TEMPERATURE EFFECT ON BINDING AFFINITY AND STOICHIOMETRY BETWEEN SOME STEROIDS AND HUMAN SERUM ALBUMIN

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ABSTRACT

The temperature dependence of the interactions between some steroids (fusidic acid, ouabain and ethynodioldiacetate) and human serum albumin (HSA) at physiological pH (7.4) were studied systematically by square-wave voltammetry. Also, these interactions were proven by means of UV–Vis. absorption spectroscopy and FT-IR spectroscopy techniques. The interaction parameters (binding constants and stoichiometries) at temperature range of 297.5-312.5 K were determined from electrochemical data. It is worthy that the temperature has played a positive role on the interactions of fusidic acid with HSA; however it has negative effective on the interaction parameters of HSA with ouabain or ethynodioldiacetate.

Keywords: steroids, human serum albumin, binding affinity, temperature effect, voltammetry.

INTRODUCTION

Steroids are organic molecules whose structure is based upon the tetracyclic ring system and also have different biological functions, for example they interfere in digestion and in solubilization of fats (colic acid), they are constituents of cell membranes (cholesterol), or can be found as hormones androgens (corticoids).[1,2] They are highly water insoluble and they are transported as complex by globulins, glycoproteins and albumin.[2] Also, the steroids are biologically inactive as long as they are associated with protein.[3]

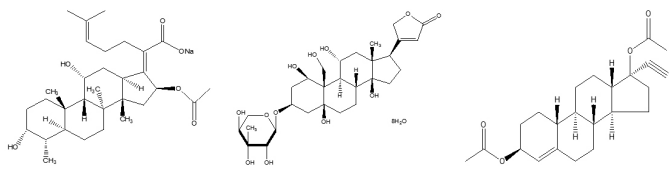
Serum albumins are the most abundant carrier proteins of blood plasma that promote the transportation and disposition of exogenous and endogenous materials in blood.[4] They are able to bind with different biologically active compounds (drugs, fatty acids, steroids, dyes, etc.) in the body.[5-7] Therefore, they are considered as model for studying the protein interactions *in vitro*.[8]

Human serum albumin (HSA) is a non-glycosylated single chained polypeptide having 67 kDa mass, which organizes to form a heart-shaped protein with approximately 67 % a-helical content.[6,9–14] It is a globular protein composed of three structurally-similar domains (I–III), each containing two sub-domains (A and B) and stabilized by 17 disulfide bridges.[6,9–17] Aromatic and heterocyclic ligands have been found to bind within two hydrophobic pockets in sub-domains IIA and IIIA, namely site I and site II.[6,9–15]

Especially, albumin was the principal carrier for steroids and regulator of access to their receptors as well as a protector of steroid receptors from occupancy by phytochemicals.[18] The numerous studies describing the interaction or binding of some steroids with serum proteins are present in the literature.[3,18–41] Also, it was reported that *in vitro* studies of steroid binding can be expected to give insight into the state of steroids in tissues and the mechanism of transport through the blood stream.[26]

Some techniques such as gel electrophoresis,[42] X-ray crystallography,[43] NMR,[44] fluorescence,[45,46] differential scanning calorimetry (DSC),[47] infrared spectroscopy,[37,40] UV/Vis. spectroscopy,[46–48] circular dichroism (CD) spectroscopy[49] and electrochemistry,[50–52] have been intensively used to study the molecular interactions. In recent years, there has been a growing interest in the electrochemical and spectroscopic investigation of molecular interations.[51,52] Also, spectroscopic and voltammetric techniques have been testified to be of high sensitivity, relatively low cost, direct monitoring and simplicity.[46–49,53–57]

Except for only the interaction of albumin and fusidic acid by zone microelectrophoresis in 0.05 M barbitone sodium buffer pH 8.4,[58] the voltammetric and spectroscopic studies on the interactions of three steroids which are presented in Scheme 1 (fusidic acid (FA), ouabain (OUB) and ethynodioldiacetate (ETH), respectively) with HSA at the physiological pH could not be traced in the literature. Therefore, the main goal of present study is to investigate the interactions of these steroids with HSA at the physiological pH, using the amperometric method, UV-Vis and FT-IR spectroscopy techniques in aqueous solution.



A B C

Scheme 1. Molecular structures of steroid compounds used in the present paper. Sodium salt of FA (A), OUB (B) and ETH (C).

EXPERIMENTAL

Reagents

HSA and steroids were purchased from Sigma and used as received. Britton-Robinson buffer solution (pH 7.4) of 0.04 M acetic, 0.04 M boric and 0.04 M phosphoric acids was used. The supporting electrolyte was B-R buffer prepared in the usual way, by adding appropriate amount of sodium hydroxide (0.2 M) to a phosphoric acid, boric acid and acetic acid mixture (0.04 M). All other reagents were of analytical reagent grade and used without further purification. A stock solution of ETH ($1.0x10^{-3}$ M) was prepared in methanol-water (50 % v/v) mixture (ETH is insoluble in water, but soluble in methanol–water mixture). The stock solutions of FA and OUB ($1.0x10^{-3}$ M) were prepared in water. The stock solutions of HSA ($1.0x10^{-5}$ M) were also prepared in water and were kept in the dark at 4 °C.

