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ORIGINAL PAPER

Characterization of the Binding of Metoprolol Tartrate and Guaifenesin Drugs to Human Serum Albumin and Human Hemoglobin Proteins by Fluorescence and Circular Dichroism Spectroscopy

Osman Duman · Sibel Tunç · Bahar Kancı Bozoğlan

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Abstract The interactions of metoprolol tartrate (MPT) and guaifenesin (GF) drugs with human serum albumin (HSA) and human hemoglobin (HMG) proteins at pH7.4 were studied by fluorescence and circular dichroism (CD) spectroscopy. Drugs quenched the fluorescence spectra of HSA and HMG proteins through a static quenching mechanism. For each protein-drug system, the values of Stern-Volmer quenching constant, bimolecular quenching constant, binding constant and number of binding site on the protein molecules were determined at 288.15, 298.15, 310.15 and 318.15 K. It was found that the binding constants of HSA-MPT and HSA-GF systems were smaller than those of HMG-MPT and HMG-GF systems. For both drugs, the affinity of HMG was much higher than that of HSA. An increase in temperature caused a negative effect on the binding reactions. The number of binding site on blood proteins for MPT and GF drugs was approximately one. Thermodynamic parameters showed that MPT interacted with HSA through electrostatic attraction forces. However, hydrogen bonds and van der Waals forces were the main interaction forces in the formation of HSA-GF, HMG-MPT and HMG-GF complexes. The binding processes between protein and drug molecules were exothermic and spontaneous owing to negative ΔH and ΔG values, respectively. The values of binding distance between protein and drug molecules were calculated from Förster resonance energy transfer theory. It was found from CD analysis that the bindings of MPT and GF drugs to HSA and HMG proteins altered the secondary structure of HSA and HMG proteins.

Keywords Human serum albumin · Hemoglobin · Interaction · Metoprolol tartrate · Guaifenesin · Fluorescence spectroscopy

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Introduction

The interactions between proteins and drugs are very significant in the pharmacokinetics, pharmacodynamics and toxicology of drugs [1]. Drug molecules can be bound by blood proteins. Unbound drug transfers easily from blood to target tissue or organ. However, for bound drug, it is difficult to reach the action site through the blood capillary walls. Thus, a bound drug loses the pharmacological activity [2, 3]. On the other hand, the binding of drugs to blood proteins is reversible [4] and there is a dynamic equilibrium between bound and free drug molecules [5]. Protein-drug complexes control the release of drugs from blood to receptors and prevent the rapid metabolism of drugs [2]. Strong binding leads to a decrease in the concentration of free drug within blood, whereas weak binding causes a low circulation time of drug [6].

Various methods have been used to investigate the binding of small ions or drug molecules to proteins. They include the use of equilibrium dialysis [7], ion selective electrodes [8, 9], UV-visible [10], fluorescence [11–13] and circular dichroism (CD) [14] spectroscopy. Equilibrium dialysis is used widely; however, it requires the analysis of free and total drug concentration and takes a long time [11]. Ion selective electrodes

have the lack of selectivity for drugs. On the other hand, spectrofluorimetric method is of various advantages. It is sensitive and easy and has a short analysis time. In addition, the use of spectrofluorimetric method allows the determination of quenching type, binding constant, number of binding site per protein molecule, binding mode and binding distance in protein-drug interactions. CD spectroscopy is an excellent method for the investigation of changes in secondary structure of protein.

Human serum albumin (HSA) and human hemoglobin are important blood proteins. The affinities of drugs for HSA and HMG proteins are very high. Generally, more than 90 % of drugs used in human bodies are bound to HSA protein [15]. The binding of drugs gives rise to a change in the conformational structure of protein, which may affect the functions of protein and also lead to various illnesses.

HSA is a single-chain consisting of 585 amino acids with one tryptophan (Trp-214 in subdomain IIA) [16]. The crystal structure of albumin reveals a heart-shaped molecule that contains an equilateral triangle with sides of ~8 nm and a depth of 3 nm [17]. HSA is responsible from the maintaining of blood pH and blood osmotic pressure. It also plays an important role in the transport and disposition of many endogenous and exogenous ligands present in blood [18]. HMG, a major component in erythrocytes, consists of two identical α-chains and two identical β-chains. Each of α-chain has 141 amino acids and each of β-chain has 146 amino acids [19]. HMG is roughly spherical molecule with 6.4×5.5×5.0 nm [20]. It carries oxygen from lung to peripheral tissues, provides electron transfer in all organs and parts of the body and adjusts the pH of blood. Moreover, HMG plays an important role in the transport of H^+ and CO_2 from tissues to lung [21, 22].

Metoprolol tartrate (MPT), 1-[4-(2 methoxyethyl) phenoxy]-3-[(1-methylethyl)amino]-2-propanol tartrate, is a selective β-blocker drug [23]. It is used widely in the treatment of cardiovascular diseases such as hypertension, angina pectoris and cardiac dysrhytmias [24]. MPT blocks the action of epinephrine and norepinephrine on the β-adrenergic receptors in the heart and reduces the cardiac work and oxygen consumption [23, 25]. Guaifenesin (GF), 3-(2-methoxyphenoxy) propane-1,2-diol, is an expectorant which reduces the thickness and stickness of mucus in trachea and bronchi and increases the removal of mucus [26, 27]. This drug is used to improve the upper respiratory infections such as sinusitis, pharyngitis and bronchitis [28].

It is very important to know the interactions of MPT and GF drugs with HSA and HMG proteins in order to understand the pharmacological behaviors of these drugs in blood stream. On the other hand; despite the common uses of MPT and GF, there are no reports in the literature about the interaction mechanisms of MPT and GF drugs with HSA and HMG proteins and the influences of these drugs on the structures of proteins. Therefore; in this study, we have investigated the binding mechanism, determined the binding constants, the numbers of binding sites per protein molecule, thermodynamic parameters and binding distances for the interactions of MPT and GF drugs with HSA and HMG proteins at 288.15, 298.15, 310.15 and 318.15 K and also examined the effect of drug molecules on the structural changes of proteins.

Experimental Section

Materials

Human serum albumin (HSA) and human hemoglobin (HMG) proteins, (±)-metoprolol (+)-tartrate (MPT), tris(hydroxymethyl)aminomethane buffer used to maintain the pH value of solutions at physiological pH and NaCl used as an ionic strength adjuster were purchased from Sigma. Guaifenesin (guaiacol glyceryl ether) (GF) was obtained from Aldrich. The chemical structures of drugs are shown in Fig. 1. The average molecular weight values of 66,500 and 64,500 g/mol were used in the preparation of HSA and HMG solutions, respectively. Prior to each experiment, all solutions were prepared freshly in tris(hydroxymethyl) aminomethane buffer solution (0.05 M, pH7.4) containing 0.1 M NaCl. Deionized water was used in the preparation of buffer solution.

