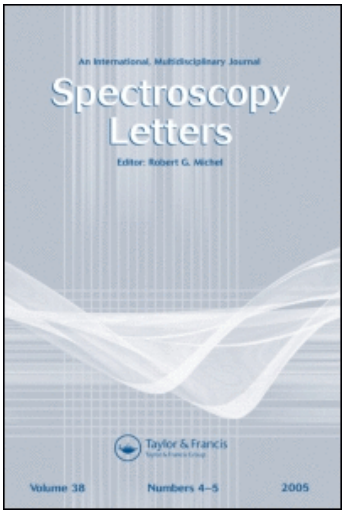

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Studies on the Binding of Barbaloin to Human Gamma Globulin

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Abstract: The binding of barbaloin to human gamma globulin (HGG) was studied *in vitro* under simulated physiological conditions by spectroscopic method including circular dichroism (CD), Fourier transformation infrared (FT-IR) spectroscopy and fluorescence spectroscopy. The binding parameters of HGG to barbaloin were studied from the fluorescence decrease of HGG by the fluorometric titrations in the presence of barbaloin. The Sips plots indicated that the binding of HGG to barbaloin at 296, 304, 310, and 317 K was characteristic of two binding sites with the average affinity constant K_o at 1.152×10^4, 1.022×10^4, 0.9618×10^4, and 0.8937×10^4, respectively. The binding process was exothermic, spontaneous, and entropy driven, as indicated by the thermodynamic analyses, and the major part of binding energy was electrostatic interaction. The secondary structure elements of free HGG and its barbaloin complexes were estimated by the FT-IR spectra and the curve-fitted results of amide I band, which were in agreement with the analyses of CD spectra.

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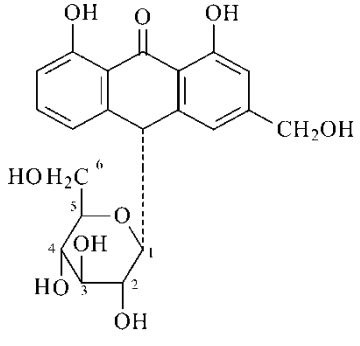
Furthermore, the average binding distance between the donor and the acceptor (3.74 nm) was obtained on the basis of the theory of Förster energy transfer.

Keywords: Barbaloin, circular dichroism (CD), drug, fluorescence quenching, Fourier transformation infrared (FT-IR) spectra, human gamma globulin (HGG), protein

INTRODUCTION

Immunoglobulin (Ig) plays a key role in human immune response.[1] As a familiar drug, intravenous immunoglobulin (IVIG) has been used in the treatment of primary and secondary antibody deficiencies for more than 25 years.[2] Recently, great attention has been paid to IVIG potential use as an adjuvant anti-neoplastic agent. Studies *in vitro* have revealed that IVIGs may stimulate the production of IL-12, an antitumor and anti-angiogenic cytokine, and enhanced NK cell activity.[3] Studies have also revealed that IVIGs can decrease the level of matrix metalloproteinase-9 (MMP-9) expression in the U937 monocyte line with a decrease in the m-RNA level of MMP-9. Matrix metalloproteinases are enzymes that participate in basement membrane degradation, a vital step in the invasion of metastatic cancer cells.[4,5] On the other hand, human immune gamma globulin (HGG) presents in the blood of adults at $9.5 \sim 12.5\,\mathrm{mg/mL}$, and as one of the human plasma proteins, it is capable of binding an extraordinarily diverse range of metabolites, drugs, organic compounds, and relevant antigens.[1,6] With remarkable binding properties, HGG can serve as an important transport proteins (carrier) for drugs. Thus, the study of the binding characteristics of HGG to medical drugs such as barbaloin is very important in drug research and in combination therapy.

Barbaloin (Scheme 1) (BAR; 19(10*H*)-anthracenore, 10-β-D-glucopyranosyl-1, 8-dihydroxy-3-hydroxymethyl) is the main medicinal composition of the Chinese traditional medicine aloe vera that belongs to the liliaceous plant. Its abilities of diminishing inflammation, disinfection, antitumor



Scheme 1. The chemical structure of barbaloin.

action, and immunological enhancement have been studied.[7,8] It is nonspecific and reversible that many of these agents can bind to serum proteins,and the binding affects their pharmacological and pharmacokinetic properties.[6] Therefore, a study on the binding of barbaloin to HGG is very significant. But the interaction of barbaloin with HGG has not been studied.

The binding of barbaloin to serum albumin has been studied,[9] but serum albumin and HGG are different proteins with different binding features to various drugs. In this paper, the binding of barbaloin to HGG was studied *in vitro* under simulated physiological conditions (pH 7.40, ionic strength 0.1) by fluorescence spectroscopy, circular dichroism (CD), and Fourier transformation infrared (FT-IR) spectrometry. The binding parameters and the thermodynamic parameters for the reaction have been calculated according to Sips plots and Gibbs–Helmholtz equation at different temperatures. The secondary structure elements of HGG and its barbaloin complexes have been estimated by FT-IR spectra and the curve-fitted results of amide I band. Furthermore, the average binding distance between barbaloin and HGG was obtained on the basis of the theory of Förster energy transfer.

MATERIALS AND METHODS

Materials

Human gamma globulin (HGG, M_r 150 kDa, purity >99%) was obtained from Sigma-Aldrich Biotechnology Company. Wogonin (standard sample, purity >99.5%) was obtained from the National Institute for Control of Pharmaceutical and Products (China). NaCl solution 1.0 mol L^{-1} was used to keep the ion strength at 0.1. Tris-HCl buffer was selected to keep the pH of the solution at 7.40. HGG solution of 1.5×10^{-5} mol L^{-1} was prepared in pH 7.40 Tris-HCl buffer solution. Barbaloin (3.0 mmol L^{-1}) solution was obtained by dissolving barbaloin in ethanol. All other chemicals were of analytical reagent grade.

