CE methods, capillary zone electrophoresis (CZE) is the most appropriate for the study of molecular interactions in cases where only very small amounts of samples are available and affinity CE (ACE) is the only method that can investigate molecular interactions quantitatively by monitoring the mobility shift of the analyte. Being a highly efficient separation technique, ACE requires only small sample volumes and has multiple running formats. ACE is characterized by an added ligand (or receptor) into the running buffer to conduct mobility shift of receptor (or ligand). In the

present work, the interaction between IH and BSA through CZE and ACE in an uncoated capillary was investigated. The peak area (in CZE) and mobility shift (in ACE) of the analyte were monitored to obtain practical data.

2. Investigations, results and discussion

Scatchard analysis is a common method to investigate K_b, and the model can be expressed as Eq. (1) in the CZE method of the current work:

$$v/[D] = -vK_b + nK_b \quad (1)$$

$$v = ([D_t] - [D])/[P_t]$$

where $[D_t]$ and $[P_t]$ are the total concentrations of the drug and protein, $[D]$ is the unbound drug concentration, K_b is the apparent binding constant, and n is the number of binding sites [5].

In ACE, the following Scatchard linear equation is used to study K_b:

$$1/\Delta\mu_A{}^L = \left[1/(K_b \cdot \Delta\mu_{max})\right] \cdot (1/[L]) + 1/\Delta\mu_{max} \quad (2)$$

where $\Delta\mu_A{}^L = \mu_A{}^L - \mu_0$ shows the mobility changes in the receptor when the running buffer is added with a certain concentration of ligand (ligand concentration » analyte concentration), $\Delta\mu_{max} = \mu_{AL} - \mu_0$. μ_0 and $\mu_A{}^L$ are the electrophoretic mobility of the receptor in the blank buffer and in the buffer containing the ligand at a certain concentration $[L]$, respectively, and μ_{AL} is the mobility of the receptor-ligand complex.

In Eq. (2), it is imperative that the mobility change in the analyte is only caused by the specific interaction with the ligand. However, the fact is that the changes in analyte electrophoretic mobility may also be attributed to the interaction of the analyte with the capillary wall, changes in temperature, or concentration changes in the separation medium. The latter nonspecific changes are usually caused by the addition of the ligand to the buffer and are most prominent for affinity systems characterized by weak analyte-ligand interactions. Generally, high ligand concentrations in the buffer are required for generating a complete binding isotherm in the weak interaction system. Many approaches have been discussed to correct the measured effective electrophoretic mobilities in the literature (Østergard et al. 2009; Vespalec and Bocek 2000; Rundlett and Armstrong 1997; Britz-Mckibbin and Chen 2002). Among these methods, the use of mobility ratios (*M)* in Scatchard plots was proposed by Bose et al. (1997). *M* was found to give more precise estimates of the K_b. In theory, *M* has been proven to be not associated with the applied voltage and the separation medium viscosity (Bose et al. 1997) and is calculated as follows:

$$M = \left(\mu_{eo} + \mu_A{}^L\right)/\mu_{eo} = t_{eo}/t_A{}^L + 1$$

where t_{eo} is the migration time of the internal standard marker and $t_A{}^L$ is the migration time of the analyte in the buffer containing the ligand at a certain concentration $[L]$. Then, the Scatchard equation is changed to Eq. (3).

$$1/\Delta M = [1/(K_b \cdot \Delta_{max})] \cdot (1/[L]) + \Delta M_{max} \quad (3)$$

where $\Delta M = M_{[L]} - M_{[L]=0}$, $\Delta M_{max} = M_{AL} - M_{[L]=0}$.

Equation (3) can be used to quantitatively characterize the interaction between the drug and protein. Linear graphs were obtained by plotting $1/\Delta M$ against $1/[L]$. Thus, the binding constant was the intercept/slope ratio of the linear graph. In the current study, the drug and protein were dissolved in electrophoresis buffer, respectively. For R_L method, Eq. (3) can be converted to Eq. (4).