Methods

Firstly, the stock solutions of HSA and HMG proteins (5.0×10^{-5}M) and MPT and GF drugs (1.0×10^{-4}M) were prepared with buffer solution and kept in a refrigerator at 4–6 °C. Then, a series of protein-drug mixture solution containing various drug concentrations was prepared from the stock solutions of proteins and drugs to investigate the interactions between protein and drug molecules. In protein-drug interaction experiments, the concentrations of HSA and HMG proteins were fixed at 5.0×10^{-6}M and 2.5×10^{-6}M, respectively, and the concentrations of drugs were varied in the range of 0.0–30.0×10^{-6}M for HSA-MPT system, 0.0–70.0×10^{-6}M for HSA-GF system, 0.0–6.0×10^{-6}M for HMG-MPT system and 0.0–10.0×10^{-6}M HMG-GF system.

The fluorescence spectra of protein-drug mixture solutions were recorded by a Varian-Carry Eclipse model fluorescence spectrophotometer using a quartz cell with 1.0 cm path length in a thermostatically controlled cell holder at 288.15, 298.15, 310.15 and 318.15±0.10 K after an equilibration time of 15 min at each temperature. An excitation wavelength of 280 nm was used for HSA and HMG protein solutions [21, 29]. The widths of excitation and emission slits were set to 5 nm in fluorescence measurements. Then,

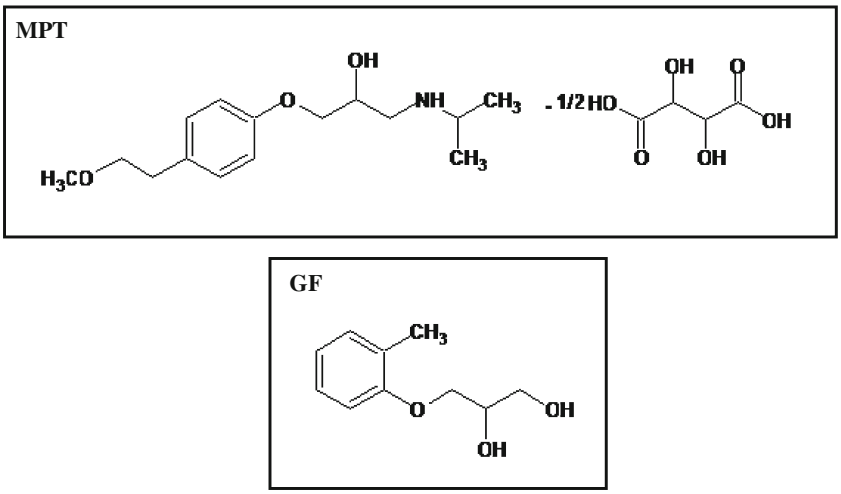

Fig. 1 Chemical structures of (±)-metoprolol (+)-tartrate (MPT) and guaifenesin (GF)

the observed fluorescence intensities were corrected to eliminate the inner filter effect of proteins and drugs. Therefore, the absorption measurements of solutions were carried out by a Varian-Cary 5000 model UV–vis-NIR spectrophotometer using a matched pair of quartz cells (path length: 1 cm) in a peltier thermostatted cell holder at 288.15, 298.15, 310.15 and 318.15±0.10 K. Then, for each system, the absorbance values were determined at the excitation and emission wavelengths of the fluorescence measurements. The corrected fluorescence intensity (F_{cor}) values were obtained by the following equation [30]:

$$F_{cor} = F_{obs} 10^{(A_{exc}+A_{em})/2} \quad (1)$$

where F_{obs} is the observed fluorescence intensity and A_{exc} and A_{em} are the absorbances of the system at the excitation and emission wavelengths, respectively. In this study, the corrected fluorescence intensity values were used.

To investigate the effect of drugs on the secondary structure of proteins, a series protein-drug solution was prepared and the CD spectra of 5.0×10^{-6}M HSA and 2.5×10^{-6}M HMG solutions containing various drug concentrations were obtained on a Jasco-J-815 model spectropolarimeter using a quartz cell with 0.05 cm path length at 298.15 K. CD measurements were carried out in the range of 200–250 nm at 1 nm intervals and CD spectra were collected with the scan speed of 20 nm/min. Moreover, each CD spectrum was the average of 3 scans.

It should be noted that the spectra of appropriate blanks corresponding to the buffer solution for pure protein and pure drug solutions and the drug-buffer solutions for protein-drug mixture solutions were subtracted from the sample spectra in all fluorescence and CD measurements.

In addition, the pH values of solutions were checked with a Jenway 3040 ion analyzer using combined glass electrode, which was calibrated with standard buffer solutions before use.

Results and Discussion

Fluorescence Spectra and Quenching Mechanism

The fluorescence spectra of HSA and HMG proteins in the absence and presence of MPT and GF drugs are shown in Figs. 2 and 3, respectively. As seen from these figures, the fluorescence emission peak is at 335 nm for HSA and at 330 nm for HMG. Tryptophan, tyrosine and phenylalanine are three fluorophores in HSA and HMG proteins. Generally, the fluorescence of HSA and HMG is almost contributed by tryptophan alone, since phenylalanine has a very low quantum yield and the fluorescence of tyrosine is almost totally quenched if it is near to an amino group, a carboxyl group or a tryptophan [31]. HSA has only one tryptophan residue (Trp-214 in subdomain IIA). On the other hand, HMG is a tetramer including two α and two β chains arranged in two identical $\alpha_1\beta_1$ and $\alpha_2\beta_2$ subunits and there are three Trp residues (α14-Trp, β15-Trp and β37-Trp) in each αβ subunit. From these amino acid residues, α14-Trp and β15-Trp residues are the outside of subunit interface. However, β37-Trp residue takes place at $\alpha_1\beta_2$ interface, which has been assigned as the primary source of fluorescence emission of HMG [19].

We can see from Fig. 2 that the addition of both drugs causes a quenching in the fluorescence spectrum of HSA. According to this result, the interactions of MPT and GF drugs with HSA are possible. After the addition of 30.0×10^{-6}M MPT, the fluorescence emission peak of HSA shifts from 335 nm to 340 nm (Fig. 2a). A similar situation is also observed in HSA-GF system (Fig. 2b). The peak position of HSA is observed at 345 nm in the presence of 70.0×10^{-6}M GF as seen from Fig. 2b.

