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The effect of structural alterations of three mammalian serum albumins on their binding properties

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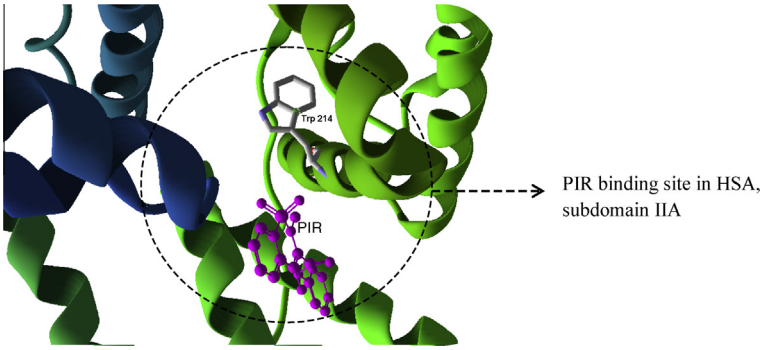
GRAPHICAL ABSTRACT



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ABSTRACT

The binding of piroxicam (PIR) to human (HSA), bovine (BSA) and sheep (SSA) serum albumin in native and destabilized/denaturated state was studied by the fluorescence quenching technique. Quenching of the intrinsic fluorescence of three analyzed serum albumins was observed due to selective exciting of tryptophanyl and tyrosil residues at 295 nm and 280 nm. Based on fluorescence emission spectra the quenching (K_Q) and binding constants (K_a) were determined. The results showed that PIR is bound mainly in IIA subdomain of HSA and is additionally able to interact with tyrosil groups located in subdomains IB, IIB or IIIA. PIR interacts only with tryptophanyl residues of BSA and SSA [Trp-214, Trp-237 (IIA) and Trp-135, Trp-158 (IB)]. The presence of denaturating factors modified the mechanism of fluorescence quenching of SSA by PIR. Linear Scatchard plots suggest that HSA, BSA and SSA bind PIR in one class of binding sites.

1. Introduction

Serum albumin (SA) is the most important plasma protein responsible for the binding, transport and disposition of various exogenous and endogenous substances such as bilirubin, hormones, unesterified fatty acids or bile acids [1–3]. Human serum albumin (HSA) molecule contains 585 amino acid residues that form a polypeptide chain stabilized by 17 disulfide bridges [4]. Based on crystallographic studies it is known that HSA contains two principal drug–binding sites. They are located in subdomains IIA and IIIA [5] and named according to the pioneering work of Sudlow et al. [6,7] as sites I and II (Fig. 1). Hydrophobic, bulky and heterocyclic molecules with negative charge in the middle of the molecule bind to site I, while aromatic carboxylic acids with negative charge at one end of the molecule bind to site II [8]. The tertiary structure of human serum albumin is homologous with bovine (BSA) or sheep albumin (SSA) in 76 and 74.7%, respectively. The similarity of BSA and SSA is more than 91% [9]. BSA and SSA contain two tryptophanyl residues while HSA has only one.

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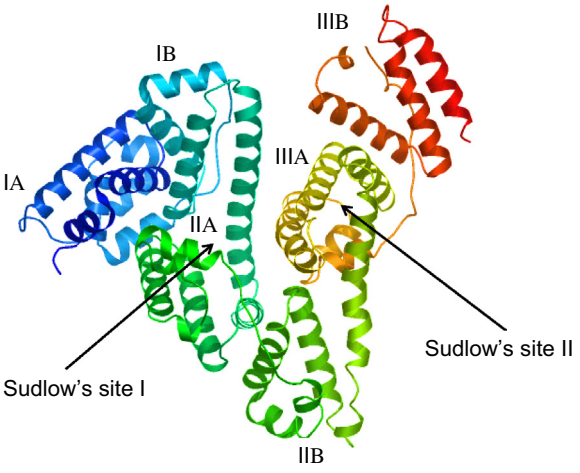

Fig. 1. Domain and binding sites location in the crystal HSA structure. Hydrophobic binding sites (Sudlow's site I and II) are indicated.

Serum albumin is able to bind various drugs reversibly and alteration in the binding ability affects drug pharmacokinetics and pharmacodynamics [8,10]. It held to be of clinical importance [11,12]. An alteration of albumin level in an organism can be the consequence of various factors e.g. a change in its rate of synthesis, an increase of catabolic rate or its redistribution from plasma to an interstitial compartment [13]. Radiation, infection or trauma have always been associated with hypoalbuminemia in human [14,15] and some other organisms [16,17]. A low level of albumin indicates liver failure, cirrhosis or chronic hepatitis, malnutrition or protein losing enteropathy. Besides the variation in albumin level in plasma, which can be an indicator of a pathological state, conformation is important for its physiological function. Tertiary structure of albumin *in vivo* can be modified by temperature [18] or by many ligands such as drugs [19–21], fatty acids [22,23], urea [24], glucose [25] or reactive aldehydes [26]. Structural modification of albumin was found in patients with liver failure, cirrhotic [27], renal diseases, uremia [28], schizophrenia [29], ischemia [30,31] and many others diseases like some type of cancer [32]. The structure of albumin in the plasma of healthy individuals can also undergo structural changes as a result of trauma [33] or intensive physical exercise [34].

Urea or guanidine hydrochloride – two denaturating agents with different mechanisms of action can cause chemical modification in the native structure of human, bovine and sheep albumin *in vitro*. The denaturating effect of urea or guanidine hydrochloride on human and bovine serum albumin has previously been described [35–37].

The aim of these experiments is to evaluate how the binding capacity and structure of HSA and two model mammalian albumins (BSA and SSA) are modified by the denaturating agents. We used piroxicam ((3E)-3-[hydroxy-(pyridin-2-ylamino)methylidene]-2-methyl-1,1-dioxobenzo[e]thiazin-4-one) (Fig. 2), one of the most popular non-steroidal anti-inflammatory drugs (NSAIDs) used in anticancer therapy of digestive system to relieve the symptoms of rheumatoid arthritis and osteoarthritis including pain, tenderness, swelling and stiffness.

