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Paclitaxel–HSA interaction. Binding sites on HSA molecule

Lilianna Trynda-Lemiesz*

Faculty of Chemistry, University of Wrocław, F. Joliot-Curie 14, 50-383 Wrocław, Poland

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Abstract—Paclitaxel (trade name Taxol®) is one of the world's most effective anticancer drugs. It is used to treat several cancers including tumours of the breast, ovary and lung. In the present work the interaction of paclitaxel with human serum albumin (HSA) in aqueous solution at physiological pH has been investigated through CD, fluorescence spectroscopy and by the antibody precipitate test. Binding of paclitaxel to albumin impact on protein structure and it influences considerably albumin binding of other molecules like warfarin, heme or bilirubin. The paclitaxel–HSA interaction causes the conformational changes with the loss of helical stability of protein and local perturbation in the domain IIA binding pocket. The relative fluorescence intensity of the paclitaxel-bound HSA decreased, suggesting that perturbation around the Trp 214 residue took place. This was confirmed by the destabilization of the warfarin binding site, which includes Trp 214, and high affinity bilirubin binding site located in subdomain IIA.

1. Introduction

The binding of drugs to serum proteins is particularly important because it affects both the activity of drugs and their disposition.[1] Serum proteins are potential drug currier of antineoplastic agents due to their accumulation in tumour tissue.[2] It has been shown that macromolecules such as albumin and globulin markedly accumulate in tumour tissues because of enhanced tumour vascular permeability and prolonged retention time in the tumour interstitium due to the obstruction of lymphatic drainage.[3]

Human serum albumin (HSA) binds a number of the relatively insoluble endogenous compounds such as unesterified fatty acids, bilirubin and bile acids and thus facilitates their transport throughout the circulation.[4] HSA is also capable of binding a wide variety of drugs,[4,5] and much of the interest in this abundant protein derives from its effects on drug delivery. Drug binding to proteins such as HSA can be an important determinant of pharmacokinetics, restricting the unbound concentration and affecting distribution and elimination. Albumin is largely α-helical, and consists of three structurally homologous domains that assemble to form a heart-shaped molecule. The principal regions of ligand binding sites of albumin are located in hydrophobic cavities in subdomains IIA and IIIA, which exhibit similar chemistry. The binding locations have been determined crystallographically for several ligands.[6] The IIIA subdomain is the most active in accommodating of many ligands, for example, digitoxin, ibuprofen and tryptophan. Aspirin show nearly equal distributions between binding sites located in IIA and IIIA subdomains, while warfarin occupies a single site in IIA. Warfarin shares this binding site with a range of other drugs (including phenylbutazone, tolbutamide and indomethacin) and thus competes with them for binding to HSA.[6,7]

The hemin binds strongly to the HSA primary binding site located at the interface between domains I and II. Further, secondary binding sites were found for hemin to be located on domains II and III.[8] The ferric heme iron is five coordinated with the Tyr 161 phenolic oxygen atom as axial ligand.[9] Bilirubin, a toxic metabolite of heme binds to albumin with high affinity at a size located at or near loop 4 in subdomain IIA.[10] Subdomain IIA, which corresponds approximately to amino acid position 190–300, has recently been shown by X-ray crystallography to be one of two principal sites on HSA for small hydrophobic ligands.[5,6] Ligand binding to one domain induces distinct conformational changes in the other domain, as both subdomains share a common

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* Tel.: +48-71-204-223; fax: +48-71-328-23-48; e-mail: ltl@wchuwr.chem.uni.wroc.pl

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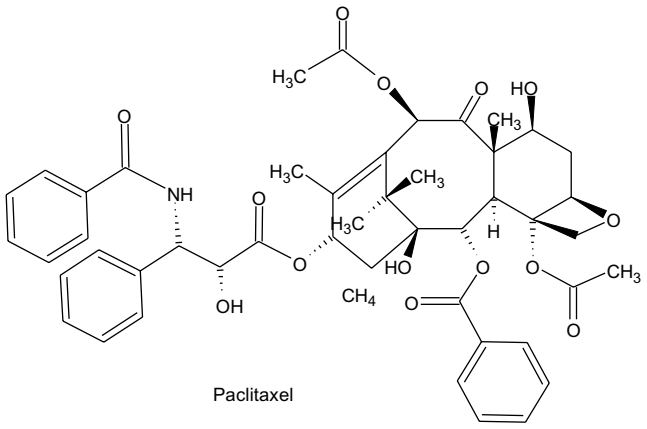

Scheme 1.

interface. Thus, the binding of particular drug molecule to serum albumin may change considerably binding abilities of HSA towards other molecules.

