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SHORT COMMUNICATION

Interaction of glycyrrhetinic acid, furosemide and hydrochlorothiazide with bovine serum albumin and their displacement interactions: capillary electrophoresis and fluorescence quenching study

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ABSTRACT: Licorice is the most widely used crude drug in traditional Chinese medicine. Glycyrrhetinic acid (GA) is the metabolite of glycyrrhizic acid, which is the main bioactive ingredient of licorice. In this work, capillary electrophoresis–frontal analysis (CE–FA) was applied to study the binding of bovine serum albumin with GA and two diuretics: furosemide (FU) and hydrochlorothiazide (HZ). The binding parameters of GA were determined by Scatchard analysis, which showed that there are two kinds of binding sites in bovine serum albumin for GA. However, the results showed that the CE–FA method was not suitable for the interaction study of FU and HZ. Therefore, utracentrifugation–CE was used to probe the binding characteristic of these two drugs and the results showed only one kind of binding site for them under the studied conditions. Displacement interactions between these drugs were also investigated by utracentrifugation–CE method and the results showed that GA hardly displaces HZ while it can slightly displace FU and FU can slightly displace HZ. For comparison, the binding of these drugs was also studied by the fluorescence quenching method and the data were processed by the Stern–Volmer quenching equation. Results showed that the binding constants were basically consistent for two methods for all drugs studied. The number of binding sites on one protein molecule was well consistent for FU and HZ while it was quite different for GA. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: capillary electrophoresis–frontal analysis; bovine serum albumin; displacement interaction; furosemide; hydrochlorothiazide; glycyrrhetinic acid; fluorescence quenching

INTRODUCTION

Serum albumin is the most abundant protein in an animal, including the human, circulatory system. It is responsible for the transport of a variety of endogenous and exogenous substances in body and plays an important role in the distribution and deposition of these substances (Yamasaki *et al.*, 1996). When drugs are absorbed, they enter the circulatory system and extensively and reversibly bind to serum albumin (Kamat and Seetharamappa, 2004). An important aspect of a drug's biodisposition profile is the extent to which it binds to plasma proteins (Reed *et al.*, 2001). It is generally accepted that bovine serum albumin (BSA) and human serum albumin (HSA) especially bind neutral and negatively charged compounds; AGP is a major binding protein for many cationic drugs and to a lesser extent it binds some anionic and neutral drugs. Lipoproteins bind to nonionic and lipophilic drugs and some anionic drugs while globulins interact inappreciably with the majority of drugs (Martínez-Gómez *et al.*, 2006). However, some results seem to contradict some of these statements according to research by Martínez-Gómez *et al.* (2006). Drug–protein interaction is of great significance in pharmacology. It can affect the biological activity (Seedher, 2000; Zlotos *et al.*, 1998) and toxicity (Kragh-Hansen, 1981; Cszerhárti and Forgács, 1995; Silva *et al.*, 2004) of the drug. The binding

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Abbreviations used: ACE, affinity capillary electrophoresis; FA, frontal analysis; FU, furosemide; GA, glycyrrhetinic acid; HZ, hydrochlorothiazide; SPR, surface plasmon resonance; TCM, traditional Chinese medicine.

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parameters are helpful in the study of pharmacokinetics and the design of dosage forms (Rieutord *et al.*, 1995; Borga and Borga, 1997). Drug–protein interactions are determining factors in the therapeutic, pharmacodynamic and toxicological drug properties (Martínez-Gómez *et al.*, 2006).

Different methodologies have been developed to evaluate drug–protein interactions, such as the traditional equilibrium dialysis (Kwong, 1985), ultracentrifugation (Kwong, 1985), ultrafiltration (Kurz, 1986), gel filtration (Ascoli *et al.*, 1995), crystallographic studies of HSA–solute complexes (Carter *et al.*, 1989) and spectroscopic measurements such as fluorescence quenching (Parikh *et al.*, 2000; Zhou *et al.*, 2007). In recent years, the use of capillary electrophoresis (CE) to study biological interactions has become increasingly popular (Martínez-Gómez *et al.*, 2006; Zhang *et al.*, 2007; Østergaard and Heegaard, 2003). However, binding parameters such as binding constants obtained by different methods are sometimes quite distinct (Zhou *et al.*, 2007). Comparative studies of the interaction between drug and protein by different methods have seldom been reported. Busch *et al.* (1997) compared five CE methods and they concluded that these methods were complementary rather than competitive. Zhang *et al.* (2007) made comparative studies of the interaction between ferulic acid and bovine serum albumin by affinity capillary electrophoresis (ACE) and surface plasmon resonance (SPR). They found that the binding constant obtained with the mobility ratio assay was only 20% of that extracted from the mobility shift assay but was in excellent agreement with that by SPR.

