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Original Article

In vitro study of drug-protein interaction using electronic absorption, fluorescence, and circular dichroism spectroscopy

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ABSTRACT

In the near future, design of a new generation of drugs targeting proteins will be required. Considering the complex bond between the drug and protein, the structure and stability of the target protein should be considered. So far, a series of *in vitro* investigations have been conducted with the aim of predicting drug-biological medium interactions. In these studies, use of spectroscopic methods, such as electronic absorption, fluorescence, and circular dichroism spectroscopy, which are briefly discussed in the present study, has been highlighted. The binding affinity of drug(s) to protein(s) and their binding mechanism(s) can be clearly determined by these methods, which reveal reactions in biological systems at low concentrations under physiological conditions. Ultraviolet-visible spectroscopy can be used as an accessible tool to measure slight changes in protein structure. Moreover, fluorescence spectroscopy provides tertiary structural information. On the other hand, circular dichroism spectroscopy in far-ultraviolet regions (180–260 nm) yields suitable information about different secondary structures of proteins. Conformational changes of proteins due to alterations such as physicochemical conditions, *in vitro* chemical modifications, and drug binding could impact ultraviolet-visible absorption, circular dichroism, and fluorescence spectra. Therefore, the study of changed spectra could reveal the structure-activity relationship of drug compounds and target proteins. In the present study, a short description of the mentioned methods, along with some related equations which are usually used to analyze and discuss the preliminary data, is presented. Overall, the obtained results could facilitate the development of biological and pharmaceutical potentials of drugs in the future.

Keywords: *In vitro* study, Drug-protein interaction, Protein structure, Electronic absorption spectroscopy, Fluorescence spectroscopy, Circular dichrohism spectroscopy

Etude *in Vitro* de l'interaction médicament-protéine par absorption électronique, fluorescence et dichroïsme circulaire

Résumé: Dans un futur proche, la conception d'une nouvelle génération de médicaments ciblant des protéines sera nécessaire. Etant donnée la complexité des liaisons entre médicaments et protéines, la structure et la stabilité de la protéine cible doivent également être prises en compte. Jusqu'alors, une série d'études *in vitro* ont été menées dans le but de prédire les interactions potentielles entre médicament et milieu biologique. Dans cette étude, plusieurs méthodes spectroscopiques comme l'absorption électronique, la fluorescence et le dichroïsme circulaire ont été utilisées dans ce sens. Ces méthodes sont capables de révéler, dans des conditions physiologiques et dans de faibles concentrations, l'affinité des liaisons médicaments-protéines survenant dans des systèmes biologiques variés. De plus, la spectroscopie à l'ultraviolet visible représente une technique accessible pour mesurer de légers changements structurels dans les protéines d'intérêts. Des données concernant les structures secondaires et tertiaires des protéines ont été respectivement obtenues par spectroscopie à ultraviolet lointain (180–260 nm) et spectroscopie à fluorescence. Notre étude montre que les changements de conformation des protéines induites par des altérations physicochimiques, des modifications chimiques *in vitro* ou des

liaisons avec des composés médicamenteux peuvent avoir un impact sur les spectres obtenus par absorption à ultraviolet-visible, par dichroïsme circulaire ou par fluorescence. Par conséquent, l'étude des spectres modifiés peut révéler la relation structure-activité entre composés médicamenteux et protéines cibles. Les différentes méthodes de spectrométries utilisées ont été brièvement décrites et les équations habituellement utilisées pour analyser et développer les résultats préliminaires sont présentées. Dans leur globalité, les résultats obtenus peuvent à l'avenir faciliter le développement de produits biologiques et pharmaceutiques.

Mots clés: Etude *in vitro,* Interaction médicament-protéine, Structure des protéines, Spectroscopie, Absorption électronique, Fluorescence, Dichroïsme circulaire

Introduction

Considering the necessity of developing drugs, which specifically target malformed cells for optimal treatment, it is essential to study the interaction between newly synthesized drugs and cell components (Miskovic et al., 2013). By the introduction of DNA intercalator drugs by Lerman in 1961, DNA became one of the most important targets for cancer treatment (Lerman, 1961). Based on numerous investigations, DNA intercalator drugs could cause various biological alterations, such as DNA damage, sister chromatid exchange, micronucleus formation, and finally induction of cytotoxic effects (Marverti et al., 2008), even in non-malignant cells (Sudharsan Raj and Heddle, 1980; Wilson et al., 1984). Therefore, new approaches by focusing on proteins as major cellular biomolecules (even in cancer therapy) provide valuable information for the design of drugs targeting proteins (Jacob and Vert, 2008; Yamanishi et al., 2008; Keiser et al., 2009). Recently, synthesis of new drugs with high bioactivity towards polymerase I inhibitors (Colis et al., 2014) and topoisomerases has been reported, without causing any DNA damage (Bandyopadhyay et al., 2012; Lin et al., 2014). In addition to nuclear proteins, critical cytosolic enzymes could be also affected by basic DNA-binding drugs. In an *in vitro* study by Attar et al. (2009), a DNA intercalator (acriflavine), which induces apoptosis and necrosis in yeast *Candida utilis* (Keyhani et al., 2009), could have direct effects on the structure and function of catalase.

