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Investigation of the interaction between valsartan and bovine serum albumin

Mariam Gaied[1]*, Lobna Abdel-Aziz[1], Laila Abdel-Fattah[2], Amira El-Kosasy[1]

[1]Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, Ain Shams University, (EGYPT)

[2]Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, (EGYPT)

E-mail : dr_mariamhany@yahoo.com

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ABSTRACT

The interaction of valsartan (VLS), an angiotensin II receptor antagonist, with plasma and bovine serum albumin (BSA) has been investigated at physiological acidity (pH 7.40) by spectroscopic (UV spectrophotometry and fluorescence) techniques. The effect of BSA on UV absorption spectra of valsartan was monitored at three concentration levels of BSA. The binding parameters of VLS-BSA were calculated using non-linear regression analysis of the Scatchard plot. The binding percentages were calculated at different dilution folds of plasma. A strong fluorescence quenching reaction of VLS to BSA was observed. The binding constants of VLS with BSA at 288, 298 and 309 K were calculated as 2.624×10^7, 2.431×10^7 and 2.254×10^7 M^{-1}, respectively. The thermodynamic parameters, $\Delta H°$ and $\Delta S°$ were obtained to be -5.35 ± 0.096 kJ mol^{-1} and 123.49 ± 0.32 J mol^{-1} K^{-1}, respectively, indicating the presence of hydrophobic forces between VLS and BSA.

KEYWORDS

Valsartan;
Bovine serum albumin;
Fluorescence quenching;
Thermodynamics.

INTRODUCTION

Bovine serum albumin (BSA) is one of the major components in plasma protein. It is in charge of the transport of a variety of endogenous and exogenous substances in body and plays an important role in the distribution and deposition of these substances[1]. When drugs are absorbed, they enter into the circulatory system and extensively and reversibly bind to serum albumin[2]. An important aspect of a drug's biodisposition profile is the extent to which it binds to plasma proteins[3]. Drug–protein interaction has significance in pharmacology. It can affect the biological activity[4,5] and toxicity[6-8] of drug. The binding parameters help in the study of pharmacokinetics and the design of dosage forms[9,10]. Hence, the studies on binding of drugs to BSA provide information of structural features that determine the therapeutic effectiveness of drugs and become an important research field in chemistry, life sciences and clinical medicine.

BSA is a single-chain 582 amino acid globular non-glycoprotein cross-linked with 17 cystine residues. It is built from three structurally homologous domains (I, II and III). Each domain is the product of two sub-domains (A and B)[11]. BSA has two tryptophans, embedded in two different domains, Trp-134 and Trp-214 IIA[12].

Different methods have been developed to evalu-

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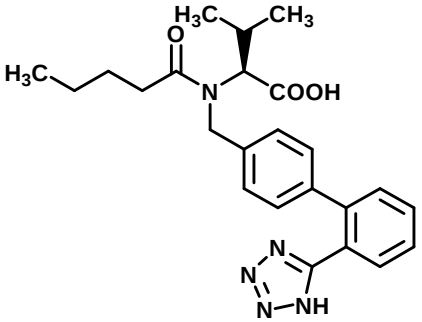

Figure 1 : Structure of Valsartan (*N*-(1-oxopentyl)-*N*-[[2'-(2H-tetrazol-5-yl)[1,1'-biphenyl]-4-yl]methyl]-l-valine)

ate drug–protein interactions, such as the traditional equilibrium dialysis[13-15], ultrafilteration[16] and chromatographic methods (HPLC[17,18] and CE[19,20]).

The spectroscopic techniques (UV/VIS absorption, fluorescence and circular dichroism) are of great help in the study of interactions between drugs and plasma proteins in general and serum albumin in particular because of their high sensitivity, rapidity and ease of implementation. They have advantages over conventional approaches employed for studying the drug–protein binding such as affinity and size exclusion chromatography, equilibrium dialysis, ultrafilteration and ultracentrifugation, which suffer from lack of sensitivity or long analysis time or both and use of protein concentrations far in excess of dissociation constant for drug–protein complex [21,22] and for drug–protein interaction studies.

