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Warfarin replaces dexamethasone in drug complexes with human serum albumin - a fluorescence spectroscopy study

S. Abarova*, A. Papageorgiou, Y. Ivanov, B. Tenchov

Department of Medical Physics and Biophysics, Medical University-Sofia, 1431 Sofia, Bulgaria

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In this work we used fluorescence spectroscopy to study the interaction of human serum albumin (HSA) with two widely used drugs, the anticoagulant warfarin (War) and the synthetic corticosteroid medication dexamethasone (Dex). Intrinsic fluorescence emission spectra of HSA in presence of the studied drugs, recorded in the range 300-500 nm at an excitation wavelength of 283 nm, showed that both drugs form complexes with HSA and act as quenchers. The HSA-drug complex formation manifested a significant decrease in the fluorescence intensity most likely due to changes in the protein fluorophore (Trp-214) environment caused by the drug binding to HSA subdomain IIA. In addition to the quenching effect, War binding caused a red shift and Dex binding caused a blue shift of the HSA emission maximum at 337 nm. Remarkably, the addition of War to already formed HSA-Dex complexes caused a red shift of the emission maximum to higher wavelengths typical of HSA-War complexes, while the addition of Dex to preformed HSA-War complexes resulted in no spectral shifts. We conclude on this basis that War has a higher binding affinity to HSA than Dex, respectively, it successfully competes with Dex for the binding site and can replace Dex in already formed HSA-Dex complexes. This effect is of certain interest as it may influence the balance between bound and freely circulating drugs in cases of simultaneous application of two drugs, thus affecting the drug pharmacokinetics, efficacy and adverse side effects.

Keywords: dexamethasone; warfarin; human serum albumin; fluorescence spectroscopy; quenching; drug binding

INTRODUCTION

Albumin, the most abundant protein in blood plasma, has high binding affinity to many endogenous and exogenous compounds and serves as their transporter in the circulation. Among the transported substances are various metabolites, drugs, and other biologically active compounds present in the blood [1]. In the present work we used fluorescence spectroscopy to study the interaction of two widely used drugs, warfarin and dexamethasone, with human serum albumin (HSA).

Warfarin (War, Fig. 1a), 3-(α-acetonylbenzyl)-4-hydroxy-coumarin, is the most widely used oral anticoagulant. Its metabolical assimilation by the human organism is excellent [2].

Dexamethasone (Dex, Fig. 1b), pregna-1,4-diene-3,20-dione,9-fluoro-11,17,21-trihydroxy-16-methyl-, (11β,16α)-, is a synthetic corticosteroid medication that is widely used in clinical treatment of Addison's disease, systemic lupus erythematosus, allergic reactions, certain skin and eye conditions, breathing problems, bowel disorders, certain cancers and autoimmune diseases (pubchem.ncbi.nlm.nih.gov/compound/ dexamethasone). Dex is easy to overdose. Its adverse side effects include seizures, gastrointestinal perforation, and heart attack. Long-term use can also cause Cushing's syndrome, osteoporosis, and cataracts [3].

Both Dex and War are known to bind to HSA [4, 5]. However, their mutual interactions upon simultaneous application and formation of complexes with HSA are not clear. In particular, the effect of War on the HSA-Dex complexes in the blood circulation is not well characterized. Therefore, investigating the interaction of Dex with HSA in the presence of War can provide useful information about the drug relative affinities to HSA and can be used as a model to clarify the properties of the drug-protein complexes in cases of simultaneous application of the two drugs.

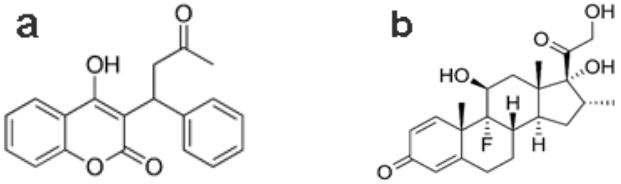

Fig. 1. Chemical structures of the drugs studied: a) warfarin, b) dexamethasone.

* To whom all correspondence should be sent:
E-mail: sabarova@abv.bg

In order to analyze the possible influence of War on the properties of the HSA-Dex complexes, we focused here on investigating the effects of War and Dex on the drug-HSA fluorescence spectra, fluorescence quenching mechanisms, binding constants and binding mode. These measurements are expected to provide information on the HSA-Dex and HSA-War relative complex stability.