Figure 3 exhibits the fluorescence spectra of HMG in the presence of various MPT and GF concentrations. As seen from Fig. 3a and b, the maximum fluorescence emission wavelength of HMG shifts from 330 nm to 340 and 342 nm

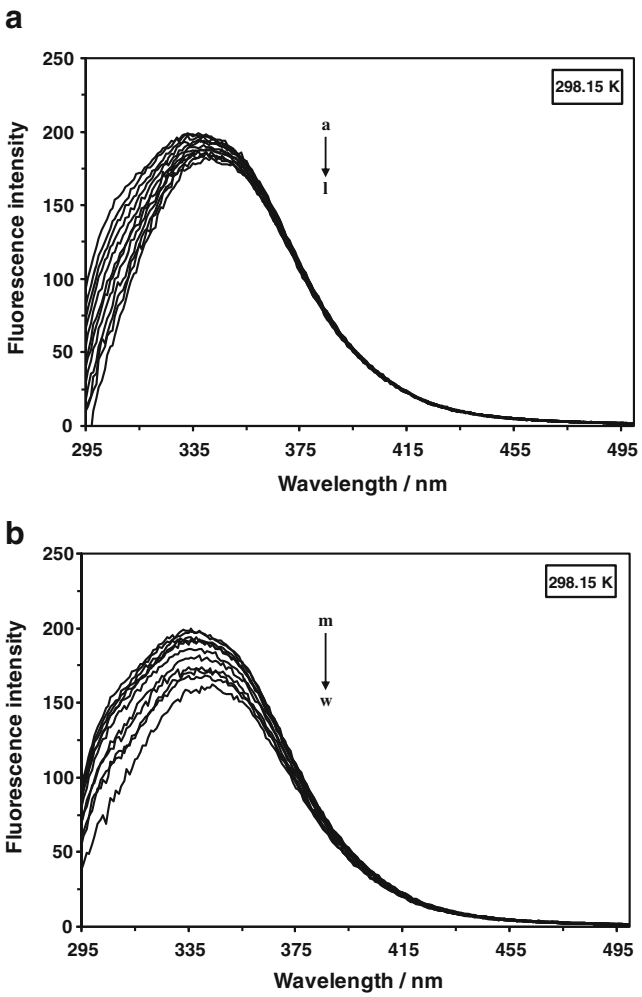

Fig. 2 Fluorescence spectra of 5.0×10^{-6}M HSA in the presence of various concentrations of MPT (**a**) and GF (**b**) at pH7.4 and 298.15 K. C_{MPT} from (a) to (l): 0.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 22.5, 25.0 and 30.0($\times10^{-6}$M), respectively. C_{GF} from (m) to (w): 0.0, 2.5, 5.0, 10.0, 15.0, 20.0, 30.0, 40.0, 50.0, 60.0 and 70.0($\times10^{-6}$M), respectively

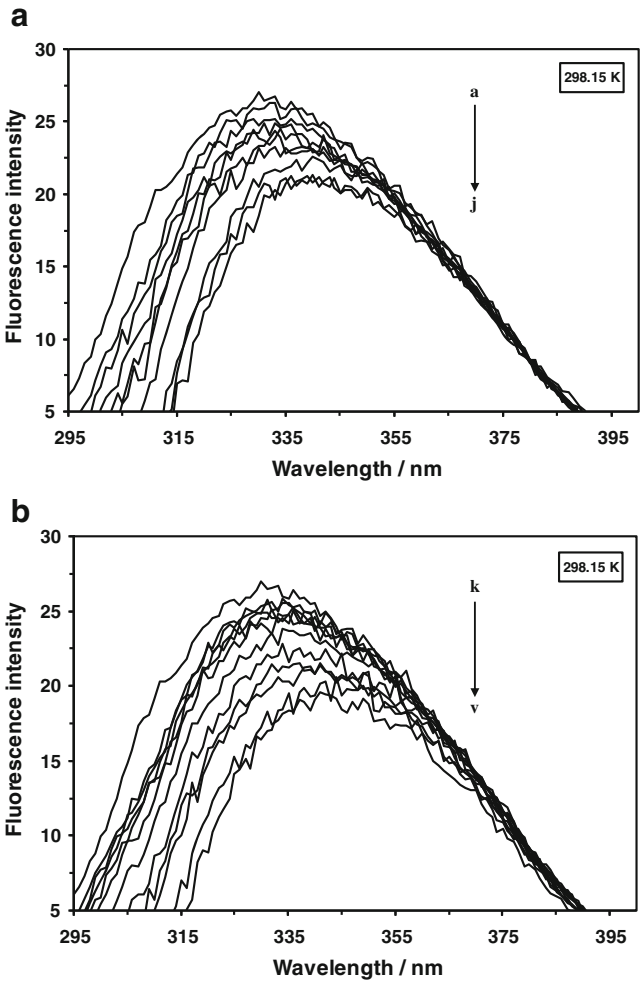

Fig. 3 Fluorescence spectra of 2.5×10^{-6}M HMG in the presence of various concentrations of MPT (**a**) and GF (**b**) at pH7.4 and 298.15 K. C_{MPT} from (a) to (j): 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0 and 6.0($\times10^{-6}$M), respectively. C_{GF} from (k) to (v): 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0 and 10.0($\times10^{-6}$M), respectively

in the presence of 30.0×10^{-6}M MPT and 70.0×10^{-6}M GF, respectively. After the addition of MPT and GF drugs, the decreases in fluorescence spectrum of HMG indicate the interactions between protein and drug molecules.

Fluorescence quenching may be dynamic (collisional) or static. In dynamic quenching, the quencher must diffuse to the fluorophore during the lifetime of excited state. Upon contact, the fluorophore returns to the ground state without emission of a photon. In static quenching, a complex is formed between the fluorophore and the quencher and this complex is nonfluorescent. Dynamic and static quenching can be distinguished by their dependence on temperature and viscosity in different way. In dynamic quenching, dynamic quenching constant exhibits an increase with increasing temperature owing to the increase in the diffusion rate. On the other hand, a higher temperature causes the dissociation of bound complexes and therefore, the value of static quenching constant decreases at higher temperatures [30].

To determine the quenching mechanism of HSA and HMG proteins by MPT and GF drugs, the fluorescence quenching data were analyzed by the Stern-Volmer equation [30]:

$$\frac{F_0}{F} = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q] \quad (2)$$

where F_0 and F are the fluorescence intensities of protein in the absence and presence of drug, respectively; k_q is the bimolecular quenching constant; τ_0 is the lifetime of the biomolecule in the absence of drug (10^{-8}s) [32], K_{SV} is the Stern-Volmer quenching constant and [Q] is the concentration of drug.

