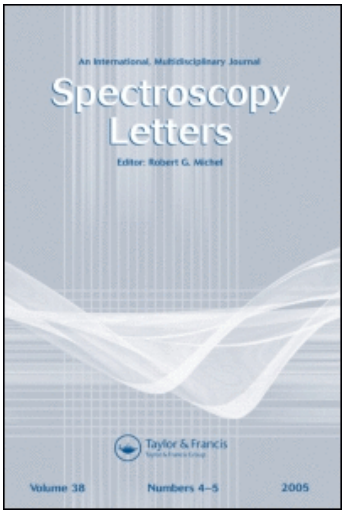

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Interaction Between Isoquercitrin and Bovine Serum Albumin by a Multispectroscopic Method

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ABSTRACT The interaction of isoquercitrin and bovine serum albumin (BSA) was investigated by means of fluorescence spectroscopy (FS), resonance light scattering spectroscopy (RLS), and ultraviolet spectroscopy (UV). The apparent binding constants (K_a) between isoquercitrin and BSA were $5.37 \times 10^5\,L\,mol^{-1}$ (293.15 K) and $2.34 \times 10^5\,L\,mol^{-1}$ (303.15 K), and the binding site values (n) were 1.18 ± 0.03. According to the Förster theory of non-radiation energy transfer, the binding distances (r) between isoquercitrin and BSA were 1.94 and 1.95 nm at 293.15 K and 303.15 K, respectively. The experimental results showed that the isoquercitrin could be inserted into the BSA, quenching the inner fluorescence by forming the isoquercitrin–BSA complex. The addition of increasing isoquercitrin to BSA solution leads to the gradual enhancement in RLS intensity, exhibiting the formation of the aggregate in solution. It was found that both static quenching and non-radiation energy transfer were the main reasons for the fluorescence quenching. The entropy change and enthalpy change were negative, which indicated that the interaction of isoquercitrin and BSA was driven mainly by van der Waals interactions and hydrogen bonds. The process of binding was a spontaneous process in which Gibbs free energy change was negative.

KEYWORDS Bovine serum albumin, fluorescence spectroscopy, interaction, isoquercitrin, resonance light scattering

INTRODUCTION

The interaction between bio-macromolecules and drugs has attracted great interest among researchers for several decades.[1–3] Among bio-macromolecules, serum albumins are the major soluble protein constituents of the circulatory system and have many physiologic functions.[4,5] Bovine serum albumin (BSA) has been one of the most extensively studied of proteins particularly because of its structural homology with human serum albumin (HSA).[6] The interaction between protein and drug molecules results in formation of a stable protein–drug complex, which may

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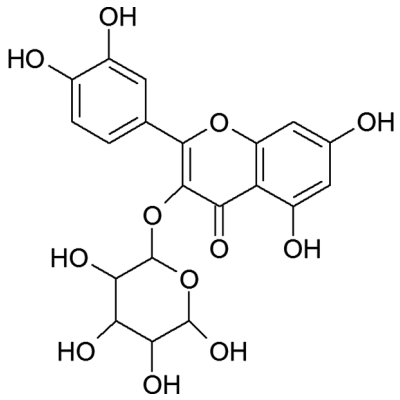

FIGURE 1 Structure of isoquercitrin.

be considered as a model for gaining general fundamental insights into drug–protein binding.[7–10]

Isoquercitrin, quercetin 3-O-glucoside (Fig. 1), is a flavonoid purified from the medicinal plant *Bridelia ferruginea*,[11] *Amelanchier alnifolia*,[12] and *Pelargonium radula*.[13] Isoquercitrin has a strong antimicrobial activity,[13] antioxidant activity,[14] and the inhibitory effects of flavonoids on free radical–induced hemolysis.[15]

Fluorescence spectroscopy is an appropriate method to determine the interaction between the small molecule ligand and bio-macromolecule.[3–10] From measurement and analysis of the emission peak, the transfer efficiency of energy, the lifetime, fluorescence polarization, and so forth, a vast amount of information will be yielded on the structural fluctuations and the microenvironment surrounding the fluorophore in the macromolecule.

Resonance light scattering (RLS), an elastic scattering, occurs when an incident beam is close to an absorption band. In recent years, RLS has been used to determine pharmaceutical and various biomacromolecules.[16–21] However, study of the interaction of isoquercitrin with BSA by RLS has not been reported. In this work, the binding reactions between isoquercitrin and BSA were investigated using multispectroscopic method, and the apparent binding constants and binding site values were also measured.