Valsartan (*N*-(1-oxopentyl)-*N*-[[2'-(2H-tetrazol-5-yl)[1,1'-biphenyl]-4-yl]methyl]-**l**-valine) (Figure 1) is an antihypertensive drug belonging to pharmacological class of angiotensin II receptor antagonists (ARA-II), characterized by their selectivity, specificity, long-acting effect on the rennin–angiotensin system and good tolerability profile [23,24]. VLS is an effective agent in selective blockade of the AT_1 receptor which is responsible for vasoconstriction, while AT_2 receptor, which is thought to have cardioprotective effects and inhibitory effects on growth is unaffected by ARA-II. This is the first report on the mechanism of interaction of VLS with BSA.

EXPERIMENTAL

Apparatus

Absorption spectra were recorded on Double beam Schimadzu (Japan) 1601 PC UV-VIS spectrophotometer connected to a computer fitted with UVPC personal spectroscopy software version 3.7, using matched quartz cuvettes in a thermostated cell holder. Measurements took place at 25°C (± 0.2). Fluorescence measurements were performed on Kontron spectrofluorimeter (Switzerland), model SFM25 with 1-cm quartz cuvettes connected to IBM compatible computer fitted with WIND 25 spectroscopy software for Windows. Calculations of binding parameters were performed using Graph pad Prism software version 4.0 for windows.

Reagents

BSA (Fraction V, approximately 99%; protease free and essentially γ-globulin free) was obtained from Sigma Chemical Company, USA. Pure Valsartan was kindly supplied by Novartis Pharma, Cairo (Egypt). Purity was reported to be 100.0 ± 0.2 %. Frozen human plasma was obtained from VACCERA, Batch no. 07/G & was stored at -20°C. Solutions of VLS and BSA were prepared in 0.05 M phosphate buffer of pH 7.40. All other materials were of analytical reagent grade and double-distilled water was used.

VLS-BSA interactions

(1) UV spectrophotometric measurements

UV absorption spectra of VLS were scanned in absence and in presence of BSA in the range of 200-400nm. BSA was used at three concentration levels: 3.77×10^{-7} M, 3.77×10^{-5} M and 7.5×10^{-5} M. At each concentration level, the concentration of BSA was kept constant ,while the concentration of VLS was increased in the range of 4.59 - 36.7 $X10^{-6}$ M, 1.15-10.3$\times10^{-5}$ M and 0.25-2.5$\times10^{-3}$ M for the three BSA concentration levels; respectively. All measurements took place at 25°C (± 0.2) against reference solution containing the same concentration of BSA as blank.

(2) Fluorescence measurements

Fluorescence spectra of BSA (2.4×10^{-7} M) were recorded in absence and presence of VLS (5×10^{-6} M - 110^{-4} M) at 288, 298 and 309 K in the range of 300–400 nm upon excitation at 269 nm in each case.

(3) VLS–plasma interactions

UV absorption spectra of VLS were scanned in absence and in presence of different dilutions of plasma

at the range of 200-400nm. Plasma was diluted in phosphate buffer pH 7.40 in the range of 2-100 dilution-folds. All measurements took place at 25°C (± 0.2) against reference solution containing the same dilution of plasma as blank.

RESULTS AND DISCUSSION

(A) VLS–BSA interactions

(1) UV spectrophotometric measurements

The UV absorption spectrum of VLS is characterized by two absorption maxima at 207 and 250 nm with molar absorptivity (ε) of 0.0362 and 0.0128 cm^{-1} M^{-1} at 207 and 250 nm; respectively. The effect of BSA on VLS UV absorption spectrum was found to vary according to the concentration of albumin (Figure 2-4). At low concentration of BSA (3.77×10^{-4} mM), the peak at 250 nm remained unchanged, however, the peak at 207 nm showed slight bathochromic and hypochromic shift. By increasing BSA concentration to higher levels (0.0377 mM), the peak at 207 nm completely disappeared, whereas the peak at 250 nm was slightly shifted to longer wavelength along with hypochromic shift. Further increase in BSA concentration (0.075 mM), lead to appearance of a new peak at 299 nm. This new peak displays slight shift to longer wavelength by increasing BSA concentration. Increasing VLS concentration at fixed BSA concentration lead to quantitative increase in absorbance. At both concentration levels of BSA, the decrease in absorbance of VLS at either wavelength was used to calculate the binding parameters using non-linear regression and transformation to the Scatchard plot performed by Graph pad Prism 4 software (Figure 5, 6).

