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RESEARCH ARTICLE

STUDY ON THE BINDING BEHAVIOR OF BOVINE SERUM ALBUMIN (BSA) AND HUMAN SERUM ALBUMIN (HSA) WITH CEFTRIAXONE: SPECTROSCOPIC INVESTIGATIONS

***Abu Teir, M.M. and Darwish, S. M.**

Biophysics Research Laboratory, Faculty of Science and Technology, Al-Quds University, Palestine

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ABSTRACT

The mechanism of the interaction between bovine serum albumin (BSA) and ceftriaxone was studied employing fluorescence, and circular dichroism (CD) spectral methods. The intrinsic fluorescence of BSA and HSA was quenched by ceftriaxone in a static quenching mode, which was authenticated by Stern-Volmercalculations. The binding constant was obtained, which indicated a spontaneous and hydrophobic interaction between ceftriaxone with BSA and ceftriaxone with HSA with ceftriaxone. The present work uses spectroscopy to elucidate the mechanism behind the interaction between BSA, HSA and ceftriaxone. The BSA and ceftriaxone complex provides a model for studying drug-protein interactions and thus may further facilitate the study of drug metabolism and transportation. The binding properties on ceftriaxone to human serum albumin (HSA) have been studied for the first time using fluorescence spectroscopy, and circular dichroism (CD) spectroscopy. The results of spectroscopic measurements suggested that the hydrophobic interaction is the predominant intermolecular force stabilizing the complex, which is in good agreement with the results of molecule modeling study.

INTRODUCTION

The interaction between biomacromolecules, especially between plasma proteins and drugs, has been an interesting research field in life sciences, chemistry, and clinical medicine (Lu *et al.,* 2010). Drug-albumin complexes may be considered as models for gaining fundamental insights into drug-protein interactions. Serum albumin is a major soluble protein constituent of the circulatory system and has many physiological functions such as acting as a plasma carrier by nonspecifically binding to several hydrophobic steroid hormones and as a transport protein for hemin and fatty acids (Zunszain *et al.,* 2003). Albumins are characterized by a low content of tryptophan and methionine and a high content of cystine and charged amino acids (Brown,, 2003; Patterson and Geller, 1977; Hirayama *et al.,* 1990). Bovine serum albumin (BSA), its molecular structure shown in Figure (1) which is an example of a mammalian albumin, has been studied extensively because of its stability, neutrality in many biochemical reactions, and low cost (Peters, 1985; Tarushi *et al.,* 2010). Brown elucidated the 607 amino acid residue, primary structure of BSA in 1975, twenty one of which are tyrosine (Tyr) residues and two of which are tryptophan (Trp) residues located at positions 134 and 212, respectively (Brown, 1975; Peters, 1985). These two Trp residues cause BSA to have intrinsic fluorescence. On the contrary, Serum albumins of the circulatory system have various physiological functions, including the maintenance of osmotic pressure, transport, distribution, and participation in the metabolism of many endogenous and exogenous ligands (e.g., drugs, metabolites, fatty acids, amino acids, and hormones), resulting in increased solubility of these compounds in the plasma, which can reduce their toxicity, and/or protect them against oxidation or other reactions (Taguchi *et al.,* 2012; Peters, 1985). The binding between drugs and serum albumin is an important factor in understanding the interaction of the organism with drugs (pharmacokinetic studies), since it influences the distribution, excretion, metabolism and interaction with the biological target, itself.HSA structure consists of three structurally-similar domains (I, II, and III), each containing two subdomains, A and B (Paul *et al.,* 2010; Carter *et al.,* 1989). Each subdomain has a main cavity for interaction with ligands and, therefore, there are a total of six main cavities for interaction. The amino acid residue tryptophan (Trp) is often used for the association studies of this albumin with endogenous and exogenous molecules by fluorescence spectroscopy techniques. The HSA structure has only one Trp, located in sub domain IIA (Trp-214), as shown in Figure 1 (Sugio *et al.,* 1999). The presence of molecules