Deionized and distilled water (specific resistivity 18 MΩ cm) used in the experiment was obtained from a water purification system (MP MINIPURE Dest up).

Apparatus

All electrochemical measurements were performed using an EG&G PAR Model 384B Polarographic analyzer controlled by a personal computer containing the ECDSOFT[59] software in conjunction with a PAR Model 303A Static Mercury Drop Electrode (SMDE). The voltammograms were recorded using a three-electrode system with a mercury working electrode with hanging mercury drop electrode (HMDE) mode, a platinum auxiliary electrode, and an $Ag/AgCl/KCl_{saturated}$ reference electrode.

The pH measurements were performed with a January 3010 pH meter equipped with a combined glass electrode. The electrochemical experiments at different temperatures were carried out by using Jacketed Cell Bottom (EG&G PAR G0193) and PolyScience Model 7306 Immersion Circulator with temperature controller (temperature stability: ±0.05 °C).

Electronic spectra were carried out at room temperature on a UNICAM V2-100 equipped with 1.0-cm quartz cells. FT–IR spectroscopy studies were conducted by KBr-disc pellet method using on a Bruker FT-IR Vertex-80v at room temperature. Spectroscopic grade KBr salt was used in pellet preparation. The KBr and sample mixtures were pressed under a pressure of 7 tons for 5 minutes to produce highly-transparent KBr-disc pellets.

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Procedure

Voltammetric measurements

A 10 mL volume containing B-R buffer solution (pH 7.4) as supporting electrolyte was added to the cell and the oxygen dissolved in the solution was removed by bubbling pure nitrogen gas through the solution for at least 5 mins before each measurement. A blanket of nitrogen gas was maintained over the electrochemical solutions at all times. Then, the blank voltammograms were recorded after the equilibrium time of 5 s by applying a potential scan. And then, the sample solution was added to the electrochemical cell and its voltammograms were recorded. The interactions of steroids with HSA were followed by titration of different HSA concentrations with a fixed concentration of the steroid solution and *vice versa*. Electrochemical experiments were carried out in the temperature range of 24.5 to 39.5 °C. For square-wave voltammetry (SWV), the experimental conditions were medium drop size (0.0154 cm^2), frequency 100 Hz, scan rate 200 mVs^{-1}, scan increment 2 mV, pulse height 20 mV and equilibrium time 5 s. On the other hand, cyclic voltammetry (CV) measurements were obtained using scan rate range of 50 to 1000 mVs^{-1}, medium drop size and equilibrium time, 5 s.

The determination of equilibrium constants and binding stoichiometries of the interactions

In a similar manner to the method reported by Sun et al.[60], the equilibrium constants (β) and binding stoichiometries (m) of steroids (STDs) with HSA have been calculated by following the changes of the peak current, assuming that HSA and STDs form the HSA–mSTD complexes (HSA + mSTD $\rightleftharpoons$ HSA–mSTD), and using equation (1):

$$\ln [\Delta I /(\Delta I_{max} - \Delta I)] = \ln \beta + m \ln [STD] \quad (1)$$

where DI is the peak current difference in the presence and absence of HSA, DI_{max} corresponds to the peak current value when the concentration of STD is much higher than that of HSA.

Preparation of HSA-STD complex solid samples

According to the ambient molar ratio (1:1 for HSA-FA and 1:2 for HSA-OUB), HSA was mixed with STDs in B-R buffer (pH 7.40) under magnetic stirring at room temperature. HSA-ETH system with molar ratio of 1:2 was prepared in methanol-B-R buffer (50 % v/v) mixture. These mixtures were stirred continuously for at least 2-hour and then waited for the volatilization of solvent. The obtained complex solid samples were separated from the mixture solution by means of filtration process. For comparison, HSA solid samples were obtained from both B-R buffer (pH 7.40) and methanol-B-R buffer (50 % (v/v), pH 7.40) mixture. In addition, the solid samples of FA and OUB were yielded from B-R buffer (pH 7.40) while ETH solid sample was obtained from methanol-B-R buffer (50 % (v/v), pH 7.40) mixture, according to the above procedure.

Spectroscopic measurements

Electronic spectra of STDs and their mixtures with HSA in B-R buffer (pH 7.4) were recorded at 200 - 400 nm range using quartz cuvettes of 1 cm path length. The changes on the electronic spectra of STDs in the presence of HSA were followed. Also, the FT-IR spectra of free solid HSA and STDs were recorded in the range of 400–4000 cm^{-1}. And then, the infrared spectra of the solid STD–HSA complexes were taken. All spectroscopic measurements were operated at room temperature (~25 °C).

RESULTS AND DISCUSSION

The voltammetric behaviours of the steroids at physiological pH:

The voltammograms of FA, OUB and ETH in B-R buffer (pH = 7.4) solution at a HMDE are shown in Fig. 1 (A, B, C, respectively). At the square-wave voltammograms of three steroids, a cathodic reduction process was observed. On the other hand, as can be seen at their cyclic voltammograms, FA and ETH have the considerable oxidation peaks while OUB has a very little anodic counterpart.