Methods

Fluorescence spectra were recorded using RF-5301PC spectrofluorophotometer (Shimadzu) with a 1-cm quartz cell. The excitation and emission band widths were both 5 nm. The temperature of sample was kept by recycled water throughout the experiment. Absorption spectra were recorded on a Tu-1901 spectrophotometer (Beijing, China).

FT-IR measurements were carried out at 296 K on a Nicolet Nexus 670 FT-IR spectrometer (America) equipped with a germanium attenuated total reflection (ATR) accessory, a DTGS KBr detector, and a KBr beam splitter. All spectra were taken via the ATR method with resolution of 4 cm^{-1} and

60 scans. Spectra processing procedures: spectra of buffer solution were collected at the same condition, then we subtracted the absorbance of buffer solution from the spectra of sample solution to get the FT-IR spectra of proteins. The subtraction criterion was that the original spectra of protein solution between 2200 cm^{-1} and 1800 cm^{-1} was featureless,[10] that is, no characteristic peaks between 2200 cm^{-1} and 1800 cm^{-1} appear and the curve is flat. The secondary structure elements of free HGG and its barbaloin complexes were estimated by the FT-IR spectra and the curve-fitted results of amide I band.

UV-CD spectra were measured at 296 K on a Jasco-20c automatic recording spectropolarimeter (Japan), and the optical path length was 0.2 cm. The induced ellipticity was defined as the ellipticity of the drug–HGG mixture minus the ellipticity of drug alone at the same wavelength. CD results were expressed in terms of mean residue ellipticity (MRE) in mdeg cm^2 $dmol^{-1}$ according to the following equation:[11]

$$MRE = \frac{Observed\ CD\ (m\ \text{deg})}{Cp \times n \times l \times 10} \quad (1)$$

where Cp is the molar concentration of the protein, n is the number of amino acid residues (670), and l is the path length (0.2 cm).

The crystal structure of HGG in complex with 5-(*para*-nitrophenyl phosphonate)-pentanoic acid was taken from the Brookhaven Protein Data Bank (entry codes 1AJ7). The potential of the 3D structure of HGG was assigned according to the Amber 4.0 force field with Kollman-all-atom charges. The initial structures of all the molecules were generated by molecular modeling software Sybyl 6.9.[12] The geometries of this drug were subsequently optimized using the Tripos force field with Gasteiger–Marsili charges. AutoDock 3.05 program[13,14] was used to calculate the interaction modes between the drug and HGG. Lamarckian genetic algorithm (LGA) implemented in Autodock was applied to calculate the possible conformation of the drug that binds to the protein. During docking progress, a maximum of 30 conformers was considered for the drug. The conformer with the lowest binding free energy was used for further analysis. All calculations were performed on Silicon Graphics Ocatane2 workstation.

Fluorometric Titration

The intrinsic fluorescence of HGG was obtained at 331 nm when exited at 282 nm. A quantitative analysis of potential interaction between barbaloin and HGG was performed by fluorometric titration. Three milliliters of solution containing 1.5 μM HGG was titrated by successively 5-min interval additions of barbaloin solution (to give a final concentration of 32.26 μM). The experiments were carried out at four temperatures (296, 304, 310, and 317 K).

The Binding Parameters

The binding parameters can be calculated using the Scatchard's procedure.[15] This method is based on the general equation (2):

$$r/D_f = nK - rK \tag{2}$$

where r is the moles of drug bound per mole of protein, D_f is the molar concentration of free drug, n is the binding site number, and K is the association binding constant. Fluorescence quenching data were analyzed according to the Sips method in the present experiment (equations 3 and 4)[1]:

$$r = \frac{n(k_o c)^{\alpha}}{l + n(k_o c)^{\alpha}} \tag{3}$$

$$\lg \frac{r}{n-r} = \alpha \lg c + \alpha \lg k_o \tag{4}$$

where r is the moles of drug bound per mole of protein, K_o is the average affinity constant accurately equal to the median affinity, c is the molar concentration of free drug, and α is the index of affinity heterogeneity of HGG. The dependence of lg $r/n - r$ on the logarithmic value of the free quencher concentration c is linear with slope equal to the value of α, and K_o can be calculated from the intercept.

Thermodynamic Parameters

Thermodynamic parameters were calculated based on the temperature dependence of the average affinity constant for barbaloin–HGG binding. The temperatures used were 296, 304, 310, and 317 K. The standard enthalpy change (ΔH°) was calculated from the slope and the standard entropy change (ΔS°) was calculated from the intercept of the plot charted by Gibbs–Helmholtz equation:

$$\ln K_o = -\Delta H^{\circ}/RT + \Delta S^{\circ}/R \tag{5}$$

where R is the gas constant and T is the absolute temperature. The free energy change (ΔG°) was calculated from the following relationship:

$$\Delta G^{\circ} = \Delta H^{\circ} - T\Delta S^{\circ} \tag{6}$$

RESULTS AND DISCUSSION

Analysis of Fluorescence Quenching of HGG by Barbaloin

When exited at 282 nm, HGG showed a characteristic emission maximum at 331 nm mainly due to its tryptophane (Trp) residues. The addition of a solution

of barbaloin to HGG solution caused a decrease in the emission at 331 nm upon excitation at 282 nm (Fig. 1) and the fluorescence emission maximum wavelength of HGG shifted to a shorter wavelength, which indicated that the chromophore of protein was placed in a more hydrophobic environment after the addition of barbaloin.[16,17]