MATERIALS AND METHODS

Fatty acid-free human serum albumin and warfarin were obtained from Sigma-Aldrich. Dexamethasone solution for injection (4 mg/ml dexamethasone phosphate in PBS, Sopharma) was purchased from a pharmacy shop. Stock solutions of HSA, War, and Dex were prepared in PBS, pH 7.4, and diluted to various lower concentrations for actual use.

Fluorescence Spectroscopy

A Scinco 2 spectrofluorimeter (South Korea) with temperature control accessory was used to measure the fluorescence spectra. Bandwidths for both excitation and emission were 5 nm. Fluorescence measurements of HSA-Dex and HSA-War mixtures were carried out by keeping a constant HSA concentration, while the Dex and War concentrations were varied in a broad range. Fluorescence spectra were recorded at three temperatures, 288, 298, and 310 K. The fluorescence emission range was 300-500 nm and the excitation wavelength was 283 nm.

UV–Vis Absorption Measurements

UV absorption spectra of HSA and its complexes with the drugs studied were measured in the range of 250–330 nm (n = 5 replicates) at 298 K.

RESULTS AND DISCUSSION

The HSA intrinsic fluorescence emission spectra recorded at three different temperatures are shown in Fig. 2. As could be expected, the HSA fluorescence intensity decreases with temperature increase. The intrinsic fluorescence of HSA is mainly due to the tryptophan residue Trp-214 in the hydrophobic cavity of HSA subdomain IIA (Sudlow's binding site I). Accordingly, if a compound binds to the latter site, it will likely affect the HSA intrinsic fluorescence [6].

Quenching of the HSA Fluorescence Spectrum by Warfarin and Dexamethasone

War is known to bind to subdomain IIA of HSA (Sudlow's binding site I) where its interaction with the protein is dominated by hydrophobic contacts. According to published data, the interaction between the War benzyl ring and the Trp-214 moiety represents a major contribution to the stability of the HSA-War complex [7-9].

As shown in Fig. 3, the HSA fluorescence is gradually quenched by addition of increasing War concentrations. The decrease of the fluorescence intensity is accompanied by a shift in the fluorescence maximum to higher wavelengths (red shift). These data suggest that War forms a complex with HSA leading to a microenvironment change in subdomain IIA and consequently to quenching of the Trp-214 fluorescence.

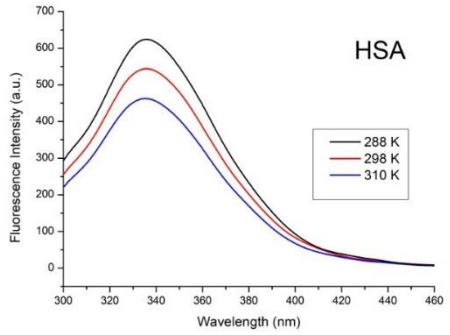

Fig. 2. Intrinsic fluorescence emission spectra of 30 μM HSA at temperatures 288, 298 and 310 K. Excitation wavelength λ_{ex} = 283 nm.

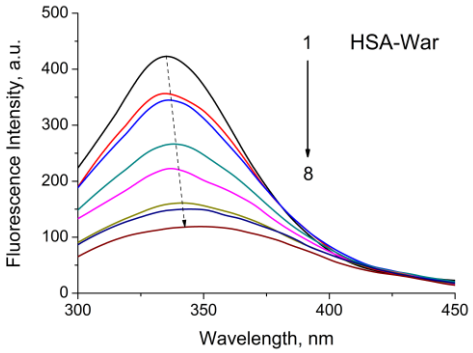

Fig. 3. Quenching of the fluorescence emission spectrum of 20 μM HSA by warfarin (War) at 298 K. Curves 1-8 correspond to War concentrations of 0, 6, 12, 18, 24, 30, 36, 42 μM, respectively. The emission maximum experiences a red shift with increase of War concentration (dashed arrow).

Addition of Dex to HSA solutions also quenched the HSA fluorescence (Fig. 4) [10]. This indicates binding of Dex to the HSA subdomain IIA where tryptophan Trp-214 is located. The increase of the Dex concentration was accompanied by a blue shift of the emission maximum (Fig. 5). Blue shifts typically result from placement of the protein fluorophore Trp-214 in a more hydrophobic environment, apparently reached in the present case after the

binding of Dex to the HSA subdomain IIA [4].