****Corresponding author: Abu Teir, M.M.***
Biophysics Research Laboratory, Faculty of Science and Technology, Al-Quds University, Palestine

that interact with HSA can modify the fluorescence of HSA and this effect depends on the concentration and average distance between these molecules and the in dole moiety of the Trpchromophore. What typically occurs is a decrease in fluorescence intensity and, therefore, it is said that the molecule is a quencher of the fluorescence Ceftriaxone is a third-generation cephalosporin antibiotic. Cephalosporins are semisynthetic antibiotics produced by fungi *Cephalosporium* and, like penicillins, are *β*-lactam antibiotics, which have broad-spectrum activity against Gram-positive and Gram-negative bacteria. Ceftriaxone is often used to chelate metal ions, and we believe this improves its antibacterial activity to study the effect of metal ions on its biological activity (Gurdal *et al.,* 1991; Auda *et al.,* 2009). The structure of ceftriaxone contains –NH2, –COOH, –CO, and N–C functional groups as shown in Figure (2) which are all electron donors. In this study a spectroscopic investigation of the binding of the ceftriaxone to HSA and BSA, steady-state fluorescence, quenching of tryptophan fluorescence and ITC measurements were carried out.

MATERIALS AND METHODS

Materials (BSA, HSA – Ceftriaxone)

Human serum albumin (HSA, 96–99% purity) and ceftriaxone disodium salt hemi(heptahydrate) in powder form were purchased from Sigma Aldrich chemical company and used without further purification. All reagents used were of analytical-reagent grade unless specified. Doubly distilled deionized water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). BSA (Sigma) was purchased from a local market without further purification to prepare a stock solution (50 μmol L^{-1}), which was kept in a brown flask at 4◦C. A 1.0 mmol L^{-1} stock solution of ceftriaxone was prepared in a calibrated flask. The phosphate buffered saline (PBS) was prepared using Na2HPO4 and NaH2PO4 for controlling pH of the system at 7.4. Other reagents were purchased from a local market.

Preparation of stock solutions

HSA was dissolved in phosphate buffer saline, at physiological pH 7.4 at (80 mg/ml) concentration. Ceftriaxone with molecular weight of (661.6 g/mol) was dissolved in phosphate buffer saline (1.21 mg/ml), the solution was placed in ultrasonic water path (SIBATA AU-3T) for six hours to ensure that all the amount of ceftriaxone was completely dissolved. The final concentrations of HSA-ceftriaxone complexes were prepared by mixing equal volume of HSA and ceftriaxone stock solution. HSA concentration in all samples was fixed at 40 mg/ml. However, the concentration of ceftriaxone in the final protein drug solutions was decreased gradually to attain the desired drug concentrations of 0.687 mM, 0.910 mM, 1.14 mM, 1.37 mM, 1.60 mM, 1.83 mM, 2.06 mM. The solution of ceftriaxone and HSA were incubated for 1 h (at 20oC) before spectroscopic measurements were taken. The same procedure was done for BSA-Ceftriaxone complexes. A 3mL solution, containing appropriate concentration of BSA, was titrated by successive additions of a 1.0mM ceftriaxone solution. Titrations were done manually by using micropipettors. The same procedure was done for the preparation of the stock solution of BSA – Ceftriaxone complex concentration.

Fluorescence

The fluorescence measurements were performed by a Nano-Drop ND-3300 Fluorospectrometer at 25 ^{o}C. The excitation had been done at the wavelength of 360 nm and the maximum emission wavelength is at 439 nm. The excitation source comes from one of three solid-state light emitting diodes (LED's). The excitation source options include: UV LED with maximum excitation 365 nm, Blue LED with excitation 470 nm, and white LED from 500 to 650 nm excitation. A 2048-element CCD array detector covering 400–750 nm, is connected by an optical fiber to the optical measurement surface. The emission spectra were recorded for free HSA (40 mg/ml) and for its complexes with ceftriaxone solutions with the concentrations of (0.687, 0.910, 1.14, 1.37, 1.60, 1.83, 2.06 mM). Repeated measurements were done for all samples and no significant differences were observed. In a typical fluorescence measurement, the fluorescence emission spectra were recorded ranging from 300 to 450nm upon the excitation wavelength at 295nm using the excitation and emission slit widths both of 5 nm. The experiments for discussing binding mechanism were conducted at temperature, 298 K, as maintained by water bath. The interaction between BSA and ceftriaxone was studied. BSA concentration was fixed at 0.5 μM, and a series of ceftriaxone standard solutions was added. Fluorescence spectra of BSA were recorded from 300 to 500nm upon excitation at 295nm.