Paclitaxel (trade name Taxol®) is a highly functionalized diterpenoid (Scheme 1) having molecular formula $C_{47}H_{51}NO_{14}$ corresponding to molecular weight of 853 Da. It is a well established antitumour drug, whose properties are based on the ability to bind and stabilize microtubules, thus leading to the block of cell replication in the late G_2–M phase of the cell cycle.[11,12] Extensive studies have indicated that an intact taxane ring and an ester side chain at C13 are essential for cytotoxic activity. In addition, it was shown that the presence of an accessible hydroxyl group at position 2″ of the ester side-chain enhances the cytotoxic activity of the drug.[13] Paclitaxel was approved in 1992 by the US Food and Drug Administration for the treatment of ovarian and breast cancer and it was shown to be active against a variety of other cancers such as lung, gastrointestinal, neck and head as well as malignant melanoma.[14,15] Pharmacokinetics of paclitaxel shows wide variability. Terminal half-life was found to be in the range of 1.3–8.6 h (mean 5 h), less then 10% drug in the unchanged form being excreted in the urine.[16] Paclitaxel is poorly soluble in an aqueous medium, but can be dissolved in organic solvents. Its solutions can be prepared in a milimolar concentration in a variety of alcohols as well as in DMSO (nonaqueous solubility is found to be ~46 mM in ethanol, aqueous solubility is ~0.6 mM).[17,18] Several reports have suggested that more than 90% of the drug binds rapidly and extensively to plasma proteins,[12,19] however none of these investigations has determined in detail the drug binding mode and drug binding site. The earlier work reported by Purcell et al. results[20] suggested the nonspecific binding of paclitaxel to albumin with an overall binding constant of $1.43 \times 10^4\ M^{-1}$ and a partial unfolding of the protein structure. However, the binding studies of Paál et al.[21] have indicated the existence of the high affinity ($K_1 = 2.4 \times 10^6\ M^{-1}$) and an intermediate affinity ($K_2 = 1.0 \times 10^5\ M^{-1}$) binding site of paclitaxel in HSA molecule. These results although different from each other have shown rather weak paclitaxel–protein interactions, and can be attributed to the presence of mainly hydrogen bonding interactions between protein donor atoms and the paclitaxel polar groups.

The interactions of drugs including paclitaxel, with HSA has a major biochemical importance, because it can produce modified variants of human serum albumin, whose binding properties can be different with respect to the native protein. On the other hand the distribution, free concentration and metabolism of drugs can be significantly altered as a result of their binding to albumin.

In this work we have performed extended studies on the interaction of human albumin with paclitaxel in aqueous solutions at physiological pH. The effects of paclitaxel binding on the structural integrity of HSA and influence on the heme, warfarin and bilirubin binding were investigated.

2. Results and discussion

2.1. Structural properties of the HSA–paclitaxel

The albumin structure is predominantly α-helical. Approximately, 67% of HSA is helical, the number of helices in the structure is 28.[5] CD spectra were used to monitor paclitaxel–albumin interaction. Stepwise CD titration of albumin with increasing amounts of paclitaxel is shown in Figure 1. The molar ratio was varied from 2 to 12.

CD spectra of HSA exhibit two negative bands in the ultraviolet region at 209 and 222 nm characteristic for an α-helical structure of protein. The binding of paclitaxel to HSA distinctly decreases both of these bands. This

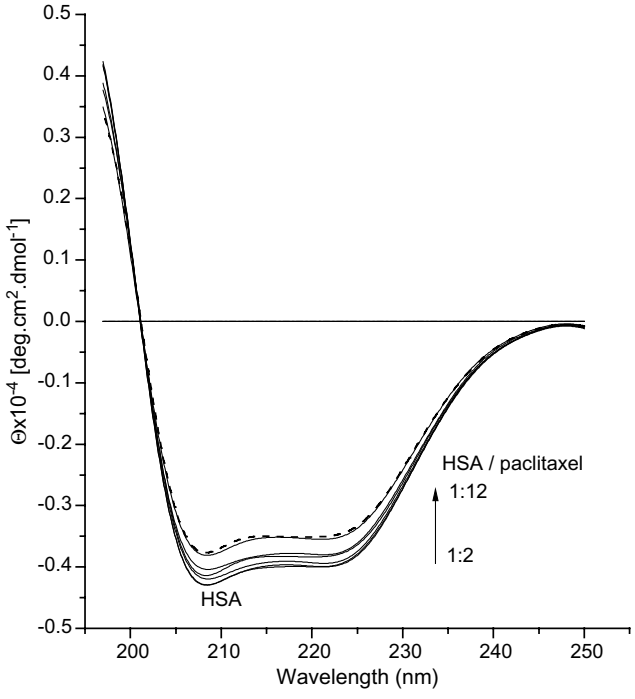

Figure 1. The effect of paclitaxel concentration on CD spectra of HSA. Concentration of HSA, 8×10^{-6} M, paclitaxel–HSA was incubated 24 h at 37 °C at molar ratio from 2:1 to 12:1.