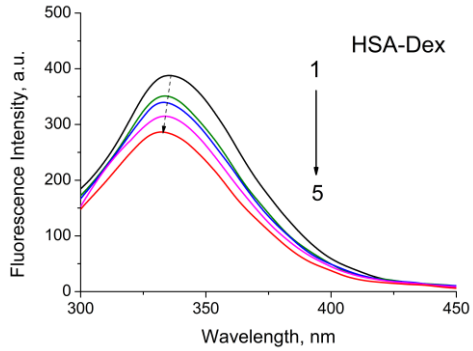

Fig. 4. Quenching of the fluorescence emission spectrum of 20 μM HSA by dexamethasone (Dex) at 298 K. Curves 1-5 correspond to Dex concentrations 0, 6, 12, 18 and 24 μM, respectively. The emission maximum experienced a blue shift with increase of Dex concentration (dashed arrow).

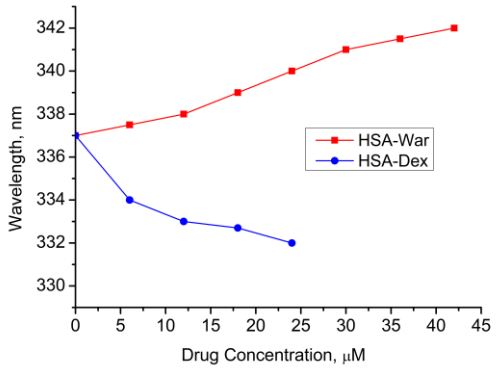

Fig. 5. Shifts in the fluorescence emission maximum upon addition of warfarin (red shift to higher wavelengths) and dexamethasone (blue shift to lower wavelengths). HSA concentration 30 μM.

Quenching Mechanism of HSA Fluorescence by War and Dex

The interactions of War and Dex with HSA were measured at three temperatures 288 K, 298 K, and 310 K and the temperature data were used to determine the nature of the drug interactions with HSA. The fluorescence quenching and the spectral shift effects at 288 K and 310 K (not shown) were similar to the results shown in Figs. 3 and 4 for 298 K.

According to its mechanism, fluorescence quenching can be dynamic or static [11]. Dynamic quenching results from collisional encounters of the quencher with the fluorophore in excited state, while static quenching results from complex formation between the fluorophore in its ground state and the quencher. Dynamic and static quenching can be distinguished from their dependence on temperature and viscosity. The decrease in emission intensity upon quenching can be described by the Stern–Volmer equation:

$$F_0/F = 1 + K_{SV}\,Q$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, Q is the concentration of the quencher, and K_{SV} is the Stern-Volmer quenching constant. Plots of F_0/F *vs.* Q are indicative for the type of quenching, i.e., they can be either linear for static or dynamic quenching, or display an upward curve for mixed quenching types [11]. For the linear plots obtained in the present study, the Stern-Volmer quenching constant K_{sv} can be calculated using the above equation. Its values for three different temperatures are given in Table 1. The K_{SV} decrease with increase of temperature shows that the HSA fluorescence quenching for both War and Dex takes place *via* a static quenching mechanism. The K_{sv} values also show the sensitivity of the fluorophore to a quencher. For the studied here HSA, quenching with War is obviously more efficient than quenching with Dex, as is clear from the K_{SV} values in Table 1.

Table 1. Stern-Volmer quenching constants K_{SV} for HSA complexes with War and Dex.

Temperature, K	HSA-War	HSA-Dex
	$K_{SV} \times 10^{-4}$ M^{-1}	$K_{SV} \times 10^{-4}$ M^{-1}
288	5.24	2.57
298	3.03	1.62
310	1.96	1.10

Displacement of Dex by War in HSA-Dex Complexes

In order to evaluate the relative stability of the HSA-War and HSA-Dex complexes we applied the following protocol. First, 5 μM HSA was reacted with 4 μM Dex and a small blue shift of about 1 nm was recorded for the HSA-Dex complex. After that, increasing concentrations of War were added, which resulted in a strong red shift of the fluorescence emission, showing that War replaced Dex in the HSA-Dex complexes (Figs. 6A and 6B). Alternatively, 5 μM HSA was reacted with 4 μM War and a small red shift of about 1 nm was recorded. After that, increasing concentrations of Dex were added, which resulted in no shifts in the emission maximum (Fig. 6B). These observations show that War binds more strongly to HSA and cannot be displaced even by large Dex concentrations, while the Dex binding is weaker than that of War and, correspondingly, War can displace

Dex from its complex with HSA. These observations were corroborated by previous findings [4] that addition of War reduced the binding constants of Dex to HSA. The observed by us displacement of Dex by War also showed that Dex most likely binds to the same site in subdomain IIA, which serves as binding locus for War with Trp-214 located near or within the binding site.

