ABSTRACT

Introduction: Human serum albumin (HSA) is a carrier for several drugs, inter alia doxorubicin and 5-fluorouracil, and it plays an important role in the regulation of their plasma concentrations. The aim of the experiment was to explore and verify the molecular interactions between doxorubicin and 5-fluorouracil and HSA using spectrofluorometric techniques.

Material and methods: The HSA solution was prepared and treated with different concentrations of doxorubicin and 5-FU. Fluorescence quenching spectra were recorded afterwards using spectrofluorometric techniques. The binding of doxorubicin at different concentrations to human serum albumin in the absence and presence of urea at pH 6.5 has been studied, as well.

Results and discussion: Both doxorubicin and 5-FU act as fluorescence quenchers. When the concentrations of HSA were constant and the concentration of doxorubicin/5-FU was gradually increased, the fluorescence intensity decreased. When compared quenching efficiency of doxorubicin and 5-FU, it is evident that doxorubicin is more potent quencher. The fluorescence emission decreases more as the concentration of doxorubicin increases, when equal concentrations of drugs are administered. Moreover, the fluorescence quenching effects of doxorubicin were shown on both native and partially unfolded form of HSA.

Conclusions: Doxorubicin and 5-FU quench the intrinsic fluorescence of HSA due to their specific molecular interactions. Doxorubicin quenching effect is stronger, due to its binding network with HSA, which might affect its biological activity and decrease its plasma concentration. 5-FU shows low affinity toward HSA molecule. There is a clear possibility that severe dose-related side effects will strike and that HSA will interact with other drugs present concomitantly. The quenching effect of doxorubicin indicates that the binding capacity of doxorubicin to the HSA (mild acidic condition, 2 M urea) decreases significantly, which is probably due to conformational changes of its binding site.

Key words: Human serum albumin; fluorescence quenching; doxorubicin; 5-fluorouracil; urea; denaturation

SAŽETAK

Uvod: Humani serum albumin (HSA) je nosač različitih lijekova, između ostalog, dok sorubicina i 5-fluorouracila, te igra važnu ulogu u regulaciji njihove koncentracije u plazmi. Oli istraživanja je da pojasni molekularne interakcije između dok sorubicina odnosno 5-fluorouracila sa HSA, spektrofluorometrijskom metodom.

Materijal i metode: Otopine HSA su pripremljene sa različitim koncentracijama dok sorubicina i 5-FU. Fluorescentni spektri snimljeni su spektrofluorometrijskom metodom. Rađena su istraživanja interakcije dok sorubicina sa humanim serum albuminom, pri različitim koncentracijama dok sorubicina, u prisustvu odnosno odsustvu uree, pri pH 6.5.

Rezultati i rasprava: I dok sorubicin i 5-FU dovode do gašenja fluorescencije. Pri konstantnoj koncentraciji HSA i pri postepenom povećanju koncentracije dok sorubicina/5-FU dolazi do smanjenja intenziteta fluorescencije. Pri poređenju učinkovitosti gašenja fluorescencije...
1. INTRODUCTION

Human serum albumin (HSA) is the most abundant plasma protein. It is a small protein with a single polypeptide chain, which is crosslinked by 17 disulfide bonds and it can bind wide range of chemically diverse exogenous and endogenous molecules.

Binding of a drug molecule to HSA significantly affects the pharmacokinetics of the drug as it increases drug solubility in plasma, decreases toxicity and protects molecule from oxidation. Therefore interaction between HSA and the drug molecule is essential for determining ADME properties (absorption, distribution, metabolism, and excretion) of the drug. It determines the free, active concentration of a drug and it provides a reservoir for a long duration of action, as well. In order to avoid undesirable drug-drug interactions and ameliorate therapy effects it is highly desirable to know the binding affinity of different drugs. Various previous studies were based on examining binding affinity of many different drugs. High-resolution crystal structures of HSA complexed with various molecules have shown that there are two main binding sites named sites I and II. Site I can bind many different molecules due to its large and flexible multichamber structure. On the other hand, ligands binding to site II are usually aromatic carboxylic acids with a negative charged group at one end of the molecule away from a hydrophobic center. Changes in the intrinsic fluorescence intensities of albumin-drug complex compared to mere albumin molecule provide considerable information regarding the binding characteristics and the therapeutic effectiveness of drugs.