In the current experiment of fluorometric titration, the Scatchard plots appeared to be nonlinear due to the affinity heterogeneity of HGG[1] (data not shown). But the Sips plots showed good linear correlation when the binding site number (n) was 2 and 4, respectively. The binding parameters for the barbaloin–HGG interaction estimated by Sips plots (Fig. 2) are summarized in Table 1. As shown in Table 1, the values of the average affinity constant (K_o) has gradually decreased with a successive increase of temperature (T) whether the binding site number (n) is two or four, indicating that the mechanism of fluorescence quenching of HGG by barbaloin is probably of static quenching.[17]

The basic building block for the antibody is a heterodimer consisting of two heavy chains and two light chains. The heavy chain contains one variable domain (V_H) and three or four constant domains (C_H) depending on the isotype. The light chain contains one V_L and one C_L. IgG proteins comprise two identical antigen-binding fragment (Fab) regions and two crystallizable fragment (Fc) regions. Fab regions consisting of V domains and C_H1-C_L are separated from the effector function region (Fc) consisting of C_H2 and C_H3 domains by a "hinge" region.[18] The overwhelming majority of binding sites of IgG for antigen exist in complement determinant region (CDR) of Fab region,and Fc region can be considered to a certain extent as

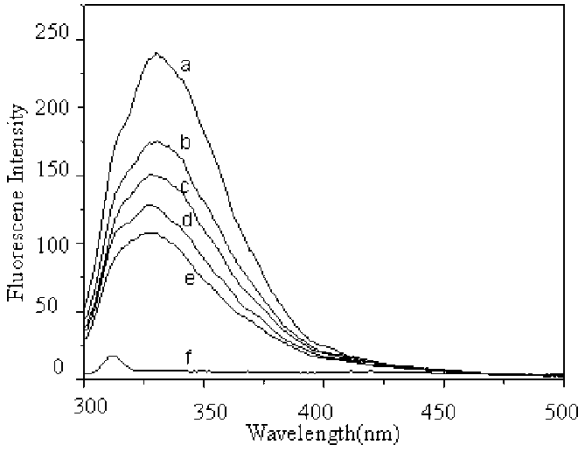


Figure 1. The fluorescence spectra of barbaloin–HGG system. The concentration of HGG was 1.5 μM while the barbaloin concentration increased from 0 to 32.26 μM from a to e. f: [barbaloin] = 32.26 μM. 296 K; pH 7.40; λ_{ex} = 282 nm, λ_{em} = 331 nm.

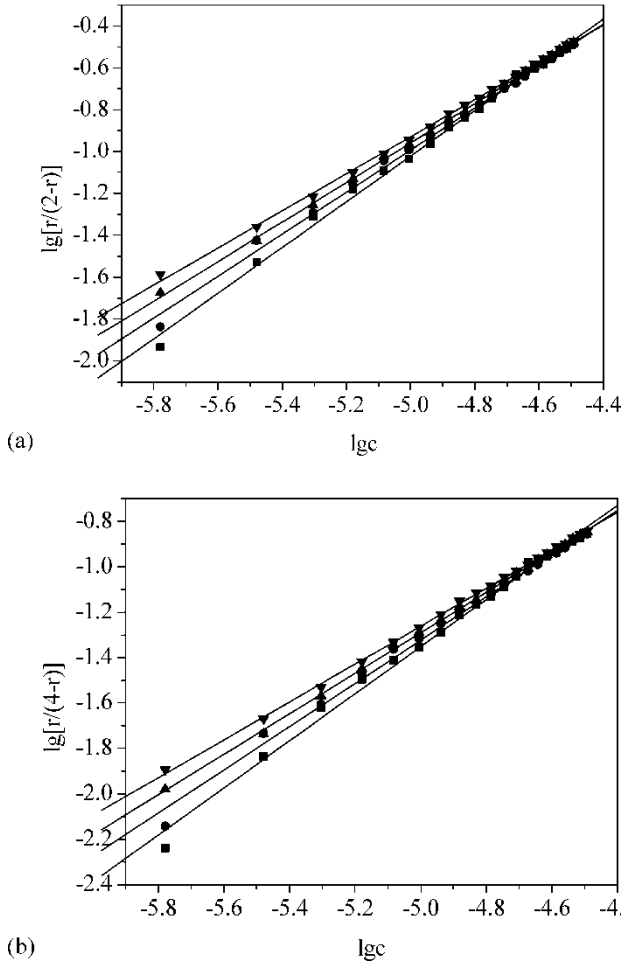


Figure 2. The Sips plots for the barbaloin–HGG system. pH 7.40, HGG concentration was 1.5 μM; ■ 296 K; ● 304 K; ▲ 310 K; ▼ 317 K; $\lambda_{ex} = 282$ nm, $\lambda_{em} = 331$ nm. The binding site number was 2 (a) and 4 (b), respectively.

a similarity to Fab region[1] and can also bind to smaller molecules.[19] Additionally, the framework region (FR), which consists of FR1, FR2, FR3, and FR4, provides CDR of Fab with a supporting framework. Recently, studies have revealed that FR3 adjacent to binding sites for antigen can also bind directly to antigens.[20,21] It is inferred that there are probably more potential binding sites of IgG for antigens/semi-antigens.[22] Table 1 shows that the Sips plots display better linear correlation whether the binding site number (n) is two or four, indicating that the binding of

Table 1. Binding parameters of barbaloin and HGG at pH 7.40 measured by fluorometric titrations

Binding parameters								
	n = 2				n = 4			
Temperature (K)	K_o (10^4)	α	R	P	K_o (10^4)	α	R	P
296	1.1522	1.0895	0.9981	<0.0001	0.4960	1.0362	0.9971	<0.0001
304	1.0221	1.0030	0.9982	<0.0001	0.4068	0.9516	0.9973	<0.0001
310	0.9618	0.9435	0.9996	<0.0001	0.3561	0.8911	0.9997	<0.0001
317	0.8937	0.8860	0.9994	<0.0001	0.3067	0.8337	0.9997	<0.0001