2. Experimental

2.1. Materials and methods

Human serum albumin fraction V (HSA, Cat. no.: 823022, Lot no.: 8234H), piroxicam (PIR, Cat. no.: R17977, Lot no.: 156277),

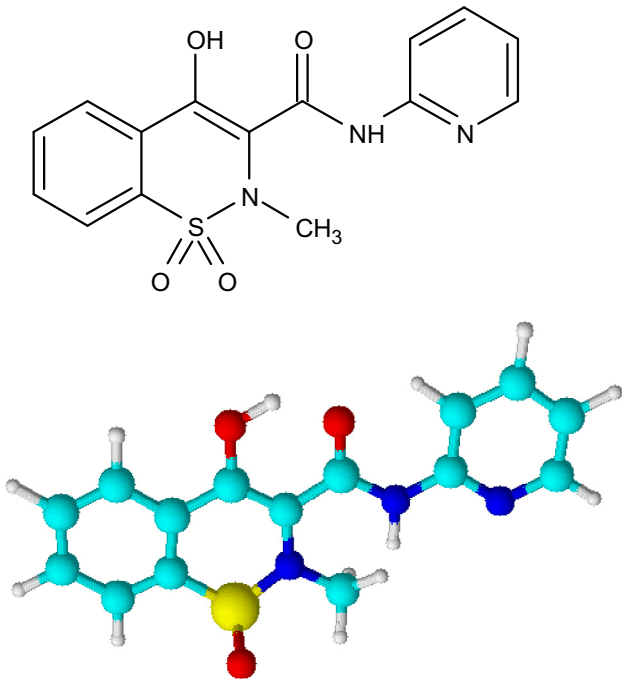

Fig. 2. Chemical structure of piroxicam (PIR).

guanidine hydrochloride (Gu.HCl, Cat. no.: 820539, Lot no.: 9398F) and urea (U, Cat. no.: 10320) were purchased from MP Biomedicals, LLC Ohio. Bovine albumin fraction V (BSA, Cat. no.: A4503) and sheep albumin fraction d (SSA, Cat. no.: A6289, Lot no.: 109F9301) obtained from Sigma–Aldrich Inc USA.

2.2. Apparatus and conditions

All solutions were prepared at physiological pH 7.47 in 0.1 M sodium phosphate buffer. The experiment was carried out at 309.6 K. UV absorption spectra and fluorescence emission spectra were recorded 60 min after preparation of the solutions.

2.2.1. Absorption spectra

Absorption spectra of HSA, BSA, SSA and PIR were recorded on a spectrometer JASCO V-530, 60 min after sample preparation. Correcting error of apparatus for wavelength (λ) is equal to ±1 and for absorbance (A) ± 0.00001. Absorption spectra were recorded in the range from 220 nm to 400 nm.

2.2.2. Fluorescence emission spectra

Fluorescence emission spectra were recorded on a KONTRON SFM Instrument AG Spectrofluorimeter. Emission fluorescence spectra were displayed in terms of relative fluorescence (RF). Correcting error of apparatus for wavelength (λ) is equal to ± 1 and for relative fluorescence (RF) ± 0.01. To excite fluorophores of all sera albumin two excitation wavelength (λ_{ex} = 280 nm and λ_{ex} = 295 nm) were used. The concentration was adjusted to produce an absorbance ($A \leqslant 0.05$) and the fluorescence spectra were not corrected for the inner filter effects [38].

Urea or guanidine hydrochloride solution was added to SA-PIR system to obtain a final concentration of denaturating agents was 1, 3, 5 and 7 M.

Association constant K_a was determined by using the Scatchard method modified by Hiratsuka [39]:

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

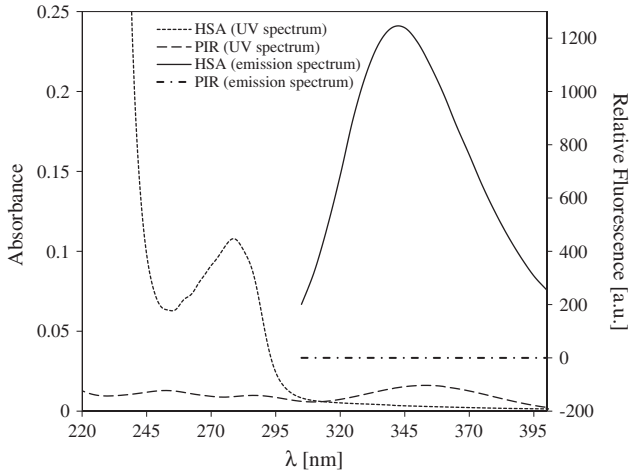

Fig. 3. UV-absorption spectra of HSA (———) and PIR (– – –); the emission fluorescence spectra of (—) and PIR (-·-·-·-) at excitation λ_{ex} = 280 nm. The HSA concentration was 1×10^{-6} M and PIR: 9×10^{-6} M.

where $r = \Delta RF/\Delta RF_{max}$ (where $\Delta RF = RF - RF_o$, $\Delta RF_{max} = RF_{max} - RF_o$) – the fractional saturation of sites; $[L_f]$ is free drug concentration, K_a is the association constant, n is the number of binding sites for the independent class of drug binding sites in the albumin molecule, which corresponds to the mean number of drug molecules bound to the independent class of drug binding sites in the albumin molecule.

The quenching effect from the fluorescence of albumin was analyzed on the basis of the Stern–Volmer equation (2), which allows us to describe ligand movement within the fluorophore microenvironment upon dynamic quenching [40]:

$$\frac{RF_o}{RF} = 1 + k_q \tau_o [Q] = 1 + K_Q [Q] \quad (2)$$

where RF_o and RF are the fluorescence intensities in the absence and presence of the quencher, respectively, K_Q is the rate quenching constant ($M^{-1}\ s^{-1}$), τ_o is the fluorescence lifetime in the absence of quencher, K_Q – the quenching constant, [Q] – the quencher (PIR) concentration in binary and ternary systems: SA-PIR, SA(U)-PIR and SA(Gu.HCl)-PIR.

Modification of Stern–Volmer equation by Lehrer [41] was used to determine the quenching constant K_Q:

$$\frac{RF_o}{\Delta RF} = \frac{1}{[Q]} \frac{1}{f_a} \frac{1}{K_Q} + \frac{1}{f_a} \quad (3)$$

where RF_o is the relative fluorescence intensities of protein in the absence of quencher, in triple systems RF_o is the fluorescence intensity of protein in the presence of urea (U) or guanidine hydrochloride (Gu.HCl) at respective concentration; ΔRF is the difference between RF_o and RF; f_a is the fractional accessible protein fluorescence; K_Q is the quenching constant; [Q] is the quencher concentration.

2.2.3. Sample preparation

All measured samples were prepared by mixing buffer solutions of serum albumin (SA) and PIR. The final concentration of human, bovine and sheep albumin was 1×10^{-6} M and the piroxicam concentration was in the range 0.25×10^{-6}–9×10^{-6} M.

2.2.4. Computer visualization of PIR binding site

A visualization of PIR binding site on HSA was performed using the Molegro Virtual Docker (MVD) 2008.3.0. computer program. Cristal structure of HSA was obtained from PDB (ID code 1AO6).

