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Some Aspect of the Interactions of Adriamycin with Human Serum Albumin

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Abstract—The interaction of adriamycin with human serum albumin (HSA) has been studied by absorption, CD, fluorescence spectroscopy, and quantitative precipitating HSA–antibody test. Our results demonstrate that adriamycin react with HSA and the binding to the protein molecule has a very distinct influence on the stability of ADR in aqueous solutions. The drug molecule binds protein as a monomer. The structural studies have shown the conformational change of HSA modified by adriamycin. The binding of ADR lowers the helicity of the native protein of ca. 15% and ca. 10% in the case of acHSA. The quantitative precipitating test supports distinct changes in the conformation upon ADR binding that decreases the ability of HSA to precipitate with its antibody. Copyright © 1996 Elsevier Science Ltd

Introduction

Anthracycline-based drugs are widely used in the treatment of various human tumour diseases. One of the most studied antibiotic in this family is the adriamycin molecule whose anticancer activity is linked with the formation of intercalative complexes with DNA[1] and inhibition of both RNA and DNA synthesis.[2,3] In the field of cancer chemotherapy, macromolecular drug carrier systems have been developed to enhance the selectivity and efficiency of the cytotoxic agent.[4] The importance of the protein binding of drugs, as well as its relation to toxicity, therapeutical activity, and pharmacokinetics is not well understood.

It is generally accepted that only free drug molecules can pass through the cell membranes to be effective against the tumour.[5,6] Thus, the anticancer activity may be strongly affected by drug–protein interactions in the blood stream.[7] This type of interaction can also influence the drug stability and toxicity during the chemotherapeutic process. The major blood protein that is able to interact with variety of xenobiotics, including anticancer drugs, is serum albumin (HSA). HSA may have a considerable impact on the activity of anticancer drugs,[8] including the most popular anthracycline drug adriamycin (ADR). The doses LD_{50} and LD_{90} evaluated for the ADR–HSA complex were distinctly higher than those for protein free-ADR.[8] This partial inactivation of drug by HSA may indicate the formation of a new compound with a similar spectrum, but reduced effect in bioassay.

The available data describing the ADR–HSA interactions are rather limited, although the major amount of ADR in the blood stream is most likely bound to HSA.[9–11] α_2, β and γ-globulins are also involved as the binding proteins for anthracyclines although to a lesser extent.[9,12] HSA is a well-known transport protein for a variety of molecules and ions, like fatty acids, hormones, metal ions of therapeutic agents,[13] due to its very unique single-polypeptide globular multi-domain structure.[14]

In this work the data obtained for the HSA–ADR system to evaluate the interactions by absorption, circular dichroism (CD), and fluorescence spectra as well as by immunological techniques are presented.

Results and Discusion

About 60% of anthracycline glycosides usually bind to serum albumins[10] and this is 62% of ADR that form a complex with HSA in human plasma. The interaction of ADR with HSA causes distinct variations of the protein conformation as well as some structural changes in the drug molecule.

Absorption spectra

The dihydroxyanthraquinone chromophore of ADR gives a very specific pattern of the UV–vis spectrum, centred at 480 nm.[15] The shape and the position of the multi-transition band of ADR depends on the phenolic groups of ADR. Thus, any change of absorption spectra could suggest the deprotonation of phenolic groups or their involvement in the interactions with other molecules.[16] The addition of HSA to ADR results in the distinct shift of ADR spectrum towards longer wavelength (from 480 to 505 nm). This clearly indicate the interaction between ADR and HSA molecules. The shift of the lowest energy band to 536 nm [Fig. 1(A)] indicates very strong involvement of phenolic hydrogen in the interaction with protein site. Such a strong shift in transition energy is observed during the deprotonation of the phenolic group.[16] Similar changes in the ADR spectrum were observed when aspirin-acetylated HSA (acHSA) was added to ADR solutions (Fig. 2).

