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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.

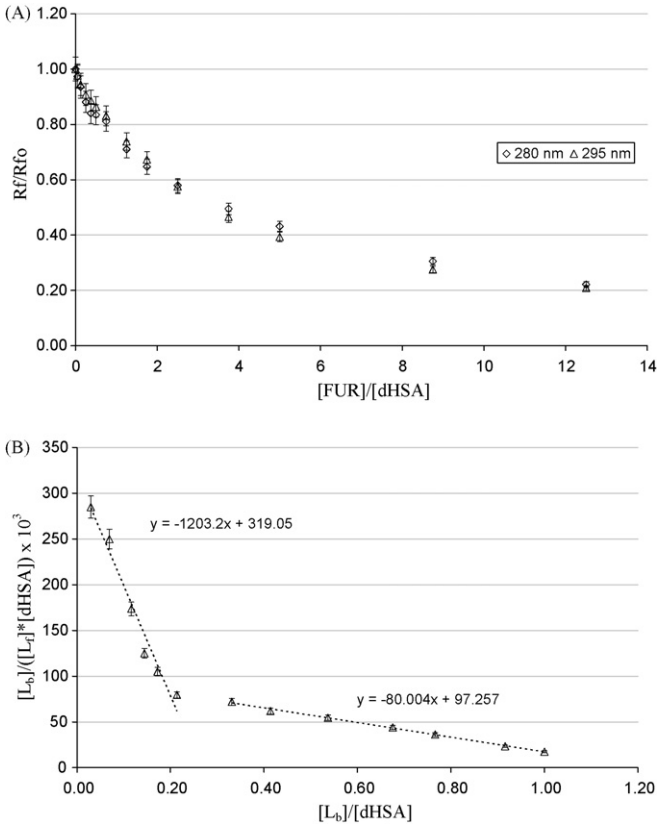

Fig. 1. Quenching curves of dHSA in the presence of FUR obtained for 280 and 295 nm excitation wavelengths (A) and Scatchard plot of FUR–dHSA complex obtained for 295 nm excitation wavelength (B).

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

Table 1
Binding constants and number of binding sites for the independent class of drug binding sites in albumin molecule determined for FUR–dHSA complex in the absence and presence of MYR, λ_{ex} 295 nm (A), 280 nm (B) and quenching constants and fractional accessible protein fluorescence for FUR–dHSA complex in the absence and presence of MYR, λ_{ex} 295 nm (C), 280 nm (D).

$\times 10^{-3}$ M^{-1}	0 M	3×10^{-6} M	5×10^{-6} M	2×10^{-5} M	8×10^{-5} M	3×10^{-4} M	5×10^{-4} M
A							
K_{aI}	1203.2	2513.4	618.1	1161.3	1265.4	572.7	745.0
K_{aII}	80.0	86.8	95.5	100.22	14.4	6.8	11.4
n_I	0.27	0.27	0.35	0.29	0.17	0.12	0.22
n_{II}	1.2	1.2	1.17	1.13	2.1	3.45	2.45
B							
K_{aI}	1612.0	706.94	1849.9	1093.9	321.9	696.9	462.3
K_{aII}	105.74	114.09	144.7	120.9	16.8	14.0	9.2
n_I	0.32	0.5	0.44	0.4	0.2	0.14	0.12
n_{II}	1.12	1.1	1.05	1.07	2.0	2.25	3.0
C							
K_{QI}	317.9	303.72	205.2	303.2	490.1	359.4	340.4
K_{QII}	52.8	44.22	56.6	67.9	18.1	11.4	9.62
f_{aI}	0.32	0.42	0.41	0.35	0.1	0.05	0.1
f_{aII}	1.05	1.23	1.06	0.94	0.71	0.75	0.96
D							
K_{QI}	247.8	131.75	134.6	214.4	214.3	452.9	439.3
K_{QII}	76.5	74.63	100.8	78.3	13.8	10.85	7.8
f_{aI}	0.46	0.76	0.94	0.53	0.15	0.09	0.06
f_{aII}	0.88	0.89	0.81	0.9	1.32	1.51	1.8