RESULTS AND DISCUSSION

Analysis of fluorescence quenching of HSA and BSA by Ceftriaxone

Fluorescence spectroscopy is one of the most widely used spectroscopic techniques in the fields of biochemistry and molecular biophysics today (Royer, 1995). Fluorescence measurements can give some information on the binding mechanism of small molecule substances to protein, including binding mode, binding constants, binding sites and intermolecular distances (Liu *et al.,* 2014). The fluorescence of HSA comes from tryptophan, tyrosine and phenylalanine residues. Actually, the intrinsic fluorescence of HSA is almost contributed by tryptophan alone (Sulkowska, 2002). Figure (3) shows the fluorescence spectra of HSA in the presence of various concentrations of ceftriaxone. It is obvious that HSA fluorescence intensity gradually decreased with the increase of ceftriaxone concentration, while the peak position shows little or no change upon increasing the concentration of ceftriaxone to HSA, indicating that ceftriaxone binds to HSA. Under the same condition, no fluorescence of ceftriaxone was observed. Which indicates that ceftriaxone could quench the auto fluorescence of HSA, and that the interaction between both ceftriaxone from one hand, and HSA from the other indeed exists, leading to a change in the microenvironment around the tryptophan residue and further exposure of tryptophan residue to the polar solvent (Wang *et al.,* 2007; Cui *et al.,* 2007). Two mechanisms, namely dynamic quenching and static quenching responsible for the fluorescence quenching, the possible quenching mechanism can be deduced from the Stern–Volmer plot. The dynamic quenching process can be described by the Stern–Volmer equation (Tian *et al.,* 2003).

