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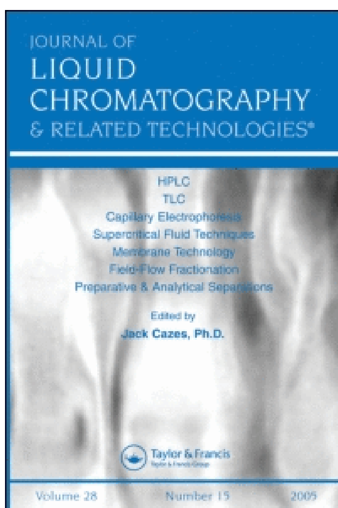

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CHARACTERIZATION OF THE INTERACTIONS BETWEEN NATURAL FLAVONOID COMPOUNDS AND BOVINE SERUM ALBUMIN BY CAPILLARY ELECTROPHORESIS AND FLUORESCENCE METHOD

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CHARACTERIZATION OF THE INTERACTIONS BETWEEN NATURAL FLAVONOID COMPOUNDS AND BOVINE SERUM ALBUMIN BY CAPILLARY ELECTROPHORESIS AND FLUORESCENCE METHOD

Tianxi He,[1,2] Qionglin Liang,[1] Yiming Wang,[1] and Guoan Luo[1]
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□ *The interactions of three flavonoid compounds isolated from natural products: Puerarin, (−)-Epicatechin, (−)-Epigallocatechin gallate with bovine serum albumin (BSA) are investigated by two methods: affinity capillary electrophoresis (ACE) and fluorescence quenching method. ACE gives binding constants (K_b) and thermodynamic parameters. The thermodynamic parameters indicate that van der Waals interactions and hydrogen bond played important roles. The result also implies that in spite of the relatively small difference in the chemical structures, large differences are observed in their binding capacity. The fluorescence quenching method gains quenching constant K_{sv}, binding site number n and K_b. The fluorescence results indicate that BSA fluorescence quenching mechanism is mainly a static quenching process. The results of the two methods show the flavonoids had a fairly strong interaction with BSA. The K_b obtained from ACE is similar with that obtained from the fluorescence method. Our work might give an insight on the high throughput screening active components in natural products.*

Keywords ACE, BSA, flavonoids, fluorescence quenching, interaction

INTRODUCTION

Investigation into the interactions of proteins and drugs are needed in pharmacokinetics, drug screening, and discovery.[1] The interaction of drug-protein has an important effect on the distribution, free concentration, and the metabolism of drugs in the blood stream. In addition, studies on the binding of a drug with protein will facilitate interpretation of the metabolism and transport process of drug. Serum albumin (e.g., Bovine serum albumin (BSA) and human serum albumin (HSA)), the most abundant protein in blood plasma, reversibly binds a wide range of endogenous

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and exogenous substances. Therefore, investigation of the active constituents of natural products with respect to serum protein binding is of imperative and fundamental importance and has been an interesting research field in life sciences, chemistry, and clinical medicine.[1–3] In this regard, a great deal of attention has been paid to the interaction of BSA with a number of active ligands in the drug discovery and development, because BSA is similar with HSA in structure, and has clinical and pharmaceutical importance.[2,3]

Natural products are attracting increasing attention because of their reliable therapeutic effects.[4,5] As a large class compounds of natural products, flavonoids are widely present in the green plant world with more than 6500 different compounds described.[6] Puerarin (P), (−)-Epicatechin (EC), and (−)-Epigallocatechin gallate (EGCG) (for chemical structures see Fig. 1) are important compounds of flavonoids. P is one of the major isoflavonoid compounds isolated from *Puerarina lobata* and EC and EGCG are the main components of green tea extracts.[7,8] They exert a wide range of the therapeutic activities in natural products, including antioxidant, antiinflammatory, antibacterial, antimicrobial, antiplatelet, blood vessel protecting.[9] Current knowledge suggests that factors such as protein binding may impair flavonoids absorption and bioavailability and even mask their antioxidant activity.[10,11] Thus the characterization of the interactions between flavonoids and serum protein is becoming very important and necessary.

ACE has been proven to be an efficient method of studying noncovalent interaction.[12–15] Advantages of this method include relatively short analysis time, multicomponent and unknown concentration analysis.[16–19] There has been some research on the interactions of serum albumin and P, EC, and EGCG. However, they were limited reports, mainly using fluorescence and other analysis techniques on the evaluating serum albumin, the analytes interactions.[20–24] Medina et al.[25] studied the interaction of EC with HSA using CE-frontal analysis. To our knowledge, the simultaneous study on interactions of P, EC, and EGCG with BSA using CE has not been reported.