noting that the Levine's method used by Parsons [18] allows us to estimate the affinity constant only when protein contains one binding site for the ligand. The application of the method when ligand has more then one binding site, i.e. furosemide leads to the overestimation of the constant value as was observed in Parsons' results [18]. The negative relation between K_a and n (Eq. (1)) leads to the decrease of one parameter when the rise of the second parameter is observed. This explains low number of FUR molecules bound with one molecule of albumin in our studies in comparison to other data. In our studies the association constants obtained for λ_{ex} 280 nm are slightly higher than those for λ_{ex} 295 nm (Table 1A and B). This can be explained by the presence of tyrosil residues in subdomain IIA which can stabilize the interaction between FUR and dHSA. However, as can be seen in Fig. 1A the participation of tyrosines in complex formation is insignificant while Trp 214 plays a major role.

The modification of the classic Stern–Volmer equation presented by Lehrer (Eq. (2)) [15] is useful to determine quenching constant K_Q and the fractional accessibility of fluorophore for the ligand (f_a) [19,20] in the heterogeneous environment when the excited molecules can be equally quenched by an exogenous quencher (K_Q is constant for all accessible fluorophores). In practice it means that plot $RFo/\Delta RF$ vs. $1/[Q]$ is linear. The value of f_a evaluated on the basis of the plot reflects the general accessibility of all albumin fluorophores. The obtained values of K_Q and f_a are presented in Table 1C and D. This results clearly show that the participation of tyrosil residues in the interaction between HSA and FUR is insignificant, which is in agreement with the previous conclusions based on the observation of the quenching curves (Fig. 1A). Using the code of practice described in our previous paper [12] K_Q for all binding sites found for the studied drug–protein complex were determined (Table 1C and D). The negative correlation observed for K_Q and f_a indicates that with the increase of the fluorophore exposition to the aqueous solution the affinity between fluorophores and the protein decreases. A similar correlation was found between K_a and n (Table 1A and B). It is probable that when the fluorophore is more exposed, the dissolved drug molecules have an easier sterical access to the binding site where the excited fluorophore is located. That is why the higher values of f_a relate to the higher n values. But on the other hand, the increased number of bound molecules of the ligand makes the close approach to the fluorophore and the energy transfer to the quencher difficult and this leads to the decrease of K_Q and K_a values. For the studied complexes the rise of accessibility of fluorophore (with the exception of the second binding site of FUR–dHSA) at 295 nm excitation wavelength was found. This suggests that the increase of f_a comes from the tyrosil residues situated in different subdomains of the macromolecule.

3.2. FUR–dHSA–MYR

According to the previous studies [12] at the concentration 3×10^{-6} M myristic acid (MYR) does not form a complex with dHSA, but its presence at the mentioned concentration induces distinct differences of K_a values between FUR–dHSA–MYR and FUR–dHSA (Table 1A and B). K_aI obtained for λ_{ex} 295 nm increased twofold, while for λ_{ex} 280 nm decreased twofold, The mean number of drug molecules bound within close vicinity of Trp 214 (λ_{ex} 295 nm) did not change, in spite of the rise of K_aI but increased when the both types of fluorophores–Trp and Tyr were observed (λ_{ex} 280 nm). The distance between FUR and Trp 214 remained unchanged (K_QI const.), but the accessibility of fluorophore (f_aI) increased. The observed rise of K_aI can be explained by the formation of an additional interaction (e.g. electrostatic or hydrogenous) as a result of the conformation changes of the binding site. The significant drop in K_aI for λ_{ex} 280 nm with its simultaneous increase for λ_{ex} 295 nm can arise from the fact that tyrosil residues situated in various subdomains are excited by 280 nm wavelength. However they cannot be quenched since the ligand cannot approach and accept their energy. Changes in parameters characterizing lower affinity binding site are not significant and they are probably a result of the reorientation of drug molecules towards the fluorophore(s) in the first binding site. The presence of MYR at 3×10^{-6} M does change the interaction in FUR–dHSA complex, in spite of the lack of the ascertained binding of MYR to albumin at the used concentration [12]. However, intermolecular interaction in the solution may lead to alterations of the affinity between FUR and dHSA.