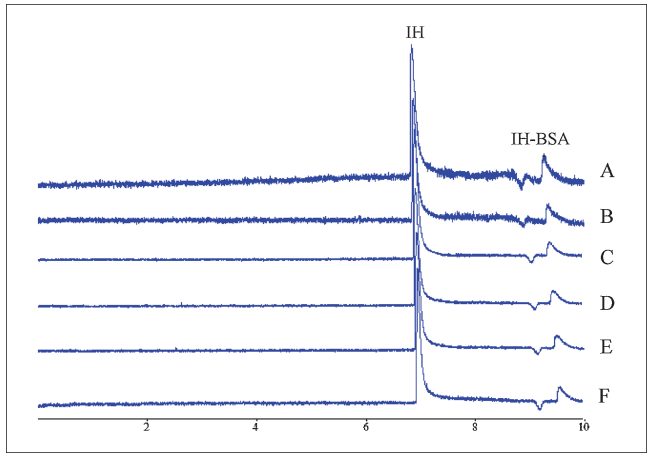

Fig. 1: Electropherograms of 200 μmol·L mixed with various concentration of IH at 310 K. (A) 800 μM IH; (B) 600 μM IH; (C) 400 μM IH; (D) 200 μM IH; (E) 100 μM IH; (F) 50 μM IH. Electrophoresis conditions: injection: 50 mbar for 3 s. Detection wavelength: 214 nm. Applied voltage: + 5 kV. Capillary: uncoated capillary (50 μm I.D., and 365 μm O.D.) of 38.5 cm (effective length 30.0 cm)

$$1/\Delta M = [1/(K_b \cdot \Delta M_{max})] \cdot (1/[D]) + 1/\Delta M_{max} \quad (4)$$

where $[D]$ is the concentration of the drug.

For the L_R method, Eq. (3) is changed to Eq. (5).

$$1/\Delta M = [1/(K_b \cdot \Delta M_{max})] \cdot (1/[P]) + 1/\Delta M_{max} \quad (5)$$

where $[P]$ is the concentration of the protein.

In the current work, K_b and the thermodynamic parameters of the binding procedure between IH and BSA were investigated using CZE and two ACE methods. Electroosmotic flow (EOF) highly depends on buffer pH. In other words, pH values remarkably influence the ionization degree of the capillary inner wall. Considering the adsorption, buffer pH should be set to pH > 6 or pH < 4. To ensure experimental practicality and an actual reflection of the drug activity in the body, buffer pH was set to 7.4 which is a near-physiological buffer condition (pH is approximately 7.35 to 7.45). Furthermore, BSA is negatively charged (pI 4.7) at this pH value and can reduce the adsorption of BSA onto the inner wall of the capillary to improve column efficiency.

2.1. Interaction between IH and BSA in CZE method

Standard samples containing IH in the range of 50 μmol·L^{-1} to 800 μmol·L^{-1} were injected individually. The relationship between peak area and IH concentration could be expressed as $y = 0.22x + 33.37$, with a coefficient of 0.9976.

Samples of mixed solutions of IH and BSA can achieve incubation at 37 °C within 2 h. Figure 1 shows the electropherograms of the mixtures containing BSA and IH at 310 K. The IH-BSA complex is retained to reach the detector when an electric field is applied, which may be attributed to the preconditions of incubation at 37 °C for 2 h. The above calibration curve was used to calculate the concentration of free IH. Fig. 2 shows the Scatchard plots at 298 K and 310 K. The linear relationship was expressed as $v/[D] = -4.07 \times 10^4 v + 6.76 \times 10^4$ (with a coefficient of 0.9914) and $v/[D] = -4.76 \times 10^4 v + 6.85 \times 10^4$ (with a coefficient of 0.9965) at 298 K and 310 K, respectively. Then, K_b was calculated to be 4.07×10^4 M^{-1} and 4.76×10^4 M^{-1} at 298 K and 310 K from the slope of the plot in Fig. 2, respectively. Higher K_b indicates that the IH-BSA combination is a fast equilibrium system (K_b is in the range of 10^4 M^{-1} to 10^6