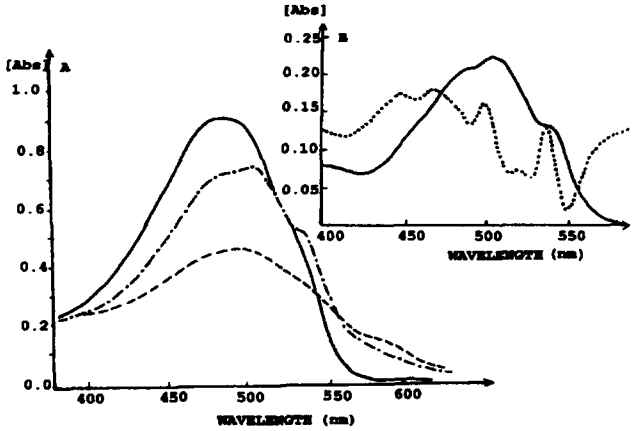

Figure 1. Visible absorption spectra of free and bound adriamycin in phosphate buffer pH 7.4. (A) Adriamycin immediately after dilution (—), adriamycin after 24 h at 37 °C (— —), ADR–HSA after 24 h of the reaction running at 37 °C (— · —). Immediately after mixing ADR and HSA a very small change is observed in visible spectrum (data not shown). Concentration of adriamycin = concentration of HSA = 9×10^{-5} M. (B) Difference spectrum of adriamycin–HSA after 24 h incubation at 37 °C recorded against a solution of free adriamycin (—), first derivative of these spectrum (···). Conditions as described in (A). First derivative of spectrum in (B) used to obtain λ_{max}.

The binding of ADR to the HSA molecule has a very distinct influence on the stability of ADR in the solutions studied. ADR hydrochloride is a very stable species, as long as it is in the solid state form, while after dissolution it can be, depending on pH concentration, buffer, or contact with oxygen, relatively easily degraded. The free ADR is stable in aqueous solution with acidic pH (3–4),[17] while at pH 7.4 in a phosphate buffer for a drug concentration around 0.05 mg/cm^3 its stability at room temperature is well below 24 h.[18] To evaluate ADR stability in the presence of HSA the HSA–ADR system was studied at 4, 24, and 37 °C using absorption spectra (Fig. 3). HSA and ADR were dissolved in 0.05 mol/dm^{-3} phosphate buffer, pH 7.4, with 0.15 mol/dm^{-3} NaCl and the drug to protein molar ratio 1:1. In the case of free ADR, the incubation of the drug at 37, 24, and 4 °C resulted in considerable degradation of 40, 30, and 15% of total ADR, respectively. In the presence of HSA this degradation was much lower and it was 13, 16, and 10% for 37, 24, and 4 °C, respectively. This shows the highest stabilization effect for 37 °C and it decreases with temperature. This behaviour becomes clear when we consider the kinetics of the HSA–ADR interactions. The equilibrium in this interactions are reached at 37 °C after 10 h, while at 24 and 4 °C 24–30 h are needed. After 48 h the amount of intact bound ADR becomes constant in all studied temperatures, while unbound drugs undergoes further degradation.

The redox properties of ADR in the free and bound state were followed by reduction of free ADR and HSA–ADR systems in anaerobic conditions with dithionite, one-electron reducing agent for the drug. The reduction of ADR leads to vanishing of the bands at 479–490 nm and increases absorption at 400–420 nm (Fig. 4). Dithionite generates sequentially semiquinone and hydroxyquinone that in the presence of oxygen may be reoxidized to the parent molecule (Fig. 4) when ADR is bound to HSA. This result suggests the involvement of anthracycline π-orbitals in the interactions with HSA.

Formation of the HSA–ADR complex protects the drug molecule against decomposition reactions in

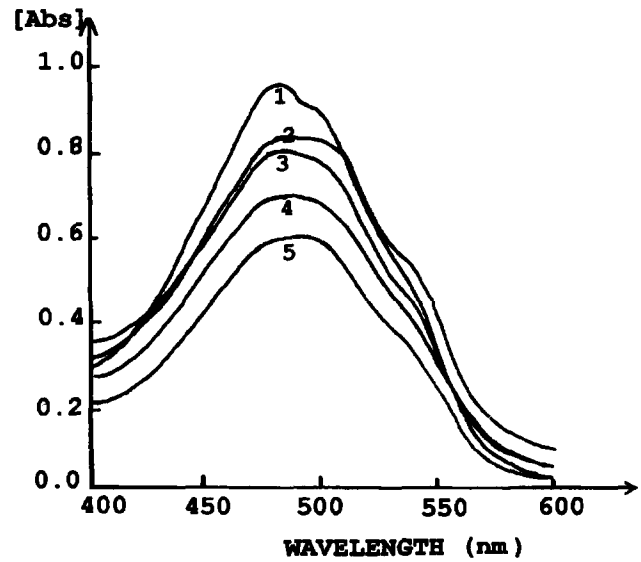

Figure 2. Absorption spectra of free adriamycin and adriamycin binding to acHSA. Adriamycin immediately after dilution. (1) Adriamycin–acHSA after 24 h of the reaction running at 37 °C, (2) adriamycin–acHSA after 24 h of the reaction running at 4 °C, (3) adriamycin after 24 h at 4 °C, (4) adriamycin after 24 h at 37 °C (5). Conditions as described in Figure 1(A).