Fluorescence quenching is considered as a useful method for measuring binding affinities. Fluorescence quenching is defined as the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecule. Thus, using the quenching of the intrinsic tryptophan fluorescence of (Trp-214) as a tool lets us study the interaction of different molecules with serum.

Doxorubicin is an anthracycline drug. It was extracted from *Streptomyces peucetius var. caesius* in the 1970s. Nowadays it is used in the treatment of several cancers including breast, lung, gastric, ovarian, thyroid, non-Hodgkin’s and Hodgkin’s lymphoma, multiple myeloma, sarcoma and pediatric cancers. Doxorubicin anticancer activity is based on intercalating into DNA, disrupting of topoisomerase-II-mediated DNA repair and generating free radicals which cause cell membrane, DNA and protein damage. Within this process, doxorubicin oxidizes to semiquinone, an unstable metabolite, which releases reactive oxygen species during converting back to doxorubicin.

The fluoropyrimidine 5-fluorouracil (5-FU) is an antimetabolite drug. It is widely used for the treatment of cancer, particularly for colorectal cancer, breast cancer and the cancers of aerodigestive tract. 5-FU exerts its anticancer effects through inhibition of thymidylate synthase (TS) and incorporation of its metabolites into RNA and DNA. Modulation strategies, such as co-treatment with leucovorin and methotrexate, have been developed to increase the anticancer activity of 5-FU. Structures of doxorubicine and 5-FU are presented in Figure 1., respectively.
The aim of this study was to explore and verify the molecular interactions between HSA and doxorubicin and 5-FU. This paper provides results of experimental study based on using spectrofluorometric techniques.

2. MATERIAL AND METHODS

The stock solutions of doxorubicin (10 mg/5mL, Pliva) and 5-FU (250 mg/5mL, Pliva) were prepared in phosphate buffer solutions (10 mM phosphate buffer, 100 mM NaCl, pH 6.5). Final concentrations were 0.2; 0.4; 0.8; 1.0; 2.0; 4.0; 6.0; 8.0; 20.0 and 42.0 μmol L⁻¹ for doxorubicin and 8.0; 20.0; 42.0 and 84.0 μmol L⁻¹ for 5-FU.

HSA was directly dissolved in distilled water to prepare stock solution, which was stored at 0-4°C. The absorbance of HSA was measured at 280 nm (Shimadzu UV-VIS Mini Spectrophotometer UV-1240) and was 2,521.

The HSA solution was treated with different concentrations of doxorubicin and 5-FU solutions to achieve final concentration of 8, 20, 42 and 84 μmol L⁻¹, respectively.

All fluorescence measurements were carried out on a spectrofluorometer (RF-5301, Shimadzu, Japan) equipped with 1.0 cm quartz cells. Emission wavelengths were set between 300 and 650 nm, whereas the excitation wavelength was 292 nm.

The entrance and exit slits for the excitation light-beam were both 1.5 nm. The resulting fluorescence was corrected for the background fluorescence of buffer and human serum albumin.

3. RESULTS AND DISCUSSION

3.1. Fluorescence spectra

Fluorescence quenching refers to any process, which decreases the fluorescence intensity of a specific fluorophore. A wide range of molecular interactions results in quenching, including excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation.

When the concentrations of HSA were constant and the concentration of doxorubicin/5-FU was gradually increased, the fluorescence intensity decreased. The family of curves obtained for HSA and doxorubicin at pH 6.5 is presented in Figure 2.

As it is presented on the Figure 2., presence of doxorubicin in HSA solution, even at low concentration, results in fluorescence quenching of the HSA molecule. The amount of fluorescence quenching depends on the concentration of doxorubicin molecules in HSA solution. Our results support results reported by Agudelo et al. who suggested that doxorubicin binds strongly to HSA via hydrophilic and hydrophobic contacts at physiological conditions. Several amino acids participate in drug-protein complexation and contacts are stabilized by H-bonding network. Contacts formed between doxorubicin and HSA are tighter than those formed with BSA, which is physiologically significant.
Similar results were observed when interaction between HSA and 5-FU was investigated. The family of curves is presented in Figure 3.