clearly indicates the considerable changes in the protein secondary structure, namely the decrease in the α-helical content in protein. The magnitude of CD change can suggest that at molar ratio paclitaxel/HSA of 10/1 a primary binding site of paclitaxel is occupied.

Fluorescence quenching of the single tryptophan residue in HSA was also used to measure drug-binding affinity (Fig. 2). Tryptophan fluorescence is the most frequently examined among the three intrinsic aromatic fluorophores in HSA molecules to obtain information about conformational changes. When HSA is excited at 298 nm a fluorescence intensity around 350 nm reflects changes of the microenvironment of tryptophan residue.[22] Figure 2 shows typical changes of fluorescence intensity of the reaction mixture in which paclitaxel and HSA were incubated for 24 h at 37 °C at molar ratio paclitaxel/HSA, 10. No change of the fluorescence intensity was observed for the control HSA solution over an incubation period. The higher excess of paclitaxel led to more effective quenching of tryptophan fluorescence. According to empirical rules for fluorescent spectra of proteins,[22] the tryptophan residue in paclitaxel–HSA system are considered to be brought to a more hydrophilic environment as a result of the paclitaxol bindings near the tryptophan residue or its proximity. The quenching of the Trp 214 fluorescence, clearly indicate that the conformation of the hydrophobic binding pocket in subdomain IIA is affected.

On the basis of the spectroscopic observations (Figs. 1 and 2) appear that paclitaxel–protein interaction with paclitaxel/HSA molar ratio ~10 leads to the binding of the drug and that binding site may be located in subdomain IIA.

2.2. Binding of warfarin to HSA-paclitaxel

To provide further information about paclitaxel binding site, the binding of warfarin as a site I marker was investigated. The warfarin site is the best characterized drug binding sites, designated as site I,[23] located in subdomains IIA near Trp 214.[6] Warfarin has a weak fluorescence at 378 nm when excited at 335 nm, and the addition of HSA induced an increase in fluorescence intensity when warfarin binds to a single site in the protein.[24] Figure 3 shows the relative fluorescence changes of warfarin with paclitaxel-treated HSA at molar ratio paclitaxel/HSA, 10. The fluorescence decrease (about 15%) of warfarin bound to protein, indicates the reduction in the warfarin binding capacity at the primary binding site of HSA. It is very likely that paclitaxel binding occurs at the Trp 214 proximity, located in subdomain IIA of the albumin structure. Crystallographic studies have shown that many of the ligands are bound primarily within two of the six subdomains, namely domains IIA and IIIA.[5] Besides of warfarin, many of other drugs (Aspirin®, 5-iodo-salicylic acid, triiodobenzoic acid) were found to bind preferentially in IIA domain.

2.3. Influence of paclitaxel on the heme and bilirubin binding to HSA

The heme–albumin and bilirubin–albumin complexes appear as an intermediate in the plasma heme and bilirubin degradation processes.

Albumin and hemopexin serve as traps for extracellular heme, to prevent its toxic effects and channel it to its