MATERIALS AND METHODS

Apparatus

Fluorescence and resonance light scattering spectra were obtained on a JASCO FP-6500 spectrofluorometer equipped with a thermostated cell compartment using quartz cuvettes (1.0 cm) (Tokyo, Japan). The UV-Vis spectra were obtained on a UV-2450 spectrophotometer using quartz cuvettes (1.0 cm) (Shimadzu, Kyoto, Japan). The pH measurements were carried out on a PHS-3C Exact Digital pH meter equipped with Phonix Ag–AgCl reference electrode (Cole-Paemer Instrument Co. IL, USA), which was calibrated with standard pH buffer solutions.

Reagents

A working solution of isoquercitrin (0.5×10^{-3} mol L^{-1}) was prepared with methanol–water solution (1:1, v/v). BSA (fraction V) was purchased from Sigma (St. Louis, MO, USA). A working solution of BSA (1.0×10^{-5} mol L^{-1}) was prepared with doubly distilled water and stored in a refrigerator. A working solution of Tween-80 (10.0 mg mL^{-1}) was dissolved with water and used as a stabilizer. Tris base (no less than 99.5% pure) was obtained from Serva (New York, USA). Tris-HCl buffer solution (0.20 mol L^{-1}) was used to keep the pH of the solution at 7.4. All other reagents and solvents were of analytical reagent grade and used without further purification unless otherwise noted. All aqueous solutions were prepared using fresh double-distilled water.

Fluorescence and Ultraviolet Spectra

Appropriate quantities of isoquercitrin solution (0.5×10^{-3} mol L^{-1}) were transferred to a 10-mL flask, and then 1.0 mL BSA solution was added and diluted to 10 mL with water. The resultant mixture was subsequently incubated at 293.15 K or 303.15 K for 10 min. The solution was scanned on the fluorophotometer in the region of 290 to 500 nm. The width of excitation and emission slits was set at 3.0 nm. The fluorescent intensity at 340 nm was determined with the excitation wavelength of 280 nm. The operations were carried out at fixed temperature (293.15 K or 303.15 K).

The UV spectra was obtained by scanning the solution on the spectrophotometer in the region of 220 to 400 nm with 1-cm quartz cells. The operations were carried out at room temperature.

Resonance Light Scattering

An appropriate aliquot of BSA working solution was added to 1.0 mL isoquercitrin working solution

and 1.0 mL Tween-80 aqueous solution and diluted to 10 mL with water. RLS spectra were obtained by synchronous scanning in the wavelength region from 250 to 750 nm on the spectrofluorophotometer. The width of excitation and emission slits was set at 3.0 nm. The operations were carried out at room temperature.

RESULTS AND DISCUSSION

Characteristics of the Fluorescence Spectra

Figure 2 shows the emission spectra of BSA in the presence of various concentrations of isoquercitrin. The fluorescence intensity of BSA decreased regularly with the increasing concentration of isoquercitrin, and there was significant λ_{em} blue shift with the addition of isoquercitrin, which indicated that isoquercitrin can quench the inner fluorescence of BSA and that the interaction between isoquercitrin and BSA has occurred. Quenching can occur by different mechanisms, usually classified as dynamic quenching and static quenching. Dynamic and static quenching can be distinguished by their different dependence on temperature and excited-state lifetime. Higher temperatures will result in faster diffusion and hence larger amounts of collisional quenching, and higher temperatures will typically result in the dissociation of weekly bound complexes and hence smaller amounts of static quenching. For the dynamic quenching, the mechanism can be described by the Stern–Volmer equation:[22,23]

$$F_0/F = 1 + K_q\tau_0\,[Q] = 1 + K_{SV}\,[Q] \qquad (1)$$

where F_0 and F represent the fluorescence intensities in the absence and in the presence of quencher, K_q is the quenching rate constant of the biomolecule, K_{SV} is the dynamic quenching constant, τ_0 is the average lifetime of the molecule without quencher, and [Q] is the concentration of the quencher. To confirm the quenching mechanism, the procedure of the fluorescence quenching was first assumed to be dynamic quenching.