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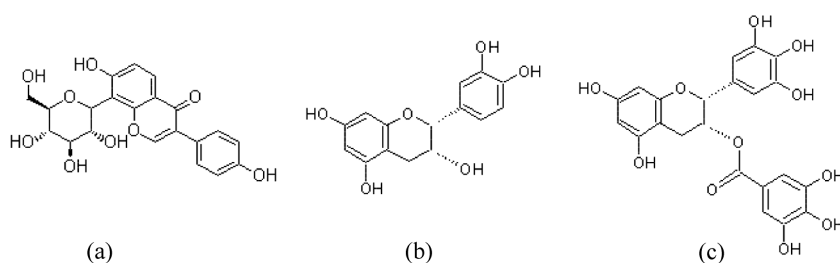

FIGURE 1 Structures of three flavonoids. (a) Puerarin (P), (b) (−)-Epicatechin (EC), (c)(−)-Epigallocatechin gallate (EGCG).

In this study, we separated P, EC, and EGCG using CZE mode and optimized experimental parameters, such as the pH and concentration of the buffer. Then, based on the CZE separation of three flavonoids, the interactions of flavonoids and BSA were studied using ACE and obtained the K_b at different temperatures. The mobility ratios (M) assay was used to deduce K_b, which effectively eliminates the viscosity change of the buffer caused by the addition of BSA. Moreover, we obtained thermodynamic constants including ΔS (entropy), ΔH (enthalpy), and ΔG (Gibbs free energy), and analyzed interaction modes of the flavonoids with BSA. We carried out fluorescence measurements for obtaining more information about the interactions between flavonoids and BSA. Fluorescence quenching mechanism, binding constants, and binding site (n) were also estimated. And the results of the two methods were similar with each other. Certainly, the study was important and worth being done for understanding of the interactions between flavonoid and protein, and the transports and metabolism process of the natural flavonoids. The results might help us to gain some insight on the possible flavonoids-serum albumin interactions and predicting the flavonoids activity. This study also demonstrated that compared to other methods (e.g.,. liquid chromatography and spectrofluorometry), the ACE-based studies are highly efficient, rapid, and amenable to more compound types.

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EXPERIMENTAL

Apparatus

All experiments were performed on a Beckman P/ACE MDQ system (Fullerton, CA, USA). A 51.0 cm (effective length 40.0 cm) × 75 μm id uncoated fused-silica capillary (Yongnian reafine chromatography equipment, P.R. China). Fluorescence measurements were performed on a Hitachi spectrofluorimeter Model F-7000 (Hitachi, Japan) equipped with a 150 W Xenon lamp and a slit width of 5 nm. UV–Vis spectra were obtained with a Rayleigh UV–Vis 1100 spectrometer (Rayleith, China) equipped with a 200 W Xenon lamp and a slit width of 0.2 nm.

Chemicals and Solutions

P, EC, and ECGC were obtained from National Institute for The Control of Pharmaceutical and Biological Products (Beijing, P.R. China). BSA (fatty acid free), Tris and HEPES, purchased from Sigma (St. Louis, MO, USA), were used as received. Boric acid, sodium borate ($NaB_4O_7 \cdot 10H_2O$), sodium dihydrogen phosphate monohydrate, H_3PO_4, HCl, and NaOH were all of analytical grade.

The sodium borate buffer of concentration range between 10 and 50 mM were selected (pH range between 7.2 and 7.8). Other several commonly used buffer systems of 25 mM (pH = 7.4), such as Tris-HCl, HEPES, and sodium phosphate buffer were prepared with corresponding reagents. All pH was adjusted with 1 M HCl or NaOH.

Individual drug stock solutions (2 mgL^{-1} P, EC, and ECCG) were prepared with methanol and sodium borate buffer (1:3, v/v). Methanol was used as an EOF marker and to enhance sample dissolution. The solutions were all stored at 4°C and protected from light. Working solutions of concentration range between 1 and 500 $\mu g/mL^{-1}$ were obtained daily by dilution of the corresponding stock solution before analysis. A 120 µM BSA stock solution was daily prepared by dissolving an appropriate BSA with 25 mM sodium borate buffer (125 mM sodium borate: 100 mM boric acid, 1:4, v/v, pH 7.4) and stored at 4°C. Working BSA solutions (0–15 µM) were obtained by diluting BSA stock solutions with sodium borate buffer. Ultra pure water (18.2 MΩ) obtained from a Millipore-Q System was used to prepare the solutions. All solutions were filtered through 0.45 µm filters and stored at 4°C.

