Journal of Pharmaceutical and Biomedical Analysis 52 (2010) 384–390

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

The influence of dietary habits and pathological conditions on the binding of theophylline to serum albumin

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ARTICLE INFO

Article history:
Received 19 April 2009
Received in revised form 1 September 2009
Accepted 5 September 2009
Available online 11 September 2009

Keywords:
Theophylline
Protein binding
Fatty acids
Fluorescence
NMR

ABSTRACT

The influence of fatty acids (FA) on theophylline (Th) binding to human serum albumin (HSA) in its high and low affinity binding sites was investigated. The content of studied FA solutions corresponds to the ones associating with different dietary habits and pathological states in vivo. Using fluorescence and ^{1}H NMR spectroscopy two high and two low affinity binding sites of Th in HSA structure were found. For each site several binding parameters in the absence and presence of FA were estimated. The results showed that the impact of FA on the affinity of HSA towards Th in high affinity binding sites is negligible whereas binding of the drug in low affinity sites decreases significantly in the presence of FA. It was observed that this effect is dependent on the number of fatty acid molecules bound to the protein while the chemical structure of fatty acids contained in the solution plays a minor role.

1. Introduction

Theophyline is a popular diuretic, cardiac stimulant and asthma medicament. It is transported in the body via the circulatory system bound with serum albumin in about 40%. The primary ligands for the albumin are fatty acids, therefore their competition in binding with drugs may occur. Several binding sites in the albumin tertiary structure for fatty acids were found, although the affinity between fatty acid and the protein mainly depends on the chemical structure of the particular acid, especially the length of the chain and the presence and the position of the double bonds [1]. The competition between ligands simultaneously bound to the protein may be due to the direct displacement in the site of binding or due to the conformational changes of albumin structure induced by the molecules of ligands bound in different subdomains. Human serum albumin is a single chain protein consisting of 585 amino acids. It has a heart shape with dimensions of 80 Å × 80 Å × 80 Å × 30 Å and is composed of three domains (I–III). Each of them is divided into two homologous subdomains A and B forming 9 loops [2–4] connected by disulfide bonds. The main sites which bind the drugs are subdomain IIA and IIIA (Sudlow's site I and site II). In the first site dicarboxylic acids and/or bulky heterocyclic molecules with a negative charge localized in the middle of the molecule are located, while the second one is occupied by the aromatic carboxylic acids with a negatively charged acidic group at the one end of the molecule away from the hydrophobic center [5].

The general affinity of albumin towards a particular drug as well as the changes of free and bound fraction concentration are important from the pharmacological point of view, since only the unbound form is pharmacologically active, while the complexed molecules are a depot of the drug. The knowledge of the location of the binding sites of the ligands and the mechanism of competition between various ligands give vital information concerning drug design.

Competition between ligands in term of plasma protein binding is the most important for drugs which binding fraction is very high and the increase of free fraction can be doubled. Fatty acids seem to be competitors which do not affect binding of drugs in such extent, however the fact they can occupy several binding sites in albumin structure can make drug–protein complex unstable. Thus, the evaluation of binding parameters for drug–albumin complex in all existing binding sites gives us the information about the changes of protein affinity towards the drug in the presence of fatty acids at increasing concentration. Because of the weak interaction between the drug and transporting protein, theophylline can be more susceptible to the influence of fatty acids in comparison to drugs which affinity towards albumin and binding fraction is higher.

In the physiological condition the main fatty acids present in the human serum are oleic (33%), palmitic (25%), linoleic (20%), arachidonic (5%), palmitoleic (3%), stearic (1.5%) and myristic (1.5%), but the composition may be altered by the changes in dietary habits or by the pathological state [1]. These differences of fatty acids

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doi:10.1016/j.jpba.2009.09.004

content in the serum can influence the formation of drug–albumin complex.

Previous studies on the influence of fatty acids on theophylline binding to albumin [6] showed that the affinity between Th and protein decreases in obese patients. Obesity is mostly associated with the elevated level of saturated fatty acids. Nowdays, the dietary supplements such as omega-3 and omega-6 fatty acids are widely used, therefore the influence of their concentration on drugs' binding should be examined.

In the current study we investigated the affinity changes of human serum albumin towards theophylline in the presence of mixtures containing different amounts and contents of saturated and unsaturated fatty acids. The particular mixture relates to the different pathological state (e.g. obesity, diabetes) or to the situation when an overdose of dietary supplements occurs. Low affinity between the drug and albumin may result in the easy replacement of theophylline by fatty acids, which are the primary albumin's ligands. Therefore, the investigation of the fatty acids' influence on the interaction in theophylline–albumin complex was performed.