The K_{SV} values of HSA-MPT, HSA-GF, HMG-MPT and HMG-GF systems at 288.15, 298.15, 310.15 and 318.15 K

were determined from the slopes of F_0/F versus [Q] plots in Fig. 4. Then, the k_q values were calculated using Eq. (2). The K_{SV} and k_q values obtained for the interactions of MPT and GF drugs with HSA and HMG proteins are given in Table 1. It is seen from this table that the K_{SV} values of HSA-MPT and HSA-GF systems have the order of $10^3 M^{-1}$ while those of HMG-MPT and HMG-GF systems are of the order of $10^4 M^{-1}$. This result shows that the interaction of MPT and GF drugs with HMG protein is more strength than that of HSA. Moreover, there is a decrease in the K_{SV} values of HSA-MPT, HSA-GF, HMG-MPT and HMG-GF systems with increasing temperature, which indicates the presence of static quenching process in the binding of MPT and GF drugs to HSA and HMG proteins. Maximum dynamic quenching constant of various quenchers for biopolymer has been reported as 2.0×10^{10} $M^{-1}s^{-1}$ [33]. As seen from Table 1, the k_q values have the orders of $10^{11} M^{-1}$ for HSA-MPT and HSA-GF systems and $10^{12} M^{-1}$ for HMG-MPT and HMG-GF systems. This result confirms the binding of MPT and GF drugs to HSA and HMG proteins by the static quenching process.

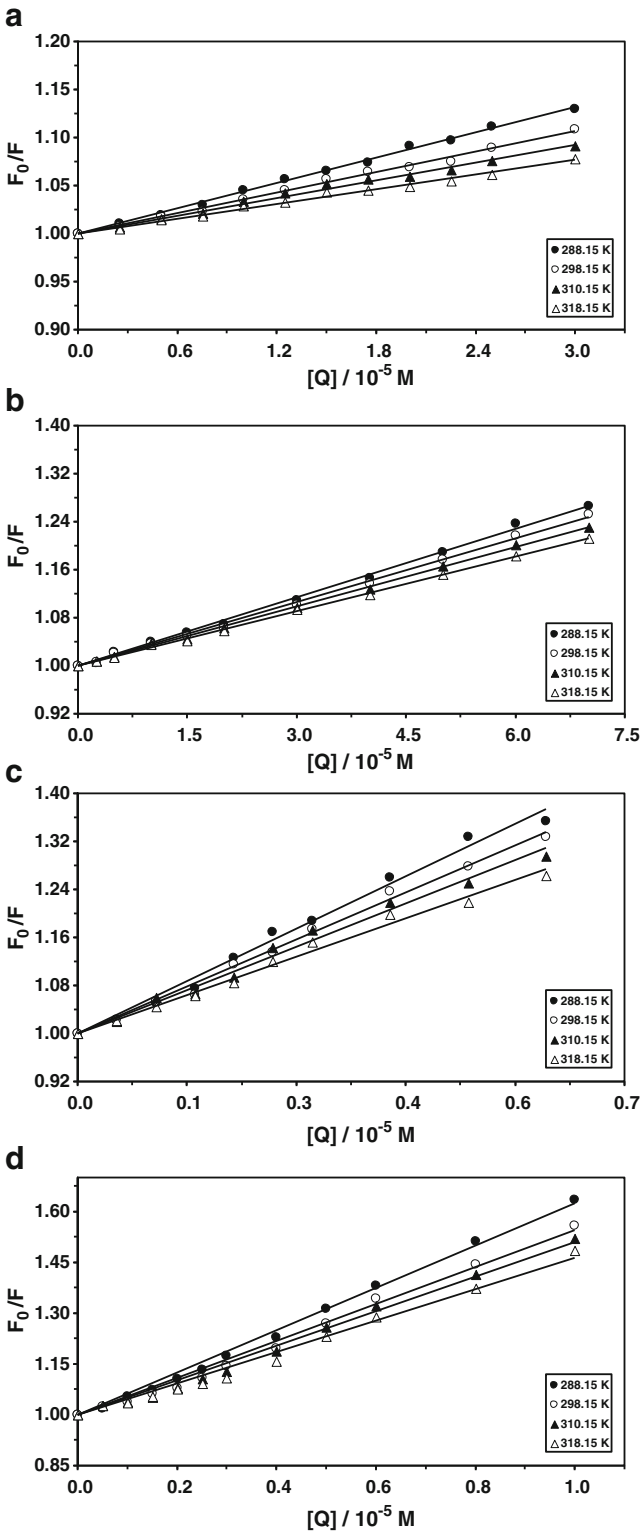

Fig. 4 Stern-Volmer plots of HSA-MPT (**a**), HSA-GF (**b**), HMG-MPT (**c**) and HMG-GF (**d**) systems

Analysis of Binding Equilibria

Fluorescence measurements exhibited that MPT and GF drugs quench the fluorescence spectra of HSA and HMG proteins through a static quenching process. For static quenching, the equilibrium reaction between protein and drug (quencher) can be given by [34]

$$P + n\,Q \rightleftarrows PQn \qquad (3)$$

Table 1 Stern-Volmer quenching (K_{SV}) constants, regression coefficients (r) and quenching rate (k_q) constants for the interactions of MPT and GF with HSA and HMG proteins at different temperatures and pH 7.4

System	T/K	K_{SV}/M^{-1}	r	$k_q/M^{-1}s^{-1}$
HSA-MPT	288.15	4.38×10^3	0.9976	4.38×10^{11}
	298.15	3.55×10^3	0.9952	3.55×10^{11}
	310.15	3.09×10^3	0.9916	3.09×10^{11}
	318.15	2.56×10^3	0.9914	2.56×10^{11}
HSA-GF	288.15	3.80×10^3	0.9976	3.80×10^{11}
	298.15	3.55×10^3	0.9975	3.55×10^{11}
	310.15	3.29×10^3	0.9987	3.29×10^{11}
	318.15	3.04×10^3	0.9991	3.04×10^{11}
HMG-MPT	288.15	6.22×10^4	0.9894	6.22×10^{12}
	298.15	5.58×10^4	0.9934	5.58×10^{12}
	310.15	5.15×10^4	0.9866	5.15×10^{12}
	318.15	4.56×10^4	0.9890	4.56×10^{12}
HMG-GF	288.15	6.24×10^4	0.9974	6.24×10^{12}
	298.15	5.43×10^4	0.9949	5.43×10^{12}
	310.15	5.10×10^4	0.9950	5.10×10^{12}
	318.15	4.62×10^4	0.9935	4.62×10^{12}