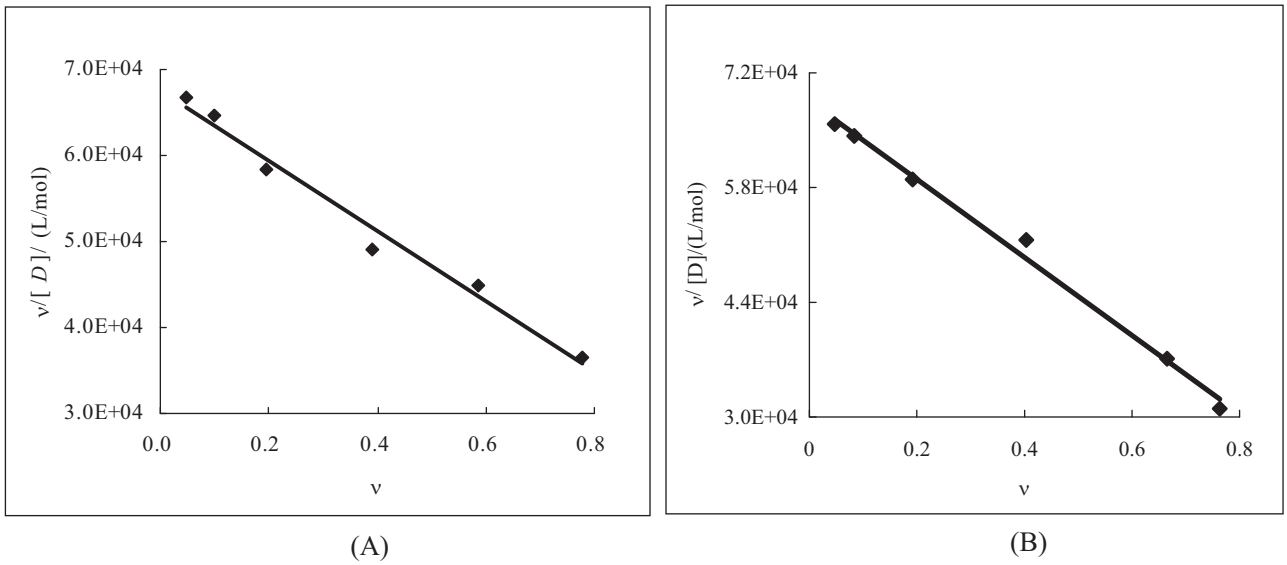

Fig. 2: Scatchard plot of the interaction between IH and BSA at (A) 298K and (B) 310K

M^{-1}). Values of 1.66 and 1.43 for the number of binding sites n were obtained when the intercept was divided by the slope.
Thermodynamic parameters of the binding procedure were calculated using the following equations:

$$\Delta G = -RT \ln K_b \quad (6)$$

$$\ln K_2/K_1 = \Delta H/R\,(1/T_1 - 1/T_2) \quad (7)$$

$$\Delta G = \Delta H - T\Delta S \quad (8)$$

where, ΔG, ΔH, and ΔS are the change in Gibbs free energy, enthalpy, and entropy, respectively. The calculated data are listed in the Table, which indicates that the interaction was a spontaneous procedure because ΔG was negative. Moreover, the main type of interaction is hydrophobic interaction because ΔH and ΔS were all positive (Ross and Subramanian 1981). In an actual drug-protein system, many types of interaction may be present simultaneously, such as ionic interaction, Van der Waals force, hydrogen bonding, and so on.

2.2. *IH and BSA ACE interaction (R_L method)*

IH was added to the running buffer to obtain different solution concentrations. A BSA sample (0.5 mg·mL^{-1}, containing 0.025% DMF) was separated six times continuously through ACE in PBS buffer. The migration time of DMF was 5.490, 5.677, 5.609, 5.628, 5.572, and 5.580 min, respectively. The relative standard deviation (RSD) of the DMF migration time was 1.12%, which confirmed that the migration time was repeatable. Thus, the system can be used.
Fig. 3 shows the representative electropherograms of the BSA sample (0.5 mg·mL^{-1}, containing 0.025% DMF) when IH is added to the running buffer at various concentrations. In the current paper, the neutral marker DMF was used to indicate the EOF. With increasing concentration of IH, the peak of BSA becomes broader and lower, the inverse peak of IH becomes larger, and the difference in migration time between BSA and EOF markers becomes larger. To quantitatively characterize the interaction between IH and BSA in ACE, Eq. (4) was used as the quantitative model. The corresponding Scatchard plot shows a linear relationship as $y = -1.81 \times 10^{-4}x - 17.73$ (with a coefficient of 0.9866) by plotting $1/\Delta M$ against $1/[D]$. Then, K_b between IH and BSA was calculated to be 9.80×10^4 M^{-1} from the ratio of the intercept/slope of the plot.
Busch et al. (1997) proposed that larger K_b may result from higher [D], which is used in calculations if complex > protein > drug mobility. Experiments in the current study are consistent with this case, that is, the complex > protein > drug mobility (Fig. 3). Although ACE has limitations, it is still used widely because the concentration and purity of the protein (sample) are not required to be very accurate. Furthermore, ACE does not require the separation of the free component and drug-protein complex. These features simplified the operation process compared with the fluorescence quenching method. Finally, the changes in ΔG of the IH-BSA combined process at 37 °C can be estimated to be −29 kJ by Eq. (6). The negative ΔG indicates that the combination of IH-BSA is a spontaneous interaction process.