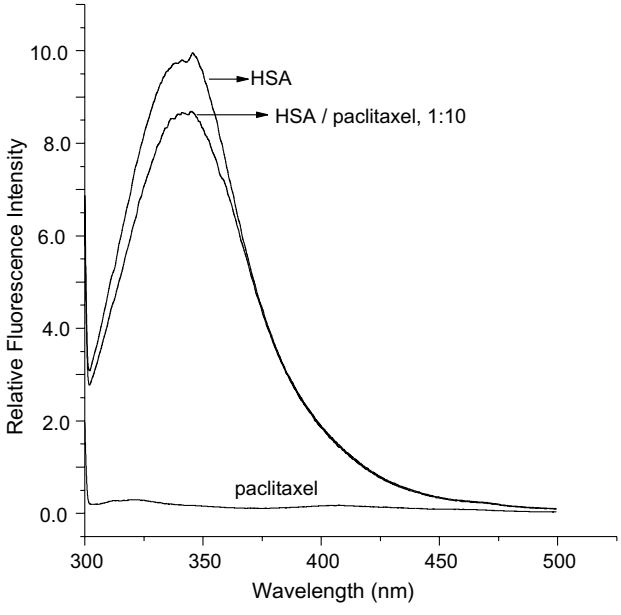

Figure 2. Tryptophan fluorescence spectral changes of HSA incubated with paclitaxel. The molar ratio paclitaxel/HSA,10:1. The excitation and emission wavelengths were 295 and 342 nm, respectively. Concentration of HSA, 8×10^{-6} M, paclitaxel–HSA was incubated 24 h at 37 °C.

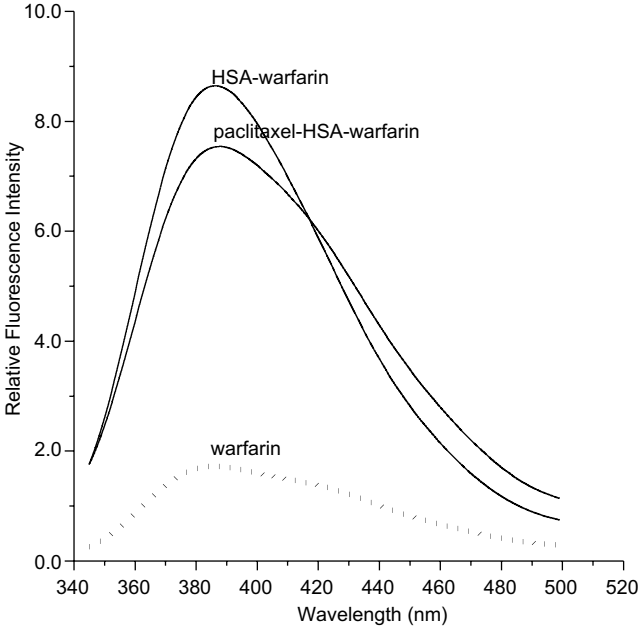

Figure 3. Relative fluorescence changes of warfarin with paclitaxel-treated HSA. Excitation at 335 nm and emission at 378 nm. Warfarin was incubated for 1 h at 37 °C with HSA and paclitaxel–HSA at molar ratio warfarin/protein 1:1. Concentration of HSA: 4×10^{-6} M, paclitaxel–HSA was incubated 24 h at 37 °C at molar ratio 10:1.

specific catabolism site on parenchymal liver cells. The hemin binding site has been located primarily at the interface between domains I and II of HSA.[8] The CD method is based on the observation that an optical activity arises from dissymmetry in the ligand induced by its binding to the protein, since the free ligand has either no asymmetric centre and therefore gives no signal in solution.

Based on the CD spectra of the heme–HSA (Fig. 4) at a low molar ligand/HSA (a primary binding site for hemin is occupied) have shown that paclitaxel binding to HSA induces only small conformational change in the hemin binding site and does not influence on the heme binding.

The high-affinity site for bilirubin has been isolated in domain II at or near loop 4 in subdomains IIA.[10] CD was also used to monitor bilirubin–albumin interaction. The results shown in Figure 5A clearly indicate that paclitaxel bound to HSA distinctly modifies the strong binding sites of bilirubin. The magnitude of Cotton effect of the bilirubin with paclitaxel-modified HSA decreased significantly (about 50%) when compared to native protein. These changes in the CD spectrum of bound bilirubin have been attributed to changes in the conformation of bound bilirubin resulting from structural changes induced in protein by paclitaxel.

The lack of the liphophilic side chain does not prevent HSA binding, since baccatin III, corresponding to the terpenoid core of paclitaxel, was even more powerful as a HSA ligand (Fig. 5B).

The binding of bilirubin to HSA and its paclitaxel modified form was also studied with a fluorescence enhancement technique.[25] Unbound bilirubin did not fluoresce but when complexed with albumin its fluorescence was enhanced. Bilirubin–albumin solution gave an emission spectrum in the wavelength range 500–600 nm with emission maxima at 540 nm when excited at 487 nm (Fig. 6) The decrease of the fluorescence intensity of the bilirubin bound with HSA-paclitaxel (about 35%) when compared to native protein confirms that paclitaxel