In previous research, changes were induced by doxorubicin, as an intercalator drug, in total superoxide dismutase activity and respiratory chain of *Salmonella typhimurium* (Keyhani et al., 2005; Khavari-Nejad et al., 2007). Overall, any conformational changes in the protein structure could alter its function. Therefore, understanding the mechanism(s) of interaction between drugs and proteins is of prime importance in the development of structure-based drug designs (Mizutani et al., 2012; Colis et al., 2014). On the other hand, the study of drug-protein interactions could be important in determining the overall distribution, solubility, half-life, excretion, activity, and toxicity of a drug (Ideker et al., 2001). For this reason, various spectroscopic methods, e.g., ultraviolet-visible (UV-Vis) spectroscopy, circular dichroism (CD) spectroscopy, fourier transform infrared spectroscopy (FTIR), and fluorimetry, along with equilibrium dialysis and electrochemistry, have been applied to both improve the binding properties and optimize the processes involving drug application (Wilting et al., 1980; Vuignier et al., 2010; Rezaei-Tavirani et al., 2012; Cui et al., 2013). The first application of spectroscopy in the study of molecular interactions between drugs and proteins was reported by Shih and Rho (1977). In their study, the chemical interaction between lysergic acid and serotonin-binding protein was confirmed by fluorescence spectroscopy. So far, numerous *in vitro* investigations have been performed, using various sensitive, reproducible, and convenient spectroscopic techniques (Kandagal et al., 2006). Minai-Tehrani *et al*. by using the abovementioned methods indicated that scopolamine (hyoscine), as an anticholinergic drug, binds to sucrase,

causes structural changes, and finally inhibits the enzymatic activity (Minai-Tehrani et al., 2010). A summary of the interactions between some classes of drugs and proteins is presented in Table 1. Since a number of drugs are injected into the blood and interact with plasma proteins, biosafety studies are of pivotal importance. By using spectroscopic methods, several researchers (Rahman et al., 1993; Naik et al., 2009; Faridbod et al., 2011; Vahedian-Movahed et al., 2011) studied the biocompatibility of a given drug on albumins, as one of the most abundant proteins in plasma (40 mg/ml in the blood), involved in many important physiological functions, including regulation of colloidal osmotic pressure and the binding and transport of substances in the bloodstream. Among various albumin sources, bovine serum albumin (BSA) and human serum albumin (HSA) have been comprehensively studied, given their medical importance, cost-effectiveness, availability, and unusual ligand-binding properties. In the present study, UV-Vis electronic absorption, fluorescence, and CD spectroscopy are briefly described to clarify drug-induced alterations in biological molecules. In addition, in order to determine the concentration, purity, and enzymatic activity, UV-Vis spectroscopy could distinguish any subtle changes in the UV or visible absorption spectrum of a protein upon drug binding.

By the use of UV-Vis spectroscopy, the number of drug molecules, which are bound to the protein, along with the binding quality, could be identified (Tayefi-Nasrabadi et al., 2006; Vahedian-Movahed et al., 2011; Naik and Nandibewoor, 2013). Additionally, fluorescence spectroscopy, as the most convenient method, provides useful information on the tertiary structure, nature of the binding site, distance between the drug and protein, affinity of the binding site towards the drug, and thermodynamic parameters (Sevilla et al., 2007; Amin and Bano, 2014; Shahabadi and Hadidi, 2014). Finally, CD spectroscopy is widely used to evaluate chiral proteins. This method in far-UV (180-260 nm) and near-UV (250-350 nm) regions provides rapid information regarding different forms of regular secondary structure and conformation of proteins, respectively (Oravcova´ et al., 1996; Sevilla et al., 2007; Shahabadi and Hadidi, 2014). Based on the discussed background, the present study was designed to provide the reader with an overview of three spectroscopic methods, used to assess the secondary structure of protein and analyze the changes in protein structure as a result of drug binding. The low-resolution structures depicted by spectroscopic methods obviously do not provide thorough structural information as X-ray crystallography or high-resolution nuclear magnetic resonance (NMR) imaging. However, spectroscopic approaches, which are mainly successful in high-affinity binding sites, can help researchers gain a general insight into newly synthesized drug(s) before being approved for human studies. In addition, many spectroscopic methods are relatively rapid and do not require various materials; therefore, they are considered appropriate for early target identification and selection. The gathered data, alone or combined with molecular modeling techniques, could be applied to explore the toxicity of synthesized drugs and help clarify the molecular mechanisms of *in vivo* toxicity.

