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Short communication

Alterations of furosemide binding to serum albumin induced by increased level of fatty acid

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ABSTRACT

Localization of high and low affinity binding sites of furosemide in human serum albumin (HSA) as well as the influence of myristic acid on the drug binding to the albumin using fluorescence quenching method was investigated. Two independent classes of binding site in subdomain IIA of HSA structure were found. Alteration of protein affinity towards the drug and the participation of tryptophanyl and tyrosil residues in drug–albumin interaction for the determined binding sites were studied. It was concluded that association of myristic acid in its low affinity binding sites which corresponds to elevated fatty acid level *in vivo*, significantly decreases albumin affinity towards furosemide.

1. Introduction

Serum albumin is a transporting protein which has the ability to bind various exo- and endogenous ligands. Binding of drugs with the albumin affects their pharmacological effect since only free fraction of drug exhibits therapeutic activity. Determination of the impact of various factors on drug–protein interaction is especially important when drug binds with albumin to a significant degree. The presence of fatty acids is one of the factors which can modify the affinity of protein towards drugs. In physiological conditions the molecule of human serum albumin (HSA) binds up to two molecules of fatty acid. However, in some diseases, i.e. infections, stress, diabetes, the level of fatty acid may increase 10-fold [1] and the number of bound fatty acids molecules can rise. Crystallographic studies showed there are eight high and low affinity binding sites for fatty acid in HSA structure [2]. HSA is a single chain protein with 585 amino acids. It is composed of three domains (I–III). Each of them comprises two subdomains A and B. The polypeptide chain is α-helix in about 67% [3–9].

In the current studies the binding of furosemide to human serum albumin in its high and low affinity binding sites was estimated. Furosemide (FUR) is a commonly used loop diuretic which bound fraction of drug in 99% at its total concentration of 10 μg/ml [1]. The influence of myristic acid (C14:0) at various concentrations on FUR–albumin complex was observed. Content of myristic acid (MYR) in plasma is significantly lower in comparison to the other fatty acids, but it was found that modification of protein via myristolation affects membrane targeting, signal transduction and apoptosis [10,11]. We have chosen MYR for the studies since its binding to albumins is well described and the current experiment is the continuation of our previous work [12,13]. The use of the fluorescence quenching method allows to determine and explain changes of albumin affinity towards the drug by direct observation of the drug's binding site(s). However, the method introduced to the studies on drug binding is only limited to the compounds which are able to accept energy from the excited fluorophores of the protein. The presence of single tryptophan of HSA (Trp 214) may give us detailed information about subdomain IIA (Sudlow's site I), however, the presence of several tyrosil residues scattered in the albumin tertiary structure (subdomains IB, IIA, IIB and IIIA) makes it impossible to point out the precise additional binding site of the drug but may suggest other sites of interaction.

The investigation of alterations of drug–protein interaction in these sites induced by various factors, e.g. competitive binding of ligands allows us to predict the risk of such competition in case of ligands with similar chemical structure. The papers already published on FUR–HSA system are based on the data obtained with the use of a technique different than the fluorescence one, i.e. ultrafiltration. These results show the general binding abilities of an albumin molecule by monitoring the free fraction of the drug. The data obtained with the use of several different techniques give us

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more specific information on the interaction in a drug–protein complex.

2. Materials and methods

Human albumin, fraction V fatty acid free (dHSA) (CAT no: 823234, LOT no: 6312A) was purchased from ICN Biomedicals Inc. Aurora, OH, USA, furosemide (FUR, CAT no: 158210, LOT no: 2508J) and myristic acid (MYR, CAT no: 100870, LOT no: 85052) from MP Biomedicals, OH, USA.

All solutions were prepared at pH 7.47, 0.1 M sodium phosphate buffer. Myristic acid was dissolved at 0.01 M NaOH and titrated with 0.1 M HCl to obtain pH 7.47. The experiment was carried out at 310 K. In the studies the quenching fluorescence method was used. Emission spectra were recorded using Kontron SFM 25 Instrument AG spectrofluorimeter and 1 cm × 1 cm × 4 cm quartz cells. To excite the protein fluorophores 280 and 295 nm wavelengths were used. The range of recorded spectra was 280(295)–400 nm. Instrumental error was ±1 nm for emission wavelength and ±0.01 for relative fluorescence (RF). Maximum deviation was 4.31%.

The concentrations of myristic acid used to investigate the influence of fatty acid on drug binding were chosen on the basis of the fluorescence alteration of the albumins by MYR described previously [12]. The subsequent concentrations of myristic acid 3×10^{-6} M, 5×10^{-6} M, 2×10^{-5} M, 8×10^{-5} M, 3×10^{-4} M and 5×10^{-4} M correspond to the following [MYR]/[albumin] molar ratios: 0.6:1, 1:1, 4:1, 16:1, 60:1 and 100:1. The concentration range of furosemide used in the studies was 2.5×10^{-7} M to 6.25×10^{-5} M, while the concentration of human serum albumin was constant and equal to 5×10^{-6} M.

Association constant K_a was calculated by using the Scatchard method modified by Hiratsuka [14]:

$$\frac{r}{[L_f]} = nK_a - K_a r \quad (1)$$

where r, number of ligands bound to one protein molecule; $[L_f]$, free (unbound) ligand concentration; n, number of binding sites for the independent class of drug binding sites in albumin molecule which corresponds to the mean number of drug molecules bound in the independent class of drug binding sites in albumin molecule; K_a, association constant.