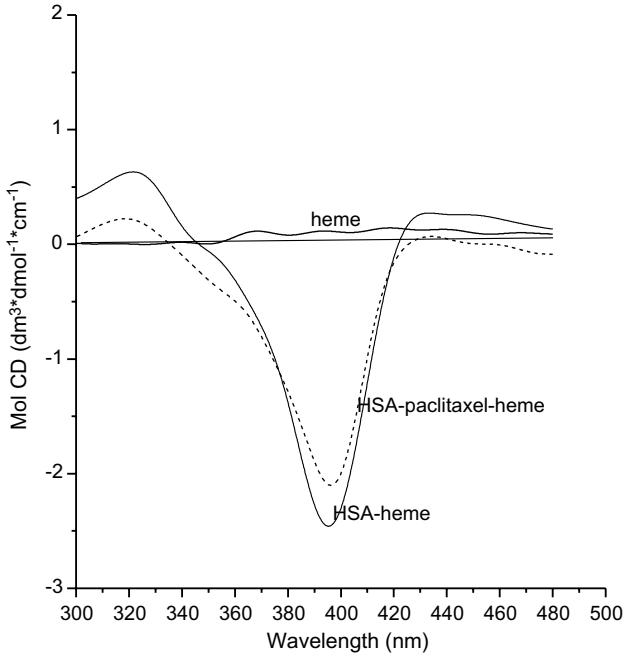

Figure 4. Effect of paclitaxel on the visible CD spectrum of HSA–heme complex. Molar ratio of heme/protein, 2:1. Concentration of HSA, 8×10^{-6} M, paclitaxel–HSA was incubated 24 h at 37 °C at molar ratio 10:1. Mol CD units are $dm^3\,mol^{-1}\,cm^{-1}$.

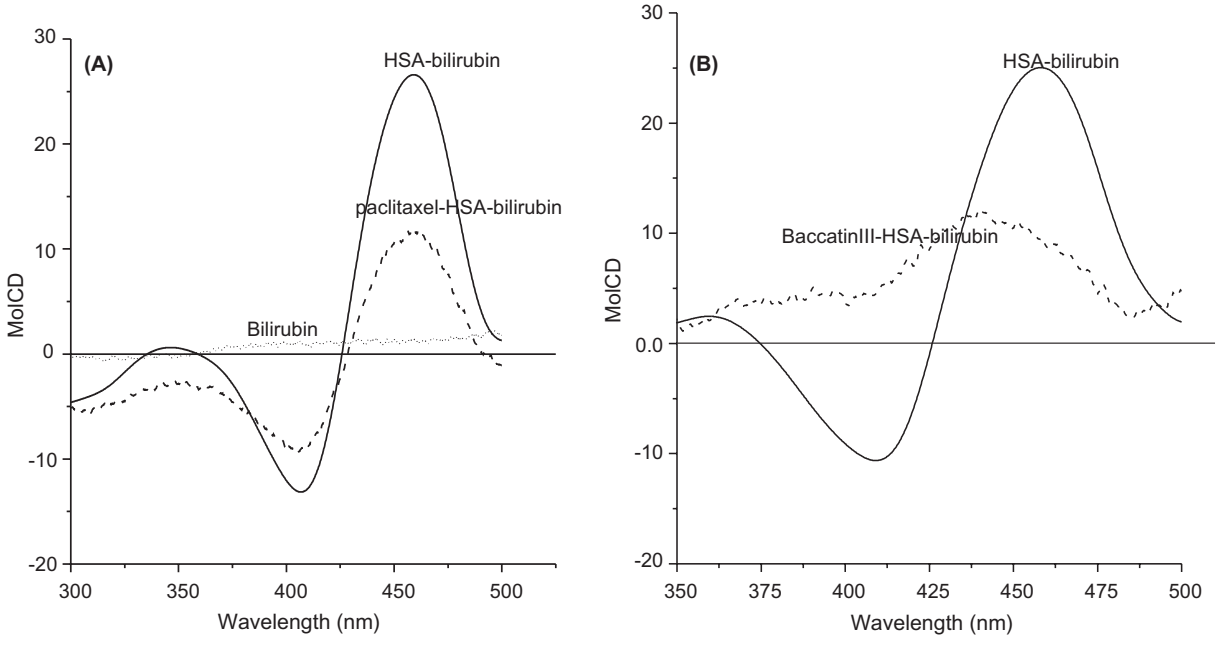

Figure 5. Effect of paclitaxel (A) and baccatin III (B) on the visible CD spectrum of HSA–bilirubin complex. Molar ratio of bilirubin/protein, 2:1. Concentration of HSA, 8×10^{-6} M, paclitaxel–HSA and baccatin III–HSA were incubated 24 h at 37 °C at molar ratio 10:1. Mol CD units are $dm^3\,mol^{-1}\,cm^{-1}$.