MATERIALS AND METHODS

Drug binding to proteins was assessed in the presence of three essential materials, i.e., protein, drug, and buffer solution. Albumins, as one of the most abundant plasma proteins from various sources (e.g., bovine and human sources), are widely used, although other proteins can be considered, as well. Overall, appropriate buffer solutions for drug-protein interaction studies should be carefully prepared. Moreover, analytical reagents and double-distilled water should be applied throughout the experiment.

UV-Vis spectroscopy. The electronic absorption spectra of the studied proteins in the absence and presence of different drug concentrations were recorded in a range of 200-700 nm on a UV-Vis spectrophotometer. The apparent dissociation constant (K_d) of the drug-protein complex, which describes the

binding strength, could be calculated according to Equation (1):

$1/\Delta A = K_d/\Delta A_{max}\ 1/[drug] + 1/\Delta A_{max}$ (1)

where ΔA is the absorbance change at a specific wavelength (e.g., ΔA_{280}) caused by a given drug concentration, ΔA_{max} denotes the absorbance change at a specific wavelength for complete formation of the complex (with an infinite drug concentration), and [drug] is the concentration of the free drug, which is assumed to be equal to the initial drug concentration. In addition, the Hill coefficient (h) could be calculated from the plot of $\log[\Delta A/(\Delta A_{max}- \Delta A)]$ versus log[drug], which suggests the number and mode of drug binding to protein (i.e., cooperative or non-cooperative) (Hadizadeh et al., 2009). To realize the stability of the drug-protein complex, the Gibbs free energy of binding ($\Delta G_{binding}$), which is related to K_d, could be calculated by Equation (2):

$\Delta G_{binding} = RT\ln(K_d)$ Equation (2)

Fluorescence spectroscopy. Intrinsic protein fluorescence after the addition of various drug concentrations was detected on the fluorescence spectrophotometer. An excitation wavelength of 297 nm specific for tryptophan fluorophores and a wavelength of 280 nm specific for tyrosine and tryptophan fluorophores were selected; the emission spectra were recorded between 300 and 450 nm. In order to distinguish the fluorescence quenching mechanism (static/dynamic quenching) with respect to temperature, the well-known Stern-Volmer plot could be analyzed by Equation (3):

$F_0/F = 1 + K_{SV}[Q]$ (3)

where F_0 and F represent fluorescence intensities in the absence and presence of the quencher, respectively , K_{SV} denotes the Stern-Volmer constant, and [Q] is the concentration of the quencher (drug). According to Equation (3), the slope of F_0/F versus [Q] could yield the Stern-Volmer constant. The fraction of total tryptophan residues accessible to the drug could be also calculated from the modified Stern-Volmer plot, also known as the Lehrer plot (Equation 4) (Hadizadeh et al., 2009):

$F_0/\Delta F = 1/K_{SV}f_a 1/[Q] + 1/f_a$ (4)

where ΔF is equal to F_0-F and f_a is the fraction of accessible flourophores; F_0, F, and [Q] were defined in the above-mentioned equation. The ordinate intercept of the $F_0/\Delta F$ plot against 1/[Q] provides the accessible fluorophores at an infinite quencher concentration.

According to Equation (5), by linearly fitting the plot of $\log(F_0\text{-}F/F)$ versus log[Q], the values of n (number of binding sites) and $K_{binding}$ (binding constant) could be measured, based on the slope and Y-axis intercept, respectively (Kandagal et al., 2006):

$\text{Log}(F_0\text{-}F/F) = \log K_{binding} + n \log[Q]$ (5)

Thermodynamic forces responsible for the binding between the protein and drug could be also evaluated, using the Van't Hoff equation (6):

$\ln K_{binding} = -\Delta H°/RT + \Delta S°/R$ (6)

where $K_{binding}$ is the binding constant at the corresponding temperature, and ΔH° and ΔS° denote the slope and intercept of linear Van't Hoff plot, respectively. Finally, the Gibbs free energy (ΔG°) could be estimated by Equation (7):