The Scatchard equation can be represented as follows[25]:

$$[ligand]_{bound} / [ligand]_{free} = n K_d - K_d [ligand]_{bound}$$

where, K_d is the equilibrium dissociation constant and n is the number of classes of binding sites.

The binding parameters are always described as B_{max} and K_d. B_{max} represents the maximum density of binding sites. K_d is the equilibrium constant. B_{max} is obtained from the intercept of the Scatchard plot and is corrected for the amount of protein used, whereas K_d is the negative reciprocal of the slope of the transformed Scatchard plot[26,27].

TABLE 1

	BSA (3.77×10^{-4} mM)	BSA (0.0377 mM)
B_{max}	8.658	8.827
K_d (μM)	21.22	5.086
r^2	0.9999	0.9998

TABLE 2 : Binding parameters of VLS using fluorescence quenching method at different temperatures

	288 K	298 K	309 K
K_{SV} (μM^{-1})	0.02266	0.01910	0.01624
n	1.68	1.701	1.686
K_b (L $mol^{?1}$)	2.624×10^7	2.431×10^7	2.254×10^7
r^2	0.9987	0.9991	0.9965

TABLE 3 : Thermodynamic parameters of BSA-valsartan system obtained from BSA fluorescence quenching method at different temperatures

T(K)	ΔH (Kj mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔG (Kj mol^{-1})	r^2
288	– 5.35 ±0.096	123.49 ± 0.32	– 40.91 ± 0.188	
298			– 42.15 ± 0.191	0.9998
309			– 43.51 ± 0.195	

At 3.77×10^{-4} mM BSA, B_{max} and K_d were calculated from Scatchard plot and were equal to 8.658 (22.966 μM/ μM of BSA) and 21.22 μM; respectively, while at 0.0377 mM BSA, B_{max} and K_d were 8.827 (234.138 μM/ mM of BSA) and 5.086 μM ; respectively (TABLE 1). It can be concluded that at the higher concentration of BSA the binding affinity increases as evidenced by lower K_d. By fitting the data into different binding models, the preferred model was found to be one-binding site model, thus proving that at each concentration level there is only one class of binding sites.

From the data obtained, it is obvious that VLS binding to BSA is concentration-dependant and there are two classes of binding sites with different binding affinities; one with low affinity seen at low BSA concentration and another with high affinity at higher BSA concentration. An excessive increase of BSA concentration leads to a different type of interaction observed as a new peak. This can be explained by the fact that, binding of ligands to proteins may lead to conformational changes in the protein structure, thus exposing more sites in the protein for interaction[28,29]. In addition, it is a well-described phenomenon that the affinity

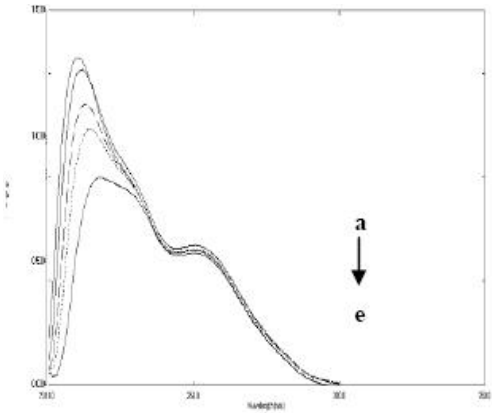

Figure 2 : Effect of low concentration of BSA on The UV absorption curve of 36 µM valsartan, a: no BSA, b-e: 0.19 µM -0.57 µM; showing bathochromic hypochromic shift at 207 nm while peak at 250 nm remains unaffected