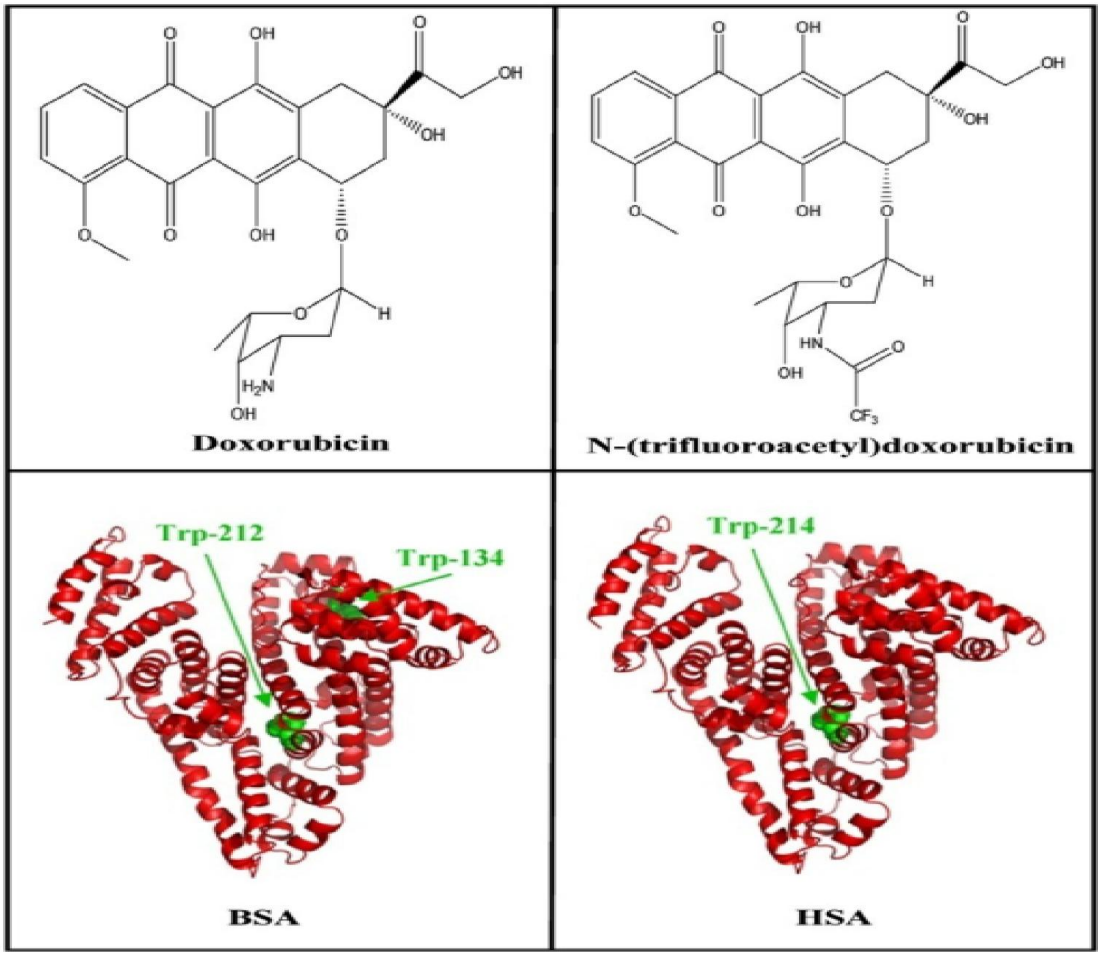

Fig. 1. Chemical structure of HSA and BSA

$$\frac{F}{F_0} = 1 + K_q\tau_0[Q] = 1 + K_{sv}[Q]$$

where F_0 and F are the fluorescence intensities of HSA in the absence and presence of the quencher, respectively. Kq is the quenching rate constant of the biomolecule, K_{SV} is the Stern–Volmer dynamic quenching constant, and $K_{SV}=K_q\tau_0$. τ_0 is the average lifetime of the biomolecule without quencher.

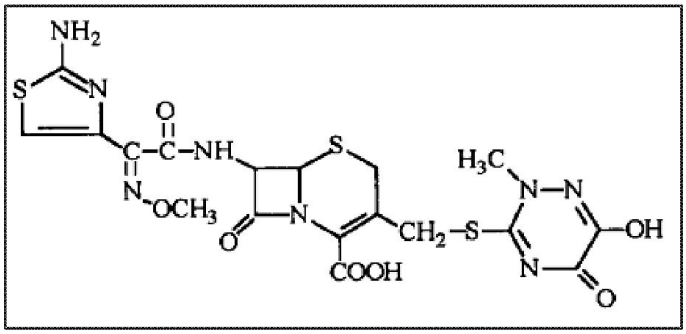

Fig. 2. Chemical structure of Ceftriaxone

The value of τ_0 of the biopolymer is $10^{-8}s^{-1}$ (Chen *et al.,* 1990) and [Q] is the concentration of quencher ceftriaxone. Fluorescence quenching can be processed via different mechanisms, usually classified as dynamic quenching and static quenching. Dynamic and static quenching can be distinguished by their response to temperature (Kandagal *et al.,* 2007).

Increased temperatures will quicken diffusion, produce large amount of collisional quenching, dissociate weakly bound complexes, and diminish static quenching. Based on this, the Stern-Volmer plots at different temperatures were drawn. As shown in Figure 4, the curves were linear. The quenching type may be static or dynamic, since the characteristic Stern-Volmer plot of combined quenching (both static and dynamic) has an upward curvature.

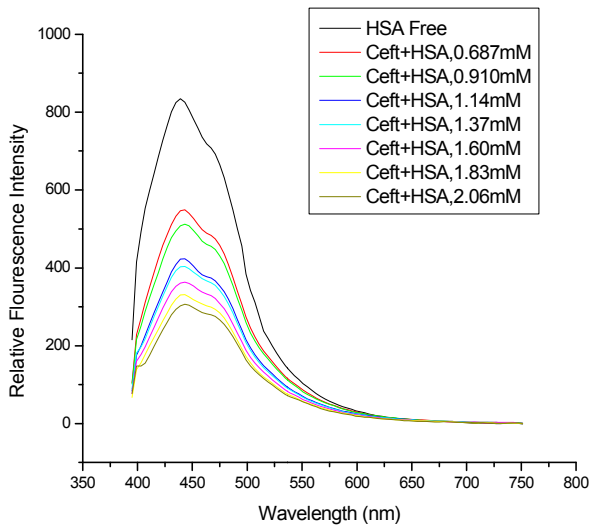

Figure 3. Fluorescence emission spectra of HSA in the absence and presence of Ceftriaxone at different concentrations