$\Delta G° = \Delta H° - T\Delta S°$ (7)

CD spectroscopy. Protein CD spectra upon drug binding (at various concentrations) in far-UV (180-260 nm) and near-UV (250–350 nm) regions were recorded with a CD spectrometer. The results are generally expressed as the mean residue ellipticity (MRE) in $deg.cm^2.dmol^{-1}$, as defined in Equation (8):

$MRE = \theta_{obs}/(10nlC_p)$ (8)

where θ_{obs} denotes CD in millidegrees, n is the number of residues in the protein, l refers to the optical path length in cm, and C_p is the mole fraction (Kelly and Price, 1997). The percentage of secondary structure fractions, such as α-helical content, was calculated, based on MRE values at a wavelength of 222 nm, using the following equation (Ahmad et al., 2006):

$\%\alpha\text{-helix} = (MRE_{222nm} - 2340/30300) \times 100$ (9)

By using the CD Spectra Deconvolution Software (i.e., CDNN version 2.1, available on http://bioinformaticbiochemtech.uni-halle.de/cdnn), the same approach could be applied for each model of CD spectrophotometer. The accuracy reported for CD

spectroscopy is 97% for helices, 75% for β-sheets, 50% for turns, and 89% for other secondary structures (Ranjbar and Gill, 2009). In general, if all measurements are performed at various temperatures, each spectrometer should be equipped with a temperature controller.

RESULTS

UV-Vis spectroscopy measurements. The electronic absorption spectrum of native horseradish peroxidase C (HRPC; 6.3 μM) in citrate buffer (0.1 M; pH: 4.0) showed a Soret band at 403 nm, a β-band at 500 nm, and a charge transfer band at 642 nm (CT1 band). After 60 min of HRPC pre-incubation with various acriflavine concentrations (15-180 μM) at room temperature, a decline was reported in the Soret absorption band at 404 nm. According to Equation (1), the interaction between the HRPC site and acriflavine by plotting $1/\Delta A_{404}$ versus 1/[acriflavine] (Figure 1a) exhibited two slopes, one corresponding to lower concentrations of acriflavine (15-60 μM) and the other representing higher acriflavine concentrations (60-180 μM). Therefore, the two calculated K_d values (K_{d1}= 58±2 μM and K_{d2}= 14.5±0.2 μM) showed at least two attachment sites with different affinities for acriflavine in the vicinity of the heme group. Based on Equation (2), the Gibbs free energy of binding ($\Delta G_{binding}$) for K_{d1} (-7068±33 cal/mol) and K_{d2} (-10509±67 cal/mol) indicated that binding sites, which were filled with higher acriflavine concentrations, had higher affinity to the drug. In addition, according to the Hill plot (Figure 1b), acriflavine bound to HRPC in two different ways. First, the plot was linear indicating the independent binding of acriflavine, and then it turned to a sigmoid trend, which shows a cooperative manner of binding. The calculated Hill coefficient was equal to one for 15-60 μM concentrations of acriflavine and three for concentrations up to 180 μM. This finding suggests that at lower concentrations of acriflavine (15-60 μM), one drug molecule bound to HRPC in a non-competitive manner, while at higher concentrations (60-180 μM), three other acriflavine molecules could bind to the enzyme in a cooperative manner.

Fluorescence spectroscopy measurements. The fluorescence quenching of beef liver catalase (BLC; 1 μM) after incubation with 18-230 μM of acriflavine in phosphate buffer (0.1 M; pH: 7.0) upon excitation at 295 nm is presented in Figure 2a. In general, the fluorescence of BLC primarily includes six tryptophan and twenty tyrosine residues (Schroeder *et al.,* 1982). At an excitation wavelength of 295 nm, the fluorescence emission is induced by tryptophan residues. In the present study, it was observed that the fluorescence intensity of the enzyme gradually decreased with the rising concentration of acriflavine, and a clear emission wavelength shift was reported (Figure 2a). BLC exhibited the characteristic fluorescence emission spectrum (maximum at 339 nm) and a slight blue shift of maximum emission wavelength (nearly 6 nm) with the addition of 230 μM of acriflavine. This indicates that the microenvironment around tryptophan residues in the BLC molecule was altered due to interaction with acriflavine. In fact, the fluorophore of the protein was placed in a more hydrophobic environment after the addition of the drug. In Figure 2b, according to Equation (3), the Stern-Volmer plot of BLC fluorescence quenching is presented after pre-incubation of the enzyme with 0-368 μM of acriflavine for 10 min. The Stern-Volmer plot followed a linear relationship at low concentrations of acriflavine, while it exhibited an upward curvature when the concentration of acriflavine exceeded 110 μM; both static and dynamic quenching occurred. To gain a clear insight into the quenching mechanism, temperature-dependent fluorescence quenching of BLC by acriflavine was carried out at 35°C and 45°C under the same conditions applied at 25 °C. The K_{SV} values at 25 °C, 35 °C, and 45°C were found to be equal to 0.046, 0.032, and 0.025 μM^{-1}, respectively. Considering the fact that K_{SV} decreased with increasing temperature, it can be concluded that quenching occurred through a static mechanism, resulting in non-fluorescent complex formation between BLC