Here, there are n possible binding sites for drug (Q) on protein (P). The binding constant (K_b) of this reaction can be written as

$$K_b = \frac{[PQ_n]}{[P][Q]^n} \tag{4}$$

where [P], [Q] and [PQ_n] are the protein, drug and protein-drug complex concentrations, respectively. If total protein concentration is [P_0], then [PQ_n] is expressed as [PQ_n]=[P_0] - [P]. Using this value for [PQ_n] in Eq. (4), we obtain the Eq. (5)

$$K_b = \frac{[P_0] - [P]}{[P][Q]^n} \tag{5}$$

If we consider that the fluorescence intensity of protein is proportional to its concentration, we can write

$$\frac{[P]}{[P_0]} = \frac{F}{F_0} \tag{6}$$

By substitution of Eq. (6) in Eq. (5) and taking logarithm, we obtain the Eq. (7)

$$\log \frac{(F_0 - F)}{F} = \log K_b + n \log[Q] \tag{7}$$

The binding constants (K_b) and the numbers of binding sites per protein molecule (n) for the protein-drug systems can be calculated from the intercepts and slopes of the plots of log ((F_0-F)/F) versus log [Q]. A typical log ((F_0-F)/F) versus log [Q] plot for the binding processes of MPT and GF drugs to HSA and HMG proteins at 298.15 K is shown in Fig. 5. The values of K_b and n determined from linear regression analysis of the data in log ((F_0-F)/F) versus log [Q] plots at 288.15, 298.15, 310.15 and 318.15 K are listed in Table 2. It is seen from Table 2 that HMG-MPT and HMG-GF systems have larger K_b values than HSA-MPT and HSA-GF systems. In other words, the binding affinity of HMG for MPT and GF drugs is much higher than that of HSA. Moreover, larger binding constants were obtained in the protein-drug binding reactions when lower temperature was used in HSA-MPT, HSA-GF, HMG-MPT and HMG-GF systems. This means that an increase in temperature has a negative effect on the binding processes. The number of binding sites, n, is approximately equal to one for all protein-drug binding reactions and does not display a significant change with temperature (Table 2). This result indicates that, for MPT and GF drugs, there is one binding site on HSA and HMG proteins.

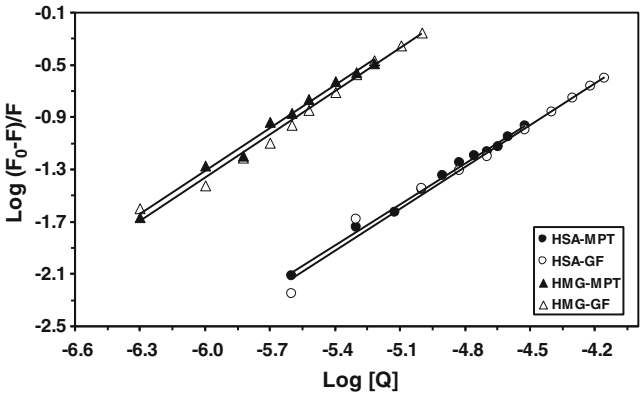

Fig. 5 Log ((F_0-F)/F) versus log [Q] plots for the binding of MPT and GF drugs to HSA and HMG proteins at 298.15 K

Thermodynamic Parameters and Nature of Binding Forces

Generally, there are four types of interaction forces, hydrogen bonds, van der Waals forces, electrostatic and hydrophobic interactions, in the binding of drug molecule to protein molecule. The binding forces between protein and drug molecules can be determined from the thermodynamic parameters (enthalpy change (ΔH), entropy change (ΔS) and Gibbs free energy change (ΔG) values) of protein-drug binding reaction. Therefore, for all protein-drug systems, the binding studies were carried out at 288.15, 298.15, 310.15 and 318.15 K.

Thermodynamic parameters can be calculated from Eqs. (8) and (9) [35]:

$$\ln K_b = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \tag{8}$$

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

Table 2 Binding constants (K_b), number of binding sites (n) and regression coefficients (r) for the HSA-MPT, HSA-GF, HMG-MPT and HMG-GF systems at different temperatures

System	T/K	K_b/M^{-1}	n	r
HSA-MPT	288.15	6.72×10^3	1.04	0.9962
	298.15	5.94×10^3	1.05	0.9951
	310.15	4.57×10^3	1.04	0.9922
	318.15	3.66×10^3	1.03	0.9869
HSA-GF	288.15	8.74×10^3	1.08	0.9791
	298.15	6.81×10^3	1.07	0.9849
	310.15	4.11×10^3	1.02	0.9973
	318.15	3.11×10^3	1.00	0.9972
HMG-MPT	288.15	4.12×10^5	1.15	0.9916
	298.15	1.95×10^5	1.10	0.9898
	310.15	1.05×10^5	1.06	0.9897
	318.15	0.640×10^5	1.03	0.9936
HMG-GF	288.15	3.62×10^5	1.15	0.9981
	298.15	1.80×10^5	1.10	0.9939
	310.15	1.27×10^5	1.08	0.9908
	318.15	0.720×10^5	1.04	0.9901

where K_b is the binding constant at temperature T and R is the universal gas constant. ΔH and ΔS values for HSA-MPT, HSA-GF, HMG-MPT and HMG-GF systems were obtained from the slopes and incepts of ln K_b versus 1/T plots (Fig. 6). Then, ΔG values were determined from Eq. (9). The calculated thermodynamic parameters for the bindings of MPT and GF drugs to HSA and HMG proteins are presented in Table 3. As seen from this table; ΔH, ΔS and ΔG values are negative for all protein-drug systems, except the ΔS value of HSA-MPT system. In addition, for HSA-GF, HMG-MPT and HMG-GF systems, ΔG values become less negative with increasing temperature. On the other hand, an increase in temperature leads to a decrease in the ΔG value of HSA-MPT system. Negative ΔG values indicate that the bindings of MPT and GF drugs to HSA and HMG proteins are spontaneous. Furthermore, the formations of HSA-MPT, HSA-GF, HMG-MPT and HMG-GF complexes are exothermic due to negative ΔH values.