HGG to barbaloin is nonspecific, and that the main binding sites of HGG for barbaloin are probably Fab regions and Fc regions. However, the average affinity constant (K_o) is higher when $n = 2$ than when $n = 4$ at the same temperature, indicating that CDR of Fab region appears to have stronger affinity than Fc region in HGG. Hence, the main binding sites of HGG to barbaloin are still the CDR of Fab region. But the changes of the linear correlation coefficient (R) at different temperatures when $n = 2$ and $n = 4$ indicate that with the increase of the temperature, especially at higher temperature, the affinity of Fc region in HGG for barbaloin appears gradually effective though the main binding sites are Fab region. On the other hand, human IgG appears to have affinity heterogeneity,[1] which is mainly due to the different affinities between the four subclasses of HGG (IgG1, IgG2, IgG3, and IgG4) binding to drugs. However, the results of affinity heterogeneity from different binding sites of the same subclass of HGG binding to drugs can also not be excluded. As shown in Table 1, the value of the index of heterogeneity (α) at the same temperature is higher when $n = 2$ than when $n = 4$, indicating that there is a slight difference of affinity heterogeneity of HGG when the main binding sites for molecules of barbaloin are the two CDRs of Fab regions of HGG. In addition, the values of the indices of heterogeneity (α) of HGG have gradually decreased with the increase of the temperature (T) whether $n = 2$ or $n = 4$ when HGG binds to the molecules of barbaloin, indicating that the difference of affinity heterogeneity of HGG either between the subclasses of HGG and/or between the Fab binding sites and the Fc binding sites increases apparently, which is probably due to the dissociating velocity of lower affinity HGG–barbaloin complexes quickening with the increase of the temperature (T). Furthermore, the values of the indices of heterogeneity of HGG both at 296 K and at 304 K go beyond of the limit 1, as shown in Table 1, indicating that the affinity distribution is not totally a successive distribution symmetric to the average affinity when barbaloin binds to HGG at lower temperatures; that is, the affinity distribution is not totally in keeping with the Gaussian distribution. Although the basis of Sips method is on the assumption that the affinity distribution is in keeping with Gaussian distribution approximately,[1] we can still approximately estimate the binding of HGG to barbaloin by Sips method in this experiment.

FT-IR and CD

In the IR region, the frequencies of bands due to the amide I, II, and III vibrations are sensitive to the secondary structure of proteins. Particularly, the amide I band is useful for the studies on secondary structure. The amide I peak position occurs in the region $1600 \sim 1700\,\mathrm{cm}^{-1}$ (mainly C═O stretch) and amide II band in the region $1600 \sim 1500\,\mathrm{cm}^{-1}$ (C–N stretch coupled with N–H bending mode). Amide I band is more sensitive to the

change of protein secondary structure than amide II.[23,24] Figure 3 shows the FT-IR spectra of free HGG and its barbaloin complexes in Tris-HCl buffer solution and at 296 K, including the subtraction spectra, the second derivative

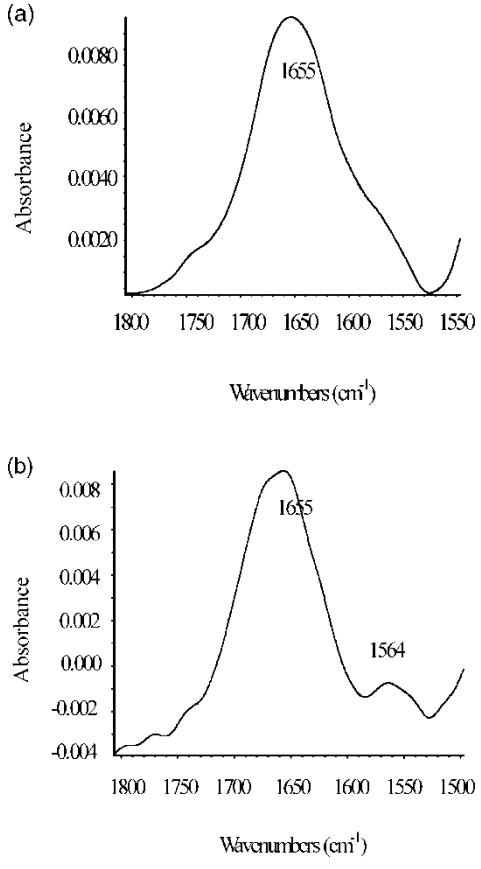


Figure 3. The FT-IR spectra of free HGG and its barbaloin complexes. pH 7.40, 296 K. (a) FT-IR spectra of 1.5 μM HGG. pH 7.40, 296 K. (b) FT-IR spectra of 3.0 μM HGG. pH 7.40, 296 K. (c) FT-IR subtraction spectra of HGG–barbaloin complexes. [(HGG solution + barbaloin solution) − barbaloin solution], $C_{HGG} = 1.5$ μM, $C_{barbaloin} = 3.0$ μM; pH 7.40, 296 K. (d) FT-IR subtraction spectra (1), deconvolution spectra (2), and second derivative spectra (3) of amide I band of 1.5 μM HGG. (e) Curve-fitted results of amide I band of 1.5 μM HGG: subtraction spectra (1); spectra after curve fitted (2); baseline of deconvolution spectra (3). (f) Subtraction spectra (1), deconvolution spectra (2), and second derivative spectra (3) of amide I band of 1.5 μM HGG and its barbaloin (3.0 μM) complexes. (g) Curve-fitted results of amide I band of 1.5 μM HGG and its barbaloin (3.0 μM) complexes: subtraction spectra (1); spectra after curve fitted (2); baseline of deconvolution spectra (3).