fluorophore and acriflavine. According to Equation (4), the ordinate intercept of $F_0/\Delta F$ plot against 1/[Q] provided the accessible fluorophores for acriflavine; the value in this study was estimated at 87%. Accordingly, from six tryptophans in each BLC subunit, five were accessible to acriflavine and could be quenched (data not shown). Based on Equation (5), for the BLC-acriflavine complex, the number of binding sites (n) was calculated to be 1.25, 1.23, and 1.07 at 25 °C, 35°C, and 45 °C, respectively. It can be inferred that nearly one independent class of binding sites was available on BLC for acriflavine. Also, the decline in $K_{binding}$ values (199.5×10^8, 126×10^8, and 79×10^8 M^{-1}, respectively) with raising temperature implies that the binding of acriflavine to protein was weakened as the temperature increased. The value of $K_{binding}$ is of the order 10^8, indicating a strong interaction between acriflavine and BLC. Finally, thermodynamic parameters were analyzed with respect to temperature, according to Equations 6 and 7 to characterize the acting forces between the drug and BLC. The negative sign obtained for Gibbs free energy ($\Delta G°$) indicates the spontaneity of the binding process. On the other hand, the negative ΔH° (-8350 cal/mol) and positive ΔS° (+9.8 cal/mol/k) values for BLC-acriflavine complex showed that both hydrogen bonds and hydrophobic interactions contributed to the binding of the drug to BLC.

CD spectroscopy measurements. The CD spectra of far-UV region taken immediately after the addition of increasing concentrations of acriflavine to BLC (5 µM) (0.1 M; pH: 7.0) are presented in Figure 3. The CD spectrum of BLC revealed a strong double-minimum potential at 210 and 222 nm, indicating an α-helical structure, characteristic of catalase. Upon the addition of acriflavine, a decline in BLC α-helix content was observed, which suggested the binding of acriflavine to amino acid residues of the main polypeptide chain. This interaction induced some conformational changes in BLC, which increased the exposure of some hydrophobic regions. Furthermore, the similar shape of CD spectra in the absence and presence of 0.1-1.5 mM of acriflavine indicated the predominantly α-helical structure of the protein, even after binding to acriflavine.

DISCUSSION

The *in vitro* study of protein-drug interaction has become a major concern in various scientific fields, especially for therapeutic purposes and development of new pharmaceuticals. For this purpose, among various analytical techniques, application of three spectral methods, including electronic absorption, fluorescence, and CD spectroscopy, can facilitate the study of subtle changes in protein conformation due to drug binding interactions. In addition to qualitative and quantitative analysis of various samples and monitoring of enzyme reactions, UV-Vis spectroscopy can be applied for resolving drug-protein interactions (Attar and Aminifar, 2014). In general, any alteration in the electronic absorption spectrum of proteins caused by this method (including chromaticity changes and maximum peak displacements) has been attributed to the formation of drug-protein complex. In the present study, valuable information was gathered on the stability of drug-protein complex, number and mode of drug binding, and nature of drug binding site(s) (Figures 1a & 1b). In this regard, based on a study by Wang *et al*. on silicotungstic acid (SiW, as an antiviral drug) and BSA, increasing the concentration of SiW (from 5 to 30 µmol/L) led to a gradual rise in the absorption peak at 277 nm and caused a blue shift in the spectrum at about 7 nm. Furthermore, in the study by Wang et al. (2007) the peak of 212 nm, which was attributed to the α-helical structure of BSA, decreased and red-shifted to 224 nm. Such results confirmed the existence of interaction between SiW and BSA and revealed the formation of a ground state complex. Overall, UV-Vis absorption spectroscopy, given its simplicity, sensitivity, reproducibility, and convenience, is regarded as an effective method for revealing the binding affinity of drugs to proteins and helps explore their binding mechanisms (Naik et al., 2009; Vahedian-