3. Results and discussion

UV and fluorescence spectroscopy were applied to study drug–binding interactions in serum albumin. Binding sites of PIR on albumin, along with binding and quenching constants and quenching mechanisms for SA-PIR are described. The influence of conformational modification on the tertiary structure of albumin upon binding of PIR was compared with the binding capacity of SA (HSA, BSA, SSA) in the SA-PIR system and in the presence of denaturating agents: urea or guanidine hydrochloride in the systems SA(U)-PIR and SA(Gu.HCl)-PIR.

The energy transfer between fluorophore and quencher is possible when the transition of a donor electron from the excited to the basic state corresponds to the absorption frequency of the acceptor. This effect is possible when the fluorescence lifetime of donor is sufficiently long and the distance between the donor and the acceptor is not larger than 7 nm (when the emission spectrum of fluorophore overlaps the absorption spectrum of a ligand (quencher) [42]. UV absorption spectra and emission fluorescence spectra of PIR and HSA excited at 280 nm are shown in Fig. 3. PIR excited at 280 nm does not fluoresce in the 300–400 nm range (spectrum -·-·-·-, Fig. 3), while HSA shows a maximum fluorescence at 340 nm and a maximum absorbance at 280 nm (spectrum —, Fig. 3). The maximum absorbance of the drug appears at 364 nm. The study of drug–albumin interaction under these conditions allows us to analyze the changes within the albumins fluorophore environment.

3.1. The structural modification of albumin as a result of denaturating factors

Proteins are stabilized by hydrophobic interactions, hydrogen bonding and van der Waals forces. Urea and its derivatives in water cause denaturation due to the weakening of the hydrogen bonds [43]. Gu.HCl, in turn, breaks intramolecular hydrogen bonds and forms a new ones in a different place between the protein and Gu.HCl molecules [37,44]. According to Veccio et al. the GuH^+ ion absorbs on a protein surface and weakens electrostatic interactions between charged site chains of amino acids [45].

The denaturation process induced by chemical or physical agents has been described in detail in the literature [45–50]. The interaction of denaturating agents with proteins causes changes in their structural conformation leading to the destabilization of the native structure [50]. This phenomenon is confirmed by the shape of the fluorescence emission spectra of HSA in the presence of U (Fig. 4A and B) or Gu.HCl (Fig. 4C and D). The spectrum of native HSA excited at 280 nm and 295 nm shows one maximum fluorescence emission (spectra ————, Fig. 4A–D). Urea at concentration 1 M and 3 M decreases the relative fluorescence of HSA causing a slight blue-shift of the maximum fluorescence emission of HSA (~2 nm: spectra ··· and —, respectively, Fig. 4A). A draft of the second maximum of the HSA fluorescence appearing in the presence of 5 M urea (spectrum-·-·-·-, Fig. 4A) at 308 nm is induced by a partial exhibition of tyrosil residues hidden in native structure, that may additionally emit fluorescence. Similar observation was reported previously during the study on the urea denaturation of HSA and BSA [35,44]. The distance between amino acids in the polypeptide chain increases making it impossible to quench the fluorescence of Tyr by Trp. During excitation of HSA(U) system at 295 nm a slight red-shift about 2 nm was observed. It points to an alteration within the fluorophore environment, which increases the polarity of Trp-214 (Fig. 4B). The presence of Gu.HCl causes a more pronounced change in the fluorescence emission spectrum of native HSA than U does (Fig. 4C and D).

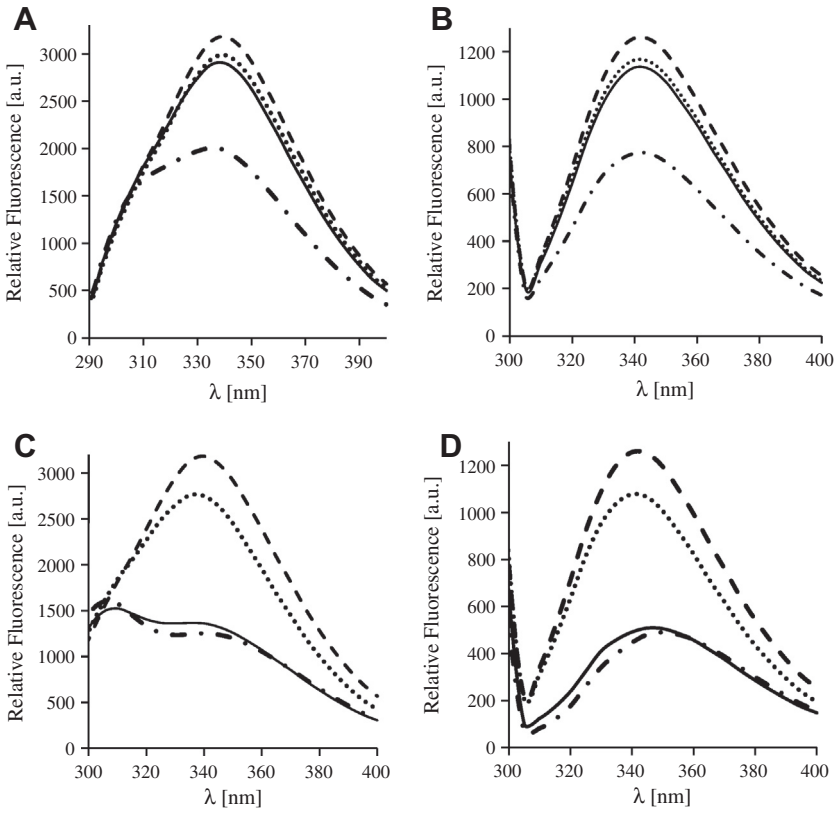

Fig. 4. Fluorescence emission spectra of HSA at concentration 1×10^{-6} M (spectra -·-·-·-) in the presence of urea (A and B) or guanidine hydrochloride (C and D) at concentration 1 M (spectra · · ·), 3 M (spectra –) and 5 M (spectra -·-·-·-), excited at 280 nm (A and C) and 295 nm (B and D).

Gu.HCl at concentration ⩾ 3 M causes the HSA fluorescence decreases more than 60% and the signal at λ_{max} at 305 nm appears. This signal derived from uncovered tyrosil groups (Fig. 4C, spectra: — and -·-·-·-) invisible on the spectrum of native HSA (Fig. 4 C, spectrum ———) attests to unfolding of native polypeptide chain.

The second emission maximum observed at 348–350 nm is derived from the emission of Trp-214. The red-shift of the HSA fluorescence at excitation 295 nm in the presence of Gu.HCl increases by above 10 nm with increasing Gu.HCl concentration. These effects point to a reduction of hydrophobicity of the Trp-214 surroundings (Fig. 4D, spectra: — and -·-·-·-).