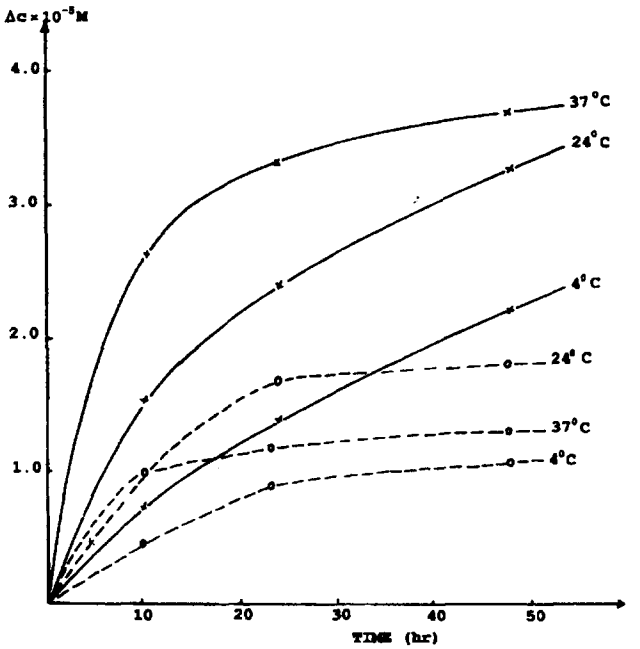

Figure 3. Stability of adriamycin in 0.05 M phosphate buffer pH 7.4. Adriamycin (—x—) and adriamycin–HSA (—o—) at temperatures of 37, 24, and 4 °C. Concentration of adriamycin = 9×10^{-5} M. Molar ratio of the reagents 1:1.

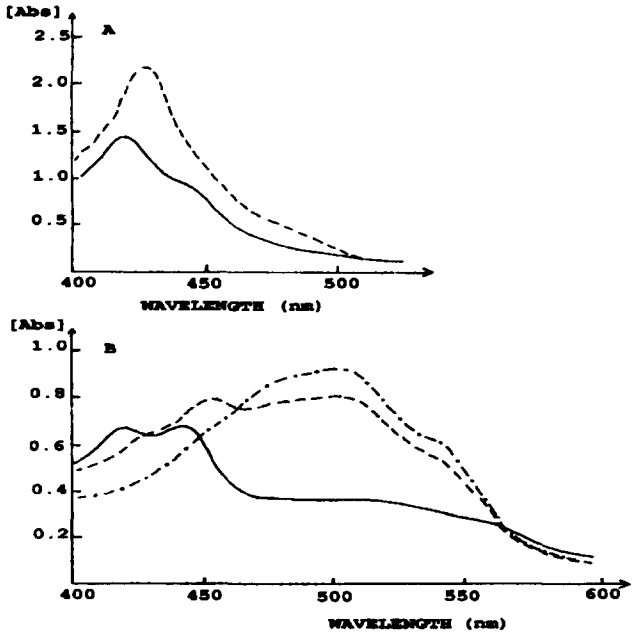

Figure 4. Absorption spectra of free adriamycin and adriamycin–HSA in phosphate buffer pH 7.4 with dithionite. (A) Adriamycin + $Na_2S_2O_4$ (—), adriamycin–HSA + $Na_2S_2O_4$ (— —). (B) Adriamycin with dithionite after 15 min bubbling with oxygen (—), ADR–HSA (1 h incubation at room temperature) with dithionite after 15 min bubbling with oxygen (— —), ADR–HSA (24 h incubation at 37 °C) with dithinite, after 15 min bubbling with oxygen (—·—). Concentration of adriamycin = 9×10^{-5} M. Molar ratio of ADR:HSA, 1:1.

aqueous solutions. It may be the effect of a more stabile reaction product of ADR and protein.

Fluorescence quenching

The interaction between ADR and HSA can be quantified by measurements of fluorescence quenching (Fig. 5). ADR exhibits a typical fluorescence spectrum with emission at 580 and 550 nm, when excited at 480 nm. In the presence of HSA this strong ADR fluorescence tends to vanish. The acetylated HSA is even more effective in quenching of ADR fluorescence than the HSA itself.