Table 1. Some of the studied drug-protein complexes with various spectroscopic methods

Drug class	Studied protein	Spectroscopic methods
Antibacterial		
1- Lomefloxacin (Vahedian-Movahed *et al*., 2011)	HSA[a]	1- Fluorescence, UV-Vis, and FTIR[c] spectroscopy
2- Sulfamethoxazole (Niak *et al*., 2009)	BSA[b]	2- Fluorescence and UV-Vis spectroscopy
Antifungal		
- Methylparaben (Naik & Nandibewoor, 2013)	BSA[b]	- Fluorescence, FTIR, and UV-Vis spectroscopy
Antihyperglycemic		
- Pioglitazone (Faodridbod *et al*., 2011)	HSA[a]	- Fluorescence spectroscopy
Anti-inflammatory		
1- Diclofenac sodium (Cui *et al*., 2013)	HSA[a]	1- Fluorescence and UV-Vis spectroscopy
2- Carprofen (Rahman *et al*., 1993)	HSA[a]	2- Fluorescence spectroscopy
Antimalarial		
- Atovaquone (Zsila *et al.,* 2010)	HSA[a]	- CD, UV/Vis, and fluorescence spectroscopy
Antioxidant		
- L-ascorbic acid (Nafisi *et al*., 2011)	BSA[b]	- FTIR and UV-Vis spectroscopy
Anti-stress		
1- Venlafaxine hydrochloride (Shahabadi *et al*., 2014)	HSA[a]	1- Fluorescence, UV-Vis, and CD spectroscopy
2- Fluoxetine (Amin *et al*., 2014)	Buffalo Brain Cystatin	2- Fluorescence and UV-Vis spectroscopy
Antitumor		
1- Gemcitabine (Shen *et al*., 2013)	BSA[b]	1- Fluorescence, UV-Vis, CD, and FTIR spectroscopy
2- Emodin (Sevilla *et al*., 2007)	BSA[b]	2- Fluorescence and CD spectroscopy
Antiviral		
- Tamiflu (Vishkaee *et al.,* 2013)	HSA[a]	UV-Vis and FTIR spectroscopy

[a]Human serum albumin [b]Bovine serum albumin [c]Fourier transform infrared spectroscopy

Table 2. Intermolecular forces involved in some drug-protein complexes (data obtained from fluorescence spectroscopic method)

Drug-protein complex	ΔS°	ΔH°	Intermolecular forces stabilizing the complex
- Venlafaxine hydrochloride-BSA (Shahabadi *et al*., 2011) - Fosfomycin disodium salt-HSA (Meti *et al*., 2014) - Pioglitazone-HSA (Faridbod *et al*., 2011) - Fulvic acid-HST[a] (Zhang *et al*., 2014)	ΔS°<0	ΔH°<0	Hydrogen bonding and van der Waals forces
- Daphnin-HSA (Zhu *et al*., 2012) - Deferiprone-HSA (Seyed Dorraji *et al*., 2014) - Docetaxel-Human hemoglobin (Cheng *et al*., 2011)	ΔS°>0	ΔH°>0	Hydrophobic interactions
- Diclofenac sodium-HSA (Cui *et al*., 2013) - Ondansetron hydrochloride-BSA, HSA (Sandhya *et al*., 2012) - Dihydropyrimidinones-HSA (Wang *et al*., 2011) - Methylparaben-BSA (Niak *et al*., 2013) - Sulfamethoxazole-HSA (Wang *et al*., 2014) - Acriflavine-BLC[b] (Attar, 2010)	ΔS°>0	ΔH°<0	Electrostatic interactions, hydrogen bonding, and hydrophobic interactions

[a]Human serum transferring [b]Beef liver catalase

Movahed et al., 2011; Cui et al., 2013; Vishkaee et al., 2013; Amin and Bano, 2014; Shahabadi and Hadidi, 2014). The fluorescence of a folded protein is a mixture of the fluorescence from individual aromatic residues (e.g., tryptophan, tyrosine, and phenylalanine) and co-factors (e.g., flavin mononucleotide, flavin adenine dinucleotide, nicotinamide adenine dinucleotide, and porphyrins) and depends mainly on the nature of the molecular neighborhood of these chromophores (Emel'yanenko and Burshtein, 1998; Gong et al., 2007). Any alterations in the protein conformation upon the addition of the drug, which would affect the chromophore environment, could change the protein fluorescence and reveal the