Modification of Stern–Volmer equation modified by Lehrer [15] was used to determine the quenching constant K_Q:

$$\frac{RFo}{\Delta RF} = \frac{1}{[L]}\frac{1}{f_a}\frac{1}{K_Q} + \frac{1}{f_a} \quad (2)$$

where RFo and RF, relative fluorescence intensity of protein in the absence and presence of quencher (L), respectively; in triple systems RFo is the fluorescence intensity of protein in the presence of myristate at respective concentration; ΔRF, the difference between RFo and RF; f_a, fractional accessible protein fluorescence; K_Q, quenching constant; $[L]$, quencher concentration.

3. Results and discussion

3.1. Drug–albumin complex

On the basis of the obtained results the capability of furosemide (FUR) to accept the energy from the excited fluorophores of defatted human serum albumin (dHSA) was observed. When 295 nm excitation wavelength is used only tryptophanyl residue (Trp 214) of the protein is excited. In case of using λ_{ex} 280 nm tyrosil residues, which may also participate in ligand–protein interaction, are additionally excited. Fig. 1A presents quenching curves of dHSA in the presence of FUR obtained for λ_{ex} 295 and 280 nm. The decrease of protein fluorescence originating the excited Trp 214 points to subdomain IIA, where the amino acid is located, as a major binding site for FUR. Whereas the effect of overlapping of the curves indicates that tyrosines do not participate in the formation of FUR–dHSA complex. Binding of FUR and other loop diuretics in the mentioned hydrophobic pocket of HSA was already previously stated [16]. To determine the number of classes of FUR binding sites in the tertiary structure of dHSA Scatchard curves modified by Hiratsuka (Eq. (1)) [14] were plotted. On the basis of the obtained results it can be concluded that FUR has two classes of binding sites in dHSA (Fig. 1B; only data for λ_{ex} 295 nm are shown in the figure). The comparison of these data with the conclusions made on the basis in Fig. 1A allows for the assumption that in dHSA both binding sites are located in subdomain IIA in the close proximity to Trp 214. The association constants and the mean number of drug molecules bound to one molecule of albumin in the given class of binding site (n) determined by using Eq. (1) for the first and the second class of FUR binding sites in dHSA structure are presented in Table 1A and B.

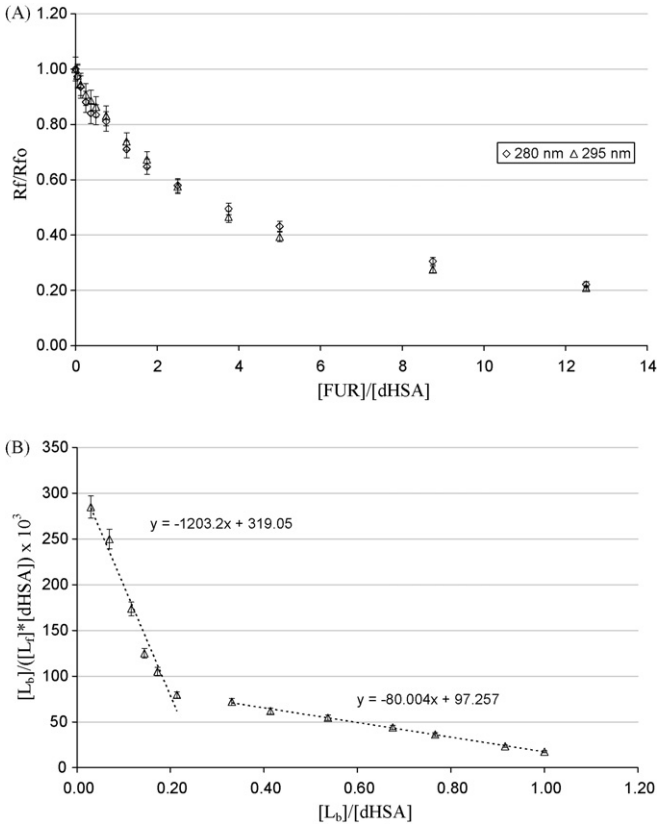

Fig. 1. Quenching curves of dHSA in the presence of FUR obtained for 280 and 295 nm excitation wavelengths (A) and Scatchard plot of FUR–dHSA complex obtained for 295 nm excitation wavelength (B).

Since the determination of binding sites for FUR in HSA is indisputable, the values of association constants and the mean number of FUR molecules bound with one molecule of albumin for that complex differ in the works of various authors [1,16–18]. These parameters calculated on the basis of the results obtained by the use of ultrafiltration method were 2.0×10^5 M^{-1} and $n = 1$ [17] and 1.9×10^5 M^{-1} and $n = 1$ [16]. The affinity constant evaluated by the use of a computer program [1] was 6.16×10^4 M^{-1}. The values of the same parameter obtained from the fluorescence measurements by using Levine's and Steiner's method were 2.72×10^6 M^{-1} and 1.66×10^5 M^{-1}, respectively [18]. All quoted authors also presented the data for the second class of binding site (3.5×10^4 M^{-1}, $n = 3.5$ [17], 3×10^4 M^{-1}, $n = 3.5$ [16], 9.18×10^2 M^{-1} [1] and 1.77×10^4 M^{-1} and $n = 5.5$ [18]) but they did not indicate its location. It is worth