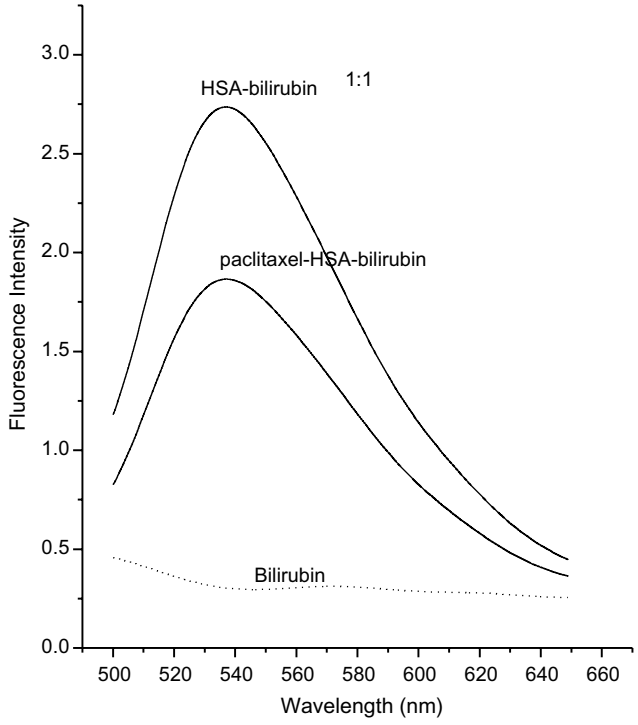

Figure 6. Change in the fluorescence intensity of bilirubin bound to HSA-paclitaxel. Molar ratio of bilirubin/protein, 2:1. Concentration of HSA: 8×10^{-6} M, paclitaxel was incubated with HSA 24 h at 37 °C, molar ratio paclitaxel/HSA, 10:1. The spectra were recorded in the wavelength range 500–640 nm after exciting the complex at 468 nm.

bound to albumin distinctly modifies the strong binding site of bilirubin.

Based on the location of the bilirubin binding site, the changes in the environment around Trp 214 can be precisely detected by using a fluorescence technique.

The fluorescence quench titration results obtained for HSA and its paclitaxel modified derivatives are shown in Figure 7. The magnitude of fluorescence quenching (relative to native albumin taken as 100%) is plotted against increasing bilirubin/albumin molar ratio (Fig. 7c). The decrease in fluorescence intensity at 342 nm observed with HSA native (about 30%) and paclitaxel–HSA (about 15%) at a bilirubin/albumin molar ratio 1:1 suggest that changes in the microenvironment around tryptophan in IIA domain are present. These results clearly indicated that paclitaxel induces structural changes in the three-dimensional conformation of albumin and local perturbations at the bilirubin binding site of HSA.