Table: Thermodynamic parameters of the IH-protein binding process

T/K	ΔG/kJ·mol^{-1}	ΔH/kJ·mol^{-1}	ΔS/kJ·mol^{-1}·K^{-1}
298	−26.30	10.02	0.12
310	−27.76	10.02	0.12

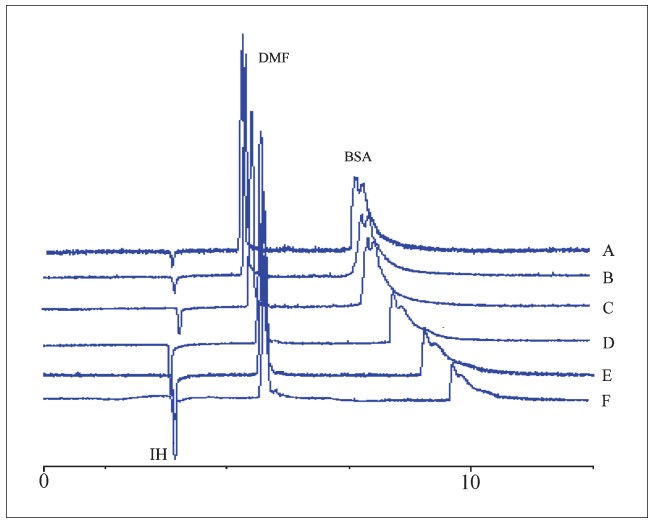

Fig. 3: The electropherogram of BSA (0.5 mg/mL, containing 0.025% DMF) when IH was added to the running buffer at various concentrations, A, 50 μM/L; B, 100 μM; C, 150 μM; D, 200 μM; E, 500 μM; F, 1000 μM. For electrophoresis conditions see Fig. 1

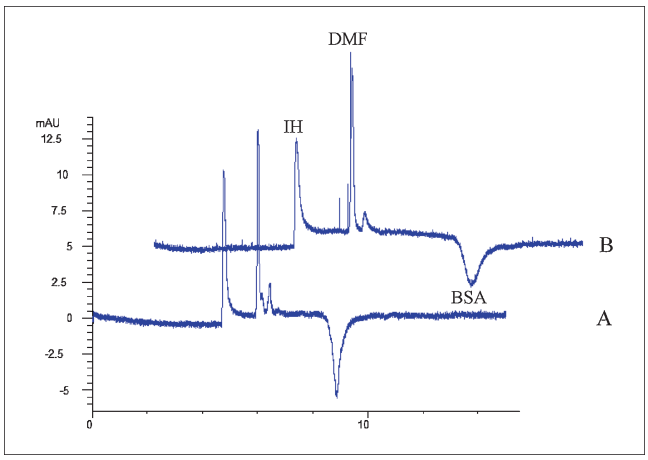

Fig. 4: Electropherograms of IH (1600 μM, containing 0.004% DMF) separated in PBS bufffer (A) with and (B) without Gly and EDTA additives