Figure 3 displays the Stern–Volmer plots of the quenching of BSA by isoquercitrin at different temperatures (293.15 K and 303.15 K). From the experimental data, the corresponding dynamic quenching constants for the interaction between isoquercitrin and BSA were $K_{SV} = 5.57 \times 10^4\,L\,mol^{-1}$ (293.15 K, R = 0.9961), and $K_{SV} = 5.54 \times 10^4\,L\,mol^{-1}$ (303.15 K, R = 0.9980), respectively. Because the fluorescence lifetime of the biopolymer is 10^{-8} s,[24–26] the quenching constants K_q at 293.15 K and 303.15 K were calculated to be 5.57×10^{12}, and $5.54 \times 10^{12}\,L\,mol^{-1}\,s^{-1}$, respectively.

According to the literature,[26,27] for dynamic quenching, the maximum scatter collision quenching constant of various quenchers with the biopolymer is $2.0 \times 10^{10}\,L\,mol^{-1}\,s^{-1}$, and the K_{SV} increases with increasing temperature. Considering that in our experiment the rate constant of the protein

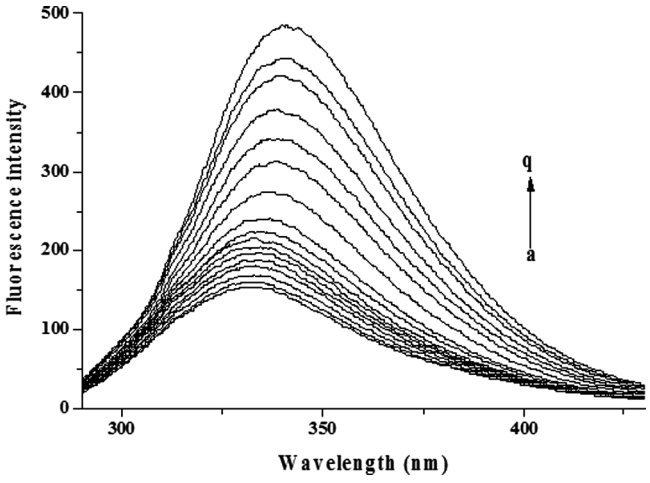

FIGURE 2 The quenching effect of isoquercitrin on BSA fluorescence. λ_{ex} = 280 nm, (a–q) BSA, 1.0×10^{-5} mol L^{-1}: 0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00, 3.25, 3.50, 3.75, 4.00 ($\times 10^{-5}$ mol L^{-1}) isoquercitrin.

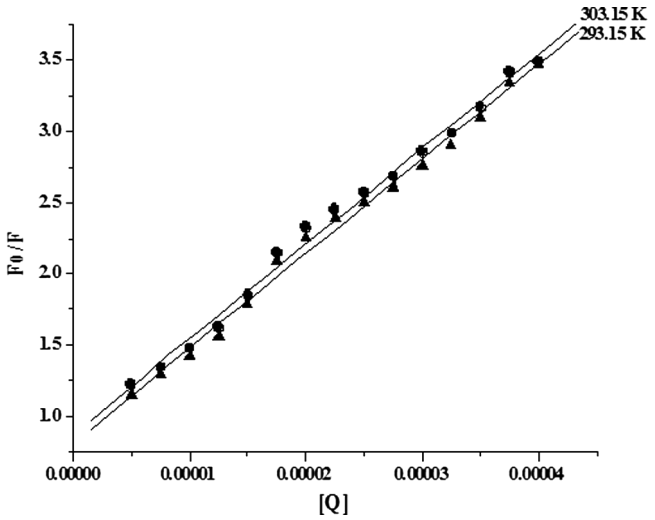

FIGURE 3 The Stern–Volmer curves of fluorescence quenching of BSA by isoquercitrin.

quenching procedure initiated by isoquercitrin is much greater than $2.0 \times 10^{10}\,L\,mol^{-1}\,s^{-1}$ and that the K_{SV} decreased with increasing temperature, it can be concluded that the quenching is not initiated by dynamic quenching but probably by static quenching resulting from the formation of isoquercitrin–BSA complex.

Characteristics of the RLS Spectra

The RLS spectrum of isoquercitrin–BSA in Tris-HCl buffer solution ($0.020\,mol\,L^{-1}$) is shown in Figure 4. It can be seen that the RLS intensity of isoquercitrin is quite weak in the whole scanning wavelength region (Fig. 4a). In contrast, upon addition of trace amount of BSA to isoquercitrin solution, a remarkably enhanced RLS with a maximum peak at 569.5 nm was observed under the same conditions (Figs. 4b–g).