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Procedures

A new capillary was rinsed for 10 min with 1 M NaOH, 10 min water, and 10 min with buffer. In between two injections, the capillary was rinsed for 2 min with 0.1 M NaOH solution, 2 min with water, and 2 min with buffer, all at 20 psi. Pressure injections were performed at 0.5 psi for 5 s. The wavelength was performed at 280 nm. The separation was done under the voltage of +20 kV. All separations were performed in triplicate. RSD of migration time (t_m) and peak area were calculated from a series of three experiments carried out with the same sample. Fluorescence measurements were carried out by keeping the concentration of BSA fixed at 2×10^{-6} M in 25 mM sodium borate buffer (pH 7.4), that of flavonoids was varied from 0 to 20×10^{-6} M. Fluorescence spectra were recorded at 298 K in the range of 300–550 nm upon excitation at 280 nm. The UV–Vis absorption spectra of the flavonoids, BSA, and the BSA-flavonoids mixtures were measured from 250 to 400 nm at 298 K.

Theory of the Estimation of Binding Constants by ACE Method

In the mobility shift (μ) assay of ACE, the $\Delta\mu_D^P$ as a function of the concentration C_P of protein yields K_b for Scatchard analysis.[23]

$$\Delta\mu_D^P / C_P = K_b \Delta\mu_D^{P^{max}} - K_b \Delta\mu_D^P \tag{1}$$

Where $\Delta\mu_D^P$ is the change in the mobility of the drug in the presence of various concentration of the protein (C_P), and $\Delta\mu_D^{P^{max}}$ is the mobility of the drug when it is saturated with the protein.

In practice, there were limitations to the method of analysis as protein was added in the buffer. The EOF and the viscosity of the buffer could change with the addition of protein and caused a large discrepancy of K_b. Therefore, Gomez et al.[26] proposed use of M to estimate K_b and calculated K_b of carbonic anhydrase B with vancomycin by the ACE method. They described that the M was independent, theoretically, of such factors as voltage, capillary length, and solution viscosity. Zhou[18] deduced the K_b between BSA and ferulic acid using the M and the μ assays and found that the K_b values from the two methods exists as a considerable discrepancy. To determine which assay was more accurate, they carried out SPR measurements and found that the K_b of SPR measurements agreed well with that of the M assay. Therefore, they considered that the M assay might eliminate the effects of EOF and viscosity and should be more reliable.

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In this study, a Scatchard equation obtained by Gomez[26] was performed on a slightly modify reference to double reciprocal analysis method and illustrated by Equation (2).

$$1/\Delta M_D^p = [1/(K_b \Delta M_{max}) \cdot 1/C_p] + 1/\Delta M_{max} \quad (2)$$

Here ΔM_D^P is the magnitude of the change in M as a function of the concentration of protein. A Scatchard analysis was performed using Equation (2) to obtain the estimated K_b by plotting $1\ \Delta_D^P$ *versus* $1/C_p$ to give a straight line with intercept $1/\Delta M_{max}$ and slope $1/(K_b\Delta M_{max})$. K_b was calculated by the use of ratio of the intercept *versus* slope.

RESULTS AND DISCUSSION

Effect of Buffer Type, pH and Borate Concentration on Separation

In our experiments, effect of buffer type, pH, and sodium borate concentration on separation were investigated. All buffers, such as HEPES, sodium phosphate, Tris-HCl, and sodium borate concentration were 25 mM and pH was 7.4. As shown in Figure 2a, using HEPES or sodium phosphate, there was an inadequate separation between EC and EGCG. Using Tris-HCl buffer, all analytes displayed a peak broadening and asymmetry. Sodium borate buffer provided the better separation efficiency, resolution, and sensitivity.