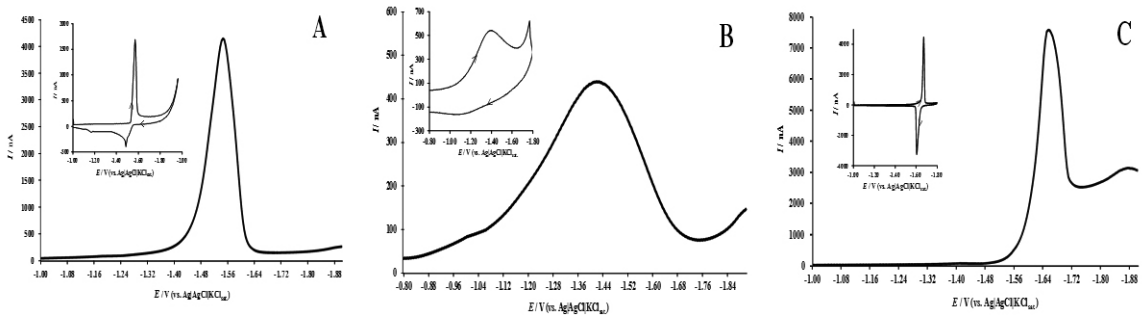

Figure 1. A) Square-wave voltammogram of $3x10^{-5}$ M FA in 0.04 M B-R buffer at pH 7.4. (Inset: Cyclic voltammogram of $3x10^{-5}$ M FA in 0.04 M B-R buffer (pH 7.4) at scan rate of 500 mVs^{-1}). **B)** Square-wave voltammogram of $5.88x10^{-6}$ M OUB in 0.04 M B-R buffer at pH 7.4. (Inset: Cyclic voltammogram of $5.88x10^{-6}$ M OUB in 0.04 M B-R buffer (pH 7.4) at scan rate of 500 mVs^{-1}). **C)** Square-wave voltammogram of $4.76x10^{-5}$ M ETH in 0.04 M B-R buffer (containing 50% of methanol (v/v)) at pH 7.4. (Inset: Cyclic voltammogram of $4.76x10^{-5}$ M ETH in 0.04 M B-R buffer pH 7.4 (containing 50% of methanol (v/v)) at scan rate of 500 mVs^{-1}). Other experimental conditions are described in the Procedure section.

The voltammetric behaviour of HSA at physiological pH:

The voltammetric behavior of HSA on HMDE was also studied with SWV and CV techniques in B-R buffer (pH 7.4). Fig. 2 shows typical voltammograms of $4.31x10^{-7}$ M HSA in the potential range of –0.4 to –1.4 V. HSA gave a reversible cathodic peak at –0.78 V for voltammetric measurements under these conditions (Fig. 2). On the other hand, for the protein, the reduction of the disulfide linkage at about –0.9 V was reported in the literature.[61-64] Moreover, it is well known that cystine contained in many proteins undergoes polarographic electroreduction of its-S-S-bond at potential of about –0.7 V (*vs*. SCE) in neutral buffer solutions.[65]

As can be seen in Fig. 2 (Inset), the difference between anodic and cathodic peak potentials ($\Delta E_p = E_{pa} - E_{pc} = 40$ mV) refers to an electrochemical processes involving the transfer of about two electrons. In a previous study on bovine serum albumin[62], the model of electrode reaction was proposed as follows:

$$R - (S - S)_n - R_{sol} \rightleftharpoons R - (S - S)_n - R_{ads} \quad (2)$$

$$R - (S - S)_n - R_{ads} + 2ne^- + 2nH^+ \rightleftharpoons 2R - (SH)_{n,ads} \quad (3)$$

As similar to above mechanism, it can be also said that the peak at –0.78 V is sourced from the reduction of disulphidic bonds on HSA molecule.

The interactions of steroids with HSA:

Voltammetry

The interactions of HSA macromolecule with STDs were studied by adding several HSA concentrations to the definite concentration solution of the steroid and obtaining square-wave voltammograms in the cathodic potentials under the conditions which are described in the experimental section. Some typical voltammograms obtained at different HSA concentrations were seen in Fig. 3. With the addition of HSA, the reductive peak currents of STDs decrease with the change of their peak potentials. The more HSA was added, the more the peak currents changed. Also, the cathodic peaks of FA and ETH shifted to less negative potentials while the reductive peak of OUB shifted to more negative potentials with increasing of HSA concentration. The changes of electrochemical responses of STDs in the presence and absence of HSA showed the interactions of STDs with HSA to form the HSA-mSTD complexes.

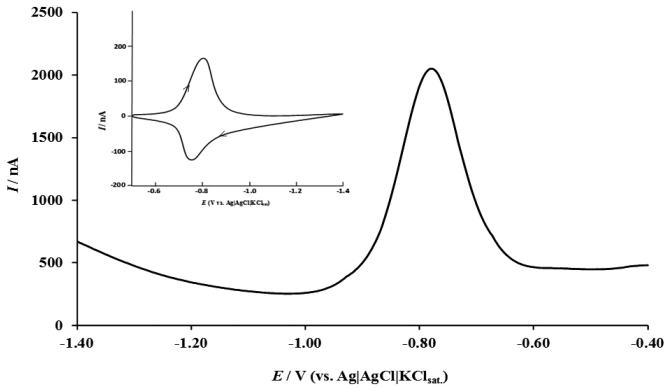

Figure 2. Square-wave voltammogram of $4.31x10^{-7}$ M HSA in 0.04 M B-R buffer at pH 7.4. (Inset: Cyclic voltammogram of $4.31x10^{-7}$ M HSA in 0.04 M B-R buffer (pH 7.4) at scan rate of 500 mVs^{-1}). Other experimental conditions are described in the Procedure section.