BSA is a frequently used model protein, as it is the most abundant protein in bovine plasma and its structure has been well characterized (Kragh-Hansen, 1981; Ou and Kwok, 2004). Moreover, the high level of sequence identity (76%) between HSA and BSA indicates that BSA undoubtedly has a very similar overall structure to HSA. Licorice has become a widely used additive in various foods and drugs (Hussain, 2003). It is also the most common traditional Chinese medicine (TCM) (Fukai *et al*., 2002); it appears in many Chinese herbal formulations. Glycyrrhizic acid (GL), one kind of saponin, is its main active ingredient (with a content of up to 10%). Glycyrrhetinic acid (GA), a β-amyrim type compound, is the *in vivo* metabolite of this acid (see Fig. 1 for its structure). GA is an inhibitor of some cancer cells and enzyme, such as choriocarcinoma (Gomez-Sanchez *et al.*, 1996), melanoma (Abe *et al.*, 1987), mastocytoma (Imanishi *et al.*, 1989) and 11β-hydroxysteroid dehydrogenase (Marandici and Monder, 1993; Monder *et al.*, 1989). GA is a potent inducer of mitochondrial permeability transition and can trigger the pro-apoptotic pathway (Salvi *et al.*, 2003). It can enhance the activity of hydrocortisone (Edwards and Teelucksingh, 1990) and retard the development of autoimmune disease (Horigome *et al.*, 2001). It also possesses hepatoprotective effects and can protect against hepatoinjury from carbon tetrachloride (Jeong *et al.*, 2002) and retrorsine (Lin *et al.*, 1999). Furosemide, an anthranilic acid derivative (see Fig. 1 for its structure), is a potent, prompt-acting diuretic used for diverse treatments in mankind, as well as in veterinary medicine (Guzmán *et al.*, 2003). Hydrochlorothiazide (Fig. 1) is a diuretic and antihypertensive agent that reduces plasma volume by increasing the excretion of sodium, chloride and water and, to a lesser extend, that of potassium ion as well (Liu *et al.*, 2007).

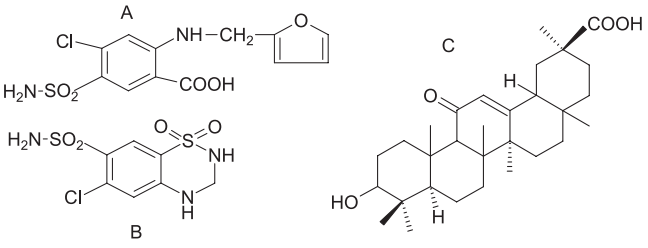

Figure 1. Structures of furosemide (A), hydrochlorothiazide (B) and glycyrrhetinic acid (C).

In this work, CE–frontal analysis (FA) method was applied to investigate the interaction of GA, furosemide (FU) and hydrochlorothiazide (HZ) with BSA. The binding parameters of GA were obtained. However the results showed that the CE–FA method was not suitable for the interaction study of FU and HZ. Therefore utracentrifugation–CE was used to probe the binding characteristic of these two drugs. For comparison, a fluorescence quenching method was also used to study the interaction of these drugs with BSA. The results showed that binding constants of drugs are basically consistent for these two methods; the numbers of binding sites are in good agreement with each other when there exists only one kind of binding site. However, the number of binding sites is quite different for these two methods when there is more than one kind of binding site. In addition, displacement interactions between these drugs were also investigated. The data gathered here would help to pharmacologically understand the use of these drugs.

MATERIALS AND METHODS

Instrumentation. A CaiLu capillary electrophoresis system (Institute of Chemistry, Chinese Academy of Sciences, Beijing) equipped with a UV detector and HW-001 Chemstation software was used throughout (Qianpu Software Co. Ltd, China). A fused uncoated capillary (52 cm × 50 μm i.d., effective length 44 cm) was employed (Yongnian Optical Fiber Co.,

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Hebei, China). A running voltage of 19 kV was used. Typical currents were 85 mA. UV detection was performed at 254, 224 and 229 nm for GA, HZ and FU, respectively. All pH measurements were made with a pHS-3 digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China). The mass of sample was accurately weighed with a microbalance (Sartorius). An ultracentrifugation tube with a molecular weight cut-off of 15 kDa (Pall Filter Co. Ltd, USA) was used to separate free drugs from serum protein and protein–drug complex.