**Figure 3.**
The fluorescence spectra of HSA in the presence of various concentrations of 5-FU in phosphate buffer (20 mM, 100 mM NaCl, pH 6.5). The concentration of 5-FU (1B2) were: 0, 8, 20, 42 and 84μM.

It is apparent that the emission spectra of HSA display a remarkable decrease by adding 5-FU. The results show that 5-FU acts as fluorescence quencher of HSA. The quenching efficiency increases greatly with a rise in the concentrations of 5-FU. These findings support previously published results, which suggested that 5-FU interacts with HSA spontaneously through Van der Waals interaction and hydrogen bonds, whereas the acting force is mainly hydrophobic.

In order to compare quenching effects of doxorubicin and 5-FU, fluorescence spectra are presented in Figure 4. When compared quenching efficiency of doxorubicin and 5-FU, it is evident that doxorubicin is more potent quencher. The fluorescence emission decreases more as the concentration of drugs are administered. This phenomenon could be explained by significant structural differences between drug molecules, which affect their interactions with HSA and cause better fitting of doxorubicin. These findings match with data reported in literature.

3.1.1. Fluorescence quenching study

Fluorescence quenching occurs by different mechanisms, often classified as dynamic or static quenching, which can be distinguished by their dependence on temperature and viscosity. In order to clarify the mechanism of HSA fluorescence quenching by doxorubicin and 5-FU, in our study the quenching experiments were carried out at 298K. The fluorescence spectra were recorded at λ_{exc} = 292 nm and λ_{em} from 300 to 650 nm. The intensity at 339 nm (tryptophan) was used to calculate the binding constant (K) according to previous literature reports.

The decrease in fluorescence intensity at λ_{max} was analyzed using the well-known 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 HSA to 51-40% drug-complex, \(K_{DOX-HSA} = 1.1 \times 10^4 \text{ M}^{-1}\). Therefore, strong interactions with HSA might decrease plasma concentration of doxorubicin and affect its therapeutic efficiency, unlike 5-FU. These findings support previously published results.
(doxorubicin/5-FU), respectively, the $K_v$ is the Stern-Volmer quenching constant and $Q$ is the concentration of the quencher, i.e. drug. Figure 5. shows Stern-Volmer plots of fluorescence quenching constant for the drug-HSA complexes at different drug concentrations. A plot of $F_0/F$ versus $[Q]$ gives a straight line with a slope of $K_v$.

\begin{align}
\log(F_0-F)/F &= \log K_v + n \log [Q] \quad (2)
\end{align}

where $K_v$ is the association constant and $n$ is the number of binding sites per HSA$^{25, 26}$. The values of $n$ and $K_v$ were obtained from the slope and intercept of the modified Stern-Volmer plot, respectively. A plot of $\log(F_0/F)$ versus $\log [Q]$ gives a straight line (Figure 6.).

\begin{align}
\log(F_0-F)/F &= \log K_v + n \log [Q] \quad (2)
\end{align}

Our results regarding number of bound molecules of doxorubicin toward HSA support previously reported data$^{12}$. The intensity at 339 nm was used to calculate the association constant and the number of binding sites per HSA for 5-FU according to previous literature reports$^{21, 25}$. A plot of $\log(F_0/F)/F$ versus $\log [Q]$ gives a straight line (Figure 7.).
These findings show that 5-FU displays lower affinity toward HSA as its number of binding sites is minor when compared with doxorubicin. These findings correlate with previous reports\(^{21,25}\).

**3.2. Fluorescence spectra after urea-induced unfolding of HSA**

Denaturing agents (e.g. urea) alter protein interacting capacities while interfering with its structure. It is previously reported that the denaturation of albumin takes place at a single, two-state transition with midpoint at about 6 M urea, due to the unfolding of its domain II\(^{27}\). Moreover, some residual 3D structure remains in the HSA even at 8 M urea\(^{27}\). In our study we investigated effects of 2M urea on HSA structure and its binding capacities. Figure 8. presents family of curves obtained by measuring fluorescence quenching by mere doxorubicin and by doxorubicin after urea-induced unfolding of HSA, respectively.