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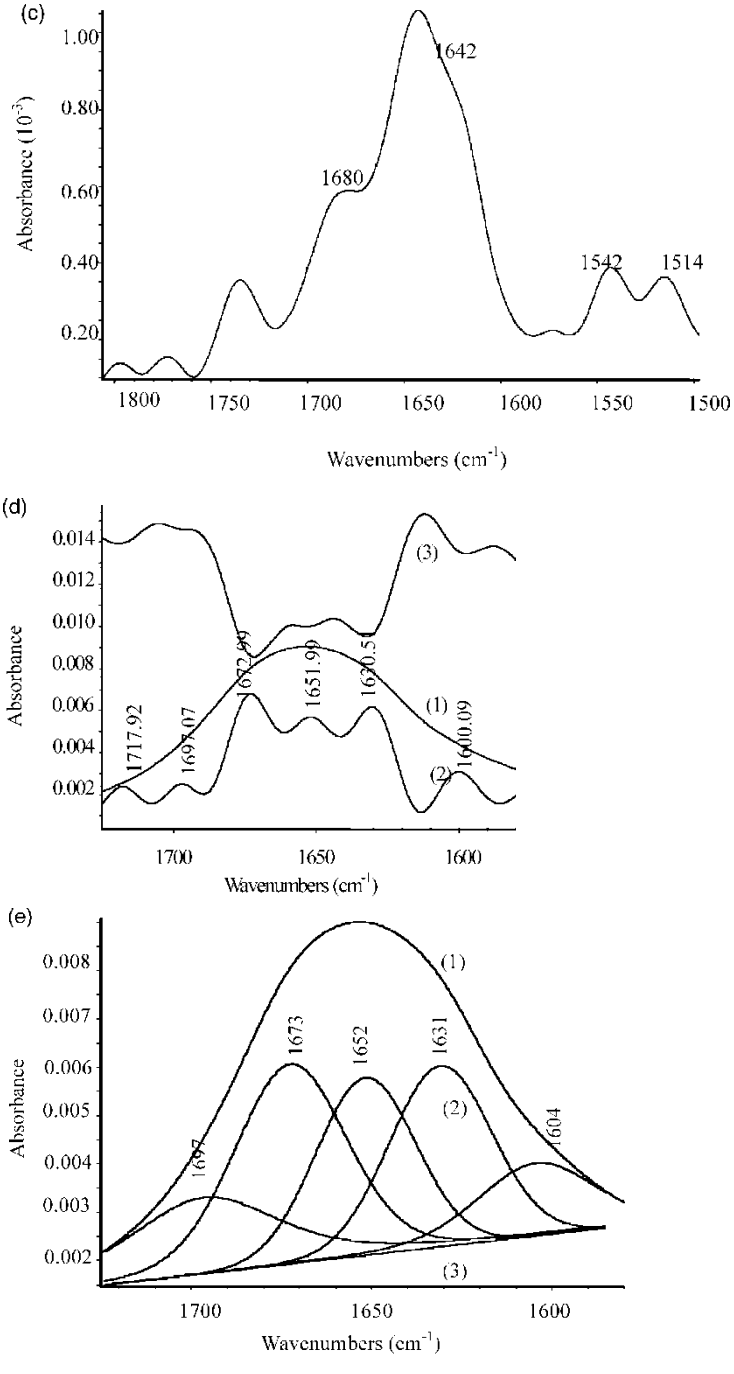

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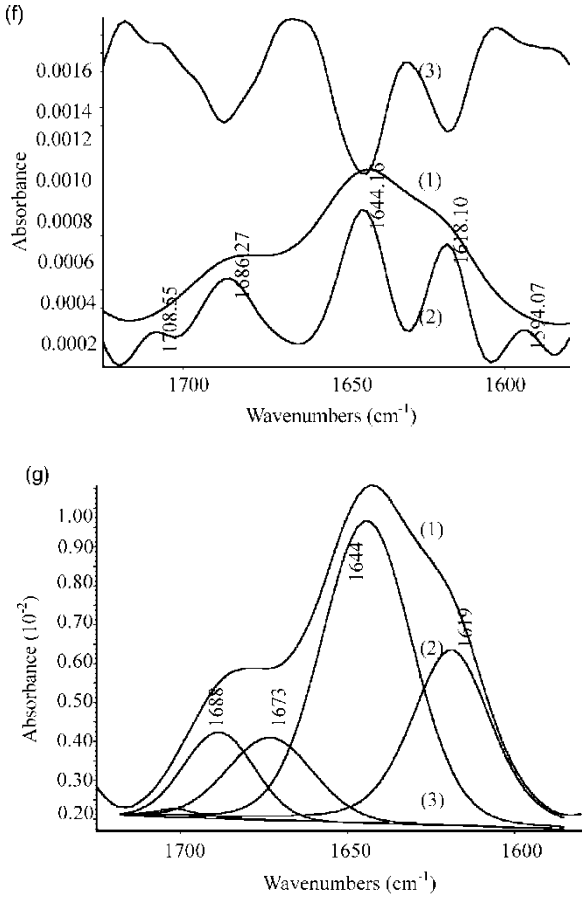

Figure 3.