Ross and Subramanian [36] have reported that negative ΔH and ΔS values indicate hydrogen bonds and van der Waals forces in the binding reactions. Electrostatic interactions are characterized by negative ΔH and positive ΔS values. Positive ΔH and ΔS values confirm hydrophobic interactions. According to the results given in Table 3, at pH7.4, electrostatic attraction forces between negatively charged HSA molecule and positively charged MPT molecule play an important role in the formation of HSA-MPT complex. According to Peters [37], the calculated net charges at pH7 for HSA are −9, −8 and +2 in domains I, II and III, respectively. Thus, the binding of MPT to HSA can be possible with a negatively charged site such as aspartic acid or glutamic acid in domains I and II. Negative ΔH and ΔS values represented in Table 3 indicate that hydrogen bonds and van der Waals forces are involved in the HSA-GF, HMG-MPT and HMG-GF binding reactions.

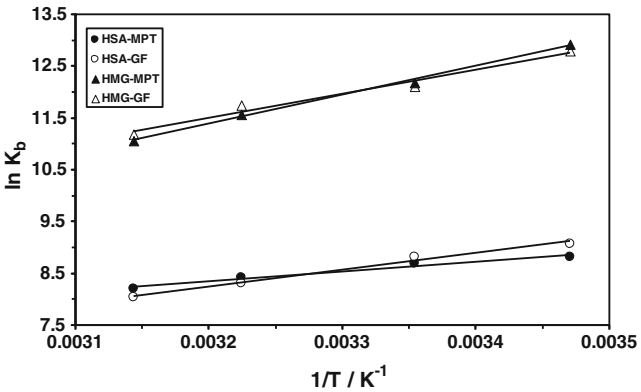

Fig. 6 Van't Hoff plots of HSA-MPT, HSA-GF, HMG-MPT and HMG-GF systems. Regression coefficients are 0.9838, 0.9931, 0.9963 and 0.9862 for HSA-MPT, HSA-GF, HMG-MPT and HMG-GF systems, respectively

Energy Transfer from HSA and HMG Proteins to MPT and GF Drugs

The distance between the tryptophan residue (donor) and the bound drug (acceptor) in protein can be calculated by Förster resonance energy transfer (FRET) theory. According to this theory, the rate of energy transfer depends on: (i) the relative orientation of the donor and acceptor dipoles, (ii) the extent of overlap of the fluorescence spectrum of the donor with the absorption spectrum of the acceptor and (iii) the distance between the donor and the acceptor [38]. The efficiency of energy transfer (E) can be calculated by the following equation;

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \tag{10}$$

where F and F_0 are the fluorescence intensities of protein molecule in the presence and absence of drug, respectively, r is the distance between the donor and acceptor and R_0 is the critical distance when the efficiency of the transfer is equal to 50 %. The value of R_0 (cm) is expressed as [30]

$$R_0^6 = 8.79 \times 10^{-25} k^2 N^{-4} \Phi J \tag{11}$$

where k^2 is the spatial orientation factor, N is the refractive index of medium, Φ is the fluorescence quantum yield of the donor and J is the overlap integral of the fluorescence spectrum of the donor with the absorption spectrum of the acceptor. J is given by the following equation;

$$J = \frac{\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda}{\Sigma F(\lambda)\Delta\lambda} \tag{12}$$

where F(λ) is the fluorescence intensity of the donor at the wavelength λ and is dimensionless, ε(λ) is the extinction coefficient of the acceptor at λ, which is in units of M^{-1} cm^{-1} and λ is wavelength whose unit is nm [30].

The spectral overlaps of the fluorescence spectra of HSA and HMG proteins with the absorption spectra of MPT and GF are shown in Figs. 7 and 8. The J values were obtained from the data presented in these figures and calculated as $8.097\times10^{-16} M^{-1}cm^3$ for HSA-MPT system, 1.571×10^{-15} $M^{-1}cm^3$ for HSA-GF system, $1.071\times10^{-15} M^{-1}cm^3$ for HMG-MPT system and $6.375\times10^{-16} M^{-1}cm^3$ for HMG-GF system. It was reported that $k^2=2/3$, $N=1.336$ and $\Phi=0.118$ for HSA [39] and $k^2=2/3$, $N=1.36$ and $\Phi=0.062$ for HMG [40]. The values of E, R_0 and r were obtained from Eqs. (10) and (11) and found to be $E=0.018$, $R_0=1.61$ nm

Table 3 Thermodynamic parameters for the bindings of MPT and GF drugs to HSA and HMG proteins

System	T/K	ΔH/(kJ.mol^{-1})	ΔS/(J.mol^{-1}.K^{-1})	ΔG/(kJ.mol^{-1})
HSA-MPT	288.15	−15.45	20.00	−21.21
	298.15			−21.41
	310.15			−21.65
	318.15			−21.81
HSA-GF	288.15	−26.92	−17.56	−21.86
	298.15			−21.68
	310.15			−21.47
	318.15			−21.33
HMG-MPT	288.15	−46.30	−53.44	−30.90
	298.15			−30.36
	310.15			−29.72
	318.15			−29.29
HMG-GF	288.15	−38.44	−27.34	−30.56
	298.15			−30.29
	310.15			−29.96
	318.15			−29.74

and r=3.14 nm for HSA-MPT system; E=0.021, R_0=1.80 nm and r=3.42 nm for HSA-GF system; E=0.119, R_0=1.50 nm and r=3.09 nm for HMG-MPT system and E=0.099, R_0=1.38 nm and r=1.99 nm for HMG-GF system. According to these results, for all protein-drug systems, the average distance between donor and

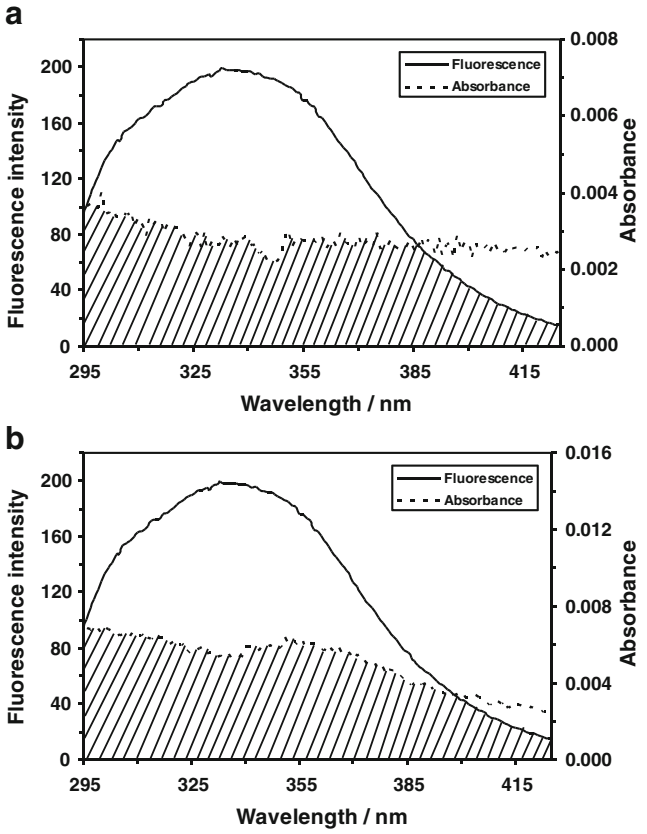

Fig. 7 Spectral overlap of the fluorescence spectrum of HSA with the absorption spectrum of MPT (**a**) and GF (**b**) (C_{HSA}=C_{MPT}=C_{GF}=5.0×10^{-6}M and T=298.15 K)