interaction between the protein and drug for forming a certain complex (Figure 2A). In the literature, fluorescence spectroscopy showed that the tertiary structure of the protein, nature of the binding site(s), distance between the drug and protein, and affinity of the binding site towards the drug could be resolved (Sevilla et al., 2007; Li et al., 2008; Yamanishi et al., 2008; Faridbod et al., 2011; Naik and Nandibewoor, 2013; Amin and Bano, 2014; Shahabadi and Hadidi, 2014). Depending on the method of interaction, fluorescence quenching, which refers to any process reducing the fluorescence intensity of a sample, can be induced by dynamic or static manners (Kandagal et al., 2006; Faridbod et al., 2011; Vahedian-Movahed et al., 2011; Cui et al., 2013). Dynamic quenching occurs when quencher molecules (drugs) have the sufficient energy to collide with the excited state of protein fluorophore and take it to the ground state. On the other hand, the formation of a non-fluorescent ground-state complex between the quencher (drug) and fluorophore gives rise to static quenching. Differentiation of the fluorescence quenching mechanism with respect to temperature could be analyzed by the Stern-Volmer constant (K_{SV}, Figure 2b). Moreover, in dynamic quenching mechanism, the K_{SV} value increases with a rise in temperature. In contrast, increased temperature is likely to decrease K_{SV} in the static quenching mechanism (Kandagal et al., 2006). Thermodynamic forces responsible for binding between the protein and drug could be also evaluated through fluorescence spectroscopy. In general, interaction forces originating from hydrogen bonds, van der Waals forces, hydrophobic interactions, and electrostatic interactions may exist between two molecules (Kandagal et al., 2006). In fact, the assessment of parameters such as $\Delta H°$ and $\Delta S°$ of binding interactions enables us to determine the type of the binding force. With regard to enthalpy and entropy changes, the model of interaction between the drug and protein can be summarized as follows: $\Delta H°>0$ and $\Delta S°>0$ indicative of hydrophobic forces, $\Delta H°<0$ and $\Delta S°<0$ indicative of van der Waals interactions and hydrogen bonds, and $\Delta H°<0$ and $\Delta S°>0$ indicative of resolved electrostatic interactions. In Table 2, intermolecular forces, which affect the stability of some studied drug-protein complexes, are summarized. Moreover, fluorescence spectroscopy is basically a probing method (Naik and Nandibewoor, 2013), revealing changes in the local environment of fluorophore. This advantage distinguishes this method from typical techniques, such as calorimetry, far-ultraviolet CD spectroscopy, and infrared spectroscopy. Compared to conventional techniques such as dialysis and ultrafiltration, fluorescence spectroscopy does not need to separate the bound and unbound drugs; this reduces the time required for the experiment (Parikh et al., 2000). In addition, due to extensive binding of highly hydrophobic drugs to the membrane, this method is preferable to dialysis and ultrafiltration techniques (Parikh et al., 2000; Faridbod et al., 2011).

Moreover, a CD spectrum, which relies on the differential absorption of left- and right-circularly polarized radiation by chiral chromophores, could help evaluate the secondary and tertiary structures of proteins (Kelly and Price, 1997; Bertucci et al., 2010). The binding of drug ligands to a given protein in an asymmetric fashion can change the CD spectra. Therefore, the shape of spectrum curves, as well as positive and negative maxima, could provide valuable information about the structure of the protein. Generally, bands present in the far-UV region (180–260 nm), corresponding to peptide bond absorption, have a ω-shaped spectrum with troughs around 222 and 208 nm as characteristic signals of α-helical structures (Figure 3) and V-shaped spectra with a trough around 220 nm, indicative of β-sheet structures (Manavalan and Johnson, 1987). CD spectroscopy in near-UV regions (250–350 nm) arises from the position of each aromatic amino acid side chain. Moreover, possible contributions of disulfide bonds or non-protein co-factors (410 nm for heme, 450 nm for flavin adenine dinucleotide, and 550 nm for Fe/S centers) yield information about the tertiary structure of the protein (Kelly and Price, 1997; Hadizadeh et al., 2009). In this regard, Mehrabi et al. (2009) evaluated the near-UV

CD spectra of human carbonic anhydrase II (hCA II) in the presence of various concentrations of celecoxib,