Chemicals and samples. All reagents were of analytical grade. BSA fraction V (Roche) was used without further purification and its molecular weight was assumed to be 66,500. Sodium dihydrogen phosphate dihydrate was obtained from JiaoZuo (Henan, China); furosemide was obtained from Sigma-Aldrich Inc. (St Louis, MO, USA); hydrochlorothiazide was obtained from Alfa Aesar (Lancaster, UK); 18β-glycyrrhetinic acid was obtained from Fluka (Spain). Figure 1 shows the structure of these drugs. HPLC-grade methanol was used to prepare drugs solutions. All solutions were filtered through 0.45 μm pore size nylon membranes and degassed prior to use. The water used was doubly distilled.

Solution preparation. The running buffer in all CE experiments was 67 mM sodium phosphate at pH 7.40 and was prepared by dissolving the appropriate amount of sodium dihydrogen phosphate dihydrate in water and adjusting the pH with 2 M NaOH monitored by pH-meter. BSA stock solutions (200 mM) were daily prepared by dissolving the corresponding amount of protein powder in phosphate buffer. Working protein solutions were obtained by dilution of BSA stock solution with phosphate. Stock standard solutions of the drugs (2 mM) were prepared in 67 mM phosphate buffer containing less than 30% v/v methanol. Working solutions were obtained by dilution with phosphate buffer from the corresponding stock solution. The methanol contents in the working solutions were always lower than 3% v/v. Standard solutions of each drug were also used to prepare calibration curves. Series of mixtures with increasing protein or drugs concentration and a fixed total concentration of drug or protein were prepared and vortexed for 30 s, then incubated at 36°C in a water bath for 2 h and allowed to equilibrate at room temperature for at least 2 h. The binding of GA was studied by CE–FA. The experiments were carried out at room temperature. All mixtures for CE were prepared in duplicate. A binding study of FU and HZ and displacement interactions between drugs was carried out using the ultracentrifugation–CE method. For binding study of FU/HZ, the equilibrated mixture of FU/HZ with BSA was transferred to an ultracentrifugation tube and then ultracentrifugated at 16,000 rpm. As for displacement interactions between drugs, the displacer was added to the equilibrated mixture. After vortexing for 30 s, it was incubated in 36°C water bath for another 2 h and allowed to equilibrate at room temperature for at least 2 h; then the mixture was separated as described above. Free drug in the centrifugate was determined by CE. The calibration curves for GA, FU and HZ were, respectively: $y = 25.808x - 9.607$, $R^2 = 0.9999$, limits of quantification (LOQ) = 7 μmol/L; $y = 32.497x + 145.87$, $R^2 = 0.9981$, LOQ = 5 μmol/L; and $y = 47.84x - 85.123$, $R^2 = 0.9995$, LOQ = 4 μmol/L.

CE procedures. New capillaries were conditioned for 30 min with 1 M HCl, 5 min with water and 30 min with 1 M NaOH at room temperature. Then they were rinsed for 5 min with water and 15 min with phosphate buffer. In order to obtain good peak shapes and reproducible migration times, the capillary was conditioned at the beginning of each day with the following sequence: (i) 2 min rinse with water, (ii) 2 min rinse with 1 M sodium hydroxide, (iii) 2 min rinse with water and (iv) 5 min rinse with running buffer. Between runs, the capillary was conditioned with water for 2 min, with 1 M NaOH for 2 min, with water for 2 min and with running buffer for 2 min. Under these conditions, the elimination of adsorption of protein in the capillary is guaranteed (Martínez-Gómez *et al.*, 2006). The pre-equilibrated samples or centrifugates were injected hydrodynamically into the capillary at a height difference of 30 cm (between inlet and outlet) for 60 s, and a running voltage of 19/14 kV was kept in the normal polarity during the electrophoretic runs.

Fluorescence procedures. Fluorescence spectra were measured using 5/2.5 (2.5/2.5 for HZ) nm slit widths (excitation/emission) and 700 V PMT. The excitation wavelength was 280 nm, and the emission was read at 290–420 nm. For fluorometric titration experiments, 2.0 mL buffer and 2.0 mL BSA stock solution (2×10^{-5} mol L^{-1}) were pipetted into a 10 mL calibrated flask, then filled with water to the scale. A 1.0 mL aliquot of this solution was transferred to a 1 cm cell and titrated by successive additions of GA or other chemical stock solution to give a certain concentration. Titrations were done manually using trace syringes, then mixed with a vortex agitator and the fluorescence intensity was measured at room temperature. The experiments were done in triplicate and the results were means of triplicate data.