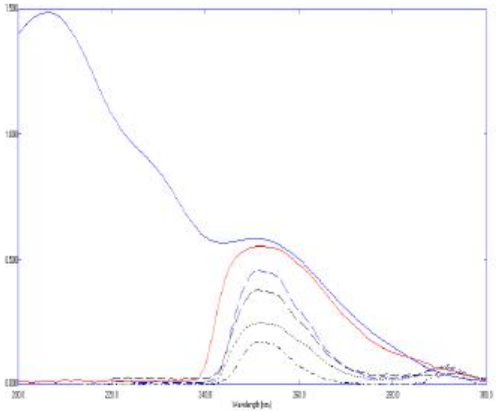

Figure 3 : Effect of intermediate concentration of BSA on The UV absorption curve of 46 µM Valsartan, a: no BSA, b-f: 25.5 µM-75.4 µM; showing disappearance of the peak maximum at 207 nm and hypochromic shift at peak maximum at 250 nm

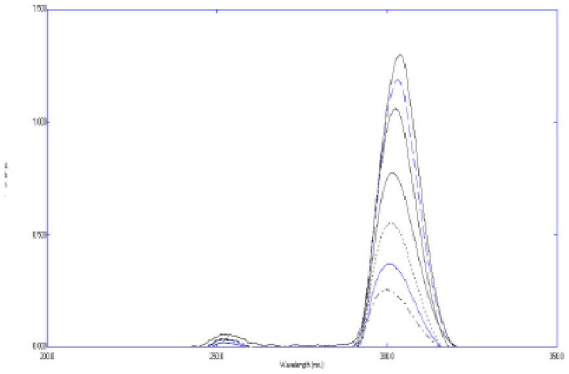

Figure 4 : UV absorption curve of Valsartan at concentration range (0.23-2.3 µM) in presence of 0.075 mM BSA at pH 7.4, showing complete disappearance of both peaks at 207 nm & 250 nm and appearance of new peak at 299 nm

of albumin for certain ligands depends on the protein concentration[30-32].

(2) Fluorescence quenching of BSA by VLS

In BSA, tryptophan and tyrosine mainly contribute to fluorescence spectra. The fluorescence decreases when some substance was added into the BSA solution. This phenomenon is called fluorescence quenching (FQ)[33,34]. FQ in serum albumin is widely used in measuring drug–protein binding affinity. The interaction of VLS to BSA at physiological acidity (pH 7.40) was evaluated by measuring the intrinsic fluorescence intensity of protein before and after addition of VLS. BSA has a strong fluorescence emission with a peak at 340 nm on excitation at 269 nm. With gradual increase in drug concentration, we observed typical FQ behavior (Figure 7). The fluorescence intensity of the protein decreases in the presence of VLS, suggesting that the microenvironment around chromophore of BSA was changed after the addition of VLS.

The fluorescence quenching data were analyzed by the Stern–Volmer equation,

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

where F_0 and F are the steady-state fluorescence intensities in absence and presence of quencher, respectively, K_{SV} is the Stern–Volmer quenching constant and [Q] is the concentration of quencher (VLS) (Figure 8). The values of K_{SV} and r^2 at different temperatures are summarized in TABLE 2. In many instances, the fluorophore can be quenched by collision (dynamic quenching) or by complex formation (static quenching) or both. A linear Stern–Volmer plot, however, does not define the quenching type, and additional information is required for this determination[35,36]. One way to distinguish dynamic from static quenching is to examine the temperature effect on the interaction of drug to BSA. These results indicate that the probable quenching mechanism of fluorescence of BSA by VLS is a static quenching procedure, because K_{SV} is decreased with increase in temperature[37,38].