We used fluorescence spectroscopy to indicate the high affinity binding site(s) of theophylline in HSA structure and to estimate the cooperativeness between bound ligands in the presence and absence of FA. Proton magnetic resonance spectroscopy (^{1}H NMR) was used to determine the weak interactions between Th and albumin in the low affinity sites as well as the condition when Th–HSA complex become saturated. For all observed class of binding sites association constants were calculated.

2. Materials and methods

2.1. Reagents

Theophylline (Th) was purchased from MP Biomedicals, Germany, human albumin, fraction V fatty acid free (HSA) from ICN Biomedicals, Inc., USA. Myristic acid (MYR), oleic acid (OA), linoleic acid (LA), palmitic acid (PA) and stearic acid (SA) from MP Biomedicals, OH, USA. 8-Anilino-1-naphthalene-sulfonic acid (ANS) was supplied by Sigma–Aldrich Chemie GmbH, Germany. Deuterium oxide 90% at.% D (D_2O), sodium deuteroxide 1 M in deuterium oxide (NaOD) and 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) were obtained from Armar Chemicals, Switzerland.

2.2. Fluorescence studies

All solutions were prepared at pH 7.47 ± 0.1 in 0.05 M sodium phosphate buffer. The measurements were performed at 310 K. Emission fluorescence spectra were recorded using Kontron SFM 25 Instrument AG spectrofluorimeter and 1 cm × 1 cm × 4 cm quartz cells.

In the studies of Th binding to serum albumins two wavelengths were used (280 and 295 nm) to excite the protein fluorophores. The range of recorded spectra was 280(295)–400 nm. For the studies on interaction between Th and ANS in subdomain IIIA the 360 nm wavelength was used to excite the ANS probe. The concentration range of theophylline used in the studies was 2×10^{-5} to 8×10^{-4} M and the concentration of human serum albumin was constant and equal to 5×10^{-6} M. For studies of Th interactions in subdomain IIIA the concentration of ANS was constant and equal to 5×10^{-6} M.

Association constant K_a was calculated by using the Scatchard method modified by Hiratsuka [7]:

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

where r is the number of ligands bound to one protein molecule; $[L_f]$ is the free (unbound) ligand concentration; n is the 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 is the association constant.

Stern–Volmer equation modified by Lehrer [8] was used to determine the quenching constant K_Q:

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

where RF_o and RF are relative fluorescence intensity of protein in the absence and presence of quencher (L), respectively; in the systems containing fatty acids RF_o is the fluorescence intensity of protein in the presence of fatty acids 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; [L] is the quencher concentration.

Hill's coefficient was calculated graphically on the basis of the following equation [9]:

$$\frac{lg\ Y}{1-Y} = n_H\ lg[L] + lg\ K \quad (3)$$

where Y is the fractional binding saturation; fraction of sites occupied with the ligand; $1 - Y$ is the number of free sites; n_H is the Hill's coefficient; K is the binding constant.

For fluorescence measurement:

$$\frac{Y}{1-Y} = \frac{Q}{Q_m - Q} \quad (4)$$

where $Q = (RF_o - RF)/RF_o$; $1/Q_m$ = intercept of OX axis on the plot $1/Q$ vs. $1/[L]$.

This form of Hill's equation is used to estimate the cooperativity in multisubunits' allosteric proteins.

2.2.1. Procedure of preparation fatty acids' solutions for fluorescence studies

In the physiological conditions serum albumin binds up to 2 mole of fatty acid (FA). Our previous fluorescence studies showed [10] that albumin forms complex with two molecules of fatty acid when [FA]/[albumin] molar ratio in the solution is between 1:1 and 1:8. To investigate the influence of FA on albumin binding of Th in semi-physiological conditions we prepared the mixture of FA corresponding to the physiological composition of fatty acids in human blood. The stock solution of mixed fatty acids for fluorescence studies was prepared in two steps.

In the first step an appropriate amount of each individual fatty acid was dissolved in 0.01 M NaOH and then titrated with 0.1 M HCl to obtain a solution of 10^{-2} M fatty acid at pH 7.47.