Data processing. CE binding parameters of drug–protein combinations were obtained by Scatchard plot [eqn (1); Ding *et al.*, 1999). In eqn (1), r is the ratio of the bound drug concentration to the protein concentration, D_f is the unbound drug concentration, K is the binding constant and n is the number of binding sites on one protein molecule. The drug–protein binding percentage (PB) was evaluated using eqn (2) (Øtergaard and Heegaard, 2003), where D_t is the total concentration of drug added to the mixture. PB was used to evaluate the displacement interaction between these drugs:

$$r/D_f = -Kr + nK \quad (1)$$

$$\text{PB}\ (\%) = 100(D_t - D_f)/D_t \quad (2)$$

The fluorescence data were analyzed using the Stern–Volmer quenching equation [eqn (3); Lakowicz, 1999] and eqn (4) (Zhou *et al.*, 2007), where $[Q]$ is the concentration of the quencher, K_{sv} is the dynamic quenching constant, K_f is the binding constant by fluorescence method, and F_0 and F are the fluorescence intensities in the absence and presence of drug.

$$F_0/F = 1 + K_{sv}[Q] \quad (3)$$

$$\lg[(F_0 - F)/F] = \lg K_f + n \lg[Q] \quad (4)$$

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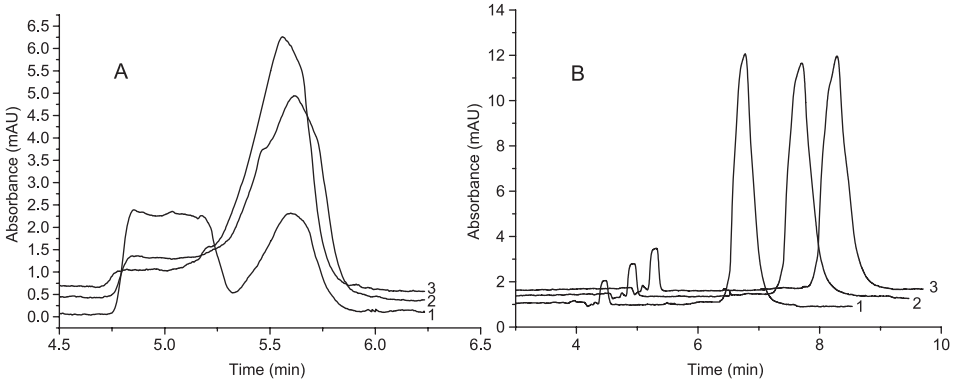

Figure 2. Electropherograms of GA–BSA (A) and HZ–BSA (B). Injected solutions contained 160 μM GA with increasing BSA concentration (**1**, 20 μM; **2**, 40 μM; **3**, 50 μM) and UV detection was performed at 254 nm; voltage applied was 19 kV. For HZ, injected solutions contained 20 μM BSA with increasing HZ concentration (**1**, 16 μM; **2**, 22 μM; **3**, 28 μM) and UV detection was performed at 224 nm; voltage applied was 19 kV.

RESULTS AND DISCUSSION

CE analysis

CE has proved to be a powerful and convenient tool in research on molecular association. The advantages include speed, ease of automation, separation power and small sample consumption. Lack of sensitivity may be the main limitation to the application of CE–FA. The output of CE–FA in terms of free ligand concentrations is thought to be appealing to researchers using conventional methods as data analysis is similar (Øtergaard and Heegaard, 2003).

In this work, GA, HZ and FU were investigated using CE–FA method for their binding characteristics with BSA. The interaction was studied under the physiological conditions. The main conditions to be optimized were voltage and sampling time. Typical electropherograms of GA, HZ and FU are shown in Figs 2 and 3. The results showed that the drug (GA) and protein zones were overlapping, while the zones of HZ and FU were completely separated from that of the protein regardless of the changes in voltage (the voltage applied was studied from 10 to 20 kV). HZ and FU seemed to represent two statuses in which one was the drug eluting faster than protein and the other was the drug eluting slower than protein. Østergaard and Heegaard (2003) thought the primary prerequisite in CE–FA was the requirement for sufficiently different migration between the free and bound forms of one of the species studied. The authors (Øtergaard and Heegaard, 2003) also stated that equilibrium was maintained when the drug and protein zones were overlapping. The electropherograms of HZ and FU did not meet this need; the equilibrium was destroyed in the elution process and the binding constant cannot be detected accurately. Our results also showed that the