It can be clearly observed that there were two peaks located at 452.0 and 569.5 nm in the RLS spectra of isoquercitrin-BSA system. The addition of increasing BSA to the isoquercitrin solution leads to the gradual enhancement in RLS intensity, exhibiting a concentration-dependent relationship. The RLS intensity correlates with the formation of the aggregate and its particle dimension in solution. Bearing this point in mind, it is inferred from the results that the added BSA may react with isoquercitrin, forming a new isoquercitrin–BSA complex that could be expected to be an aggregate. The newly formed isoquercitrin–BSA complex may be ascribed to the higher electrostatic attraction between isoquercitrin and BSA. Moreover, the dimension of isoquercitrin–BSA particles may be much less than the incident wavelength, and thus the enhanced light-scattering signal occurs under the given conditions.

Binding Constant and Binding Sites

For static quenching, the relationship between fluorescence quenching intensity and the concentration of quenchers can be described by the binding constant equation:[27]

$$\lg[(F_0 - F)/F] = \lg K_a + n \lg[Q] \qquad (2)$$

where K_a is the binding constant, and n is the number of binding sites per BSA. After the fluorescence quenching intensities on BSA at 340 nm were measured, the double-logarithm algorithm was assessed by Eq. (2).

Figure 5 shows the double-logarithm curve, and Table 1 gives the corresponding calculated results. The apparent binding constants (K_A) between isoquercitrin and BSA were $5.37 \times 10^5\,L\,mol^{-1}$ (293.15 K) and $2.34 \times 10^5\,L\,mol^{-1}$ (303.15 K). The binding site value (n) was 1.18 ± 0.03. The correlation coefficients are larger than 0.99, indicating that the interaction between isoquercitrin and BSA agrees well with the site binding model underlying Eq. (2). The result illustrated that there is a strong binding force between isoquercitrin and BSA.

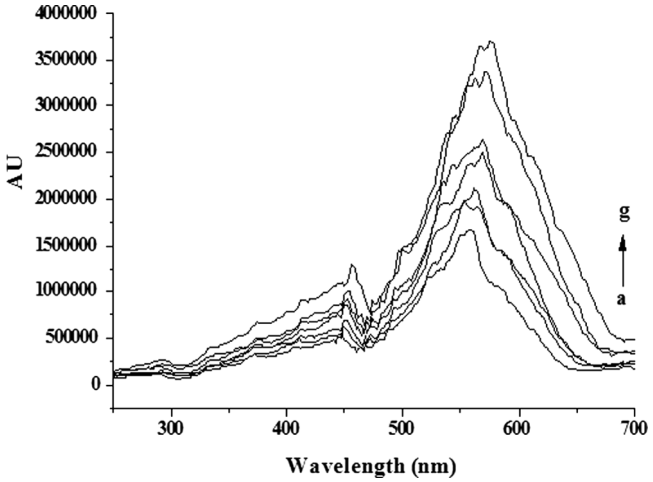

FIGURE 4 RLS spectra of five isoquercitrin–BSA systems: (a–g) isoquercitrin solution, $1.0 \times 10^{-4}\,mol\,L^{-1}$ and Tween-80 ($1.0\,mg\,mL^{-1}$): 0.0, 0.5, 1.0, 1.5, 2.0, $2.5 \times 10^{-6}\,mol\,L^{-1}$ BSA.

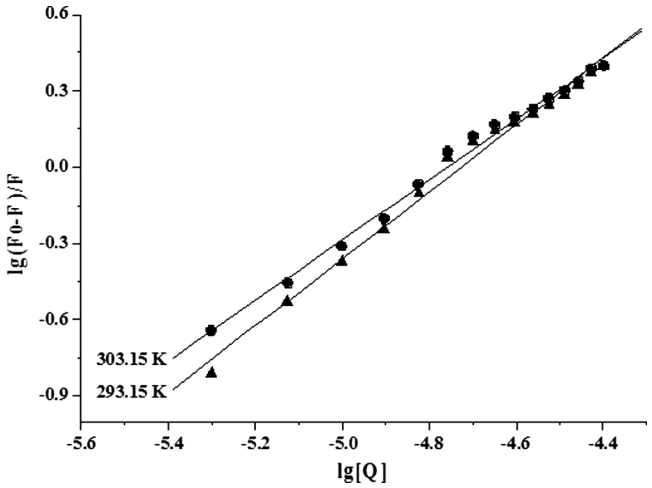

FIGURE 5 Double-log plot of isoquercitrin quenching effect on BSA fluorescence at different temperatures.