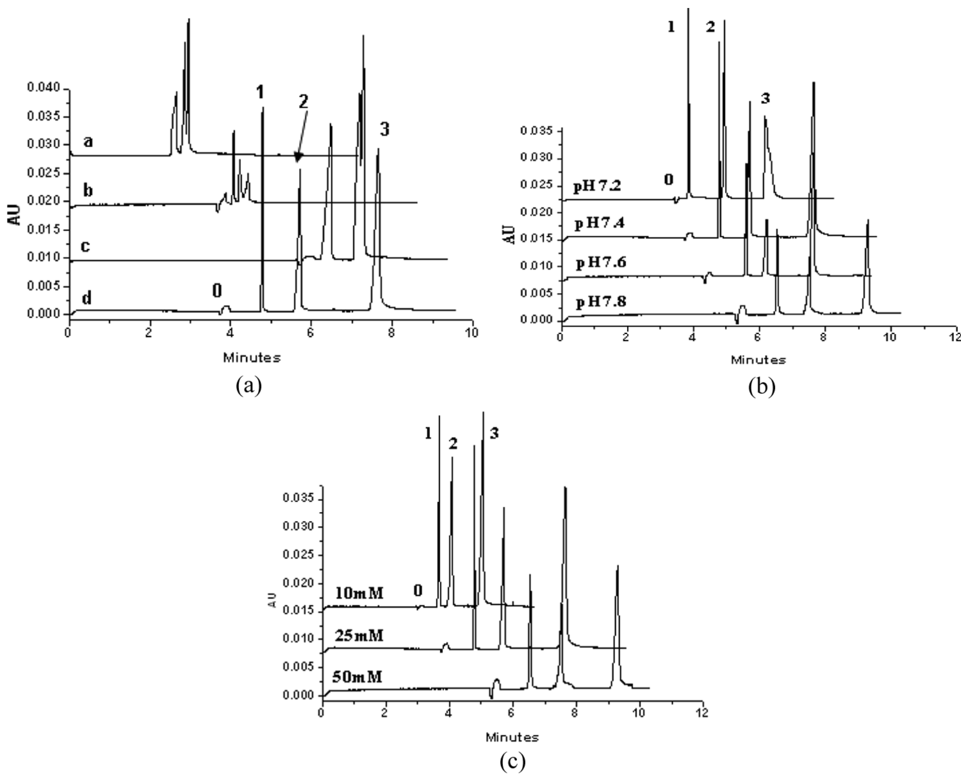

FIGURE 2 Electropherograms on the effect of buffer type at pH 7.4 (a), effect of pH with 25 mM borate buffer (b) and effect of borate concentration at pH 7.4 (c) on separation. (a) a-25 mM HEPES; b-25 mM Tris-HCl; c-25 mM sodium phosphate; d-25 mM sodium borate. Injection, 0.5 psi × 5 s; separation voltage, 20 kV; capillary temperature, 298 K; UV detection at 280 nm. Peak representation 0 = EOF; 1 = P, 2 = EC, 3 = EGCG.

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The effect of pH was studied by varying it from 7.2 to 7.8 fixed sodium borate concentrations (25 mM). As can be seen from Figure 2b, the t_m is increased with an increase of pH. At pH 7.2, these compounds could be baseline separated; however, EGCG displayed peak tailing. The t_m became longer and sensitivity decreased at pH 7.6–7.8. Thus, pH 7.4 sodium borate was selected as the buffer. In addition, effect of the concentration of the sodium borate buffer (pH 7.4) was shown in Figure 2c. The results indicated that the t_m increased with an increase of the concentration of the buffer. Although the low concentration sodium borate led to short t_m, moderate concentration buffers were desirable for the suppression of interaction between protein and silianol groups on the capillary wall. Considering the t_m, the resolution, sensitivity, and concentration of buffer, 25 mM sodium borate was chosen as the buffer to alleviate the adsorption of proteins onto the capillary wall.

TABLE 1 The Calibration Equations, Concentration Range, Repeatability (RSD) of t_m and Peak Area, LOD and Recovery for the Studied Flavonoids

Analytes	Regression Equation[a]	R	Concentration Range (μg/mL^{-1})	RSD (%) (t_m)	RSD (%) (area)	LOQ (μg/mL)	Recovery (%) (RSD)
P	Y = 380.7X + 2088	0.994	5–500	0.36	4.06	3	101.4 (2.06)
EC	Y = 247.8X + 2723	0.999	5–500	0.57	2.68	3	99.6 (1.56)
EGCG	Y = 546.6X + 6653	0.998	10–500	0.63	3.11	8	100.2 (1.89)

[a]X and Y in the regression equations were at μg/ml and AU corresponding to the concentration and peak area, respectively.

Validation of the Method

Under the optimized conditions, a series of concentrations of flavonoids were tested to determine the linearity. The linear range, regression equation, correlation coefficient (R), LOD and recovery were listed in Table 1. Good linear relationships were obtained in the concentration range of $5.0 - 500\,\mu g/mL^{-1}$ (the concentration range of this research) for P, EC, and $10.0 - 500\,\mu g/mL^{-1}$ for EGCG. The linearity was satisfactory with the R for all flavonoids greater than 0.994. The LOD was $3\,\mu g/mL^{-1}$ for P and EC, and $8\,\mu g/mL^{-1}$ for EGCG. The electropherograms of the flavonoids and the flavonoids at the LOD level were illustrated in Figure 3.