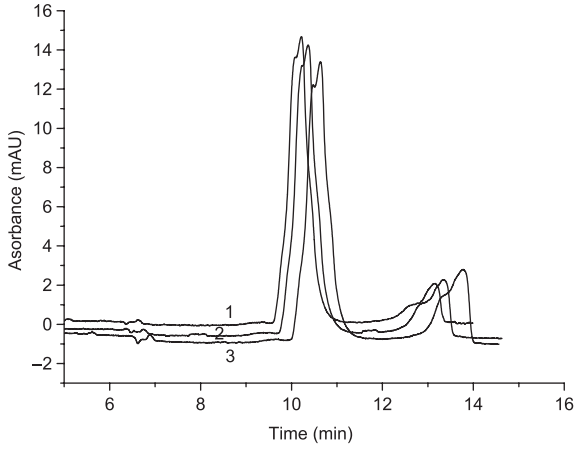

Figure 3. Electropherograms of FU–BSA. Injected solutions contained 30 μM BSA with increasing FU concentration (**1**, 40 μM; **2**, 50 μM; **3**, 60 μM). UV detection was performed at 229 nm and voltage applied was 14 kV.

detected concentration of HZ in pre-equilibrated drug–protein solution by CE–FA was equal to that added, which meant that the HZ–BSA complex had completely disassociated in the elution process. The concentration of FU detected by CE–FA was less than 30% of that by utracentrifugation–CE, which meant the FU–BSA complex had partially disassociated in the elution process. Moreover the concentration of FU was hard to detect accurately due to the difficulty of obtaining a plateau. However the result of GA by CE–FA was in good agreement with that by utracentrifugation and CE detection. Therefore the binding parameters of GA were determined by CE–FA while that of FU and HZ were obtained by utracentrifugation and CE detection.

To bring about the CE–FA, a relative larger volume of sample (60–200 nL) should be introduced into the capillary. As the injected sample forms a plug in the

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capillary, the detected signal is plateau-shaped, and absorbance strength linearly depends on the concentration of analyte in the zone. Øtergaard and Heegaard (2003) stated that plateau peaks might not be an absolute requirement, but they also thought that the presence of plateau peaks might lead to a more robust method of analysis. In this study, a stable plateau was obtained when the sampling time was more than 55 s. Therefore a sampling time of 60 s was adopted. The free concentration of GA was calculated by the height of plateau [about 5 min in Fig. 1(A)] through a calibration curve. FU and HZ were first centrifugated by ultracentrifugation tube after pre-equilibrium. The free concentration of drug in the filtrate was detected by CE. The ultracentrifugation tube was tested for the adsorption of these drugs and the adsorption was found to be negligible. The concentration was calculated by calibration curve. The bind curves of GA, FU and HZ are shown in Fig. 4 and a Scatchard plot of them is shown in Fig. 5. According to above data, the binding parameters by CE are listed in Table 1. Obviously, according to Scatchard plot ($r/D_f = -K_r + nK$), the intercept on the x-axis is the number of binding sites. The plot apparently has two linear parts which represent two different binding sites. Therefore, there exist two kinds of binding sites in BSA for GA for which the number of binding sites is 3.0 and 5.3 for high-affinity and low-affinity sites, respectively. There is only one kind of binding site for FU (n = 0.94) and HZ (n = 0.95) in the studied conditions. The binding constant of GA and FU was up to 10^5 and that of HZ was 10^4.

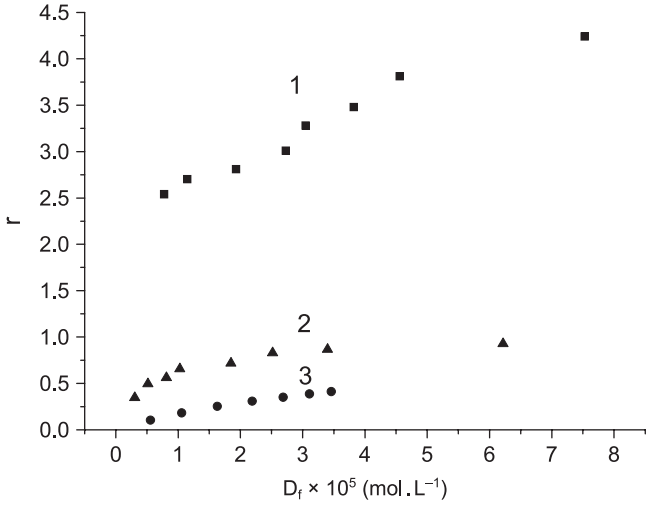

Figure 4. Binding curves of drugs. Curves 1, 2 and 3 are GA, FU and HZ, respectively. For detection conditions refer to Figs 2 and 3. When GA was determined, the concentration of GA was fixed at 160 μM and the concentration of BSA was 20, 30, 35, 40, 45, 50, 55 and 60 μM. When HZ was determined, the concentration of BSA was fixed at 20 μM and concentration of HZ was 16, 22, 28, 34, 40, 46 and 52 μM, respectively. When FU was determined, the concentration of BSA was fixed at 30 μM and the concentration of FU was 15, 20, 25, 30, 40, 50, 60 and 90 μM, respectively.