TABLE 1 The Binding Parameters for the System of Isoquercitrin–BSA

Temp./K	Binding constant/L mol^{-1}	Binding site	Correlation coefficient
293.15	5.37×10^5	1.21	0.9941
303.15	2.34×10^5	1.18	0.9958

Characteristics of the UV Spectra

Figure 6 shows the ultraviolet absorption spectra of BSA in the presence of various concentrations of isoquercitrin. The BSA solution showed a weak absorption peak at 281.0 nm. The addition of isoquercitrin to the BSA solution leads to two new absorption peaks at 266 nm and 360 nm. The addition of increasing isoquercitrin to the BSA solution leads to the gradual enhancement in UV intensity and exhibits a concentration-dependent relationship.

Binding Distance Between the Drug and the Amino Acid Residues of BSA

According to the Förster non-radiation energy transfer theory,[28,29] the energy-transfer effect is related not only to the distance between the acceptor and donor but also to the critical energy-transfer distance (R_0)

$$E = R_0^6/(R_0^6 + r^6) \tag{3}$$

$$E = 1 - F/F_0 \tag{4}$$

where E is the energy transfer efficiency, R_0 is the critical distance when the transfer efficiency is 50%, and r is the binding distance between donor and acceptor

$$R_0^6 = 8.8 \times 10^{-25} K^2 N^{-4} \Phi J \tag{5}$$

where K^2 is the spatial orientation factor of the dipole, N is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor, and J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor.[30] Therefore,

$$J = (\Sigma I_P(\lambda)\varepsilon_D(\lambda)\lambda^4\Delta\lambda)/(\Sigma I_P(\lambda)\Delta\lambda). \tag{6}$$

Here, $I_P(\lambda)$ is the fluorescence intensity of the fluorescence donor at wavelength λ and $\varepsilon_D(\lambda)$ the molar absorptivity of the acceptor at wavelength λ.

The overlap of the absorption spectrum of isoquercitrin and the fluorescence emission spectrum of BSA is shown in Figure 7. The overlap integral was calculated to be 2.81×10^{-15} cm^3 L mol^{-1} at 293.15 K by integrating the spectra for 300–400 nm in Figure 5. The critical distance, R_0, corresponding with 50% energy transfer from BSA to isoquercitrin,

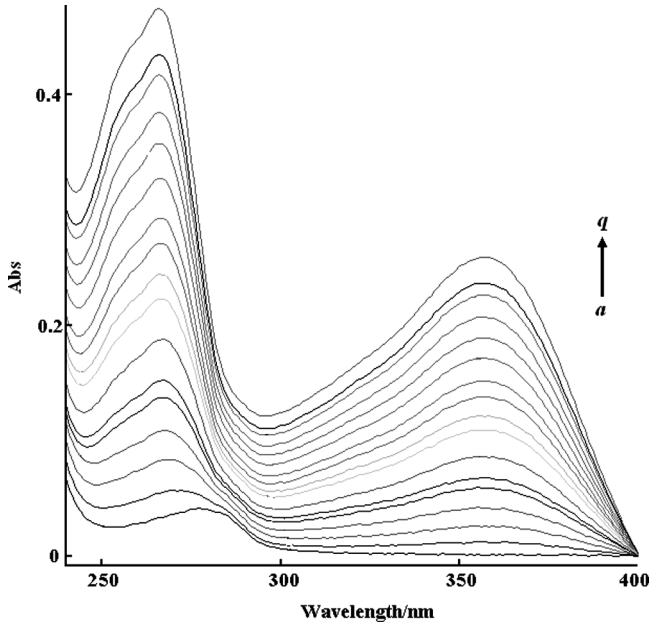

FIGURE 6 UV absorption spectra of isoquercitrin–BSA system. (a–k) BSA, 1.0×10^{-5} mol L^{-1}: 0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00, 3.25, 3.50, 3.75, 4.00 ($\times 10^{-5}$ mol L^{-1}) isoquercitrin.