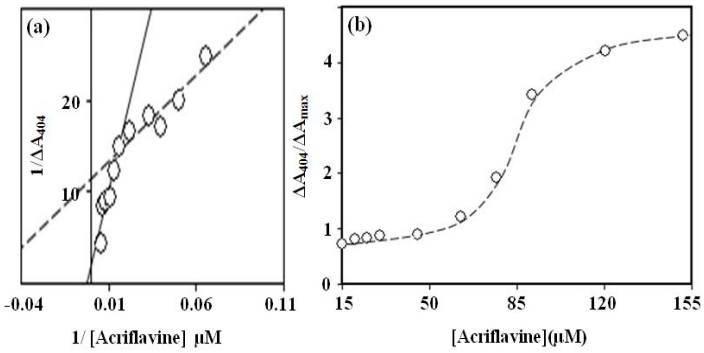
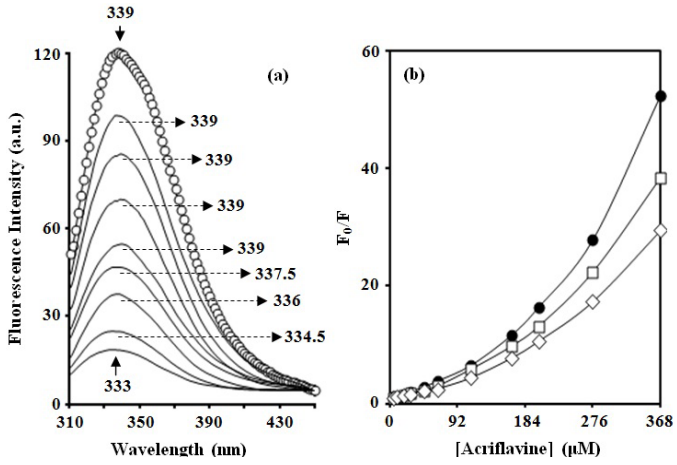
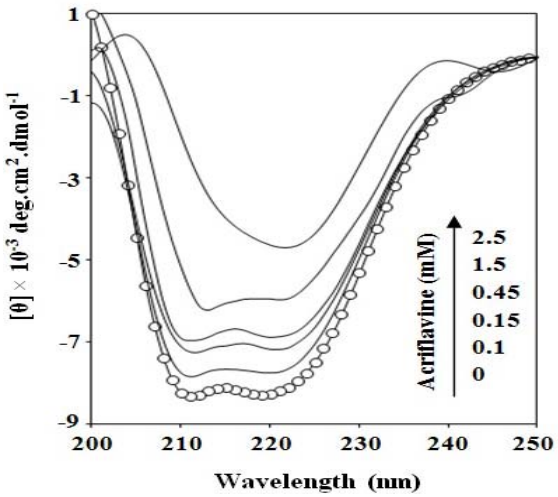


Figure 1. (a) Inverse plots of $1/\Delta A_{404}$ vs. 1/[acriflavine], giving apparent dissociation constants K_{d1} (filled line) and K_{d2} (dotted line). K_{d1} and K_{d2} were found at 15-60 µM and 60-180 µM concentrations of acriflavine, respectively. **(b)** Plot of the ratio of $\Delta A_{404}/\Delta A_{max}$ vs. [acriflavine]. ΔA_{404} indicates the absorbance change caused by a given acriflavine concentration at the specified wavelength. ΔA_{max} is the absorbance change for the complete formation of HRPC-acriflavine complex as seen at the wavelength.





a sulfonamide drug via weak near-UV CD signals. The results indicated the high mobility of aromatic side chains, which can be attributed to the reduced flexibility of hCA II tertiary structure upon celecoxib binding. Therefore, CD spectroscopy is a well-known spectroscopic method, which plays a major role in complementing structural approaches (with higher resolutions) of highly sensitive optical techniques, such as X-ray crystallography and NMR imaging. The principal advantages of CD spectroscopy include the limited number of required samples due its non-destructive properties, high speed of data collection, time-resolved measurements, and evaluation of the dynamic aspects of protein structure. CD spectroscopy with its high sensitivity to sample perturbations is dependent on drug binding interactions, chemical agents, solvent composition, detergents, temperature, and pH. This method allows proteins to be assessed both quantitatively and qualitatively. In addition, CD spectroscopy is a relatively simple method for studying a large number of membrane proteins, which are difficult to crystallize (for identifying the crystallographic structure) or are too complex for NMR imaging. The main limitation of CD spectroscopy is that it only offers structural information with a relatively low resolution and provides scarce detailed information on the quaternary structure of proteins. Overall, despite some limitations of the discussed spectroscopic methods, the obtained results would be of great value in understanding the mechanism(s) involved in drug interactions with proteins. Moreover, the findings could be useful in describing the structure-activity relationship of compounds and target proteins and could be applied as a practical guideline for further clinical studies.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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