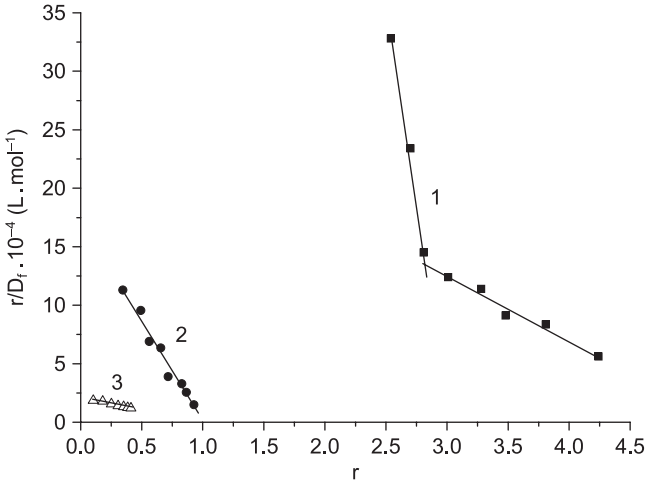

Figure 5. Scatchard plot for binding data of drugs. Curves 1, 2 and 3 are GA, FU and HZ, respectively. For detection conditions refer to Fig. 4.

The displacement interactions between these drugs were investigated. The results of displacement were expressed as binding percentage (PB). The results are listed in Tables 2–4. As can be seen from these tables, GA hardly displaces HZ while it can slightly displace

Table 1. Binding parameters by CE methods

	Number of binding sites		Binding constant	
Drugs	n_1	n_2	$K_1 \times 10^{-5}$	$K_2 \times 10^{-4}$
GA	3.0	5.3	6.70	5.49
FU	0.94		2.33	
HZ	0.95		0.22	

Table 2. Displacement of FU by GA

Concentration of GA (μM)	Concentration of bound FU (μM)	PB (%)
0	26.4	52.8
50	24.7	49.4
100	24.1	48.1

Note: mixture of 50 μM FU and 20 μM BSA at pH 7.40 phosphate buffer.

Table 3. Displacement of HZ by GA

Concentration of GA (μM)	Concentration of bound HZ (μM)	PB (%)
0	9.7	24.3
20	10.6	26.6
40	11.1	27.6
80	10.7	26.7

Note: mixture of 40 μM HZ and 20 μM BSA at pH 7.40 phosphate buffer.

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Table 4. Displacement of HZ by FU

Concentration of FU (μM)	Concentration of bound HZ (μM)	PB (%)
0	9.7	24.3
40	8.2	20.4
80	6.4	16.0

Note: mixture of 40 μM HZ and 20 μM BSA at pH 7.40 phosphate buffer.

FU and FU can slightly displace HZ. From Fig. 1, one can see that GA and FU have the same carboxyl group while FU and HZ have the same groups—one sulfamoyl and one chloro. Substituent groups play an important role in the drugs binding. Drugs sharing the same group tend to bind to the same sites in BSA; the binding sites of FU maybe partially overlap with those of GA and HZ while the binding sites of GA and HZ do not overlap. Therefore GA hardly displaces HZ while it can slightly displace FU and FU can slightly displace HZ.

Fluorescence quenching analysis

Owing to its exceptional sensitivity, selectivity, convenience and abundant theoretical foundation (Gao *et al.*, 2004), fluorescence spectroscopy is a popular method for studying drug–protein interactions (Kang *et al.*, 2004). It can provide binding information and reflect the conformation changes of proteins in various environments, which the CE method cannot obtain. The fluorescence of BSA comes from the tyrosine, tryptophan and phenylalanine residues. The spectrum of BSA is sensitive to the micro-environment of these chromophores and it allows non-intrusive measurements of protein in low concentrations under physiological conditions. Therefore, spectroscopic methods are usually applied to the study of the conformation of serum protein. The maximum emission wavelength (λ_{max}) is also very useful in estimating the hydrophobicity of the trytophan resides. λ_{max} at 330–332 indicates that trytophan resides are located in the nonpolar region, that is, they are buried in a hydrophobic cavity in BSA; λ_{max} at 350–352 nm suggests that tryptophan residues are exposed to water, i.e. the hydrophobic cavity in BSA is disagglomerated and the structure of BAS is looser.