When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the equation[39].

$$\text{Log}(F_0\text{-}F)/F = \text{logK} + \text{nlog[Q]}$$

where K and n are the binding constant and the number of binding sites, respectively. Thus, a plot (figure 9) of log $(F_0\text{-}F)/F$ versus log [Q] yielded the K and n values

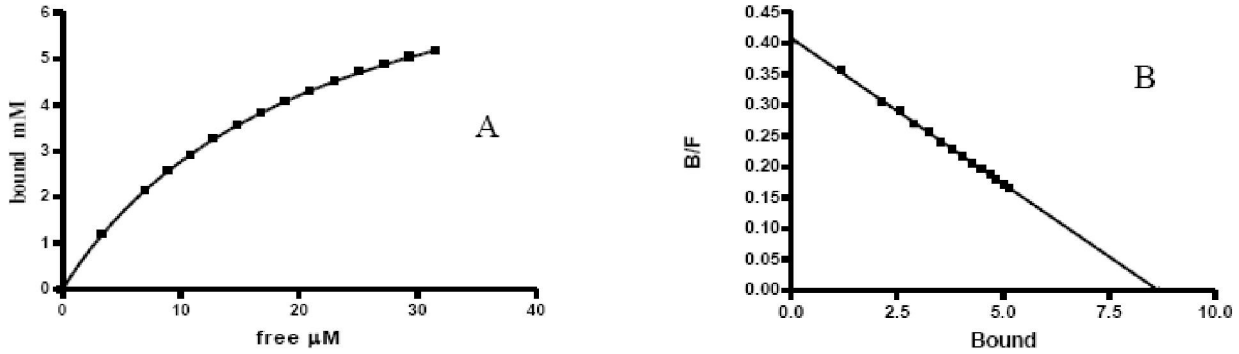

Figure 5 : Graphical Presentation of Valsartan-BSA Interaction at 207 nm, using varying concentrations of valsartan (4.59-36.7 µM) in presence of 0.37 µM BSA; A: binding isotherm showing the degree of binding versus the free valsartan concentration, B: Scatchard plot obtained by nonlinear regression analysis of A

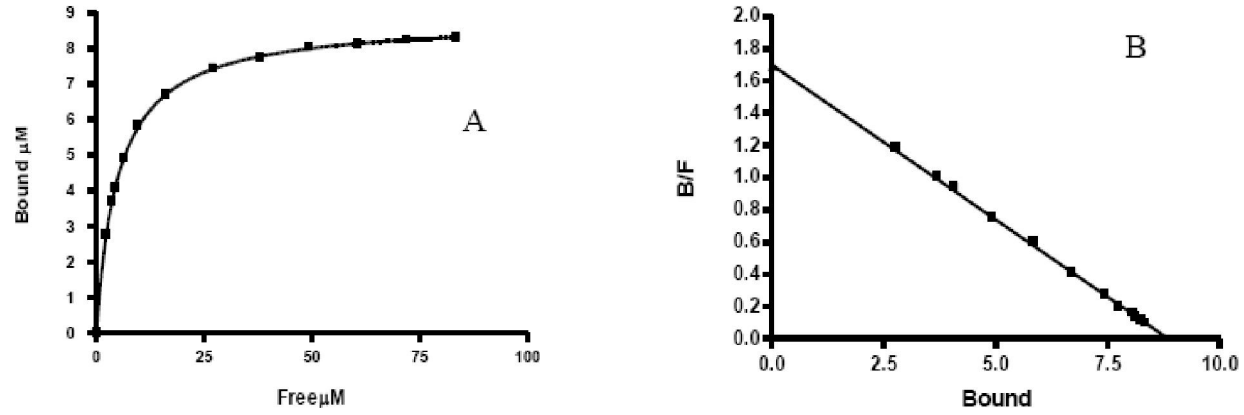

Figure 6 : Graphical presentation of Valsartan-BSA interaction at 250 nm, using varying concentrations of valsartan (11.5 - 103 µM) in presence of 37.7 µM BSA; A: binding isotherm showing the degree of binding versus the free valsartan concentration, B: Scatchard plot obtained by non-linear regression analysis of A

to be 2.624×10^7 L mol^{-1}and 1.68, 2.431×10^7 L mol^{-1} and 1.701, and 2.254×10^7 L mol^{-1} and 1.686, at 288, 298 and 309 K; respectively. The value of n approximately equals to 2, indicating the existence of 2 classes of binding sites of different affinities in BSA for VLS, which agrees well with the findings of the binding study using UV- absorption of VLS.