These solutions were used to prepare five stock solutions containing different proportions of all studied fatty acids in phosphate buffer pH 7.47. A semi-physiological solution contained 40% of oleic acid, 25% of linoleic acid, 30% of palmitic acid, 2.5% of myristic acid and 2.5% of stearic acid. Total concentration of fatty acids in the stock solution was 2×10^{-4} M.

The next two solutions had a 4- and 8-fold higher concentration of saturated fatty acids (PA, MYR and SA). Total concentration of these solutions was 4.08×10^{-5} and 7.07×10^{-5} M.

The last two solutions contained a 4- and 8-fold increased concentration of unsaturated fatty acids (OA and LA) in comparison to the semi-physiological solution. Total concentration of these solutions was 5.91×10^{-5} and 1.11×10^{-4} M.

All fatty acid stock solutions were gently heated and placed in an ultrasound bath. This procedure allowed to achieve a homogeneous suspension of solutions containing a high concentration of fatty acids.

2.3. ^1H NMR studies

All stock solutions of theophylline and albumin were prepared at pH 7.47 ± 0.1 in 0.05 M sodium phosphate buffer in D_2O. The concentration of Th and HSA was 2×10^{-3} to 4×10^{-2} and 2.5×10^{-4} M, respectively. All measurements were done at 310 K. Proton magnetic resonance ^1H NMR spectra were recorded on Bruker Avance 400 spectrometer, probe was tuned at 400 MHz and 5 mm tubes were used. For water suppression the presaturation method was used. Chemical shifts of theophylline proton resonance signals were measured in relation to the DSS signal at 0.05 ppm. The proton resonance assignment was performed on the basis of the simulated spectrum of theophylline (Fig. 5) obtained with the use of ACD/HNMR Predictor (v7.07).

Association constant was determined on the basis of the following equation [11]:

$$\frac{1}{\delta - \delta_f} = \frac{1}{(\delta_b - \delta_f)[P]_0}([L]_0 + K_d) \quad (5)$$

where $[L]_0$ is the total ligand concentration; $[P]_0$ is the total protein concentration; K_d is the dissociation constant ($-K_d$ value is an intercept of *OX* axis on the plot $1/(\delta - \delta_f)$ vs. [L]).

2.3.1. Procedure of preparation fatty acids' solutions for NMR studies

Total concentration of the semi-physiological stock solution used for NMR studies was 1.0×10^{-2} M. The high concentration was necessary to reach [FA]/[HSA] molar ratio 4:1 To obtain this solution an appropriate amount of each FA was added simultaneously, dissolved in 0.01 M NaOD and titrated with 0.1 M HCl in D_2O. An analogous procedure was used to prepare the stock solutions containing the increased concentration of saturated and unsaturated FA.

3. Results and discussion

3.1. Fluorescence studies

3.1.1. Binding of theophylline to HSA (Th–HSA) in the absence of fatty acids

Studies on theophylline (Th) binding to serum albumin showed that the drug occupies two binding sites in the protein structure [12,13]. Location of these sites was determined only for bovine serum albumin (BSA) [13]. To find the binding site(s) in human serum albumin (HSA) two wavelengths—295 and 280 nm were used in order to excite the fluorophores of the albumin. The use of 295 nm excitation wavelength allows us to observe the tryptophanyl residues of the protein only, whereas 280 nm wavelength also excites tyrosil residues. Human serum albumin has several tyrosil residues located in different subdomains of the protein and only one tryptophanyl residue (Trp 214) in subdomain IIA known as Sudlow's binding site I.

The quenching curve of HSA in the presence of Th at increasing concentration (Fig. 1) shows the decrease in albumin's fluorescence during excitation with both, 295 and 280 nm wavelength. The drop in the curve plotted for 295 nm wavelength indicates that Th interacts with Trp 214 which is located in subdomain IIA and accepts the fluorophore's energy. The Scatchard plot (Fig. 2) shows a nonlinear dependence of Th–HSA complex observed for λ_{ex} 295 nm. The run of the plot is specific for the two-site model. This suggests that Th has two independent classes of binding sites in this subdomain which differ in the affinity of the observed fluorophore towards the drug. The following binding parameters: association and quenching constants, K_a and K_Q, respectively, the mean number of drug molecules bound with 1 mole of macromolecule in a particular binding site n and Hill's coefficient n_H is collected in Table 1.