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spectra, the deconvolution spectra, and the curve-fitted results. As shown in Fig. 3, free HGG shows a 1655 cm^{-1} peak position of amide I and a 1564 cm^{-1} peak position of amide II. After addition of barbaloin, the FT-IR subtraction spectra of HGG–barbaloin complexes show two peak positions of amide I at 1680 cm^{-1} and 1642 cm^{-1} and two peak positions of amide II at 1542 cm^{-1} and 1514 cm^{-1}. The results indicate that the secondary structure of HGG has changed because of the interaction of barbaloin with HGG. The FT-IR spectra peak position of amide II of 3.0 μM HGG appears more obvious than that of 1.5 μM HGG, which may be due to their difference of concentrations. According to the FT-IR spectra and the curve-fitted results of amide I band, the secondary structure elements of HGG in the absence and in the presence of barbaloin were estimated (Table 2). As shown in Table 2, the content of β-sheet structure composition of HGG decreased from

Table 2. Secondary structure elements of HGG and its barbaloin complexes estimated by FT-IR spectra and the curve-fitted Results of amide I band, pH 7.40, 296 K

	Secondary structure elements of HGG (%)		
System	Remainders	Turn	β-Sheet
1.5 μM HGG	22.48	43.72	33.80
1.5 μM HGG + 3.0 μM barbaloin	45.53	29.17	25.30

33.80% to 25.30%, the content of turn structure composition of HGG decreased from 43.72% to 29.17%, while the content of "remainders" structure elements of HGG including α-helix structure,random coiling structure, and so forth, increased from 22.48% to 45.53% when the molar concentration ratio of HGG to barbaloin was 1 to 2.

To obtain an insight into the change of secondary structure of HGG, UV-CD spectra were also studied for the barbaloin–HGG system. In ultraviolet region, such spectra relate to the polypeptide backbone structures. The comparison of the CD spectra of barbaloin–HGG with free HGG is shown in Fig. 4. As shown, the CD spectra of HGG exhibited a negative minimum at 215 nm that represents the typical β-structure.[25] The interaction between barbaloin and HGG caused a decrease of CD spectra of HGG at 215 nm in band intensity with a slight change of shape but not a significant shift of peak at all wavelengths of the UV-CD when the molar concentration ratio of HGG to barbaloin was 1 to 2, indicating that this drug induced a decrease of the β-structure elements of HGG, which are in good agreement with the analyses of FT-IR spectra.

Binding Mode

The thermodynamic parameters were calculated from the linear relationship between ln K_o and the reciprocal absolute temperature (Fig. 5, Table 3). There is a positive linear dependence of ln K_o on $1/T$ that is consistent with an exothermic molecular association of process. It is clear from the values of standard entropy changes (ΔS°) and standard enthalpy changes (ΔH°) as shown in Table 3 that binding of barbaloin to HGG was an exothermic process accompanied by a positive value of ΔS° and a negative value of ΔG°. The binding process was spontaneous as evidenced by the negative sign of the values of ΔG°. As for typical hydrophobic interaction, both ΔH° and ΔS° are positive, while negative values of ΔH° and ΔS° arise from van der Waals force and hydrogen bonding formation in low dielectric media. However, negative value of ΔH° and positive value of ΔS°, which largely contribute to ΔG°, play a main role in electrostatic interactions.[26,27] Therefore, the binding of barbaloin to

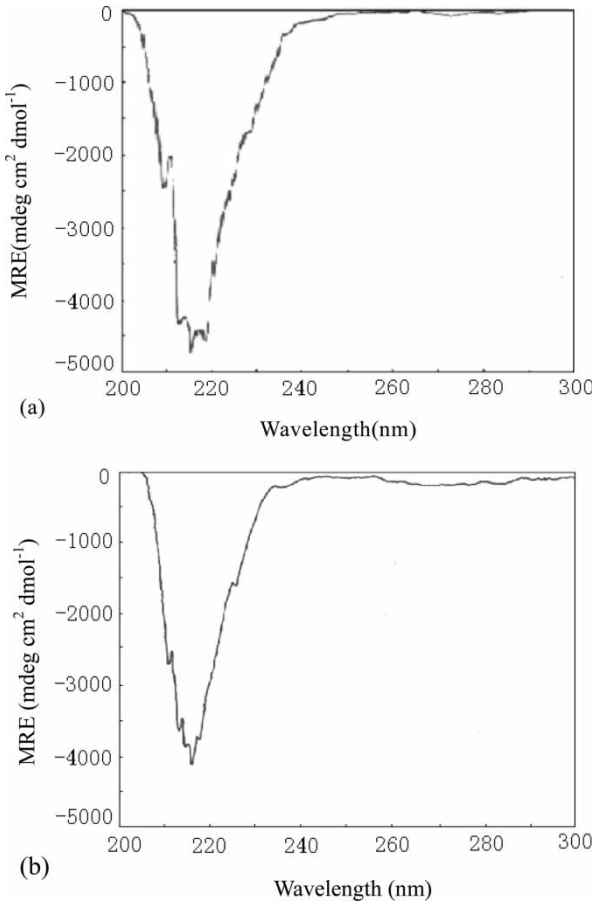

Figure 4. The CD spectra of the HGG–barbaloin system. (a) 3.0×10^{-5} mol L^{-1} HGG, (b) 3.0×10^{-5} mol L^{-1} HGG + 6.0×10^{-5} mol L^{-1} barbaloin; pH 7.40, 296 K. The number of the amino-acid residues of HGG was taken as 670.

HGG might involve electrostatic interaction strongly, but the hydrophobic interaction can also not be excluded as evidenced by the positive values of ΔS°. Under the corrent study conditions such as pH around 7.4 and the ion strength at 0.1, many amino acid residues in HGG that were not at their isoelectric points might well be ionized, and barbaloin itself might also be ionized due to its hydroxyls, hence electrostatic interaction played a major role in the binding. Furthermore, it was found that the major contribution to ΔG° derived from the ΔS° term rather than from ΔH° term, so the binding process was entropy driven, and the increase of entropy was based on the destruction of the iceberg structure induced by the hydrophobic interaction.