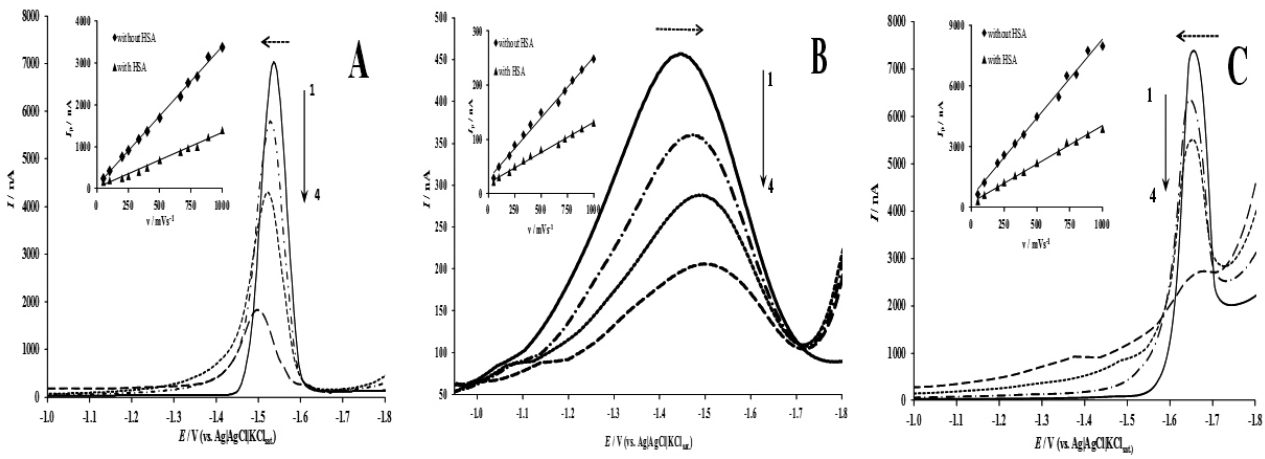

Figure 3. The square-wave voltammograms of STDs at various concentrations of HSA. **A)** 1: $5.0x10^{-5}$ M FA, 2: 1 + $2.1x10^{-7}$ M HSA, 3: 1 + $4.2x10^{-7}$ M HSA, 4: 1 + $6.3x10^{-7}$ M HSA in 0.04 M B-R buffer (pH 7.4) (Inset: the dependence of the peak current (I_p) of $3.0x10^{-6}$ M FA with the potential scan rate (ν) in the absence and presence of $6.3x10^{-7}$ M HSA). **B)** 1: $7.84x10^{-6}$ M OUB, 2: 1 + $1.1x10^{-8}$ M HSA, 3: 1 + $2.2x10^{-8}$ M HSA, 4: 1 + $3.3x10^{-8}$ M HSA in 0.04 M B-R buffer (pH 7.4) (Inset: the dependence of the peak current (I_p) of $1.0x10^{-6}$ M OUB with the potential scan rate (ν) in the absence and presence of $4.2x10^{-8}$ M HSA). **C)** 1: $4.76x10^{-5}$ M ETH, 2: 1 + $2.2x10^{-8}$ M HSA, 3: 1 + $4.4x10^{-8}$ M HSA, 4: 1 + $7.7x10^{-8}$ M HSA in 0.04 M B-R buffer (containing 50% of methanol (v/v)) of pH 7.4 (Inset: the dependence of the peak current (I_p) of $4.76x10^{-5}$ M ETH with the potential scan rate (ν) in the absence and presence of $4.5x10^{-8}$ M HSA). Other experimental conditions are described in the Procedure section. The arrow with dashed line shows the shift in the peak potential.

There may be three different explanations for the decreases at the reductive peak currents of STDs in the presence of HSA:[66,67] (1) the competitive adsorption between the STDs and HSA; (2) the formation of an electrochemically active compound and changes of the electrochemical parameters; (3) the formation of electroinactive complex without the changes of the electrochemical parameters. On the other hand, an adsorbable substance acts usually as an inhibitor of an electrode reaction and shifts reduction process to negative potential.[68] The positive shifting in the peak potentials of FA and ETH by the addition of HSA could be an evidence for the exclusion of the presence of competitive adsorption on the electrode surface. Moreover, Li and co-workers[69] have investigated the interactions of many electroactive small molecules with biomolecules such as albumin. They reported[69] that in lower concentration of protein and shorter accumulation times, the coverage of the electrode surface is only about 10% of the total electrode area, so the competitive adsorption hardly exists.[67,69] Therefore, the interactions of STDs with HSA formed the electroactive complexes, which could be reduced on the mercury electrode surface. STDs and HSA are in equilibrium with the complexes. Therefore, the followed current responses are the result of the reduction of both the free STD and the complexed STD molecules. In the presence of HSA, the equilibrium concentrations of free STDs in solution are decreased, which resulted in the decrease of their peak currents. In addition, the changes at their current responses may be also due to the decrease of the apparent diffusion coefficients of STDs, when they are complexed with HSA.