3.2. Piroxicam–albumin complex – fluorescence spectroscopy studies

3.2.1. The quenching effect and binding site of PIR

The quenching of albumin fluorescence by PIR may be explained by the energy transfer from the fluorophore/s of human, bovine and sheep serum albumin (Fig. 5A–C, respectively). Only tryptophanyl residues (Trp-214 in HSA, Trp-135 and Trp-214 in BSA and in SSA: Trp-158 and Trp-237 in SSA) are excited at 295 nm, while excitation at 280 nm excites also tyrosil group/s (17 Tyrs in HSA, 20 in BSA and 21 in SSA). Consequently both types of fluorophore may participate in ligand-albumin interaction. The quenching curves for the HSA-PIR system obtained for λ_{ex} = 280 and λ_{ex} = 295 nm do not overlap as shown in Fig. 5A. This suggests that Trp-214 and the tyrosil group/s may together participate in the interactions HSA-PIR. The difference in the quenching effect of HSA fluorescence by PIR using two excitations wavelength: 280 and 295 nm shows that the binding site for PIR on HSA is located in IIA subdomain where Trp-214 and Tyr-263 were found. Additionally the binding site for PIR on HSA may be located in IB, IIB or the IIIA subdomain where Tyr residues are found (Tyr-138, Tyr-140, Tyr-148, Tyr-150, Tyr-319, Tyr-332, Tyr-334, Tyr-341, Tyr-353, Tyr-370, Tyr -401, Tyr-411, Tyr-497). A computer visualization of PIR binding site close to the Trp-214 residue in the tertiary structure of HSA is shown in Fig. 6.

It was a different case for the BSA-PIR and SSA-PIR system where the quenching curve for excitation 280 and 295 nm was almost the same (Fig. 5B and C). This means that Tyr residues of BSA and SSA do not participate in the interactions with PIR. The drug is binding solely close to the tryptophanyl residue/s in BSA and SSA. A comparison of the quenching curves for BSA-PIR with HSA-PIR excited at 295 nm made it possible to identify the BSA tryptophanyl residue/s involved in the binding of PIR. The large differences observed between the quenching curves for those systems (data not shown) allow us to assume that the binding site of PIR in BSA molecule is mainly within subdomain IIA and IB in the vicinity of Trp-214 and Trp-135, respectively. 21 Tyr residues do not interact with PIR. It was found that mainly Trp-214 (subdomain IIA) and in less extension Trp-158 (subdomain IB of SSA) may interact with PIR. Probably piroxicam forms "sandwich-type" complex in the IIA and IB subdomain between its aromatic rings and the protein's tryptophan/s and tyrosine/s aromatic ring/s. The chemical modification of tertiary structure of HSA, BSA and SSA by urea or guanidine hydrochloride in concentration 1, 3 and 5 M causes the reduction of quenching effect of SA fluorescence by PIR (Table 1A–D). This is possible due to the unfolding of the albumin polypeptide chain, which causes the amino acid residues to become more exposed to denaturant activity. Thus the unfolding facilitates the binding of ligand in other (so far unavailable) binding sites. The denaturating agents (U and Gu.HCl) react with albumin in subdomain IIA. Thus a reduction in SA quenching fluorescence by PIR may be a consequence of competition between U or Gu.HCl with PIR for a common binding site (Table 1A and B for

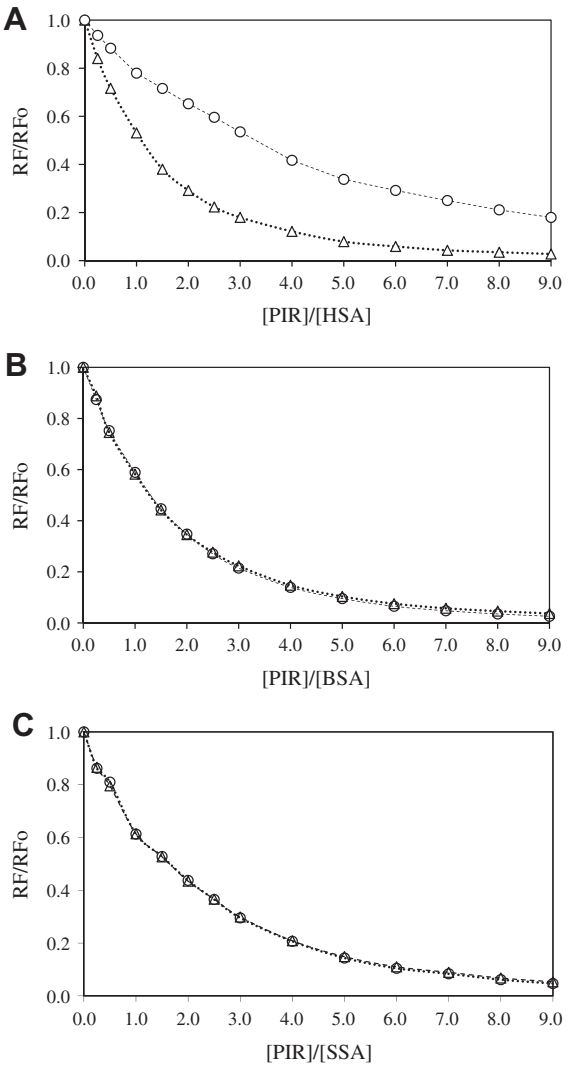

Fig. 5. Quenching curves of the HSA (A), BSA (B) and SSA (C) in the presence of piroxicam obtained for 280 nm and 295 nm excitation wavelength.

U and Table 1C and D for Gu.HCl) as a result of destruction of hydrophobic pocket/s with binding capacity.

The increase of the urea or Gu.HCl concentration in the HSA-PIR, BSA-PIR and SSA-PIR complexes caused a decrease of the quenching fluorescence intensity of albumin by PIR. At excitation 280 nm a higher number of excited fluorophores (Trp/s, Tyr/s) engaged in the interaction with PIR and hence a higher quenching effect was observed for SA-PIR, SA(U)-PIR and SA(Gu.HCl)-PIR.

3.2.2. Binding strength of PIR to SA

The linear Scatchard plots presented in Fig. 7 show that PIR occupies in native protein only one class of binding sites in HSA (Fig. 7A), BSA (Fig. 7B), as well as in SSA (Fig. 7C). Destabilization or denaturation of polypeptide chain did not change the shape of Scatchard plots. It means that PIR binds to structural altered albumins, similarly as in native, in one class of binding sites.