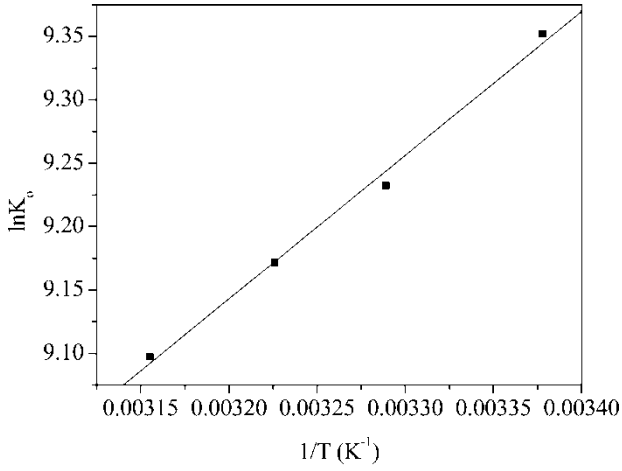

Figure 5. The Gibbs–Helmholtz plot, pH 7.40. The binding site number was taken as 2.

The Binding Distance Between Barbaloin and HGG

According to the theory of Förster energy transfer,[28] the efficiency of energy transfer, E, is given by:

$$E = 1 - F/F_o = R_o^6/(R_o^6 + r^6) \tag{7}$$

where r is the donor–acceptor distance and R_o is the distance at 50% transfer efficiency.[29] F_o and F are the fluorescence intensity of HGG in the absence of and in the presence of quencher, respectively.

$$R_o^6 = 8.8 \times 10^{-25} K^2 n^{-4} \phi J \tag{8}$$

where K^2 is the orientation factor related to the geometry of the donor–acceptor of dipole and $K^2 = 2/3$ for random orientation as in fluid solution; n (=1.336) is the refractive index of medium; ϕ is the fluorescence

Table 3. Thermodynamic parameters of barbaloin–HGG interaction at pH 7.40, n = 2

Temperature (K)	ΔG° (KJ/mol)	ΔH° (KJ/mol)	ΔS° (J/mol K)
296	−23.00		
304	−23.36	−9.432	45.83
310	−23.64		
317	−23.96		

quantum yield of the donor, its value was taken as 0.118[19]; and J is the overlap integral of the fluorescence emission spectra of the donor and the absorption spectra of the acceptor. J is given by:

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4\Delta\lambda / \sum F(\lambda)\Delta\lambda \quad (9)$$

where $F(\lambda)$ is the fluorescence intensity of fluorescence reagent when the wavelength is λ; $\varepsilon(\lambda)$ is the molar absorbance coefficient at the wavelength of λ. From these relationships, J, E, and R_o can be calculated, so the value of r can also be calculated.

Figure 6 is the overlap of the fluorescence spectra of HGG and the absorption spectra of barbaloin when the molar ratio of barbaloin to HGG was 2 to 1. The value of J calculated according to the above relationship is 1.654×10^{-14} $cm^3 L\ mol^{-1}$. So the value of R_o is 2.35 nm, and the value of r is 3.74 nm. This result indicates further that the binding reaction of barbaloin to HGG was through energy transfer, which would quench the fluorescence of chromophore (mainly the tryptophane residue) in HGG. IgG comprises 12 domains, each light chain has two domains (V_L and C_L), and each heavy chain has four domains (V_H, C_H1, C_H2, and C_H3).

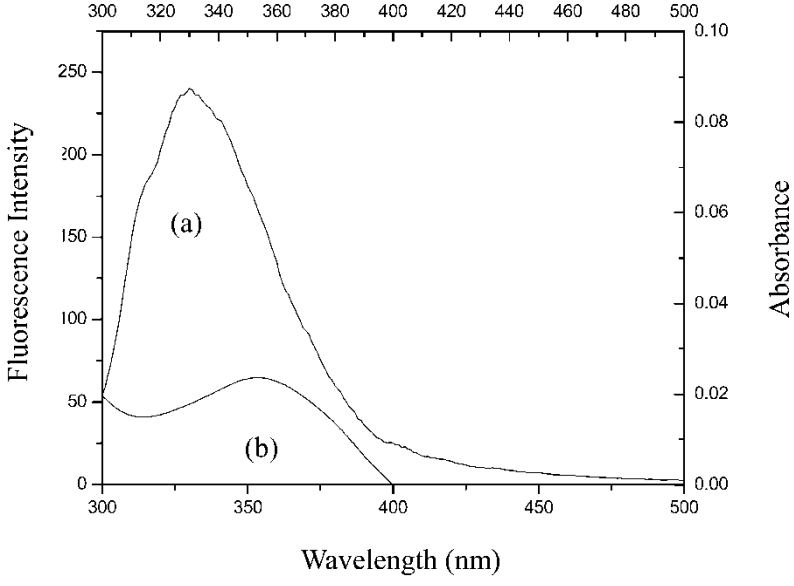

Figure 6. The overlap of the fluorescence spectra of HGG and the absorption spectra of barbaloin. $\lambda_{ex} = 282$ nm, $\lambda_{em} = 331$ nm; pH 7.40, 297 K; HGG concentration = 1.5 μM, barbaloin concentration = 3.0 μM. (a) The fluorescence spectra of HGG; (b) the absorption spectra of barbaloin.