The fluorescence decreases when drugs are added to BSA solutions. This phenomenon is called fluorescence quenching (FQ), although a variety of molecular interactions can result in quenching, including excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation and collisional quenching. Fluorescence quenching is usually divided into two types: static quenching and dynamic quenching. FQ in serum albumin is widely used in measuring drug–protein binding affinity. The interaction of drugs with BSA at physiological acidity (pH 7.40) was evaluated by measuring the intrinsic fluorescence intensity of protein before and after addition of drugs. Figures 6 and 7 show the fluorescence spectra of BSA in the presence of drugs. Quenching of BSA by drugs was studied in the concentrations of drugs of 2.5–140, 2.5–100 and 2.0–24 μM for GA, FU and HZ, respectively. The Stern–Volmer curves for quenching of BSA by drugs are shown in Figs 8 and 9. For GA, there are two types of binding sites in which the turning point is [GA]:[BSA] ≈ 3. This result is in good agreement with that obtained by CE. About 87.6% of fluorescence was quenched at 140 μM GA concentration and thus caused 7 nm of blue shift for maximum emission wavelength (from 340 to 334 nm), which indicated that tryptophan was located in a more hydrophobic environment after addition of GA. For FU, distinct positive cooperativity was observed when [FU]:[BSA] ≈ 10. About 97.2% of fluorescence was quenched at 100 μM drug concentration and thus caused up to 29 nm of red shift for

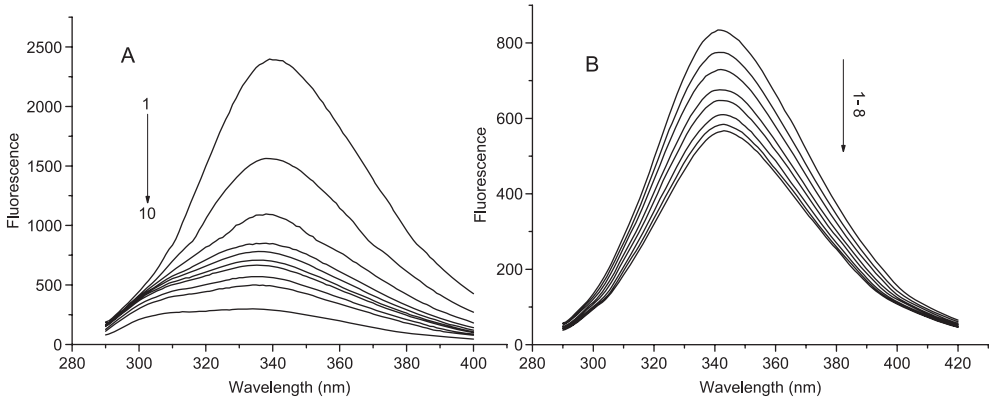

Figure 6. Effect of GA (A) and HZ (B) on florescence spectra of BSA. The concentration of BSA is 4 μM. In (A), the concentration of GA increases from top to bottom (0, 2.5, 5, 7.5, 10, 15, 20, 40, 60 and 140 μM). In (B), the concentration of HZ increases from top to bottom. (0, 2, 4, 8, 12, 16, 20 and 24 μM).

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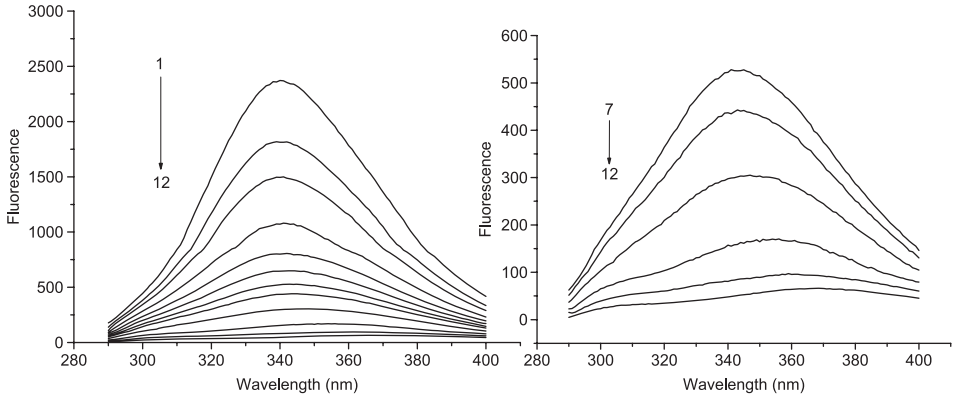

Figure 7. Effect of FU on florescence spectra of BSA. The concentration of BSA is 4 μM. The concentration of FU increases from top to bottom. (0, 2.5, 5, 10, 15, 20, 25, 30, 40, 60, 80 and 100 μM). Note: the right part is a partial enlargement of the left part.