The repeatability was estimated by repetitive determination ($n = 5$) of $80\,\mu g/mL^{-1}$ analytes under the same conditions. The RSD of t_m were between 0.36% and 0.63%, and RSD of the peak area were between

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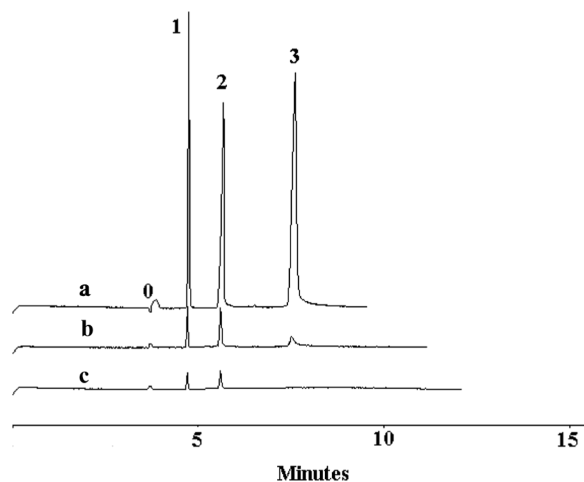

FIGURE 3 Electropherogram of flavonoids at $80\,\mu g/mL^{-1}$ (a), and electrophoregram of flavonoids at the LOD level ($8\,\mu g/mL^{-1}$) for EGCG (b), and electrophoregram of flavonoids at the LOQ level ($3\,\mu g/mL^{-1}$) for P and EC (c) using the running buffer of 25 mM brote (pH 7.4). Other conditions are the same as in Figure 2.

2.68% and 4.06%. The interday precision was evaluated by analyzing 80 μg/ mL^{-1} analytes on five different days, and the RSD of t_m and peak area were less than 2.01% and 8.79%, respectively. The mean recoveries of the analytes were between 99.6% and 101.4% with the RSD less than 2.06% by the standard addition method. These data indicated that the proposed method was precise and reliable.

Estimation of the Binding Constants by ACE

The binding constants of the three flavonoids with BSA could be estimated simultaneously during ACE process. The t_m of each flavonoids were measured in the present of different BSA concentrations of the buffers. A set of electropherograms of the analytes versus BSA concentration change at 310 K were shown in Figure 4. A negative peak was observed indicating the migration of methanol in the buffer. Therefore, the negative peak was regarded as an EOF marker. Intuitively, an increase of the BSA concentration could lead to an increase of the t_m. The peak shapes gradually became asymmetry as the BSA concentrations increased. Broadening of the EC and EGCG peak shape began to appear at 9 μM BSA and became the serious leading peak more than 15 μM for EGCG. Hence, we selected

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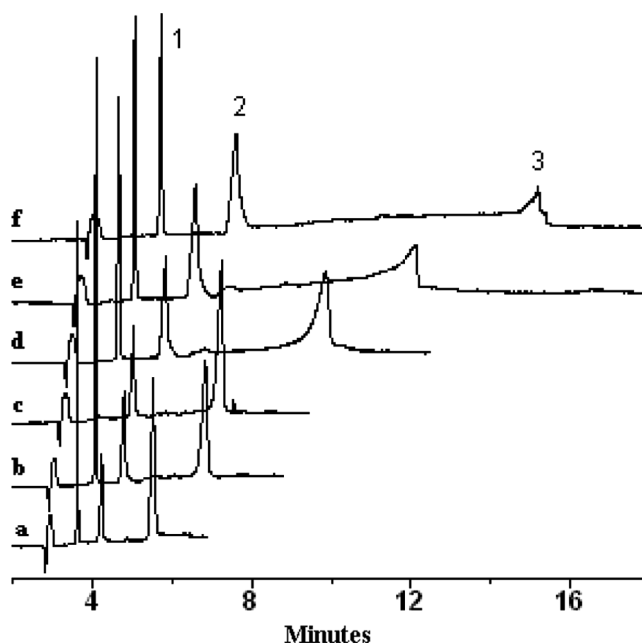

FIGURE 4 Electropherograms of the t_m shift of the flavoniods *versus* BSA concentration change at 310 K. *a, b, c, d, e, f* represent BSA concentration of 0, 3.0, 6.0, 9.0, 12, 15×10^{-6} M, respectively. Other conditions are the same as in Figure 2.