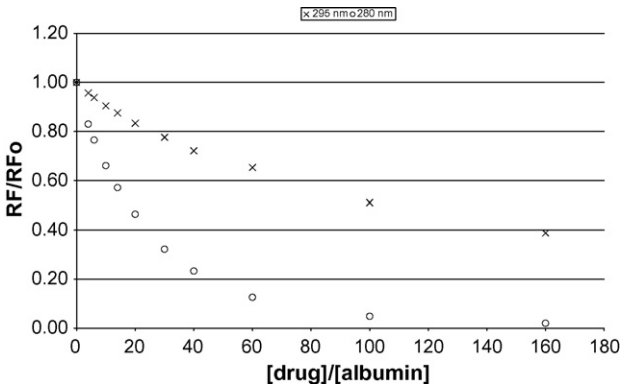

Fig. 1. Quenching curves of HSA in the presence of Th obtained for 295 and 280 nm excitation wavelengths; HSA 5×10^{-6} M and Th 2×10^{-5} to 8×10^{-4} M.

The observed changes in the run of the quenching curves obtained for 295 and 280 nm excitation wavelengths indicate the significant participation of tyrosines in the interaction between Th and HSA. Of all tyrosil residues of albumin two play a distinct role in ligand binding. These are Tyr 401 and 411 located in subdomain IIIA (Sudlow's binding site II). The significant participation of tyrosil residues observed in the interaction suggests that Th may also occupy subdomain IIIA. To confirm or deny drug binding in this site we conducted competitive studies with 1-anilino-9-naphthalene sulfonate (ANS) which is a fluorescent dye with the highest affinity towards subdomain IIIA [14]. ANS also binds in subdomain IIA, however its predominant affinity to subdomain IIIA allows us to use it at low concentration as a selective dye for Sudlow's site II [15]. ANS excited with λ_{ex} 360 nm shows fluorescent abilities ($\lambda_{max\ em}$ 460 nm) when it is bound with protein. Displacement of ANS by the drug is seen as the decrease of the dye's fluorescence. The lack of changes in ANS fluorescence in the presence of theophylline at increasing concentration shown in Fig. 3 suggests that Th does not bind in subdomain IIIA. Though the result of the experiment seems to be indisputable, the big difference between the association constants of these two ligands (K_a ANS 0.87×10^6 M^{-1} [14], K_a Th 8.5×10^3 M^{-1} (Table 1)) may suggest that an interaction of theophylline with albumin occurs but is too weak to displace ANS in its primary binding site.

The Scatchard plot obtained for Th–HSA complex for λ_{ex} 280 nm (Fig. 2) indicates the existence of one class of theophylline binding site in albumin. The comparison of these data with the ones obtained for λ_{ex} 295 nm, which suggested Th binding in two classes of sites, leads us to the conclusion that tyrosines stabilize the

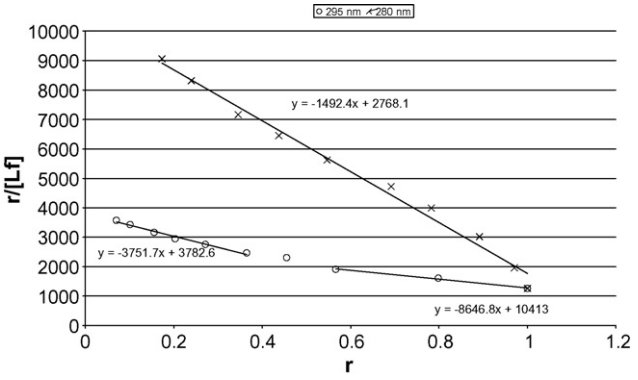

Fig. 2. Scatchard plot of Th–HSA complex obtained for 295 and 280 nm excitation wavelengths; HSA 5×10^{-6} M and Th 2×10^{-5} to 8×10^{-4} M.

Table 1
Binding and quenching constants (K_a and K_Q, respectively), number of binding sites (n) and Hill's coefficient (n_H) for the independent classes of binding sites of the drug in albumin molecule determined for Th–HSA complex in the absence and presence of various amounts of saturated (SFA) and unsaturated (USFA) fatty acids, λ_{ex} 295 and 280 nm.