Addition of MYR at concentration 5×10^{-6} M results in the decrease of K_aI (λ_{ex} 295 nm) at about 50% in comparison with K_aI obtained for FUR–dHSA complex and at about 75% against previously described FUR–dHSA–MYR system (3×10^{-6} M). The association constant K_aI evaluated for λ_{ex} 280 nm shows a slight increase in comparison to the complex containing only FUR and

dHSA and a significant rise (about 2.5-fold) relating due to the previous ternary system (Table 1B). In the presence of MYR at concentration 5×10^{-6} M the decrease of K_aI calculated for λ_{ex} 295 nm accompanies the increase of nI and K_QI (Table 1A and C), while the rise of K_aI obtained for λ_{ex} 280 nm corresponds to the drop in nI, and to a slight increase of K_QI as well as to the growth of fluorophore accessibility (Table 1B and D). Such constellation of the parameters may suggest the interaction of FUR with tyrosil residues located in albumin subdomains different than IIA, i.e. IIIA. The stability of these couplings is probably too weak to be seen on the Scatchard plot as a separate class of binding sites. Similar phenomenon was described for aspirin–dBSA complex, where the interaction of drug with subdomain IB was not observed on the Scatchard plot as an additional class but as an increase of K_a value obtained for the primary binding site [12]. The parameters which describe interaction of Trp 214 (λ_{ex} 295 nm) with the drug in the second class of binding site shows no significant changes in comparison to the binary and previous ternary systems (Table 1A and C). Similarly as in the case of the primary binding site, the increased contribution of tyrosil residues on the stabilization of the complex is reflected in a slight rise of K_a and K_Q. The fractional accessibility of fluorophore as well as the number of bound ligand molecules decreases. This makes the energy transfer between the excited fluorophores and the quencher easier and allows FUR to form more stable complexes.

It was previously concluded [12] that MYR at concentration 2×10^{-5} M interact with dHSA and forms complexes in two high affinity binding sites. One of the bound acid molecules MYR4 is located in subdomain IIIA while the second one, MYR5, in subdomain IIIB [2]. Crystallographic studies did not show any significant changes of albumin conformation when MYR4 and MYR5 molecules were bound with protein. However, binding of the third MYR molecule induces the rotation of domain I and III [2]. From the comparison of the FUR–dHSA–MYR system (MYR 2×10^{-5} M) where MYR 4 and MYR 5 are bound in domain III of dHSA to the FUR–dHSA–MYR system (MYR 5×10^{-6} M) where no MYR molecule is bound with albumin it results that the value of K_aI increases twofold for λ_{ex} 295 nm and decreases when tyrosil residues were additionally excited (λ_{ex} 280 nm) (Table 1A and B). The rise of K_QI indicates that the distance between energy donor (Trp 214) and the acceptor became smaller. A similar effect was found on comparing the FUR–dHSA–MYR system (MYR 3×10^{-6} M) to the complex formed in the absence of fatty acid (FUR–dHSA). As it was previously explained for MYR 3×10^{-6} M, the increase of K_aI at λ_{ex} 295 nm and its decrease at λ_{ex} 280 nm is due to the lack of quenching of energy emitted by tyrosines. Although, part of the tyrosine residues are located in subdomains occupied by MYR 4 (Tyr 401, 411 and 497), the myristic acid does not have chromophores which could accept the energy from the excited fluorophores. Moreover, the rest of tyrosines is situated in subdomains IB and IIB which do not interact with mentioned ligands. The slight enhancement of Trp affinity and the weakness of Tyr affinity toward the drug can be found in the secondary binding site (the increase of K_aII and K_QII for λ_{ex} 295 nm and the decrease for λ_{ex} 280 nm).