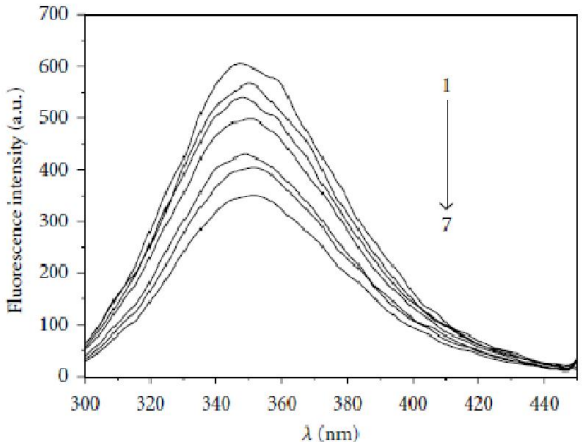

Fig. 4. Fluorescence emission spectra of BSA in the absence and presence of Ceftriaxone at different concentrations

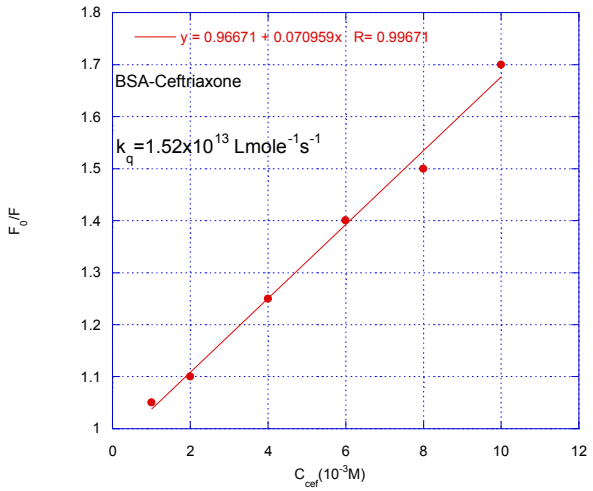

Fig. 5. The Stern–Volmer plot for Ceftriaxone–BSA complexes

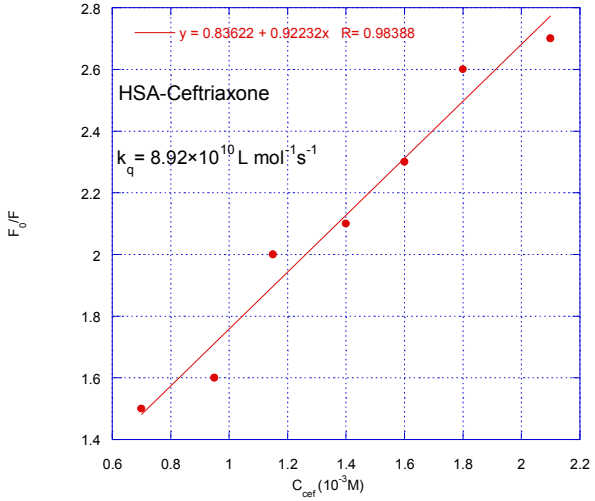

Fig. 6. The Stern–Volmer plot for Ceftriaxone–HSA complexes

Table 1. *KSV* and *kq*values at 298 K for BSA and HSA

	Temperature (K)	K_{sv}(L.mole^{-1})	K_q(L.mole^{-1}s^{-1})
BSA	298	1.52×10^5	$1.52\text{x}10^{13}$
HSA	298	8.92×10^2	$8.92\text{x}10^{10}$

Furthermore, the slope of the Stern- Volmer plots increased with the temperature. In this case, the quenching mode of ceftriaxone on the fluorescence of BSA was due to static quenching. The binding constant of ceftriaxone with BSA and with HSA was studied and it was shown that the binding of ceftriaxone with BSA stronger than that with HSA.