	λ_{ex}	K_{aI} ($\times 10^{-3}$ M^{-1})	K_{aII} ($\times 10^{-3}$ M^{-1})	K_{QI} ($\times 10^{-3}$ M^{-1})	K_{QII} ($\times 10^{-3}$ M^{-1})	n_I	n_{II}	n_{HI}	n_{HI}
Th–HSA	295 nm	3.8	1.5	4.2	1.4	1.0	1.9	1.30	0.9
	280 nm	8.6	N/A	8.3	N/A	1.2	N/A	0.90	N/A
Th–HSA–FA_{phys}	295 nm	N/A	N/A	17.0	0.9	N/A	N/A	3.50	1.3
	280 nm	6.5	N/A	7.5	N/A	1.3	N/A	0.96	N/A
Th–HSA–FA_{4S}	295 nm	N/A	N/A	11.0	0.4	N/A	N/A	1.40	1.7
	280 nm	8.3	N/A	8.5	N/A	1.2	N/A	0.90	N/A
Th–HSA–FA_{8S}	295 nm	1.5	N/A	1.2	N/A	1.8	N/A	1.00	N/A
	280 nm	7.5	N/A	7.9	N/A	1.2	N/A	0.96	N/A
Th–HSA–FA_{4US}	295 nm	1.0	N/A	1.3	N/A	2.2	N/A	1.10	N/A
	280 nm	7.2	N/A	7.8	N/A	1.3	N/A	0.90	N/A
Th–HSA–FA_{8US}	295 nm	0.6	N/A	0.3	N/A	3.2	N/A	1.10	N/A
	280 nm	6.8	N/A	7.4	N/A	1.3	N/A	0.95	N/A

interaction between drug molecules and the albumin in the class of binding sites previously described. This causes the rise of the affinity of Th molecules towards subdomain IIA and results in the equalization of association constants in both classes (Table 1). It is observed as a one site model on the Scatchard plot (Fig. 2).

The high affinity between tyrosil residues of albumin and Th may suggest that Th also binds in subdomain IIIA despite of the lack of ANS fluorescence changes. If the interaction between Th and Tyr 401 and/or 411 located in Sudlow's site II is of the same order as Th interaction in site I then the binding site in subdomain IIIA will belong to the same class of binding sites and will not be represented as a separate line in the Scatchard dependence. However, further studies should be conducted to investigate the binding interaction of Th in this subdomain.

3.1.2. The influence of physiological composition of fatty acids on Th–HSA complex (Th–HSA–FA_{phys})

Binding of Th to HSA showed significant differences in the presence of fatty acids' mixture reflecting the physiological conditions when compared to Th–HSA complex in the absence of fatty acids. The Scatchard plot (Fig. 4) obtained for this system when λ_{ex} 295 nm was used is characteristic for cooperative binding. This is confirmed by the value of Hill's coefficient which is equal to 1.6. In analogy to Th–HSA system in the absence of FA we estimated the quenching constant and Hill's coefficient for two classes of binding sites. The results indicate that both classes show cooperativeness in ligand binding, however it is more distinct in the class with higher affinity. This can be explained by the binding of two molecules of fatty acids (FA4 and FA5) in subdomain IIIA [10,16]. These molecules alter the structure of the albumin and consequently can change the position of the bound Th molecules. The rise of K_{QI} and the decrease of K_{QII} in comparison to Th–HSA complex (Table 1) explains the translocation of Th molecules in reference to Trp 214. Molecules which belong to the first class are shifted closer to this amino acid, whereas those bound with lower affinity (second class) are moved further from the tryptophanyl residue.

The run of the Scatchard plot is linear (data not shown), which points to the existence of one class of binding sites when participation of tyrosil residues was also observed (λ_{ex} 280 nm). The number of Th molecules bound to albumin remains the same when compared to Th–HSA complex, while K_a and K_Q decrease slightly. When all molecules of theophylline interact with the albumin with the same affinity, almost no cooperation in the ligand binding is being observed ($n_H \sim 1$).

3.1.3. The influence of the increased concentration of saturated fatty acids on Th–HSA complex (Th–HSA–SFA)

The 4-fold increase of saturated fatty acid (SFA) concentration results in the drop of Hill's coefficient in the first class of binding sites referring to Th–HSA–FA_{phys} system (Table 1). However, the run of the Scatchard plot is still characteristic for cooperative binding when λ_{ex} 280 nm was used (data not shown). This means that cooperativeness between Th molecules in this class and other ligands occurs but is weaker. The decrease of K_Q values indicates that the distance between the drug and Trp 214 becomes greater in the case of both classes of binding sites (Table 1).