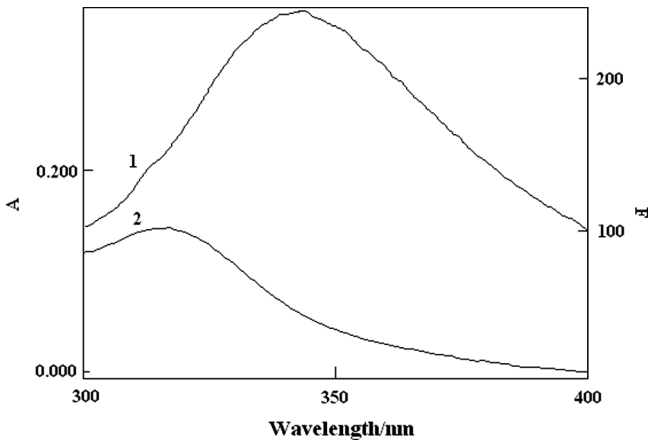

FIGURE 7 Overlap spectra of isoquercitrin UV absorption spectra (1) and BSA's fluorescence emission spectra (2): $C_{BSA} = 1.0 \times 10^{-5}$ mol L^{-1}, $C_{isoquercitrin} = 1.0 \times 10^{-5}$ mol L^{-1}.

was calculated to be 1.98 nm from Eq. (5) when $K^2 = 2/3$, $N = 1.336$, and $\Phi = 0.118$.[28] The binding distance, r, between isoquercitrin and the amino acid residue in BSA was found to be 1.94 and 1.95 nm at 293.15 K and 303.15 K, respectively, which are much smaller than 7 nm, a criterion value for energy transfer phenomenon to occur, suggesting that the energy transfer from BSA to isoquercitrin may occur with high possibility.

Thermodynamic Parameters and Nature of the Binding Forces

The interaction forces between drug and biomolecule may involve hydrophobic forces, electrostatic interactions, van der Waals interactions, hydrogen bonds, and so forth. According to the data of enthalpy change (ΔH) and entropy change (ΔS), a model of interaction between drug and biomolecule can be concluded:[31] (1) $\Delta H > 0$ and $\Delta S > 0$, hydrophobic forces; (2) $\Delta H < 0$ and $\Delta S < 0$, van der Waals interactions and hydrogen bonds; (3) $\Delta H < 0$ and $\Delta S > 0$, electrostatic interactions. To elucidate the interaction of isoquercitrin with BSA, we calculated the thermodynamic parameters from Eqs. (7)–(9). If the temperature does not vary significantly, the enthalpy change (ΔH) can be regarded as a constant. The free energy change (ΔG) can be estimated from the following equation, based on the binding constants at different temperatures:

$$\Delta G = -RT \ln K \tag{7}$$

where R is the gas constant, T is the experimental temperature, and K is the binding constant at the corresponding T. Then the enthalpy change (ΔH) and entropy change (ΔS) can be calculated from Eqs. (8) and (9):

$$\ln K_2/K_1 = [1/T_1 - 1/T_2]\ \Delta H/R \tag{8}$$

where K_1 and K_2 are the binding constant at the experiment temperatures T_1 and T_2, respectively:

$$\Delta G = \Delta H - \mathrm{T}\Delta S \tag{9}$$

The thermodynamic parameters for the interaction of isoquercitrin with BSA are shown in Table 2. The negative sign for ΔG means that the interaction process is spontaneous. The free energy of binding of drugs to BSA consists of two main contributions: (a) from electrostatic interactions and (b) from hydrophobic forces. The negative ΔH and ΔS values indicated that van der Waals interactions and hydrogen bonds may play a major role in the binding between isoquercitrin and BSA.[31]

TABLE 2 The Thermodynamic Parameters of Isoquercitrin–BSA Binding Procedure

Temp./K	ΔH/kJ mol^{-1}	ΔG/kJ mol^{-1}	ΔS/J mol^{-1} K^{-1}
293.15		−32.16	−96.44
303.15	−60.43	−31.19	−96.44

CONCLUSIONS

The binding interactions of isoquercitrin with BSA in dilute aqueous solution were studied using fluorescence spectra, resonance light scattering spectra, and absorbance spectra. The apparent binding constants (K_a) between isoquercitrin and BSA were 5.37×10^5 L mol^{-1} (293.15 K) and 2.34×10^5 L mol^{-1} (303.15 K), and the binding site values (n) were 1.18 ± 0.03. According to the Förster theory of non-radiation energy transfer, the binding distances (r) between isoquercitrin and BSA were 1.94 and 1.95 nm at 293.15 K and 303.15 K, respectively. The entropy change and enthalpy change were negative, which indicated that the interaction of isoquercitrin and BSA was driven mainly by van der Waals interactions and hydrogen bonds. The process of binding was a spontaneous process in which Gibbs free energy change was negative.

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