2.3. Interaction between IH and BSA ACE (L_R method)

L_R method is the basic ACE model in drug screening. However, adding protein to buffer is still a challenge because of its adsorption onto the capillary inner surface which induces peak broadening and tailing. Thus, the L_R method is still limited in drug-protein interaction research despite its importance in drug screening. Comparably, the adsorption of BSA sample could be ignored in R_L method because of its very little amount. In L_R method, BSA was added to the running buffer at various concentrations. As mentioned above, protein added to buffer may adsorb onto the capillary surface in L_R method. Modifying and controlling the surface properties of capillary is commonly used for the elimination of protein adsorption, such as extreme buffer pH or capillary surface modification. In the current study, Gly and EDTA were added to the running buffer to improve peak shape. Gly is an amphoteric ion which can compete with BSA to adsorb onto the capillary inner wall and suppress protein adsorption in the pH value of experiments. Furthermore, Gly has no interference in the detection wavelength. EDTA is a chelating reagent which can reduce the metal ions in the solution. Peak shapes were improved obviously when Gly and EDTA were added to the buffer (Fig. 4). To investigate the effect of additives on experimental reproducibility, samples of IH (1600 μM, containing 0.004% DMF) were continuously separated in PBS buffer (containing 0.25 M Gly and 0.5 mM EDTA) for seven times. The migration time of DMF was 5.135, 5.109, 5.129, 5.318, 5.379, 5.379, and 5.375 min, respectively. The RSD was 2.47%.

Fig. 5 shows the representative electropherograms of the IH sample (1600 μM, containing 0.004% DMF) when BSA was added to the running buffer (containing 0.25 M Gly and 0.5 mM EDTA) at various concentrations. With increasing BSA concentration, the peak of IH becomes smaller, the inverse peak of BSA becomes larger, and the difference in migration time between IH and EOF marker becomes larger. Similar with R_L method, Eq. (5) was also used as the quantitative model. The corresponding Scatchard plot shows a straight line (with a coefficient of 0.9803) after plotting $1/\Delta M$ against $1/[D]$. The linear relationship was expressed as $y = -1.33 \times 10^{-4}x - 12.29$. K_b between IH and BSA that was calculated to be 9.24×10^4 M^{-1} from the ratio of the intercept/slope shows that it is in good agreement with that detected through R_L method (9.80×10^4 M^{-1}), which showed better consistency between the two ACE methods. The changes in ΔG of IH-BSA are also approximately −29 kJ which was calculated through L_R method. The linear coefficient of the plot obtained through R_L method is better than that of the L_R method. The result of K_b in ACE is larger than the value obtained through the CZE method, which was approximately twofold of that in

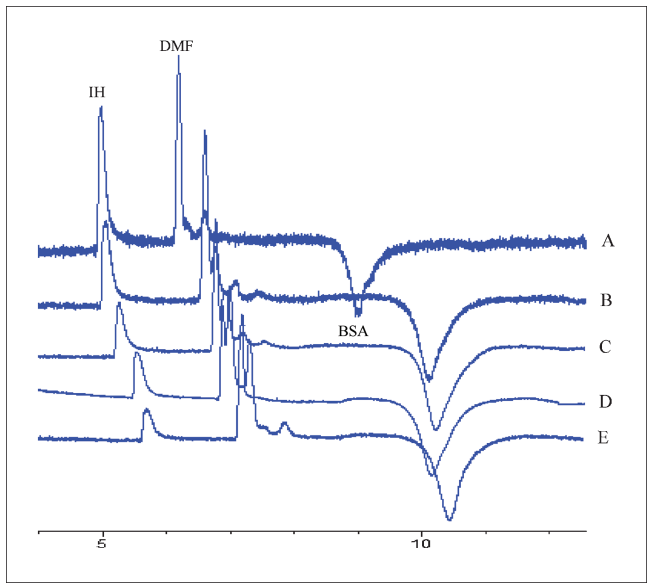

Fig. 5: The electropherograms of IH (1600 μM, containing 0.004% DMF) when BSA was added to the running buffer (containing 0.25 M Gly and 0.5 mM EDTA) at various concentrations, A, 2.5 mM; B, 5Mm; C, 7.5Mm; D, 10 mM; E, 12.5 mM. For electrophoresis conditions see Fig. 1

CZE. The difference is mainly attributed to the difference in the two methods. CZE is quantified by using peak areas and ACE is by the ratios of mobility. The different ranges of the ratio of IH to BSA may be the other reason for the difference. Comparably, CZE needs smaller amounts of reagents and can also provide the number of binding sites and thermodynamic parameters of the binding process.