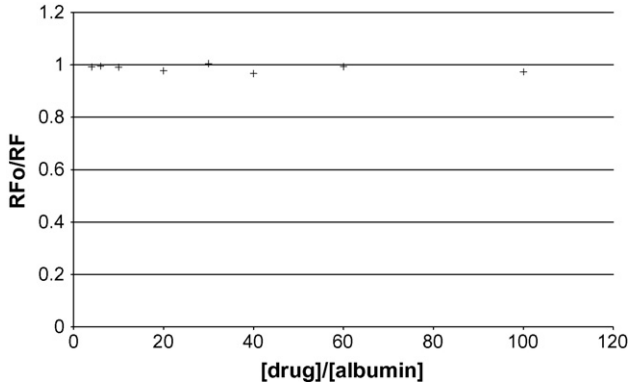

Fig. 3. The influence of Th at increasing concentration on ANS fluorescence. [ANS]/[HSA] molar ratio 1:1; HSA and ANS 5×10^{-6} M, Th 2×10^{-5} to 8×10^{-4} M; λ_{ex} 360 nm.

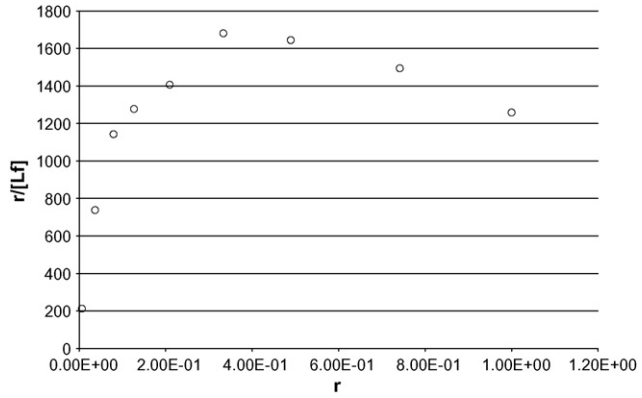

Fig. 4. Scatchard plot of Th–HSA–FA_{phys} complex. HSA 5×10^{-6} M, Th 2×10^{-5} to 8×10^{-4} M, FA 2×10^{-5} M; λ_{ex} 295 nm.

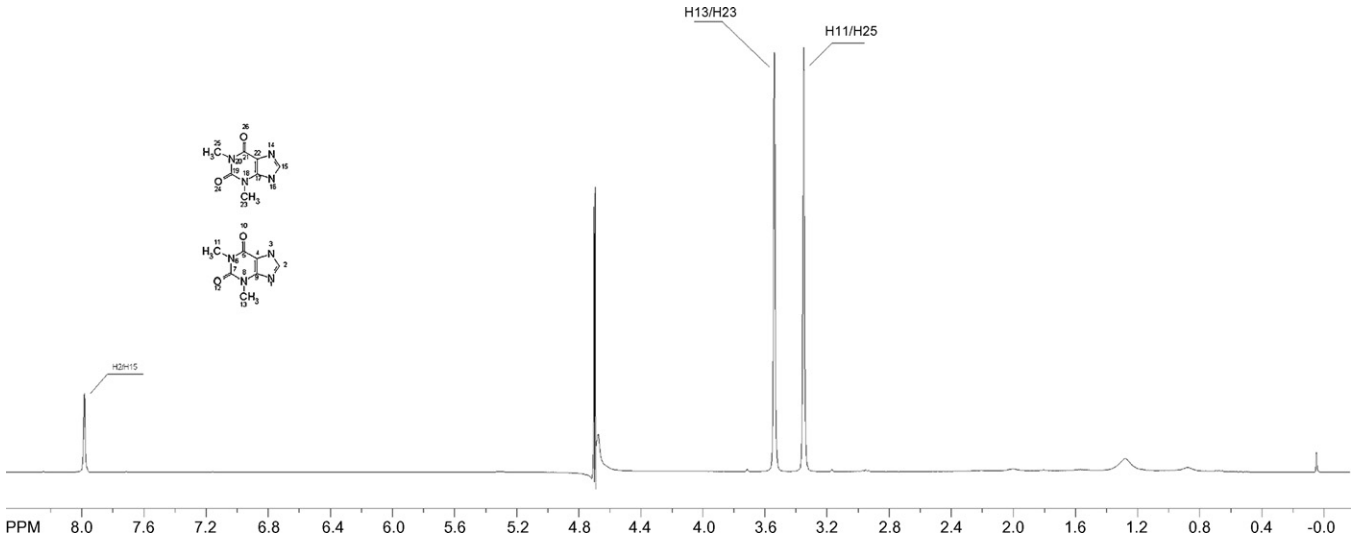

Fig. 5. ^{1}H NMR spectrum (400 MHz) of theophylline and chemical structure of the drug.