TABLE 2 Binding Constants and Quenching Constants of Flavonoids to BSA

Flavonoids	T/K	$K_{Ref.}{}^{a}/10^4\,M^{-1}$	$^{A}K_b/10^4\,M^{-1}$	$K_{sv}/10^4\,M^{-1}$	$K_Q/10^{12}\,M^{-1}s^{-1}$	$^{F}K_b/10^4\,M^{-1}$	n
P	288.00	2.48(18°C)[24]	4.648				
	293.00	1.13[25]	4.057				
	298.00	5.04[26]	3.092	4.074	4.074	1.575	0.86
	303.00	1.54[25]	2.238				
	310.00	2.22[24]	1.333				
EC	288.00		3.526				
	293.00		2.658				
	298.00		2.051	3.926	3.926	1.116	0.84
	303.00		1.624				
	310.00		0.9113				
EGCG	288.00		6.361				
	293.00		4.826				
	298.00		3.845	6.708	6.708	1.795	0.85
	303.00		2.924				
	310.00		1.684				

[a]Reported reference values using fluorescence method.
$^{A}K_b$ and $^{F}K_b$ are binding constants obtained by ACE and Fluorescence methods, respectively.

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BSA concentration between 0 and 15 μM to study interaction of flavonoids and BSA.

The obtained K_b values for three flavonoids with BSA by ACE methods were listed in Table 2. At 298 K, the K_b values of $3.092 \times 10^4\,M^{-1}$, $2.051 \times 10^4\,M^{-1}$ and $3.845 \times 10^4\,M^{-1}$ for P, EC, and EGCG, respectively, with correlation coefficients R^2 greater than 0.9840.

Fluorescence Studies

In order to obtain more information about the structural perturbation of BSA, fluorescence measurements, the synchronous fluorescence and UV-Vis absorption spectra were performed. The interaction of flavonoids with BSA was evaluated by monitoring the intrinsic fluorescence intensity changes of BSA upon addition of flavonoids.

Characteristics of the Fluorescence Spectra

When different amounts of EGCG solution were added with a fixed concentration of BSA, a gradual decrease in the fluorescence intensity of BSA was observed (the results are provided in Figure 5a) in the present study. These observations can be rationalized in terms of interactions between EGCG and BSA and formation of complexes between the two analytes. The P and EC have also taken place in the same experimental

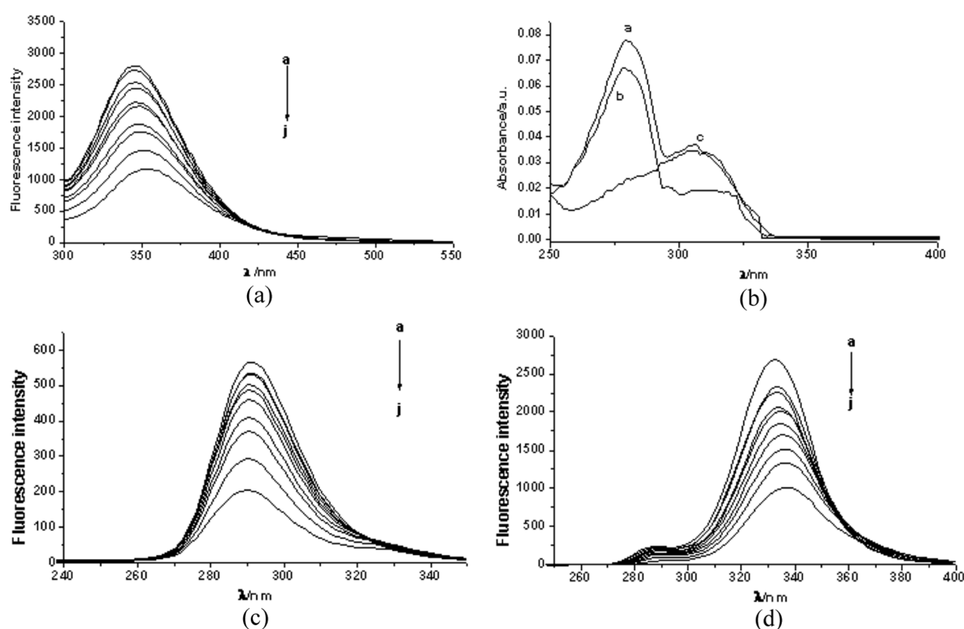

FIGURE 5 (a) Fluorescence spectra of the BSA–EGCG system. The concentration of BSA was 2.0×10^{-6} M and EGCG concentration increased from 0 to 20×10^{-6} M at 298 K. The arrow indicates the increase in EGCG concentration. (b) The UV absorption spectrum of BSA in the absence and presence of EGCG. a—BSA-EGCG, b—BSA, c—EGCG. Synchronous fluorescence spectra under similar conditions (c) keeping $\Delta\lambda = 15$ nm, (d) keeping $\Delta\lambda = 60$ nm. BSA and EGCG were at the same concentration (2.0×10^{-6} M).