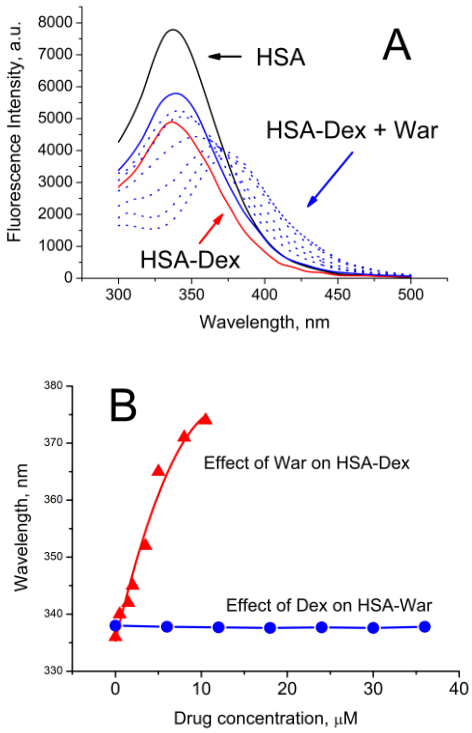

Fig. 6. A. Red shifts in the emission spectra of 5 μM HSA-4 μM Dex complexes upon addition of increasing War concentrations from 0.5 to 10.5 μM; B. Red spectral shifts induced by addition of War to HSA-Dex complexes (red triangles) and absence of spectral shifts upon addition of Dex to HSA-War complexes.

The influence of War and Dex on the UV-Vis Spectra of HSA

The UV absorption spectra exhibited by proteins are due to absorption by Trp (280 nm), Tyr (280 nm) and Phe (257 nm), also by the peptide bonds (225 nm) [12-14]. Here we measured the UV-Vis absorption spectra of HSA and HSA-drug complexes in order to identify and confirm the mechanism of the quenching process. We found that the UV absorption of HSA increased upon addition of both War and HSA (Fig. 7). As dynamic quenching only affects the excited states of the fluorophores, it results in no change in the absorption spectra. Thus, UV absorption increase upon addition of War and Dex to HSA indicates formation of HSA complexes with the drugs. These data confirmed once again that War and Dex quenched the HSA intrinsic fluorescence by means of a static quenching mechanism, in agreement with our previous work and other published studies [10, 15].

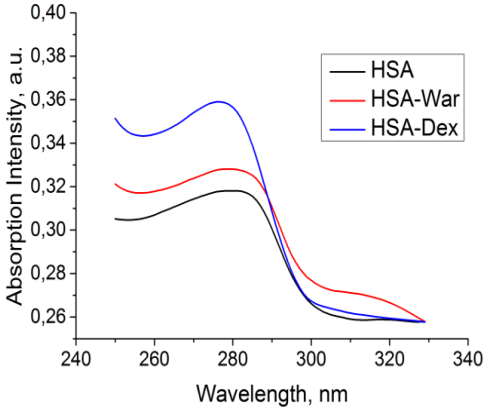

Fig. 7. UV absorption spectra of HSA, HSA-War and HSA-Dex complexes. $C_{HSA} = C_{War} = C_{Dex} = 4$ μM.

CONCLUSIONS

Measurements of HSA intrinsic fluorescence showed that two widely used drugs, War and Dex, form complexes with HSA, manifested in strong fluorescence quenching. In addition to their quenching effect, War and Dex also caused red and blue shifts, respectively, of the HSA emission maximum at 337 nm.

Based on these spectral shifts, we applied a new, rather sensitive protocol to evaluate the relative stability of the HSA-drug complexes and found that War displays a higher affinity to HSA and can displace Dex from the HSA-Dex complexes, while Dex cannot displace War from the HSA-War complexes even at rather high Dex concentrations. This effect is of certain interest as it sheds light on the balance between bound and freely circulating drugs in cases of simultaneous application of two drugs, thus affecting the drug pharmacokinetics, efficacy and adverse side effects.

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