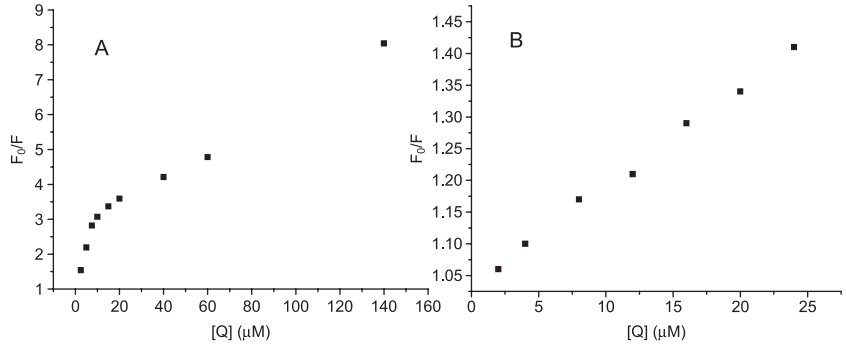

Figure 8. Stern–Volmer curves for quenching of GA and HZ to BSA. The data are from Fig. 6.

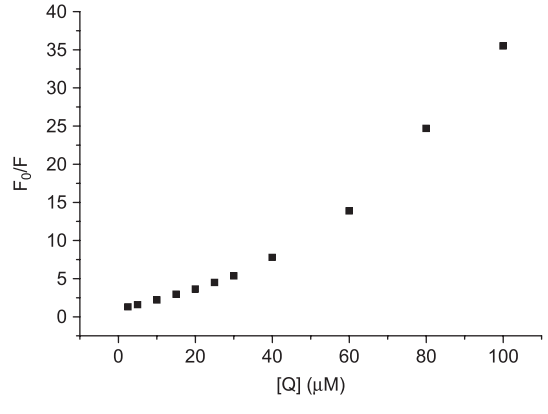

Figure 9. Stern–Volmer curves for quenching of FU to BSA. The data are from Fig. 7.

maximum emission wavelength (from 340 to 369 nm), which indicated that tryptophan was located in a more hydrophilic environment after addition of FU. Furthermore, when the concentration ratio [FU]:[BSA] was equal to 12.5, the maximum emission wavelength changed to 351 nm, suggesting that tryptophan residues were exposed to water, i.e. the hydrophobic cavity in BSA is disagglomerated and the structure of BAS is looser (Brustein *et al.*, 1973). As for HZ, only one type of binding sites was observed and there was no cooperativity in the studied condition. The maximum emission wavelength shifted from 341 to 343 nm. Therefore, the addition of these drugs can cause conformation change of BSA to some extent. The results of eqns (3) and (4) are listed in Table 5. Compared with the

Table 5. Binding parameters by fluorescence quenching method

Drugs	$K_{sv} \times 10^{-5}$	Correlation coefficient	$K_f \times 10^{-5}$	Correlation coefficient	n
GA	2.56 (0.37)	0.9999 (0.9981)	8.99	0.9992	1.11
FU	1.42	0.9981	2.55	0.9993	1.06
HZ	0.19	0.9928	0.39	0.9963	1.02

The figure in parentheses is the value of a second site.

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data from Tables 1 and 5, one can see that binding constants of drugs are basically consistent for these two methods. However, the number of binding sites is quite different for them. The number of binding sites is in good agreement when there is only one kind of binding site while the number of binding sites is quite different for these two methods when there is more than one kind of binding site.

CONCLUSIONS

In this work, the interaction of GA, FU and HZ with BSA was studied by CE and fluorescence quenching. The binding constant by fluorescence quenching is 8.99, 2.55 and 0.39×10^5 for GA, FU and HZ, respectively. These results are basically consistent with that found by CE, in which the binding constants are 6.70, 2.33 and 0.22×10^5 for GA, FU and HZ, respectively. The quenching data showed that binding of these drugs could cause conformational change to some extent. The number of binding sites was in good agreement when there was only one kind of binding site. However, the number of binding sites was quite different for these two methods when there was more than one kind of binding site. In addition, displacement interactions between these drugs were also investigated and the results showed that strong binding drugs were not always displaced by weak binding drugs. Displacement interaction between drugs depends on both binding abilities and the sites in which they bind. The data gathered here will help pharmacological understanding of the use of these drugs.

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