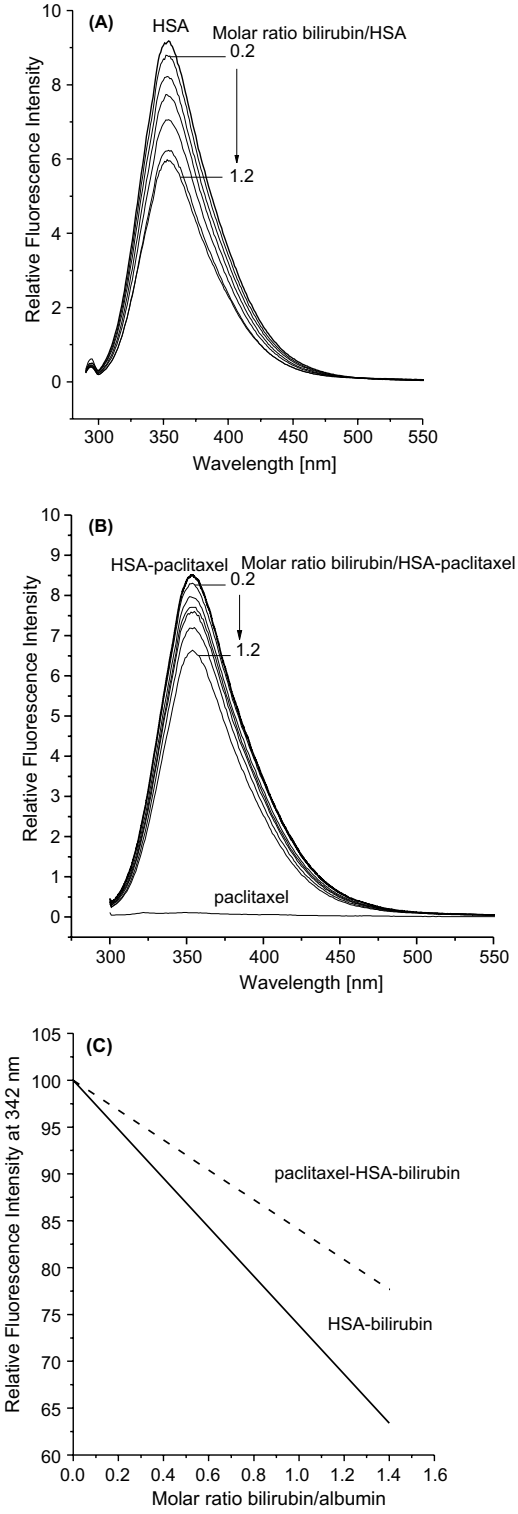

Figure 7. Fluorescence quench titration results of HSA (A) and HSA-paclitaxel (B) at increasing bilirubin to protein molar ratios obtained in phosphate buffer pH 7.4. The molar ratio was varied from 0.2 to 1.2 in each case and the excitation and emission wavelengths were 295 and 342 nm, respectively. The data were plotted as percent fluorescence versus bilirubin to protein molar ratio (C). Concentration of HSA: 8×10^{-6} M, paclitaxel was incubated with HSA 24 h at 37 °C at molar ratio paclitaxel/HSA, 10:1.

2.4. Effect of paclitaxel on antigenicity of HSA

It is now well established that the immunochemical reactivity of native protein antigens is highly influenced not only by the modification of the antigenic sites. The immunochemical reactivity is highly influence by changes in their conformation[26] and the immune response to native protein is directed against its unaltered three-dimensional structure.

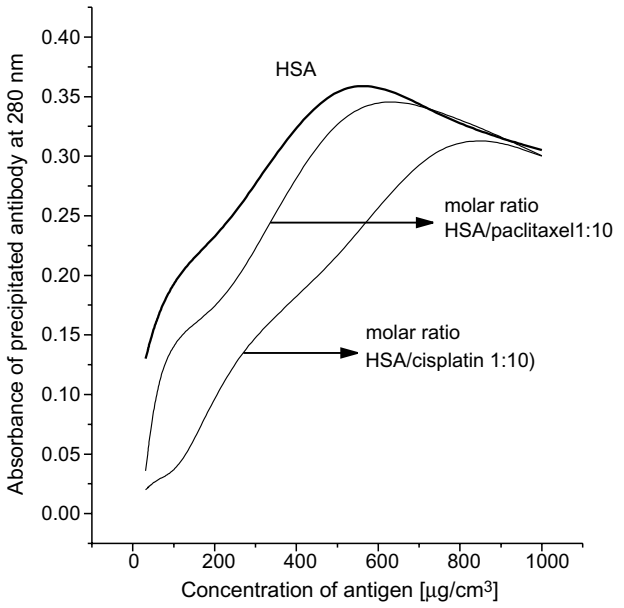

Figure 8. Quantitative precipitation of HSA after paclitaxel and cisplatin modification, with anti-HSA serum. Molar ratio paclitaxel/HSA and cisplatin/HSA, 10:1.

Quantitative precipitating HSA antibody test shows a reduction in the amount of precipitated antibody when HSA-paclitaxel was reacted with anti-HSA (Fig. 8).

The decrease in antigenic properties can be connected to the unfolding of the antigen structure, which brings about perturbation of the complementarity of the antigen–antibody reactive sites. Recent chemical and immunochemical studies[27] have reported five major antigenic sites of bovine and human serum albumin. Antigenic determinants usually include charged and/or aromatic residues such as Asp, Glu, Lys, Arg, Tyr and Trp.

The conformational changes caused by drug binding in subdomain II (in which paclitaxel is likely to be bound) may lead to increasing of asymmetry of the molecule and lowering of antigenicity. The reduction of the ability to precipitate with anti-HSA antibodies is not very significant, similar results were observed when acetylated or deaminated albumin was reacted with anti-HSA.[28] The comparative study of the paclitaxel and cisplatin influence on the antigenic properties of HSA has indicated that coordination of cisplatin to albumin, which strongly modified the protein structure by aggregation of molecule into dimmer and large decrease secondary structure,[29] greatly change antigenic determinants of HSA.