CD spectra

CD spectra of the anthracycline ring system, induced by optically active sugar moiety, are very characteristic for ADR, the protonation states of aromatic ring functions (phenolic groups) or the interactions involving drug molecules (Fig. 6).[19,20,16] Protein-free ADR at pH 7.4 exhibits three distinct bands at 468, 360, and 290 nm, characteristic for the monomeric form of ADR. The binding of ADR to HSA does not affect distinctly the band at 468 nm, suggesting that the drug molecule binds protein as a monomer.

Changes induced on the HSA structure by ADR

The conformational properties of the adriamycin-modified HSA in phosphate buffer pH 7.4 were studied by CD. The HSA–ADR and acHSA–ADR systems were compared with the native and acetylated HSA at

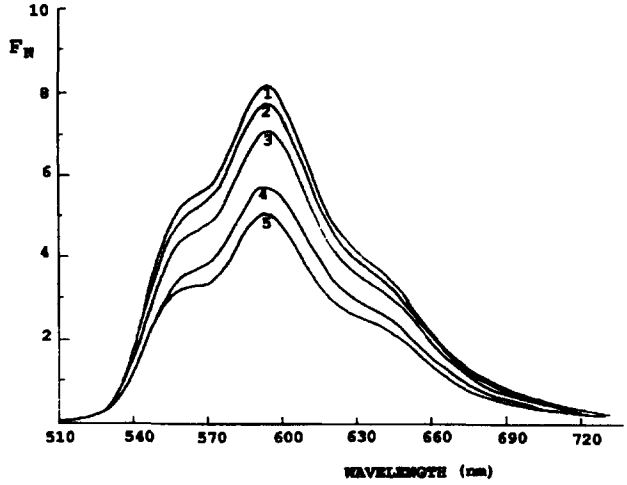

Figure 5. Fluorescence spectral changes of adriamycin incubated with HSA and acHSA. Adriamycin in phosphate buffer pH 7.4 after 24 h at 4 °C (1) and 37 °C (3) ADR–HSA after 24 h of the reaction running at 4 °C (2) and 37 °C (4), ADR–acHSA after 24 h of the reaction running at 37 °C (5). Concentration of adriamycin = 9×10^{-5} M. Molar ratio of adriamycin:HSA, 1:1.

the same conditions. The binding of ADR to HSA has a distinct effect on the HSA structure, among other things it decreases α-helical content in the protein (Fig. 7), up to 15% for HSA and up to 10% for acHSA. It should be mentioned, however, that in the case of acHSA the ellipticity is much lower than that in HSA.[21] Thus, the effect of ADR binding on the acHSA structure is of the same order as that found in the ADR–HSA system.

The conformational changes in the HSA structure induced by ADR binding affect the protein ability to react with its antibody. The quantitative precipitating test (Fig. 8) support the distinct changes in the protein conformation upon ADR binding that decreases the

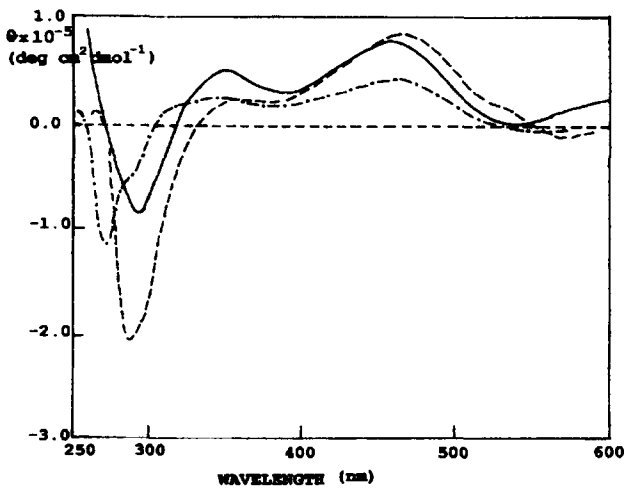

Figure 6. CD spectra in the 600–250 nm region of adriamycin (—), adr–HSA (— —), ADR–acHSA (—·—) at the molar ratio 1:1 in phosphate buffer pH 7.4, after 24 h reaction running at 4 °C. Concentration of adriamycin = 9×10^{-5} M.