DISCUSSION

From the results of the interaction of the ceftriaxone with BSA and HSA and as shown in the Figure (3) and Figure (4), the fluorescence quenching of BSA caused by ceftriaxone was attributed to the static quenching (Kandagal *et al.,* 2007; Abu Teir *et al.,* 2011, 2012). Thus, it is proposed that the complex formation takes a major role rather than dynamic collision for the interaction between BSA and ceftriaxone. The Stern-Volmer quenching constant Ksv indicates the sensitivity of the fluorophore to a quencher. Linear curve was plotted according to the Stern–Volmer equation as shown in Fig. (5) for ceftriaxone- BSA complexes. The Stern–Volmer quenching constant Ksv was obtained by the slope of the curve obtained in Fig. (5), and its value equals (1.52×10^5L mol-1). From the equation above the value of $Ksv=Kq\tau_0$, from which we can calculate the value of Kq using the fluorescence life time of 10^{-8} s for BSA, to obtain Kq value of (1.52×10^{13} L mol^{-1}s^{-1}) for ceftriaxone- BSA complexes shown in table (1). The Stern-Volmer quenching constant Ksv indicates the sensitivity of the fluorophore to a quencher. Linear curve was plotted according to the Stern–Volmer equation as shown in Fig.(6) for ceftriaxone- HSA complexes. The Stern–Volmer quenching constant Ksv was obtained by the slope of the curve obtained in Fig.(6), and its value equals (8.92×10^2L mol^{-1}). From the equation above the value of $Ksv=Kq\tau_0$, from which we can calculate the value of Kq using the fluorescence life time of 10^{-8} s for HSA, to obtain Kq value of (8.92×10^{10} L mol^{-1}s^{-1}) for ceftriaxone- HSA complexes shown in table (1). Generally, the maximum dynamic quenching constant, Kq of various kinds of quenchers with biopolymer is (2.0×10^{10} L mol^{-1}s^{-1}) (Lakowicz, 1973). Obviously, the values of kq were greater than that of the maximum dynamic quenching constant.

This suggested that the fluorescence quenching was not the result of dynamic quenching, but the consequence of static quenching (Chen *et al.,* 1990; Wang *et al.,* 2008). From the results of the quenching constant, it was clear that the fluorescence quenching of BSA caused by ceftriaxone is much larger than that of the fluorescence quenching of HSA caused by ceftriaxone. This is related to the positions of the intrinsic Tryptophan residue in the BSA (Trp 212 and Trp 134) while in the HAS only one position of the intrinsic Tryptophan residue Trp214, that means the quenching in the case of BSA-Ceftriaxone will be much greater than that of HAS-Ceftriaxone case. The excitation had be done for the BSAof a wavelength of 295 nm and BSA exhibits a strong fluorescence emission with band peak at 348 nm. While for HAS The excitation had been done at the wavelength of 360 nm and the maximum emission wavelength is at 439 nm as shown in the Figures. Besides that, the binding constant of the ceftriaxone with HSA was found $K=1.02\text{x}10^3$ M^{-1} while the binding constant of the ceftriaxone with BSA was found $K=1.99\text{x}10^5$ M^{-1}, so it was clear that the binding of the ceftriaxone with BSA was much stronger than that with HSA, therefore the interaction between ceftriaxone with BSA more than that with HAS (Qiaoli Yue *et al.,* 2012; Abu Teir *et al.,* 2014).

Conclusion

From the Fluorescence study we determined values for the binding constant and the quenching constant for ceftriaxone-HSA complexes and ceftriaxone-BSA complexes. The results indicate that the intrinsic fluorescence of HSA and BSA was quenched by ceftriaxone through static quenching mechanism. The results of the fluorescence quenching caused by the ceftriaxone shows that the quenching of BSA is greater than that of the HSA. Beside that it was shown that the binding of ceftriaxone with BSA is much stronger than that with HSA.

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