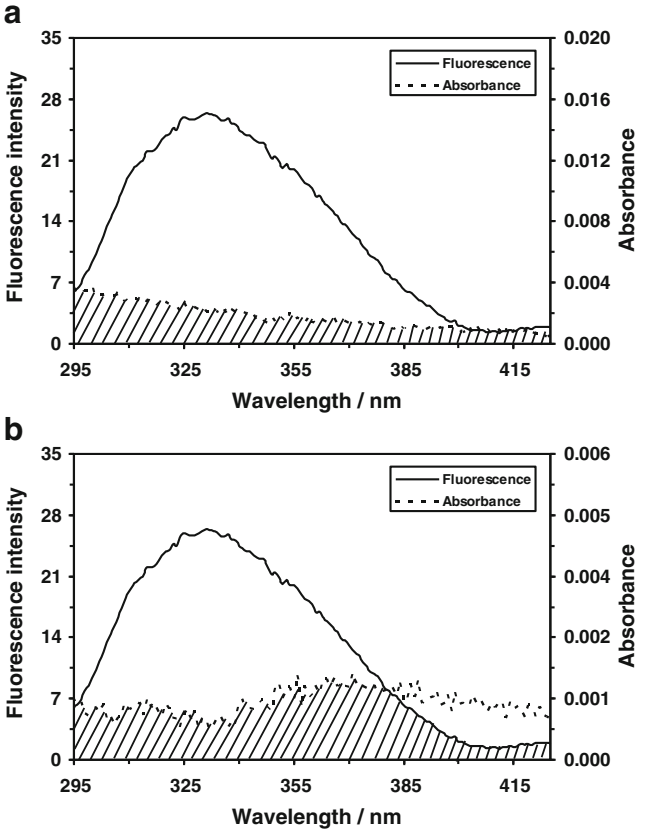

Fig. 8 Spectral overlap of the fluorescence spectrum of HMG with the absorption spectrum of MPT (**a**) and GF (**b**) (C_{HMG}=C_{MPT}=C_{GF}=2.5×10^{-6}M and T=298.15 K)

acceptor is less than 7 nm and is not outside the range of $r=0.5R_0$ to $r=2R_0$ [30]. In this case, it is possible the energy transfers from HSA and HMG proteins to MPT and GF drugs during the formation of protein-drug complexes. In addition, these results support that MPT and GF drugs quench the fluorescence of proteins by a static quenching mechanism [41].

Circular Dichroism (CD) Spectra

One of the most important parameters in protein-drug interactions is to determine the change in the conformational structure of protein, which may affect the functional properties of protein. For this reason, the CD spectra of proteins for the HSA-MPT, HSA-GF, HMG-MPT and HMG-GF systems were obtained to evaluate the influence of drug bindings on the secondary structures of HSA and HMG proteins. In the presence of various MPT and GF concentrations, the CD spectra of HSA and HMG proteins are shown in Figs. 9 and 10,

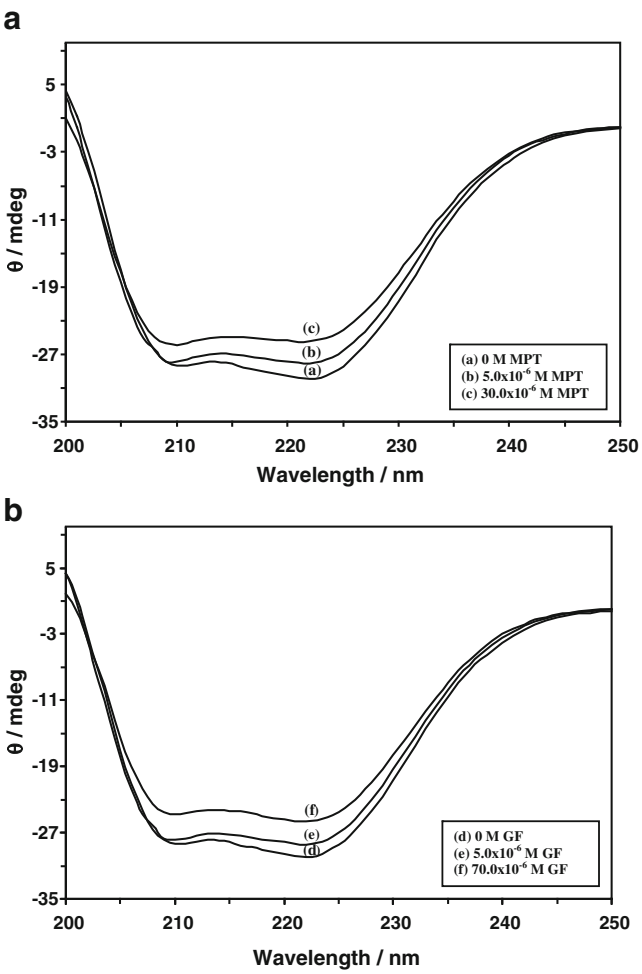

Fig. 9 CD spectra of 5.0×10^{-6}M HSA in the presence of various concentrations of MPT (**a**) and GF (**b**) at 298.15 K