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phenomena. Fluorescence quenching can be described by the Stern–Volmer equation:[27]

$$F_0/F = 1 + K_Q\tau_0[Q] = 1 + K_{SV}[Q] \quad (5)$$

where F_0 and F are the steady-state fluorescence intensities in the absence and presence of the quencher, respectively. K_Q is the quenching rate constant and K_{SV} is the Stern–Volmer dynamic quenching constant. $[Q]$ is the concentration of the quencher, and τ_0 is about 10^{-8} s for most biomolecules, and represents the average lifetime of the biomolecules without quencher.[28] The regression equation of EGCG was shown in Figure 6a and the Stern–Volmer quenching constants (Table 2) calculated from the slope of the regression curve were all greater than 2.0×10^{10} $\text{Lmol}^{-1}\,\text{s}^{-1}$, indicating that the fluorescence quenching was mainly arisen from static quenching by complex formation instead of dynamic quenching.[29] To clarify the probable fluorescence quenching mechanism of BSA by flavonoids, the UV-Vis absorption spectra of BSA, the flavonoids and the mixture of BSA-flavonoids at the same concentration were recorded. The obvious enhancement of absorbency intensity (A) and the change of

absorption spectra also verified the formation of a new complex between BSA and EGCG, as shown in Figure 5b. P and EC had also the same phenomena. These experimental results also showed that the possible quenching mechanism was static and forms the complex between flavonoids and BSA.

To explore the conformation change of BSA by the addition of EGCG, we measured synchronous fluorescence spectra of BSA with various amounts of EGCG. The synchronous fluorescence spectra gave information about the molecular environment in the vicinity of the chromophoremolecules.[30,31] The effect of EGCG on BSA synchronous fluorescence spectroscopy was shown in Figures 5c–d. There was no significant change in emission maxima of BSA upon addition of EGCG. A weak blue shift (−1.2 nm) and a red shift (2 nm) could be observed in Figures 5c–d, respectively. The blue shift led to increased hydrophobicity of tyrosine (Tyr), red shift was made to decrease the polarity of tryptophan (Try), but Tyr residues intensity was less clear than Try residues. It indicated that red shift played a decisive role and brought about the increase of the polarity. The observations suggested that a change in the conformation of BSA took place and EGCG molecules were only located at close proximity to Try residues. It confirmed that the interaction of EGCG with BSA did affect somewhat the conformation of Try residue microregions. As can be obtained from the experimental results, the maximum emission wavelength (λ_{max}) of P shifted 2 nm to red light and the λ_{max} of EC had no change when $\Delta\lambda = 60$ nm, however, the λ_{max} of P and EC had no significant change when $\Delta\lambda = 15$ nm. These results revealed that the conformation of BSA had changed when BSA binds to P. But no conformation change occurred when BSA bounded to EC.

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Calculated Apparent Binding Constant and Binding Sites

When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules could be described by the equation:[32]

$$\log(F_0 - F)/F = \log K_b + n\log[Q] \quad (6)$$

Where K_b is the binding constant, and n is the number of binding sites. After the fluorescence quenching intensities on BSA at 346 nm were measured, the double logarithm algorithm was assessed by Equation (6)

The plot of $\log(F_0 - F)/F$ versus $\log[Q]$ for a representative system of flavonoids, EGCG-BSA was shown in Figure 6b. The values of K_a and n were summarized in Table 2. It was noticed that the binding constant values were all at about $10^4\,M^{-1}$ level between the flavonoids and BSA.

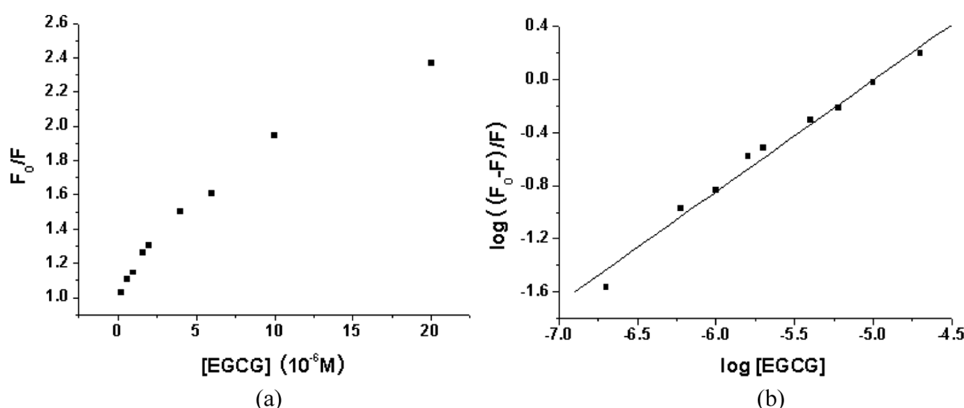

FIGURE 6 Stern–Volmer plot for the binding of BSA–EGCG at 298 K (a) and plot of $\log(F_0 - F)/F$ vs. $\log[Q]$ with the concentration of EGCG to BSA molar ratios ranged from 0 to 10.0 (b).