All the domains of IgG have two cysteine (Cys) residues which form the intra-domain disulfide bond and at least one tryptophane (Trp) residue, which can prevent from hydrolysis by proteinase.[1] Studies of X-ray analysis of crystal structure on the light-chain dimer Mcgλ have proved that V_L has a constant Trp-37 residue and C_L has a constant Trp-152 residue, both of which are adjacent to disulfide bonds. Studies of X-ray analysis of crystal structure on the interaction of IgG1Fab′ (New) with $VitK_1OH$ have proved that the end of $VitK_1OH$ is adjacent to the Trp-54 of CDR of IgG1Fab′ (New).[30] The crystal structure of HGG in complex with 5-(*para*-nitrophenyl phosphonate)-pentanoic acid taken from the Brookhaven Protein Data Bank (entry codes 1AJ7) in Fig. 7 shows that Trp-35 is highly adjacent to molecule of barbaloin. In this experiment, barbaloin binds probably to the Trp-35 residue and will quench the fluorescence of HGG. However, there are other tryptophane residues in HGG. So, the distance (r) calculated here is actually the average value between the bound barbaloin and the tryptophane residues of HGG.

Molecular Modeling Study of the Interaction Between HGG and Barbaloin

HGG has two heavy chains consisting of about 450 amino acid residues and two light chains consisting of about 210 $\sim$ 230 amino acid residues. Studies

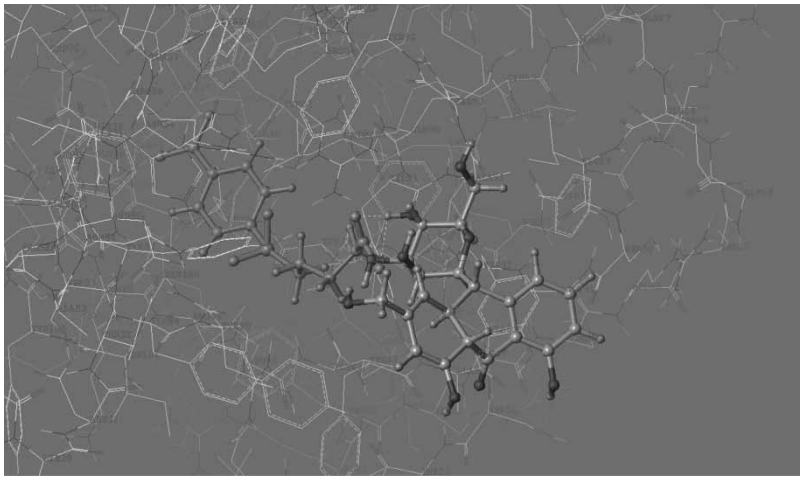

Figure 7. The interaction mode between barbaloin and HGG; barbaloin is located in CDR of Fab of HGG. Only residues around 8 Å of the ligand are displayed. The residues of HGG are represented using line and the ligand structure is represented using ball and stick model. The green model represents template molecule. The hydrogen bonds are indicated by yellow dash.

of X-ray analysis of crystal structure on IgG1 (New) have proved that the antigen-binding site distributes over a "shallow cavity" (cleft) with the size of 1.5 nm × 0.6 nm × 0.6 nm, which is composed of about $10 \sim 12$ amino acid residues of the complement-determinant region (CDR) of Fab, and semi-antigen, such as barbaloin, can inlay into the cleft and associate with the amino acid residues of CDR in the form of complementary structure through hydrogen bond, van der Waals force, electrostatic interaction, hydrophobic interaction, and so forth.[30] There are two such structurally "shallow cavities" (clefts) in HGG, two binding sites for antigens/semi-antigens, which appear to be of higher affinity and specificity and that many drugs can bind to, though there are two binding sites for complements in HGG that appear to be of lower affinity and that drugs can also bind to as indicated by the binding parameters calculated at the beginning of this paper. The binding of barbaloin to HGG may involve electrostatic interaction strongly, but the hydrophobic interaction cannot be excluded as evidenced by the thermodynamic parameters calculated at the beginning of this paper, too. However, the interaction between barbaloin and HGG via hydrogen bonds can also not be excluded as indicated by the illustration (Fig. 7). Here, partial binding parameters of the HGG–barbaloin system were calculated through Silicon Graphics Ocatane2 workstation. Figure 7 shows clearly that there are interactions of hydrogen bonds between barbaloin and the amino acid residues of HGG. As shown, glucopyranosyl 3-OH substituent of barbaloin donating hydrogen forms a hydrogen bond with Ser-34 of HGG; Ser-34 and Trp-35 of HGG respectively form two hydrogen bonds with glucopyranosyl 4-OH substituent of barbaloin.

Comparison of the Binding Properties of HGG and Serum Albumin to Barbaloin

By the same studying methods as barbaloin, the binding of HGG to isofraxidin and wogonin, two active components from different plant medicines, were investigated respectively, and it was found that both average binding constants presented an order of magnitude of about 10^4. There are different binding modes and different binding parameters including the binding constants and the binding site number between the interaction of HGG and serum albumin with drugs due to their different structures and different conformations. All the binding constants of serum albumin binding to the three drugs (wogonin, isofraxidin, and barbaloin) presented to be about 10^5,[6,9,31] indicating that generally the binding of serum albumin to drugs is stronger than that of HGG to them. This gives the information that there will be a competitive reaction between serum albumin and HGG binding to the same drug in blood.

CONCLUSIONS

The interaction of barbaloin with HGG has been studied by fluorescence quenching, CD, and FT-IR method. From the above experiments, we can obtain that barbaloin binds to HGG with an average affinity constant of 1.152×10^4 (296 K). The molecule of barbaloin is mainly located in CDR of Fab of HGG, and the β-structure elements of HGG will decrease in the presence of barbaloin. The main interaction is of electrostatic force, but the hydrophobic interaction and hydrogen bond cannot be excluded. The distance of the donor and the acceptor is 3.74 nm.

There will be a competitive reaction between serum albumin and HGG binding to the same drug in blood. In addition, the binding of barbaloin to HGG has caused a change of the conformation of HGG. However, the conformational change of HGG by drug may cause a change of immune function of HGG *in vivo*. These problems should be studied further.

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