The structural modification of HSA, BSA or SSA molecule due to denaturants action, causes a change in their binding ability as evinced the change in binding constants value (Tables 2A and 2B).

HSA seems to be the most sensitive albumin within studied proteins to the chemical denaturants action. The presume of U at concentration 5 M causes that the binding constant decreased almost trice (Table 2A), while 5 M Gu.HCl decreases it about 8-fold (Table 2B). The smaller the binding constants were the bigger number of independent class binding sites (n), (Tables 2A and 2B). The unfolding of the polypeptide chain of albumin tertiary structure has an impact on previous statement. It was found that the binding constants decreased with increasing concentration of the denaturating agents resulting in a reduction in a stability of the HSA-PIR, BSA-PIR and SSA-PIR complexes.

The decrease in the strength of the drug binding to albumin caused by the change in its tertiary structure under U or Gu.HCl actions indicates the ligand binding hydrophobic pocket is altered. It can be inferred from the values of n that there is one independent class of binding sites of PIR on both native and destabilized/denatured by U and Gu.HCl structure of HSA, BSA and SSA (Tables 2A and 2B).

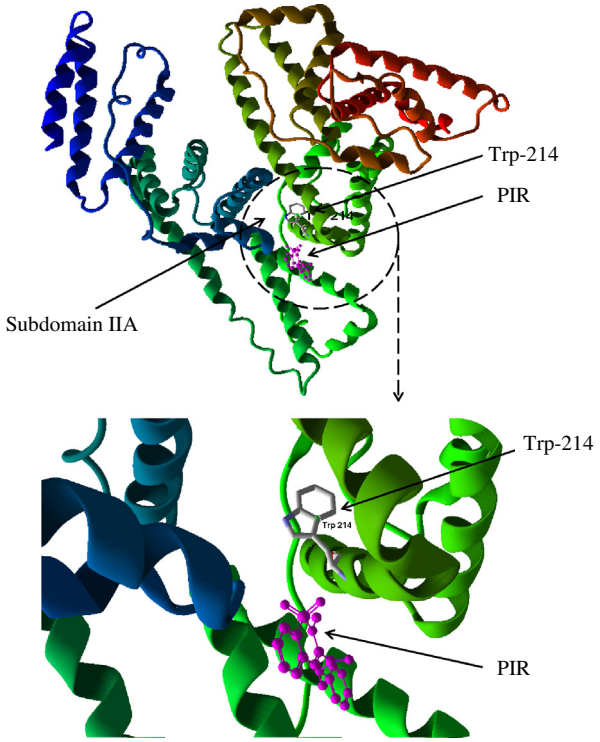

Fig. 6. Visualization of PIR binding site in subdomain IIA near Trp-214 in native HSA molecule.

Table 1
Quenching effect of HSA, BSA or SSA fluorescence by PIR in the presence or in the absence of urea (A and B) and guanidine hydrochloride (C and D), for excitation λ_{ex} = 280 nm (a and c) and λ_{ex} = 295 nm (b and d), SA:PIR molar ratio 9:1).

Quenching effect Q (%)	0 M	1 M	3 M	5 M
a. urea/280 nm				
$Q_{HSA-PIR}$	97.24	97.11	96.38	90.30
$Q_{BSA-PIR}$	96.31	94.83	92.32	85.05
$Q_{SSA-PIR}$	95.32	92.35	87.24	84.59
b. urea/295 nm				
$Q_{HSA-PIR}$	82.10	80.40	74.25	71.47
$Q_{BSA-PIR}$	96.21	93.79	92.23	85.01
$Q_{SSA-PIR}$	94.67	92.12	78.21	83.89
c. Gu.HCl/280 nm				
$Q_{HSA-PIR}$	97.24	92.95	89.38	87.77
$Q_{BSA-PIR}$	96.31	87.85	85.51	83.18
$Q_{SSA-PIR}$	95.32	90.32	89.50	88.73
d. Gu.HCl/295 nm				
$Q_{HSA-PIR}$	82.10	81.45	78.96	76.11
$Q_{BSA-PIR}$	96.21	87.11	84.10	80.02
$Q_{SSA-PIR}$	94.67	89.43	84.05	82.65

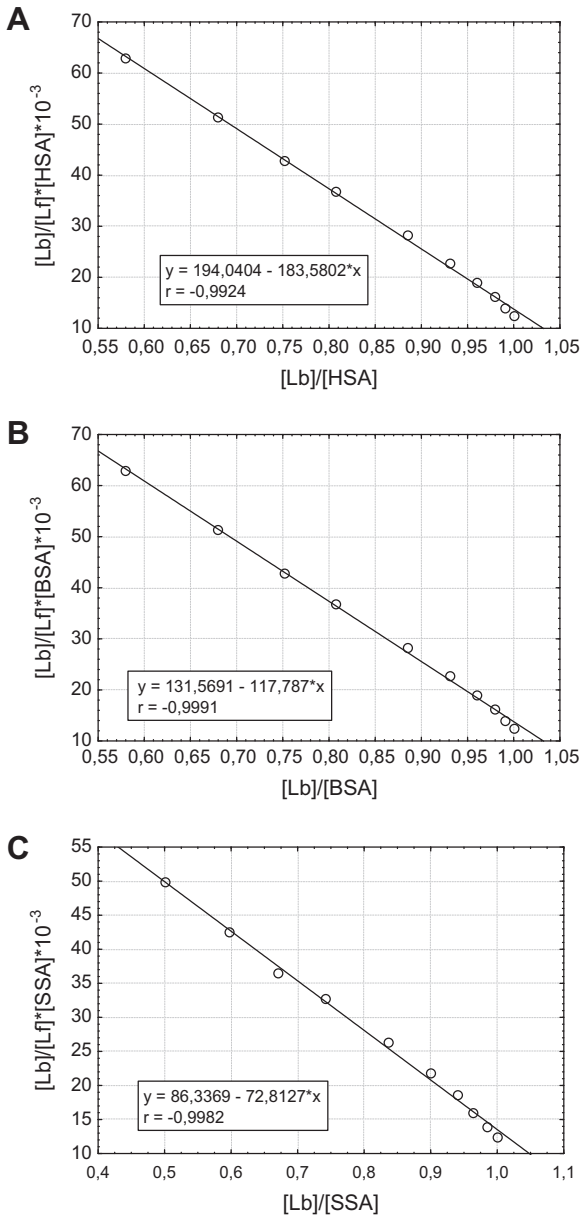

Fig. 7. Scatchard plots for HSA-PIR (A), BSA-PIR (B) and SSA-PIR complexes (C), excitation wavelength 280 nm.