2.4. Conclusion

The interaction between the cardiovascular drug IH and BSA was determined for the first time. CZE and two ACE methods (R_L and L_R) were applied to study the interactions. In the CZE method, K_b of IH and BSA at 298 K and 310 K were obtained through Scatchard analysis. K_b was 4.07×10^4 M^{-1} (298 K) and 4.76×10^4 M^{-1} (310 K). The number of binding sites (n) was approximately one. Furthermore, thermodynamic parameters, such as ΔG, ΔH, and ΔS of the binding procedure were also obtained from the K_b of the two temperatures. At 298 K, ΔG, ΔH, and ΔS were −26.30 kJ·mol^{-1}, 10.02 kJ·mol^{-1}, and 0.12 kJ·mol^{-1}·K^{-1}, respectively. ΔG at 310 K was −27.76 kJ·mol^{-1}, ΔH and ΔS at 310 K were identical with that at 298 K. Results indicate that the combination of IH-BSA is a spontaneous interaction process. The main type of interaction is hydrophobic interaction. In ACE method, M, a more reliable parameter, was used to estimate K_b. The constant between IH and BSA could be estimated at 9.24×10^4 M^{-1} and 9.80×10^4 M^{-1} from the ratio of the intercept/slope using L_R and R_L methods, respectively. ΔG in IH-BSA combined process is approximately −29 kJ calculated using the two ACE methods. Results show that CZE and ACE methods provide highly efficient, fast, and quantitative methods for investigating the interaction between IH and proteins. These results indicate that CE method can be employed as a reliable alternative to other drugs. Furthermore, these methods can be applied to discover cardiovascular drugs.

3. Experimental

3.1. Chemical and reagents

BSA was purchased from Merck (Darmstadt, Germany). IH was obtained from Shanghai Harvest Pharmaceutical Co., Ltd. (Shanghai, China).

N,N-dimethylformamide (DMF) was purchased from Kewei company of Tianjin University (Tianjin, China). Disodium ethylenediaminetetraacetic acid (EDTA) was obtained from Xi'an Chemical Reagents Factory (Xi'an, China), and glycine (Gly) was purchased from Sigma (St. Louis, MO, USA). All chemicals were analytical grade unless otherwise indicated.

3.2. Apparatus

All experiments were performed on an HP^{3D} CE system (Agilent, USA) equipped with a P/ACE station software version. An uncoated fused silica capillary tube (50 μm I.D. and 365 μm O.D.) was purchased from Reafine Chromatograph Device (Hebei, China). Its total and effective lengths were 38.5 and 30.0 cm, respectively. The temperature was set to 25 °C or 37 °C, and the absorbance was monitored at 214 nm. New capillaries were treated using 1 M NaOH, 1 M HCl, and deionized water for 20 min, respectively.

3.3. Procedures

For CZE, the conditions were as follows: the temperature of the cartridge was 25 °C or 37 °C. The capillary was filled with blank buffer (20 mM phosphate buffer, PBS, pH 7.4). Samples of IH (50 μmol·L^{-1} to 800 μmol·L^{-1}) or mixed solutions of IH and BSA were injected using the pressure injection mode at 50 mbar for 3 s. The concentration of BSA in the above mixed samples was fixed at 200 μmol·L^{-1} and the concentration of IH varied from 50 μmol·L^{-1} to 800 μmol·L^{-1}. The applied voltage was + 5 kV and the detection wavelength was 214 nm. After each run, the capillary was flushed with 0.1 M NaOH, deionized water, and running buffer for 5 min, respectively.

For ACE, the conditions were as follows: the temperature of the cartridge was 37 °C. The capillary was filled with running buffer containing IH or BSA. When drug is added to the buffer in varying concentrations, it is called the R_L method. In this method, samples containing 0.5 mg·mL^{-1} BSA and 0.025% DMF are injected using the pressure injection mode at 50 mbar for 3 s. Various contents of IH were dissolved in 20 mM PBS buffer (pH 7.4) to form the running buffer (50, 100, 150, 200, 500, and 1000 μM). The applied voltage was + 5 kV. Between each run, the capillary was flushed with 0.1 M NaOH, deionized water, and running buffer for 5 min, respectively.

If protein is added to the buffer in varying concentrations, it is called the L_R method. In this method, samples containing 1.6 mM IH and 0.004% DMF are injected using the pressure injection mode at 50 mbar for 3 s. Various contents of BSA were dissolved in 20 mM PBS (pH 7.4, containing 0.25 M Gly and 0.5 mM EDTA) to form the running buffer (2.5, 5.0, 7.5, 10.0, and 12.5 mM). Other electrophoretic conditions were identical with the above R_L method.

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