The presence of MYR at concentration 8×10^{-5} M correlates to the binding of MYR2 at the interface between three subdomains, IA–IB–IIA [12]. Although, MYR2 molecule does not interact directly with Trp 214 and tyrosines present in subdomain IIA but with Arg 257 and Ser 287 [2] it dramatically affects the affinity between protein and drug in its secondary binding site. The values of K_aII calculated for both excitation wavelengths decrease *ca*. 85%. Simultaneously, the number of bound ligand molecules is doubled and the exposition of Trp 214 is lowered.

Further binding of MYR to dHSA in its low affinity sites (MYR1, MYR3, MYR6, MYR7 and MYR8) causes continuation of the decrease of interaction between FUR and the albumin in the secondary binding sites. The number of drug molecules associated with the protein is threefold higher than the initial value. However, the interaction between donor and acceptor are significantly weaker (K_aII is eightfold lower than the initial value; the capability of energy transfer from Trp 214 is reduced 2.5 times). The value of K_aI decreases by about 40–50% although in this case the number of bound drug molecules also falls. For the results obtained using 280 nm excitation wavelength the decrease of K_aI and K_aII was 70 and 90%, respectively. This leads to the conclusion that the presence of bound molecules of MYR induces the weakness of affinity between FUR and tyrosil residues located outside the subdomain IIA. Binding of some MYR molecules to various sites on albumin molecule may suppress the fluorescence emitted by the excited tyrosines.

Changes of FUR binding to HSA in high and low affinity binding sites induced by the presence of fatty acid were described by Schwartz et al. [1]. They noted that together with the increase of concentration of oleic and linoleic acid sodium salts and linoleic acid the gradual increase of concentration of free drug faction occurred. A different effect of oleic acid on FUR–HSA interaction was described by Takamura et al. [17]. He stated that oleic acid inhibits the drug association when the number of bound acid molecules exceeds 4. He also pointed to the competitive interaction as the mechanism leading to the decrease of albumin affinity toward FUR in the primary binding site. Takamura also noted the fall of the concentration of free drug fraction when the number of MYR bound molecules was above 4. However, all the results concern the binding affinity of the entire albumin molecule. The authors [17] observed a similar influence on drug binding to HSA for linoleic and stearic acid. The effect decreased in the following order: oleic acid > linoleic acid > stearic acid.

In the current studies when the changes in all FUR binding sites were analyzed it was shown that binding of the first two molecules of MYR in domain III does not change the number of drug molecules bound with albumin (Table 1A and B). Whereas binding of the third high affinity MYR molecule with dHSA results in the rise of the number of bound drug molecule in subdomain IIA but in 90–95% these are molecules which form lower affinity complexes. On the basis of f_a values it can be concluded that the increase of MYR concentration correlates with the decrease of availability of fluorophores. The observed simultaneous reduction of the number of drug molecule bound in the high affinity binding site, its rise in low affinity sites and the weakness of interaction in drug–protein complex seems to be undesirable from the pharmacological point of view. In the situation when the additional agent influencing the drug binding appears the risk of excessive complex dissociation occurs. It may lead to a sudden increase of the free fraction of FUR. Considering the fact that furosemide *in vivo* remains bound with albumin in 99%, even the dissociation of a small amount of drug can result in a considerable increment of free and pharmacologically active fraction of FUR and the appearance of adverse effects. On the other hand, when no additional agent affects FUR binding and the excessive number of bound drug molecules remain in the complex it may be necessary to elevate the dosage of FUR to reach the therapeutic concentration.

4. Conclusions

The binding of fatty acid with human serum albumin induces various changes in the interaction between FUR and protein. The effect is different for the determined binding sites. However, it can be concluded that the association of fatty acid molecules in their low affinity binding sites which corresponds to the elevated level of fatty acid *in vivo*, significantly decreases the albumin affinity towards furosemide. Considering the fact that furosemide remains

bound with serum albumin in 99%, the great variability of the albumin affinity towards the drug may lead to the appearance of adverse effects in patients with an elevated level of fatty acids. Although our studies do not correlate directly to clinical practice they indicate that a monitoring therapy should be considered in patients with hyperlipidemia.

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