Table 2A
Binding constants and number of binding sites for the independent class of drug binding sites in albumin molecule determined for HSA-PIR, BSA-PIR and SSA-PIR complex in the absence and presence of urea, λ_{ex} = 280 nm (a), λ_{ex} = 295 nm (b).

$\times 10^{-5}$ M^{-1}	0 M	1 M	3 M	5 M
a. urea/280 nm				
$K_{a\ HSA}$	1.835	1.654	1.068	0.717
$K_{a\ BSA}$	1.177	0.759	0.511	0.273
$K_{a\ SSA}$	0.728	0.550	0.357	0.343
n_{HSA}	1.06	1.07	1.11	1.13
n_{BSA}	1.12	1.18	1.26	1.45
n_{SSA}	1.19	1.24	1.36	1.37
b. urea/295 nm				
$K_{a\ HSA}$	1.827	1.653	1.079	0.772
$K_{a\ BSA}$	1.110	0.841	0.515	0.351
$K_{a\ SSA}$	0.704	0.641	0.365	0.321
n_{HSA}	1.06	1.08	1.12	1.16
n_{BSA}	1.13	1.16	1.28	1.37
n_{SSA}	1.19	1.20	1.35	1.40

Table 2B
Binding constants and number of binding sites for the independent class of drug binding sites in albumin molecule determined for HSA-PIR, BSA-PIR and SSA-PIR complex in the absence and presence of guanidine hydrochloride, λ_{ex} = 280 nm (a), λ_{ex} = 295 nm (b).

$\times 10^{-5}$ M^{-1}	0 M	1 M	3 M	5 M
a. Gu.HCl/280 nm				
$K_{a\ HSA}$	1.835	0.667	0.242	0.240
$K_{a\ BSA}$	1.177	0.441	0.287	0.270
$K_{a\ SSA}$	0.728	0.455	0.424	0.361
n_{HSA}	1.06	1.18	1.51	1.58
n_{BSA}	1.12	1.13	1.15	1.15
n_{SSA}	1.19	1.25	1.30	1.35
b. Gu.HCl/295 nm				
$K_{a\ HSA}$	1.827	0.573	0.407	0.217
$K_{a\ BSA}$	1.110	0.475	0.258	0.239
$K_{a\ SSA}$	0.704	0.428	0.427	0.326
n_{HSA}	1.06	1.23	1.35	1.72
n_{BSA}	1.13	1.12	1.15	1.15
n_{SSA}	1.19	1.30	1.31	1.41

Table 3A
Quenching constants for HSA-PIR, BSA-PIR and SSA-PIR complex in the absence and presence of 1, 3 and 5 M urea, λ_{ex} = 280 nm (a), λ_{ex} = 295 nm (b).

$\times 10^{-4}$ M^{-1}	0 M	1 M	3 M	5 M
a. urea/280 nm				
$K_{Q\ HSA}$	8.19	7.35	6.61	5.17
$K_{Q\ BSA}$	5.47	4.92	4.09	2.49
$K_{Q\ SSA}$	4.67	3.97	2.21	1.75
b. urea/295 nm				
$K_{Q\ HSA}$	7.88	7.07	6.06	5.77
$K_{Q\ BSA}$	5.31	4.83	2.55	1.87
$K_{Q\ SSA}$	4.63	2.75	2.16	1.26

Table 3B
Quenching constants for HSA-PIR, BSA-PIR and SSA-PIR complex in the absence and presence of Gu.HCl, λ_{ex} = 280 nm (a), λ_{ex} = 295 nm (b).

$\times 10^{-4}$ M^{-1}	0 M	1 M	3 M	5 M
a. Gu.HCl/280 nm				
$K_{Q\ HSA}$	8.19	3.95	1.89	1.53
$K_{Q\ BSA}$	5.47	2.79	1.97	1.87
$K_{Q\ SSA}$	4.67	2.83	2.74	2.27
b. Gu.HCl/295 nm				
$K_{Q\ HSA}$	7.88	3.44	2.63	1.31
$K_{Q\ BSA}$	5.31	2.98	1.92	1.65
$K_{Q\ SSA}$	4.63	2.73	2.69	2.09

3.2.3. Mechanism of the SA fluorescence quenching by PIR

In the current study the fluorescence quenching data were analyzed using the Stern–Volmer equations (Eqs. (2) and (3)). The probable quenching mechanism of fluorescence of HSA, BSA and SSA by PIR was proposed based on the dependence of quenching constants (K_Q) on the concentration of the denaturation factors. The K_Q values decrease with increase in urea and Gu.HCl concentration thereby indicating the presence of static quenching (Table 3A and 3B).

In contrast, Akbay et al. [51] obtained linear Stern–Volmer plots (for destabilized/denaturated albumins) during the interaction of coumarin derivatives and BSA. Analysis proves the dynamic mechanism of fluorescence quenching. A positive deviation on Stern–Volmer curve was found for PIR-native SA complexes (Fig. 8A–C, plots □□□□). The deviation of the unmodified Stern–Volmer curve points to the existence of a mixed fluorescence

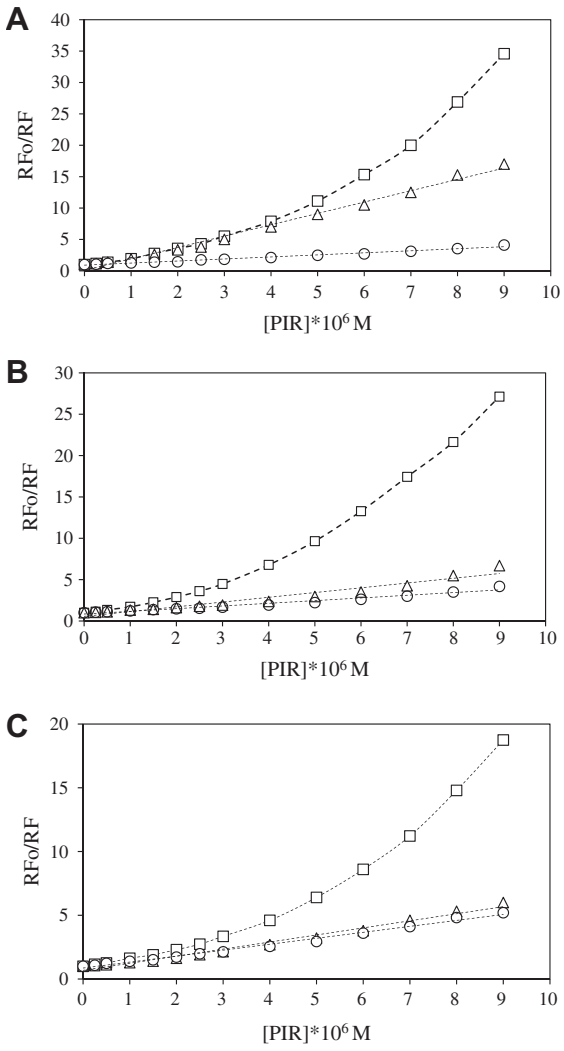

Fig. 8. Non-modyfied Stern–Volmer plots for SA-PIR (□□□), SA(U)-PIR (ΔΔΔ), SA(Gu.HCl)-PIR complex (○○○), respectively for HSA (A), BSA (B) and SSA (C), λ_{ex} = 280 nm.