The acting forces between drug and biomolecules include hydrogen bonds, van der Waals forces, electrostatic forces, hydrophobic interaction forces [290-292]. Thus, one could obtain some important parameters with the help of Van't Hoff plot and thermodynamic equation, that is,

$$\mathbf{Log\ K = -\Delta H^\circ / 2.303RT + \Delta S^\circ / 2.303R}$$

$$\mathbf{\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ,}$$

Here, ΔH°, ΔG°, ΔS° are standard enthalpy change, standard free energy change and standard entropy change, respectively. The fluorescence quenching study was carried out at 288, 298 and 309 K. At these temperatures, BSA does not undergo any structural degradation. The Van't Hoff (plot of log K versus 1/T) (Figure 10) enabled the determination of ΔH°, ΔS° and ΔG°, and these values are summarized in TABLE 3. Nemethy and Scheraga[40], Timasheff[41] and Ross and Subramanian[42] have characterized the sign and magnitude of the thermodynamic parameter associated with various individual kinds of interaction that may take place in protein association processes. The binding process is exothermic reaction accompanied by a positive ΔS° value. From the point of view of water structure, for a drug–protein interaction, a positive ΔS° value is frequently regarded as an evidence for a hydrophobic interaction[43,44] because the water molecules that are arranged in an orderly fashion around the drug and protein acquire a more random configuration. Nevertheless, it has been pointed out that specific electrostatic interactions between ionic species in aqueous solution are characterized by a positive value of ΔS° and a nega-

tive ΔH° value. A negative ΔH° value may be also obtained in case of hydrogen bonding in the binding reaction[45]. The negative value for ΔG° indicates the spontaneity of the binding of VLS with BSA. Meanwhile, it was found that the main contribution to ΔG° value arises from the ΔS^0 rather than from ΔH°, so hydrophobic forces most likely play a major role in the binding of VLS to BSA, but the relatively smaller enthalpy change means that hydrogen bonding and electrostatic interaction also cannot be excluded.

(B) VLS–plasma interactions

The absorption maximum at 207 nm has completely disappeared even by diluting plasma up to 100 folds. When plasma was diluted 60 or more folds the peak at 250 nm was not affected. Increasing plasma concentration (fewer folds of dilution) lead to slight red shift to longer wavelength accompanied by decrease in absorbance (bathochromic hypochromic shift). Plasma, at 10 folds dilution and less, lead to disappearance of the peak at 250 nm and appearance of a new absorption maximum at λ 303 nm. The new peak displayed quantitative increase in absorbance by increasing the concentration of VLS at a fixed plasma level.

The binding percentage was calculated at each plasma dilution using the decrease in absorbance at 250 nm (Figure 11). The binding percentages (TABLE 4) were determined in triplicate assays and calculated according to the following equation[46,47]:

$$\%\ \text{unbound (free)} = A_{\text{drug in plasma}} / A_{\text{same drug conc. without plasma}} 100$$

$$\%\ \text{bound} = 100 - \%\ \text{free}$$

It was found that percent bound drug increased by increasing plasma concentration (fewer folds of dilution), without affecting the binding pattern emphasizing that plasma proteins concentration is directly correlated with the percentage of the bound fraction. It is important to note that, in vivo the percent bound does not increase but approaches a plateau because of the faster clearance of the free fraction.

CONCLUSION

The binding pattern and parameters of VLS with BSA were calculated using the UV absorption spectra of VLS and confirmed by fluorescence quenching of intrinsic fluorescence of BSA. In conclusion, there are 2 classes of binding sites, of different affinities in albumin to which VLS can be bound. Hydrophobic forces most likely play a major role in the binding of VLS to BSA, however, hydrogen bonding and electrostatic interaction cannot be excluded. The percentage of binding with plasma was calculated and was found comparable to the stated in literature.

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