In the absence of HSA, the peak currents ($I_{p,0}$) of STDs increased with scan rate (v) (Fig. 3, Insets). Furthermore, the plots between log $I_{p,0}$ *versus* log v were linear and showed the slope values of 0.92, 0.72 and 0.84 for FA, OUB and ETH, respectively. When a diffusion process takes place, a slope of 0.5 is obtained; whereas for an adsorption process, a slope of 1 is obtained. These intermediate slope values suggest a 'mixed' diffusion–adsorption process,[70] thus, it can be said that the electrochemical reductions of these steroids also went through same process under these experimental conditions.

In the presence of HSA, the peak currents (I_p) of STDs also increased linearly with increasing of the scan rate (v) (Fig. 3, Insets). However, the slopes of the I_p-v plots in the presence of HSA are smaller than those of the I_p-v plots in the absence of HSA (Fig. 3, Insets). This case may be derived from the decreases in the concentrations of free STDs and in the transport rate of the STD-HSA complexes to the mercury electrode surface.

Uv-vis spectra

The difference between the electronic spectra of free HSA, free STDs and the HSA-STDs mixtures should be also studied to confirm whether the HSA-STD complexes are formed or not. The absorption spectra of free HSA, free STDs and their mixtures are shown in Fig. 4 (A, B and C, respectively). At B–R buffer solution (pH 7.4), HSA showed two absorption peaks at 219 nm

and 278 nm. The strong absorption peak at 219 nm corresponds to absorption of the protein backbone.[71] The weak absorption peak at 278 nm appears due to the aromatic amino acids.[71-74] Moreover, the literature reported that the peak in the 219 nm region resulted from the $\pi \rightarrow \pi^*$ transition of HSA's characteristic polypeptide backbone structure C=O and was related to the changes in the conformation of peptide backbone associated with helix-coil transformation in the difference spectra of proteins.[75] In addition, the weak absorption peak at 278 nm region was involved in the polarity of the microenvironment around tryptophan (Trp) and tyrosine (Tyr) residues of HSA.[76] On the other hand, STDs (FA, OUB and ETH) have only one absorption peak at 209 nm region resulted from the $\pi \rightarrow \pi^*$ transition.

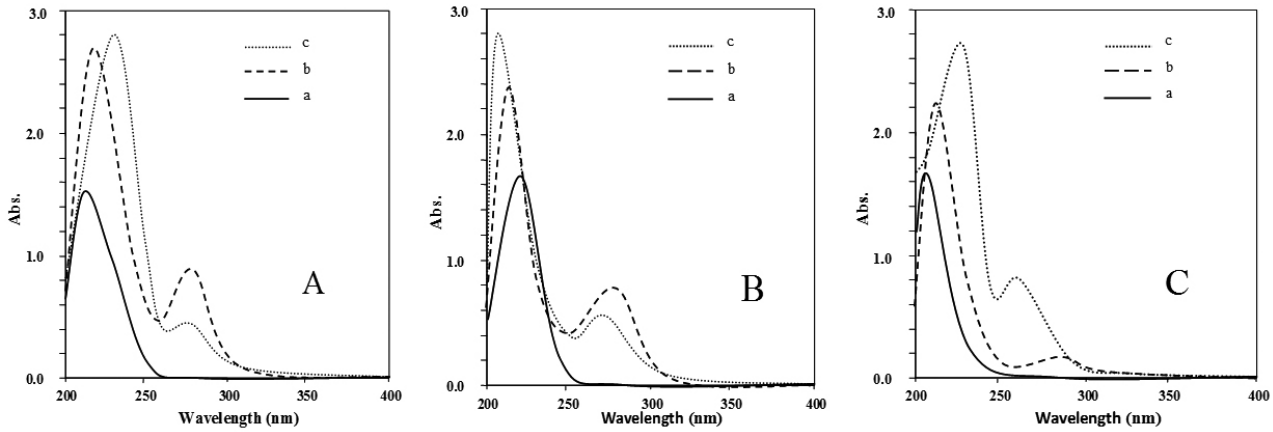

Figure 4. A) (a) The UV absorption spectrum of FA ($5.0x10^{-5}$ M); (b) the UV absorption spectrum of HSA ($2.1x10^{-6}$ M); (c), the UV absorption spectrum of FA + HSA, $C_{HSA} = 2.1x10^{-6}$ M, $C_{FA} = 5.0x10^{-5}$ M (in 0.04 M B-R buffer of pH 7.4). **B)** (a) The UV absorption spectrum of OUB ($6.7x10^{-5}$ M); (b) the UV absorption spectrum of HSA ($1.1x10^{-6}$ M); (c), the UV absorption spectrum of OUB + HSA, $C_{HSA} = 1.1x10^{-6}$ M, $C_{OUB} = 6.7x10^{-5}$ M (in 0.04 M B-R buffer of pH 7.4). **C)**(a) The UV absorption spectrum of ETH ($1.0x10^{-4}$ M); (b) the UV absorption spectrum of HSA ($1.5x10^{-6}$ M); (c), the UV absorption spectrum of ETH + HSA, $C_{HSA} = 1.5x10^{-6}$ M, $C_{ETH} = 1.0x10^{-4}$ M (in 0.04 M B-R buffer of pH 7.4 (containing 50% of methanol (v/v)).