quenching mechanism (static and dynamic simultaneously). It is assumed that during dynamic quenching the ligand may penetrates into the surrounding of protein molecule and the fluorescence quenching is caused by the collision of the quencher molecule and fluorophore/s [52]. Static quenching proves that all excited amino acids residues are equally available for ligand or alternatively a sole amino acid residue dominates the fluorescence. Eftink and Ghiron [40] observed positive deviation of the unmodified Stern–Volmer curves using the 6.7 M Gu.HCl denaturation of pepsine, trypsine and aldolase. The determining of the fluorescence quenching mechanism provides information about complex formation. The fluorescence quenching process depends on the nature of the fluorophore/s and the quencher molecule [54–57]. It was found that static quenching appears to be based on non-fluorescent protein-ligand complex creation or on the energy transfer from the fluorophore by PIR. However, the dynamic is caused by the interaction between the excited molecule and unexcited one. Silva et al. suggested that an alteration of the binding ability is a function of temperature due to the conformational disorders in albumin in the presence of chlorpromazine [58]. Eftink and Ghiron claimed that together with the number of occupied binding sites and growth in protein molecule by ligand the fluorescence intensity of the fluorophore/s decreases [40,53]. A linear shape of Stern–Volmer unmodified curve implies a dynamic fluorescence quenching of SSA by PIR in the presence of urea or Gu.HCl (Fig. 8A–C, plots ΔΔΔΔ and ○○○○, respectively). A positive deviation from the linear course observed for SA-PIR complexes indicates both static and dynamic quenching of SA fluorescence by PIR. An increase of Stern–Volmer curve slope together with the ligand concentration in the complex growth indicates an increasing number of ligand molecules bound in the surrounding of fluorophore/s.

4. Conclusions

Results showed that PIR binds to HSA in IIA subdomain and additionally it is able to interact with tyrosil groups located in subdomain IB, IIB or IIIA. PIR interacts only within tryptophanyl residues (Trp-214, Trp-237 (IIA) and Trp-135, Trp-158 (IB)) of BSA and SSA and does not interact with Tyr groups. The presence of denaturating factors modifies the fluorescence quenching mechanism of the SSA-PIR complexes. Linear Scatchard plots suggest that PIR binds to HSA, BSA and SSA in one class of binding sites.

Many pathological processes including ischemia, diabetes, atherosclerosis, chronic liver disease, kidney disease, schizophrenia, acute and chronic inflammatory conditions in many living organisms are accompanied by disorders of the spatial structure of HSA. It was found that the albumin structure alters in different physiological states e.g. as a consequence of aging and the experience of trauma.

In this work it was proven that the structural abnormalities of HSA and two model mammalian albumins cause an alteration in their binding properties. Thus an individual plan of dosage of drugs in elderly patients and patients burdened with disease-inducing structural modifications of HSA is suggested. It seems that study *in vivo* with albumin, similar to that described above, should help to achieve the desired therapeutic effect and prolong the life of patients afflicted with pathologies that alter the structure of albumin.

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References

[1] U. Kragh-Hansen, Pharmacol. Rev. 33 (1981) 17–53.
[2] T. Peters, Adv. Protein Chem. 37 (1985) 161–245.
[3] U. Kragh-Hansen, V.T.G. Chuang, M. Otagiri, Biol. Pharm. Bull. 25 (2002) 695–704.
[4] D.C. Carter, J.X. Ho, Adv. Protein Chem. 45 (1994) 153–203.
[5] X.M. He, D. Carter, Nature 358 (1992) 209–215.
[6] G. Sudlow, D.J. Birkett, D. Wade, Mol. Pharmacol. 11 (1975) 824–832.
[7] G. Sudlow, D.J. Birkett, D.N. Wade, Mol. Pharmacol. 12 (1976) 1052–1061.
[8] J.R. Simard, P.A. Zunszain, J.A. Hamilton, S. Curry, J. Mol. Biol. 361 (2006) 336–351.
[9] T. Peters, All About Albumin, Biochemistry, Genetics and Medicinal Applications, Academic Press, New York, 1996.
[10] J. Ghuman, P.A. Zunszain, I. Pepitas, A.A. Bhattacharyja, M. Otagiri, S. Curry, J. Mol. Biol. 353 (2005) 38–52.
[11] N.H.G. Holford, L.Z. Benet, in: B.G. Katzung (Ed.), Basic and Clinical Pharmacology, Appleton and Lange, Stamford, CT, 1998, pp. 34–39.
[12] W.E. Lindup, in: J.W. Bridges, L.F. Chasseaud, G.G. Gibson (Eds.), Progress in Drug Metabolism, vol. 10, Taylor & Francis, London, 1987, pp. 141–185.
[13] B. Ruot, D. Breuillé, F. Rambourdin, G. Bayle, P. Capitan, C. Obled, Am. J. Physiol. Endocrinol. Metab. 279 (2000) 244–251.
[14] M.A. Rothschild, M. Oratz, S.S. Schreiber, Hepatology 8 (1988) 385–401.
[15] K.C. Fearon, J.S. Falconer, C. Slater, D.C. McMillan, J.A. Ross, T. Preston, Ann. Surg. 227 (1998) 249–254.
[16] L. Voisin, D. Breuillé, B. Ruot, C. Rallière, F. Rambourdin, M. Dalle, C. Obled, Am. J. Physiol. Regul. Integr. Comp. Physiol. 275 (1998) R1412–R1419.
[17] G. Schreiber, A.R. Aldred, T. Thomas, H.E. Birch, P.W. Dickson, T. Guo-Fen, P.C. Heinrich, W. Northemann, G.J. Howlett, F.A. de Jong, A. Mitchell, Inflammation 10 (1986) 59–66.