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The obtained value of n for flavonoids-BSA were noticed to be almost unity indicating that there was one independent class of binding sites on BSA for three flavonoids.

From ACE and fluorescence experimental results, it could be seen that the binding affinity of BSA to EGCG was stronger than that of BSA to P and EC. It could be explained by the difference of the chemical structure. All molecules of EGCG, P, and EC have a hydroxyl group and ketone group for P and EGCG. EGCG, P, and EC have eight, five, and four hydroxyl groups, respectively. The hydroxyl group in a drug molecule could bind with the polypeptide chain of BSA by forming hydrogen bonds.[33] So the binding affinity of BSA to EGCG, which had more hydroxyl groups and more chances to form hydrogen bonds, was stronger than that of P and EC. Comparison of the binding constants of the three flavonoids, revealing the order EGCG > P > EC, also allowed the conclusion that the hydroxyl group made an important contribution to the binding process. Furthermore, comparison was carried out between the results of this study and others published by fluorescence method.[20,22] The slight difference in binding constants between ACE and fluorescence methods may be resulted from the following two reasons. First, the principles and experimental conditions of these methods for determination of the binding constant were different. Second, it is common that the results of different plotting methods are not identical because the transformation of variables before regression analysis inevitably will result in a weighting of data.

Determination of Thermodynamic Parameters

The thermodynamic parameters, ΔH, ΔS, and ΔG of the reaction, are important for presumption and confirming binding mode of

TABLE 3 Thermodynamic Parameters of Interactions Between the Flavonoids and BSA Obtained by ACE

Flavonoids	Linear Equations	R	ΔH/kJ mol^{-1}	ΔS/J mol^{-1} K^{-1}	ΔG/298 K, kJ mol^{-1}
P	$Y = 5.143 \times 10^3 X - 7.004$	0.9837	−42.76	−57.53	−25.62
EC	$Y = 5.323 \times 10^3 X - 7.970$	0.9907	−44.26	−65.97	−24.60
EGCG	$Y = 5.255 \times 10^3 X - 7.138$	0.9908	−43.69	−58.84	−26.16

drug-protein.[34] Therefore, in order to further characterize the acting forces between flavonoids and BSA, the temperatures dependent thermodynamic parameters were analyzed. Considering that ΔH did not vary significantly over the temperature range studied, the thermodynamic parameters could be evaluated using the following equations:

$$ln\, K_b = (-\Delta H/RT) + \Delta S/R \quad (7)$$

$$\Delta G = -RTLn\, K_b \quad (8)$$

$$\Delta S = (\Delta H - \Delta G)/T \quad (9)$$

Here K_b is the binding constant and R is the gas constant. The binding studies were carried out at 288, 293, 298, 303, and 310 K, since there was not any structural degradation of BSA in this temperature range. The K_b had been used in the Van't Hoff Equation (7), and a plot of $ln\, K_b$ versus 1/T provided ΔH associated with the interactions. The values for ΔG and ΔS could then be obtained from Equations (8) and (9).

The obtained thermodynamic parameters for the binding of flavonoids with BSA at 25°C by ACE method were listed in Table 3. Van der Waals interaction was the most important factor contributing to the observed negative ΔH and ΔS and, hence, to the stability of protein association complex. Negative ΔH value was also observed whenever there was hydrogen bonding in the protein drug binding and the binding reaction was an exothermic process.[34] The negative ΔH values obtained could not be attributed to electrostatic interactions since for electrostatic interactions, ΔH was very small, almost zero.[34] As a result, we concluded that both van der Waals interaction and hydrogen bonds played major roles in the binding processes of the three flavonoids to BSA.

CONCLUSION

Natural products attract increasing attention because of their reliable therapeutic effects. It is becoming important to study the interactions of the active components of natural products with biological molecules. Such

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studies can help people to understand pharmacokinetic and pharmacodynamic properties of natural products before clinical studies. In the present work, the interactions of three flavonoids to BSA were carried out employing ACE and the fluorescence method. The results of these two methods were similar with each other and might gain some information of flavonoids binding to serum protein. Results also showed that both van der Waals interaction and hydrogen bonds played pivotal roles in the binding process of these flavonoids to BSA. Compared to fluorescence and other methods, the ACE approach is simple, highly efficient, and high throughout, and a cost effective method for the study of drug protein interactions, especially suitable for the actual samples of natural products.

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