In the presence of FA, the peak absorbance of HSA at 278 nm was decreased, but no obvious change in the peak position was observed. In the presence of OUB, the intensity of the absorbance peak at 278 nm both decreased and shifted slightly toward shorter wavelength of 251 nm. However, in the presence of ETH, the absorption band of HSA at 278 nm shifted to 260 nm and its intensity was increased. The range of 240 - 300 nm has been generally used in the study of HSA structure and conformation.[77]

In the presence of STDs, the changes at absorption band of HSA at 219 nm are different. As seen in Figs. 4A and 4C, in the presence of FA and ETH, absorption band of HSA at 219 nm shifts to longer wavelengths (red shift). However, its maximum absorption band shifted towards to shorter wavelength (blue shift) with the addition of OUB (Fig. 4B).

These changes on the absorption spectra indicated that there are binding interactions between STDs and HSA, which induce the conformational change of HSA[71] and also the polarity change of the microenvironment around Trp and Tyr residues of the protein.[78]

Infrared spectra

FT-IR spectroscopy is one of the few techniques that is established in the determination of protein secondary structure[79,80] and also used to study the binding of small molecules to HSA.[40]

Infrared spectra of proteins exhibit a number of amide bands, which represent different vibrations of the peptide moiety. The amide group of proteins and polypeptides presents characteristic vibrational modes (amide modes) that are sensitive to the protein conformation and largely been constrained to group frequency interpretations. Amide I (1700–1600 cm^{-1} region) is primarily due to the C=O stretching vibration, amide II (1600–1480 cm^{-1} region) to the coupling of the N-H in-plane bending and C-N stretching modes.[78,81] In general, amide I band is the most widely used in studies of protein secondary structures as it is more sensitive to the changes than amide II band.[78,82,83] However, it is well known that at infrared analysis on the interactions of HSA with small molecules, although the significant changes at the positions of the amide bands are not observed, there are very small shifts in their numbers.[37,40]

The FT-IR spectra of free HSA, STDs and HSA-STDs are exhibited in Figs. 5 - 7. These FT-IR spectra clearly showed that the peak position of amide I band at 1657 cm^{-1} was moved to 1656 cm^{-1}, while the amide II band was shifted from 1558 to 1550 cm^{-1} for FA-HSA and from 1558 to 1549 cm^{-1} for OUB-HSA. On the other hand, for ETH-HSA, the peak position of amide I band was shifted from 1659 to 1661 cm^{-1}, while the amide II band was not changed.

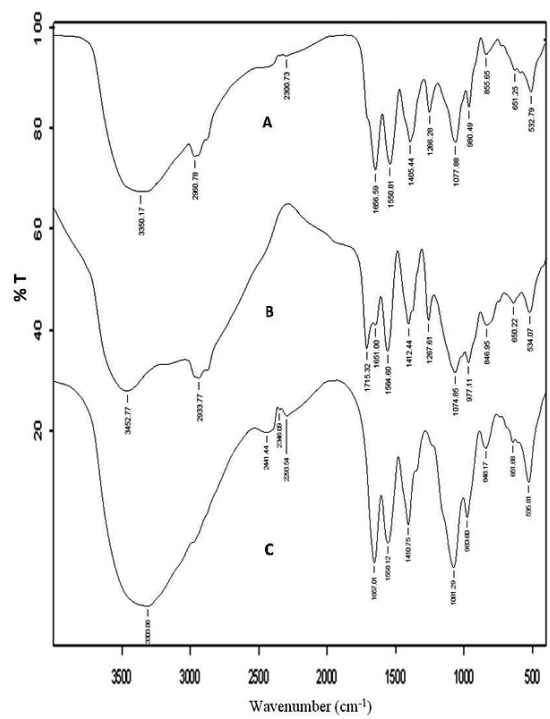

Figure 5. FT-IR spectra of A) FA-HSA, B) FA and C) HSA.

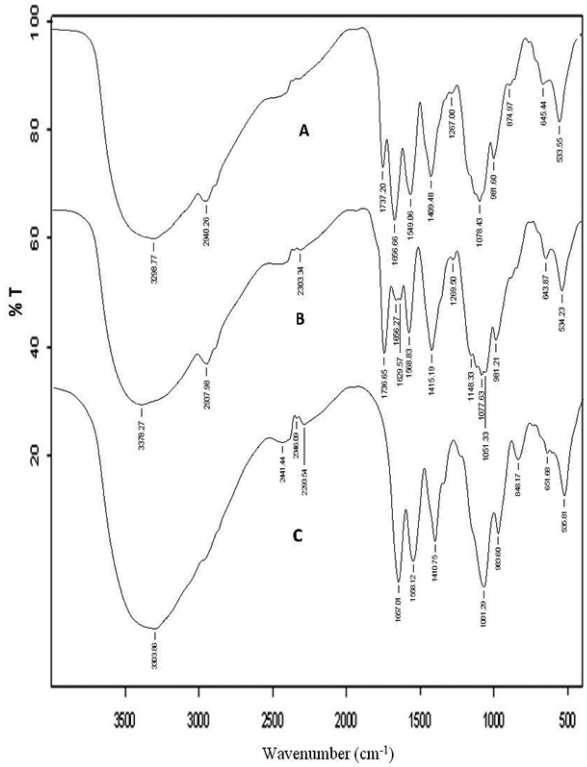

Figure 6. FT-IR spectra of A) OUB-HSA, B) OUB and C) HSA.