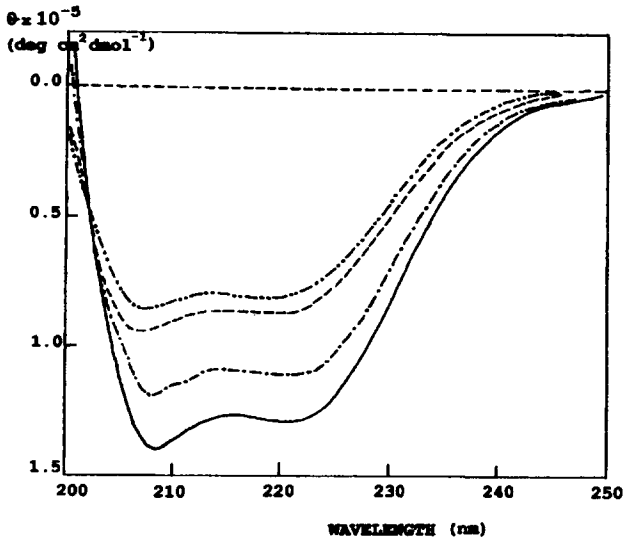

Figure 7. UV CD spectra in the 250–200 nm region of native HSA (—), acHSA (— —), ADR-HSA (—·—·), and ADR-acHSA (—··—··) at the molar ratio 1:1, after 24 h of reaction running at 4 °C.

ability of HSA to precipitate with its antibody. The decrease in antigenic properties can be connected to the unfolding of the antigen structure, which brings about perturbation of complementarity of antigen–antibody reactive sites.

The results presented above clearly indicate that adriamycin binds effectively to HSA, considerably changing its structure and antigenic properties. The binding of adriamycin to HSA has a very distinct influence on stability and redox properties of adriamycin in aqueous solutions.

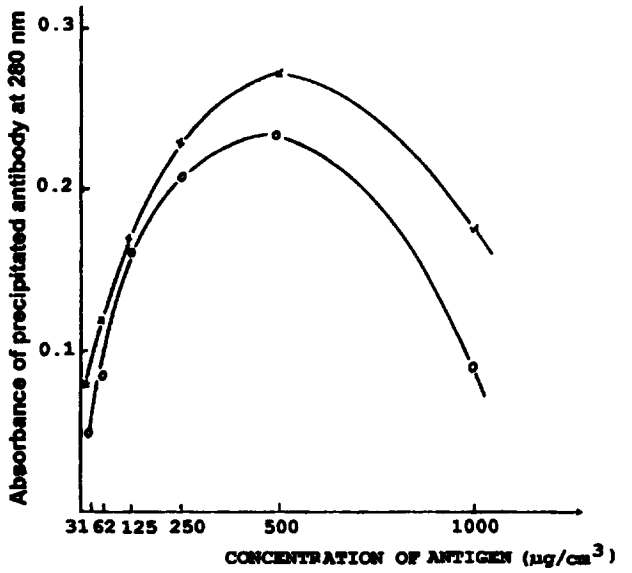

Figure 8. Quantitative precipitation of HSA and adriamycin modified HSA with anti-HSA serum. HSA (—x—), ADR-HSA (—o—).

Materials and Methods

Materials

HSA and antiserum from rabbit were obtained from Polish Chemicals (POCH). Protein was purified as described earlier.[22] HSA concentration was determined by absorption at 280 nm.[23] Acetylated HSA was prepared according to the procedure of Pincard et al.[24] ADR hydrochloric salt was obtained from FarmItalia Carlo Erba. The concentration of drug was determined at 480 nm using $\varepsilon = 11\,500$ mol^{-1}/cm^1/dm^3.[25] As ADR is sensitive to light and oxygen, a stock solution was prepared just before the use. All other reagents were of analytical grade and deionized bidistilled water was used through the experiments.

Methods

Absorption spectra were recorded on a Beckman DU 650 spectrometer and CD spectra on a Jasco JK-600 spectropolarimeter. Fluorescence measurements were carried out on a SLM AMINCO SPF-500 spectrofluorimeter with the excitation and emission wavelength set at 475 and 560 nm, respectively. A quantitative precipitation test was performed in the following way: the increasing amounts of antigen (125–500 μg) dissolved in 0.05 M borate buffer at pH 8 were added to 0.5 cm^3 of undiluted antiserum. Control tubes contained borate buffer instead of antigen solution. The contents of the 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, then redissolved in 3 cm^3 of 0.1 M NaOH, and the absorbance was measured at 280 nm in a 1 cm cell. Excess 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.

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