3. Conclusions

Human serum albumin is the main constituent of plasma proteins responsible for the binding and transport of many molecules including drugs. Drug binding to HSA is a major problem in pharmaceutical research because the binding to albumin influences the effective drug concentration that can be obtained at the target site. The information about binding of paclitaxel to albumin and the location of high affinity binding site of this drug on the protein molecule is of special importance as the multi-drug therapy (combination of taxol, cisplatin or carboplatin and anthracyclines) has become the preferred chemotherapy regimen. All this drugs binds extensively to albumin and might compete for the same binding sites.

Crystallographic analysis of HSA revelated that the protein contains three homologous α-helical domains (I, II and III), each of which contains two subdomains (A and B). The ligand binding versatility of HSA has intrigued researchers for a long time, and various attempts have been made to localize the high affinity binding sites in these domains and subdomains.

The principal regions of ligand binding sites of albumin are located in hydrophobic cavities in subdomains IIA and IIIA, which exhibit similar chemistry. The results presented above indicate that paclitaxel bind to HSA considerably changing protein conformation, regarding both the secondary structure and the local conformation. The binding of paclitaxel with albumin presumably occurs through the core eight-membered taxane ring. The relative fluorescence intensity of the paclitaxel-bound HSA decreased, suggesting that perturbation around the Trp 214 residue took place. This was confirmed by the destabilization of the warfarin binding site located in subdomain IIA. CD and fluorescence spectroscopic results showed marked reductions (about 50% decrease in the CD Cotton effect intensity, and ~35% decrease of the fluorescence intensity) in the affinity of albumin for bilirubin upon paclitaxel binding. These results suggested that high affinity binding site of paclitaxel molecule is in the vicinity of Trp 214, which forms part of the wall in one of the two main drug-binding cavities of HSA (site I, subdomain IIA).

4. Experimental

Human serum albumin (HSA) (essentially fatty acids free) was obtained from Fluka Chem. Co., rabbit antiserum to human albumin used in the quantitative precipitation test was obtained from IGN Biomedicals, bilirubin and warfarin were purchased from Sigma. Paclitaxel and baccatin III were obtained from Sigma. The stock solutions of paclitaxel and baccatin III were prepared by dissolving in 10% methanol solution in double distilled water and were used in all experiments from freshly prepared 0.2 mM solutions. In the final step a stock solution was diluted with the buffer and added dropwise to the protein solution.

HSA concentration was determined by absorption spectrum, taking the absorbance of a 1 mg/cm^3 solution at 280 nm as 0.55.[30] Hemin chloride was used as obtain from Serva. Its concentration was evaluated spectrophotometrically in 0.01 M NaOH, using absorption co-efficient of 58.4 mM^{-1} cm^{-1} at 385 nm.[31] All procedures

involving bilirubin were carried out under minimal light. The appropriate amount of the substance was dissolved in 0.01 M NaOH, rapidly diluted 10-fold and used in this form within 1 h. Concentrations were determined spectrophotometrically using an absorption co-efficient of 52 mM^{-1} cm^{-1} at 437 nm.[31] In all of the experiments, a sodium phosphate buffer (0.05 M, pH 7.4) containing 0.1 M NaCl was used.

CD spectra were recorded on a JASCO J-700 spectropolarimeter over the range of 190–250 and 300–600 nm, using 0.1 and 1.0 cm cuvettes, respectively. Fluorescence measurements were carried out on an SLM AMINco SPF-500 spectrofluorimeter with the excitation and emission wavelength set at 298 and 350 nm (HSA), 335 and 378 nm (warfarin), 487 and 530 nm (bilirubin), respectively.

Quantitative precipitation test was performed in following way: the increasing amounts of antigen (125–500 μg of HSA and HSA-modified with paclitaxel) dissolved in 0.05 M borate buffer at pH 8 were added to 0.5 mL of undiluted antiserum. Control tubes contained borate buffer instead of antigen solution. The contents of tubes were mixed and incubated at 37 °C for 1 h and then at 4 °C for 24 h. The precipitates were washed three times with cold borate buffer at 4 °C, and then redissolved in 3 mL of 0.1 M NaOH, and the absorbance was measured at 280 nm in a 1 cm cell. Excess of antigen or antibody present in the supernatants obtained after separation of the antibody antigen precipitate was detected by adding antigen to one-half of the supernatant and antiserum to the other half. The contents of the tubes were mixed, incubated at 37 °C for 1 h, stored overnight and centrifuged. The amount of a precipitate was measured as described above.

Acknowledgements

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