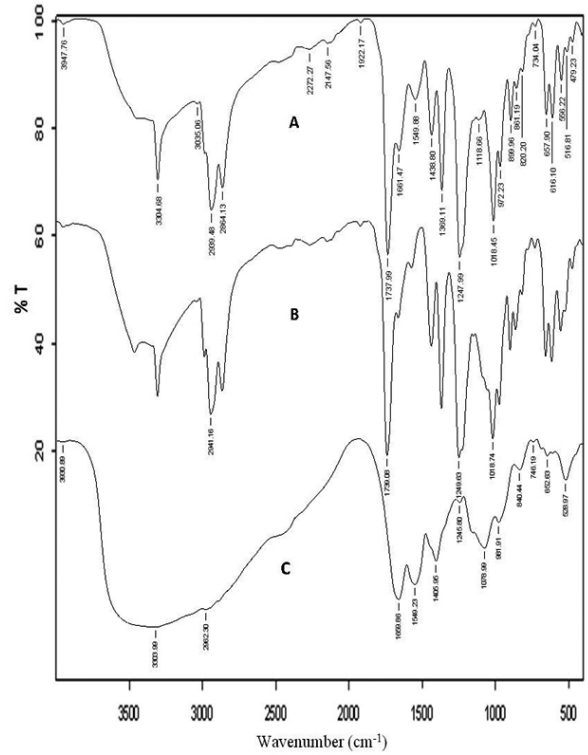

Figure 7. FT-IR spectra of A) ETH-HSA, B) ETH and C) HSA.

Due to the acetyl $\nu_{C=O}$ vibration, FA, OUB and ETH have the characteristic absorption bands at 1715, 1736 and 1739 cm^{-1}, respectively. In the FT-IR spectra of STDs upon interaction with HSA, this band was disappeared (for FA-HSA) or shifted to 1737 cm^{-1} and its strenght was decreased (for OUB-HSA and ETH-HSA).

In addition, other some bands of HSA, for example the bands at 848 and 1078 cm^{-1} were disappeared at the spectrums of OUB-HSA and ETH-HSA, respectively. In the case of FA-HSA, the bands of HSA at 1410 and 848 cm^{-1} shifted to 1405 and 855 cm^{-1}, respectively.

According to the changes of these band positions and strengths, it may suggest that STDs were interacted with HSA through the C=O and/or C-N groups in its polypeptide chains and also changed the secondary structure of the protein.

The determination of binding constants and stoichiometries for the interactions between the STDs and HSA at different temperatures:

First of all, the relationships of the cathodic peak currents of STD with their concentrations were studied in the presence and absence of HSA at different temperatures. Typical plots of the peak currents (I_p) and peak differences (ΔI_p) of FA, OUB and ETH *versus* their concentrations (C) in the presence and absence of $4.6x10^{-8}$ M HSA at 33.5 °C are seen in Fig. 8.

To investigate the intensity of the interactions between the STDs and HSA, the equilibrium constants were determined by Eq. (1). By plotting of ln [ΔI / ($\Delta I_{max} - \Delta I$)] *vs* ln C (C is concentration for FA, OUB and ETH), β can obtained from the slope and also stoichiometries (m) were determined from intercept of resulted curves, as shown in Fig. 9. The values of βs and m for the STDs-HSA complexes were given in Table 1. The values of β and m for FA-HSA system increases whereas the β and m values of OUB-HSA and ETH-HSA systems decrease while the temperature increases, revealing the influence of temperature on stability of the complexes (Table 1).

Table 1. The equilibrium constants (β) and stoichiometries (m) for HSA–*m*FA, HSA–*m*OUB and HSA–*m*ETH systems calculated from the results of square-wave voltammetry at different temperatures.

T(K)	HSA–*m*FA system		HSA–*m*OUB system		HSA–*m*ETH system	
	m	***β* (M^{-m})**	***m***	***β* (M^{-m})**	***m***	***β* (M^{-m})**
297.5	1.1	$3.11x10^{5}$	2.3	$5.90x10^{12}$	2.1	$1.09x10^{11}$
300.5	1.5	$1.02x10^{10}$	2.1	$9.43x10^{11}$	1.8	$2.00x10^{10}$
303.5	1.5	$2.13x10^{10}$	1.8	$2.85x10^{10}$	1.4	$1.84x10^{7}$
306.5	2.1	$4.48x10^{10}$	1.7	$6.55x10^{9}$	1.0	$3.82x10^{5}$
309.5	2.1	$2.16x10^{11}$	1.6	$2.42x10^{9}$	0.6	$6.94x10^{3}$
312.5	2.3	$1.12x10^{12}$	1.4	$7.97x10^{7}$	0.5	$9.67x10^{2}$

Hydrogen bonds and hydrophobic interaction were found to be the predominant intermolecular forces stabilizing the drug-protein.[84] It is well known that hydrogen bonding decreases; however, hydrophobic forces increase as the temperature increases.[85–87] In addition, HSA recognizes a wide variety of agents and transports them in the blood stream.[88,89] It comprises three homologous domains (denoted I, II, and III).[9,88] Each domain is a product of two subdomains, A and B, with common structural motifs. The principal regions of ligand bindings to HSA are located in hydrophobic cavities in subdomains IIA (binding site I) and IIIA (binding site II). The binding site I is dominated by the strong hydrophobic interactions. On the other hand, binding site II mainly involves ion (dipole)–dipole, van der Waals, and/or hydrogen-bonding interactions.[9,88,90–95] So, it can be concluded that FA binds to HSA by the hydrophobic forces at site I whereas OUB and ETH interact with site II by the means of van der Waals or hydrogen bonds.