[18] M. Rezaei-Tavirani, S.H. Moghaddamnia, B. Ranjbar, M. Amani, S.A. Marashi, J. Biochem. Mol. Biol. 39 (5) (2006) 530–536.
[19] M.A. Cheema, P. Taboada, S. Barbosa, E. Castro, M. Siddiq, V. Mosquera, Biomacromolecules 8 (2007) 2576–2585.
[20] N. Chadborn, J. Bryant, A.J. Bain, P. O'Shea, Biophys. J. 76 (1999) 2198–2207.
[21] R. Subramanyam, A. Gollapudi, P. Bonigala, M. Chinnaboina, D.G. Amooru, Photobiol. Photochem. 94 (2009) 8–12.
[22] B. Bojko, A. Sułkowska, M. Maciążek-Jurczyk, J. Równicka, W.W. Sułkowski, J. Pharm. Biomed. Anal. 52 (2010) 384–390.
[23] B. Bojko, A. Sułkowska, M. Maciążek-Jurczyk, J. Równicka, W.W. Sułkowski, Spectrochim. Acta A 76 (2010) 6–11.
[24] N. Gull, P. Sen, Kabir-Ud-Din, R.H. Khan, J. Biochem. 141 (2007) 261–268.
[25] M. Koga, S. Kasayama, Endocr. J. 57 (2010) 751–762.
[26] K. Mera, K. Takeo, M. Izumi, T. Maruyama, R. Nagai, M. Otagiri, J. Pharm. Sci. 99 (2010) 1614–1625.
[27] A. Watanabe, S. Matsuzaki, H. Moriwaki, K. Suzuki, S. Nishiguchi, Nutrition 20 (2004) 351–357.
[28] A.I. Ivanov, E.A. Korolenko, E.V. Korolik, S.P. Firsov, R.G. Zhbankov, M.K. Marchewka, H. Ratajczak, Arch. Biochem. Biophys. 408 (2002) 69–77.
[29] T.A. Gryzunov, T.I. Syrejshchikova, M.N. Komarova, E.Y. Misionzhnik, M.G. Uzbekov, A.V. Molodetskich, G.E. Dobretsov, M.N. Yakimenko, Nucl. Instrum. Methods Phys. Res. A 448 (2000) 478–482.
[30] J. Falkensammer, T. Stojakovic, K. Huber, A. Hammerer-Lercher, I. Gruber, H. Scharnagl, G. Fraedrich, W. Santner, M. Schocke, A. Greiner, Clin. Chem. Lab. Med. 45 (2007) 535–540.
[31] S. Turedi, A. Gunduz, A. Mentese, S.C. Karahan, S.E. Yilmaz, O. Eroglu, I. Nuhoglu, I. Turan, M. Topba, Am. J. Emerg. Med. 25 (2007) 770–773.
[32] S.C. Kazmierczak, A. Gurachevsky, G. Matthes, V. Muravsky, Clin. Chem. 52 (2006) 2129–2134.
[33] M. Can, S. Demirtas, O. Polat, A. Yildiz, Emerg. Med. J. 23 (2006) 537–539.
[34] G. Lippi, G.L. Salvagno, M. Montagnana, F. Schena, F. Ballestrieri, G.C. Guidi, Clin. Chim. Acta. 367 (2006) 175–180.
[35] J. Równicka, A. Sułkowska, J. Pożycka, B. Bojko, W.W. Sułkowski, J. Mol. Struct. 792–793C (2006) 243–248.
[36] J. Równicka-Zubik, A. Sułkowska, B. Bojko, M. Maciążek-Jurczyk, J. Pożycka, D. Pentak, W.W. Sułkowski, J. Photochem. Photobiol. B. 97 (2009) 54–59.
[37] J. Równicka-Zubik, A. Sułkowska, J. Pożycka, K. Gaździcka, B. Bojko, M. Maciążek-Jurczyk, W.W. Sułkowski, J. Mol. Struct. 924–926 (2009) 371–377.
[38] R.F. Steinem, I. Weinryb, E.P. Kirby (Eds.), Fluorescence Instrumentation and Methodology, Inc., Maryland, 1970, pp. 39–42.
[39] T. Hiratsuka, J. Biol. Chem. 265 (1990) 18786–18790.
[40] M.R. Eftink, C.A. Ghiron, Biochemistry 15 (1976) 672–680.
[41] S.S. Lehrer, Biochemistry 10 (1971) 3254–3263.
[42] B. Valeur, Molecular Fluorescence Principles and Applications, Willey/VCH, London/Weinheim, 2002.
[43] A. Michnik A, Mikrokalorymetryczne badania przemian konformacji albuminy poddanej działaniu wybranych czynników fizykochemicznych. Wydawnictwo Uniwersytetu Śląskiego, Katowice, 2009.
[44] A. Aschi, N. Mbarek, M. Othman, A. Gharbi, Mater. Sci. Eng. C 28 (2008) 594–600.
[45] P. Veccio, G. Graziano, V. Granata, G. Barone, L. Mandrich, M. Rossi, G. Manco, Biochem. J. 367 (2002) 857–863.
[46] S. Tayyab, M.U. Siddiqui, N. Ahmad, Biochem. Ed. 23 (1995) 162–164.
[47] S. Muzammil, Y. Kumar, S. Tayyab, Proteins 40 (2000) 29–38.
[48] B.J. Bennion, V. Daggett, Proc. Natl. Acad. Sci. USA 100 (2003) 5142–5147.
[49] M.Y. Khan, S.K. Agarwal, S. Hangloo, J. Biochem. 102 (1987) 313–317.
[50] D.M. Togashi, A.G. Ryder, J. Fluoresc. 16 (2006) 153–160.
[51] A. Shrake, P.D. Ross, Biopolymers 32 (1992) 925–940.
[52] N. Akbay, D. Topkaya, Y. Ergün, S. Alp, E. Gök, J. Anal. Chem. 65 (2010) 382–387.
[53] M.R. Eftink, C.A. Ghiron, Proc. Natl. Acad. Sci. USA 72 (1975) 3290–3294.
[54] J. Thipperudrappa, D.S. Biradar, M.T. Lagare, S.M. Hanagodimath, S.R. Inamdar, J.S. Kadadevaramath, J. Photochem. Photobiol. A Chem. 177 (2006) 89–93.
[55] J. Thipperudrappa, D.S. Biradar, S.M. Hanagodimath, J. Lumin. 124 (2007) 45–50.
[56] H.M. Suresh Kumar, R.S. Kunabenchi, J.S. Biradar, N.N. Math, J.S. Kadadevarmath, S.R. Inamdar, J. Lumin. 116 (2006) 35–42.
[57] H.M. Suresh Kumar, R.S. Kunabenchi, S.V. Nishti, J.S. Biradar, J.S. Kadadevarmath, Spectros. Lett. 42 (2009) 226–234.
[58] D. Silva, C.M. Cortez, S.R. Louro, Spectrochim. Acta. A Mol. Biomol. Spectrosc. 60 (2004) 1215–1223.