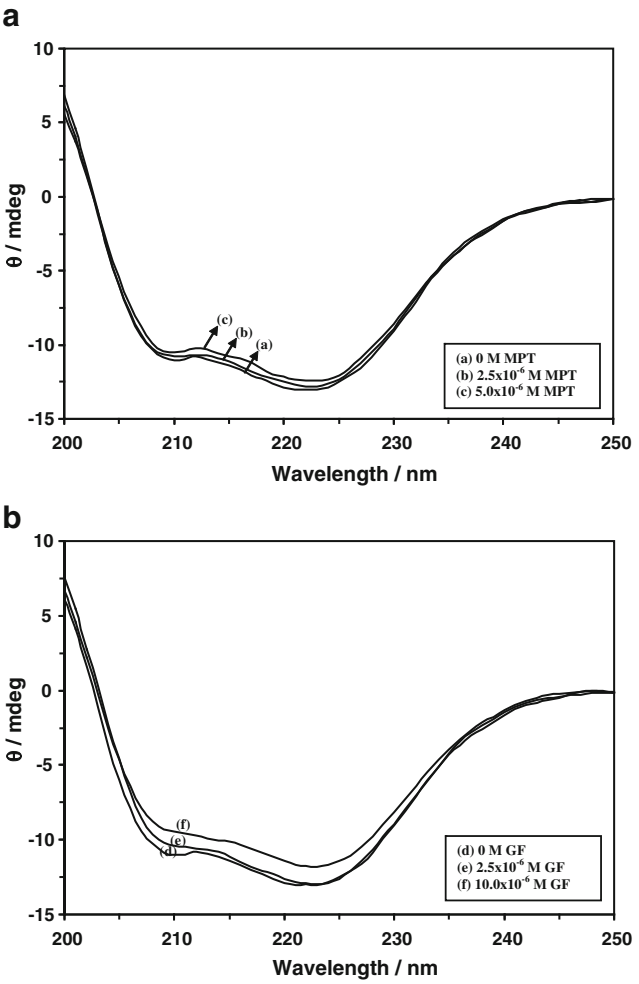

Fig. 10 CD spectra of 2.5×10^{-6}M HMG in the absence and presence of various concentrations of MPT (**a**) and GF (**b**) at 298.15 K

respectively. As seen from these figures, the CD spectra of HSA and HMG have two negative bands approximately at 209 and 222 nm, which are characteristic of the α-helix structure of proteins, owing to n→π* transition in the peptide bond of α-helix structure [30]. If there is a change in the α-helix structure of protein due to the bonding of drug to protein, the CD spectrum of protein varies depending on the degree of interaction. Generally, for both proteins, the intensities of negative bands at 209 and 222 nm exhibit an increase with the addition of MPT and GF. The CD results can be expressed in terms of mean residue ellipticity (MRE) in deg.dm^2.dmol^{-1} according to the following equation [30]:

$$MRE = \frac{\theta_{obs}(m\,\mathrm{deg})}{C_p n l 10} \tag{13}$$

where θ_{obs} is the observed CD, C_p is the molar concentration of protein, n is the number of amino acid residue in protein (n=585 and 574 for HSA and HMG, respectively) and l is the path length of the quartz cell.

The α-helix contents of HSA and HMG proteins are calculated from MRE values at 208 nm by the following equation [30]:

$$\alpha - helix(\%) = \left[\frac{(-MRE_{208} - 4000)}{(33000 - 4000)}\right] \times 100 \qquad (14)$$

where MRE_{208} is the MRE value at 208 nm, 4000 is the MRE of the α-form and random coil conformation at 208 nm and 33000 is the MRE value of a pure α-helix at 208 nm [29]. From the above equations, the α-helix amounts of HSA and HMG proteins in the absence and presence of MPT and GF drugs were calculated and the results were given in Table 4. As seen from this table, when the MPT and GF concentrations are increased from 0.0 to 5.0×10^{-6}M, the α-helix amount of HSA decreases slightly from 48.9 % to 48.4 % and 47.8 %, respectively. This decrease continues with the addition of higher MPT and GF concentrations. The addition of 2.5×10^{-6} and 8.0×10^{-6}M MPT into the 2.5×10^{-6}M HMG solution causes the decrease of 0.2 % and 1.9 %, respectively, in the α-helix amount of HMG (Table 4). In HMG-GF system, there is a decrease in the α-helix amount of protein. The impact of GF on the α-helix amount of HMG is higher than that of MPT. These results indicate that the bindings of drug molecules to HSA and HMG proteins change the hydrogen bonding networks of proteins.

Similar results have been reported by other authors. Lu et al. [42] have investigated the interaction of resveratrol with HSA and hemoglobin proteins and indicated that the α-helix amount of 2.0×10^{-6}M protein in the presence of 0.0 M, 2.0×10^{-6}M and 4.0×10^{-6}M resveratrol is 49.65 %, 45.51 % and 40.34 % for HSA and 36.55 %, 34.82 % and 33.10 % for hemoglobin. The interaction between hemoglobin and Ni^{2+} has been studied by Wang et al. [43]. They have reported that the α-helix value of 3.0×10^{-7}M hemoglobin is 36.6 % and it decreases to 32.9 % in the presence of 5.0×10^{-5}M Ni^{2+}.

Table 4 The α-helix amounts of HSA and HMG in the presence of MPT and GF at 298.15 K

System	[Protein]/10^{-6}M	[Drug]/10^{-6}M	α-helix (%)
HSA-MPT	5.0	0.0	48.9
	5.0	5.0	48.4
	5.0	30.0	45.0
HSA-GF	5.0	0.0	48.9
	5.0	5.0	47.8
	5.0	70.0	41.8
HMG-MPT	2.5	0.0	35.6
	2.5	2.5	35.4
	2.5	8.0	33.7
HMG-GF	2.5	0.0	35.6
	2.5	2.5	32.5
	2.5	10.0	28.4

Conclusions

The interaction of MPT and GF drugs with HMG protein was stronger than HSA-MPT and HSA-GF interactions. The values of Stern-Volmer quenching constant, bimolecular quenching constant and binding constant for HSA-MPT, HSA-GF, HMG-MPT and HMG-GF systems at pH7.4 decreased with increasing temperature. The number of binding site on protein molecule was determined to be one for each protein-drug system. Negative ΔH and positive ΔS values indicated that electrostatic attraction forces played an important role in HSA-MPT binding reaction. However, negative ΔH and ΔS values obtained for other protein-drug systems indicated that the formation of HSA-GF, HMG-MPT and HMG-GF complexes occurred via hydrogen bonds and van der Waals forces. In addition, ΔG values were negative for all systems; therefore, the binding processes were spontaneous. During the formation of protein-drug complexes, energy transfers from HSA and HMG proteins to MPT and GF drugs occurred by a static quenching mechanism. The average binding distances between donor and acceptor molecules were found to be 3.14 nm for HSA-MPT system, 3.42 nm for HSA-GF system, 3.09 nm for HMG-MPT system and 1.99 nm for HMG-GF system. Moreover, the interactions of MPT and GF drugs with blood proteins caused a decrease in the α-helix amount of HSA and HMG proteins.

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