CONCLUSIONS

In the present study, the effect of temperature on the interactions of some steroids (FA, OUB and ETH) with HSA at physiological pH (7.4) has been

studied by using square-wave voltammetry. According to the obtained data, temperature has a positive effect for the interaction intensity between FA and HSA whereas it exhibits a negative impact on the intermolecular binding strengths of others (OUB and ETH) with HSA. This case may be sourced from the differences of intermolecular forces between the steroids (FA, OUB and ETH) and HSA. In addition, it has been observed that their binding stoichiometries are depending on the intensity of affinity between those molecules. On the other hand, the spectroscopic data have also been supported the interactions of these steroids with HSA.

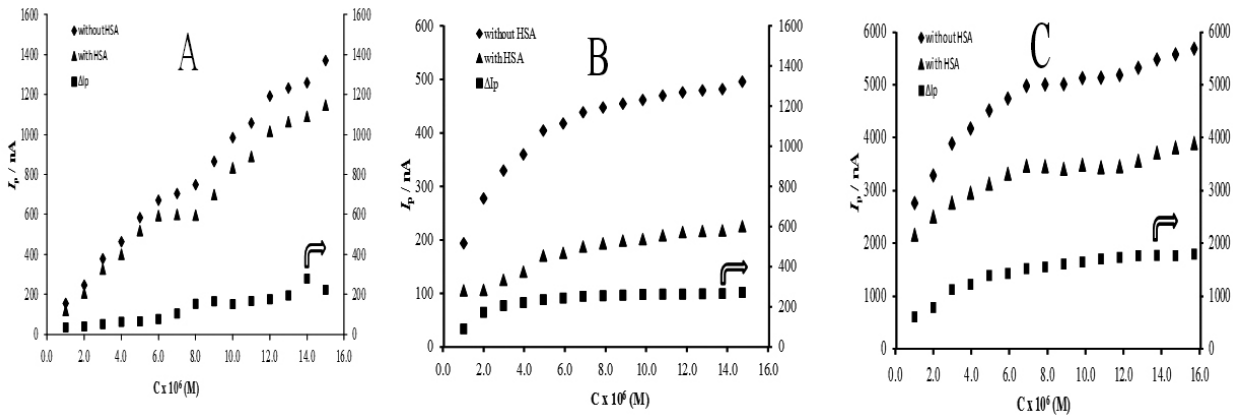

Figure 8. Typical dependence of the peak current (I_p) and peak difference (ΔI_p) of **A)** FA **B)** OUB and **C)** ETH with their concentrations (C) in the presence and absence of $4.6´10^{-8}$ M HSA at 33.5 °C.

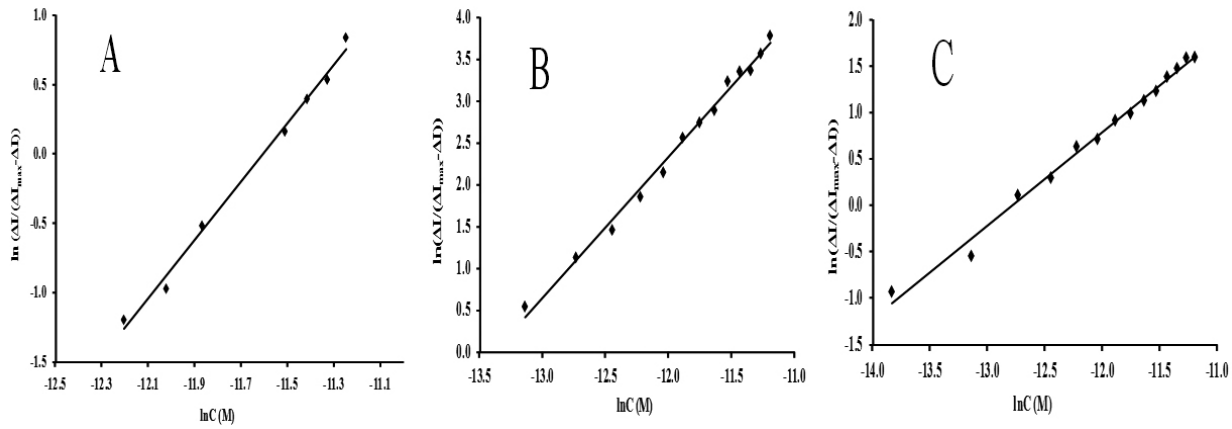

Figure 9. Typical plots of ln [D*I* /(DI_{max} – D*I*)] *vs* ln C for **A)** FA-HSA **B)** OUB-HSA and **C)** ETH-HSA systems at 33.5 °C (C: [FA], [OUB], [ETH]).

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