Firstly, it is apparent that urea-induced denaturation leads to HSA fluorescence quenching. Secondly, the fluorescence quenching effects of doxorubicin were shown not only on the native but also on the partially unfolded form of HSA. The quenching effect indicates that the binding capacity of doxorubicin to the denatured HSA decreases significantly, which is probably due to conformational changes of its binding site.

The free concentration of a drug is often affected by interaction with co-administered drugs or by pathological conditions that can modify to a significant extent the binding properties of the carrier leading to relevant clinical impacts. This phenomenon is significant for drugs that have a relatively narrow therapeutic index. Therefore, characterization of albumin binding sites and their enantioselectivity is of great value, as well as the study of the changes in the binding properties of the protein arising by interaction between different ligands. Our work represents an analysis which could be exploited in future investigations on proteins in solution, in the binding of drugs or endogenous compounds as well as in the study of allosteric effects, cooperation or anticooperation mechanisms. On top of that, we used pH 6.5, which is close to physiological pH, where HSA fibrillation naturally occurs\(^{28,29}\). That way we managed to imitate physiological processes regarding HSA alterations and its interactions. Similar studies might lead to development of novel drugs with less side effects and better pharmacokinetics in the future.

Even though doxorubicin is an effective chemotherapeutic agent, its antineoplastic application is limited due to severe injuries of non-targeted tissues and diminished quality of patients’ life\(^{12}\). Moreover, the use of doxorubicin has been limited by irreversible cardiotoxicity, which is dose-related\(^{12}\). Therefore, nano-particle delivery systems of doxorubicin are new promising approach to site-specific drug delivery, which increases its antineoplastic efficacy and decreases its side-effects\(^{23,24}\). Accordingly, exploring its pharmacokinetic and pharmacodynamic profile is crucial for further improvement of its pharmaceutical form and quality of patients’ life. Further, it is known that pH strongly alters conformation of proteins and drug characteristics, consequently affecting their interactions and biological activity. Detailed studies are required in order to asses how slight
pH changes affect interactions between doxorubicin and HSA and its biological activity in general. In addition, different denaturing agents should be set in focus of attention as well, as they strongly alter protein conformation and its behaviour, which is essential for drug interactions. On the other hand, it is known that concomitant presence of more than one drug molecule alters their biological activity potentially increasing negative side effects. Studies regarding antineoplastic drug interactions are essential for improving life quality of patients with cancer, as their metabolic systems are significantly changed. Therefore diminishing side effects by combining proper drugs in proper doses is very important.

5-FU is widely recognized as effective treatment for tumours of the head, neck and breast and it exhibits an antibacterial activity, as well. According to its low affinity toward HSA its plasma concentrations increase rapidly after oral administration and can cause severe side effects. Therefore, high percentage of free drug molecules can interact with other drug molecules in vivo and cause treatment-related severe toxicity. Given all above, further studies regarding 5-FU and its pharmacokinetic profile are required.

4. CONCLUSION

Drug interactions at molecular level in most case significantly affect the apparent distribution volume of the drugs and their elimination rate. Therefore, studies regarding the interaction of drugs with carrier proteins are essential for comprehensive improving of their pharmacokinetic and pharmacodynamic profile. The experimental results of our study suggested that doxorubicin and 5-FU quench the intrinsic fluorescence of HSA due to their specific molecular interactions. Doxorubicin quenching effect is stronger, due to its binding network with HSA, which might affect its biological activity and decrease its plasma concentration. On the other hand, 5-FU shows low affinity toward HSA molecule, meaning that the percentage of free drug molecules increases rapidly after administration. Therefore, there is a clear possibility that severe dose-related side effects will strike and that 5-FU will interact with other drugs present concomitantly. Further studies are required for elucidating other characteristics of these drugs.

REFERENCES