Similarly as in the previous systems (Th–HSA and Th–HSA–FA_{phys}) interaction of Th with tyrosines leads to the equalization of affinity in all observed binding sites. The Scatchard plot exhibits a linear relation (data not shown) and K_a and n obtained from the equation describing this dependence are 8.3×10^3 M^{-1} and 1.2, respectively.

Further increase of SFA (2-fold higher in reference to the above described system) results in significant changes in affinity between Th and Trp 214. All drug molecules bound in subdomain IIA have the same access to the studied amino acid residue. The run of the Scatchard plot is linear and fits the one site model of binding. Hill's coefficient is 1.0, a fact that points to the lack of ligands cooperativeness. The equalization of binding properties of albumin in subdomain IIA arises from the fact that at the used FA concentration, the third molecule of fatty acid binds to HSA (FA2) [10]. This molecule occupies subdomain IIA [16], which makes this hydrophobic pocket less spacious and probably inhibits the movement abilities of drug molecules inside the subdomain.

On observing fluorescence of both, tryptophanyl and tyrosil residues no significant change between those two systems containing SFA at increased concentration was noted. The values of K_Q and K_a are a slightly lower; the value of n remains 1.2 and of n_H shows no cooperativeness between bound ligands.

Since the results of studies done with the use of λ_{ex} 295 and 280 nm indicate the existence of one class of binding site, it seems reasonable to assume that the comparison of binding parameters obtained for both excitation wavelengths may show us the degree of tyrosine participation in drug–albumin interaction (Table 1). Quenching and association constants are about five times higher in case of λ_{ex} 280 nm and the mean number of Th molecules bound with albumin is by about 30% lower. Hence we conclude that a smaller number of drug molecules are capable of approaching tyrosines than tryptophan. However the small distance between Th and Tyr allows for a more effective energy transfer which in consequence results in a more stable interaction.

3.1.4. The influence of the increased concentration of unsaturated fatty acids on Th–HSA complex (Th–HSA–USFA)

The presence of unsaturated fatty acids (UFA) at concentration 4-fold higher than in the semi-physiological condition corresponds to the situation when FA2 molecule binds within subdomain IIA of albumin. The Scatchard plot shows the linear dependence indicating that all Th molecules bind with albumin with the same affinity (data not shown). The association and quenching constants are 1.0×10^3 and 1.3×10^3 M^{-1}, respectively when 295 nm excitation wavelength is used. Analogically, K_a and K_Q values are 7.2×10^3 and 7.8×10^3 M^{-1} for λ_{ex} 280 nm. The number of Th molecules interacting with Trp 214 equals 2.2 and decreases when tyrosil residues are taken into consideration (1.3). Hill's coefficient in both cases is ~1. This value indicates that no cooperative ligand binding occurs in the complex.

Since the initial content of USFA is about 2-fold higher than that of SFA, the described rise of USFA (4-fold) gives a concentration similar to the one reached when SFA concentration is increased 8-fold. The comparison of binding parameters (Table 1) shows that changes in the affinity of albumin towards theophylline induced by fatty acids are the result of the presence of fatty acid itself whereas the composition of the mixture is of minor importance.

When the concentration of USFA is 8-fold higher than in semi-physiological conditions the decrease of both constants, K_a and K_Q with the simultaneous increase of the number of bound molecules of the drug can be observed. This occurs when the participation of tryptophanyl residue is studied (Table 1). Hence it can be concluded that a greater number of drug molecules may interact with Trp, although the distance between these ligands and the fluorophore increases. All binding parameters estimated for this Th–HSA–USFA complex for λ_{ex} 280 nm are very similar to the ones obtained for Th–HSA–USFA complex in the case when fatty acids concentration was 4-fold higher than in physiological condition. These results indicate that participation of tyrosines in the drug–albumin interaction stabilizes the forming complex and make it resistant to the influence of the high concentration of fatty acids.

3.2. ^{1}H NMR studies

Proton magnetic resonance spectrum ^{1}H NMR of theophylline and chemical structure of the drug is shown in Fig. 5. On the basis of the chemical shift of Th proton resonance signals the dependence δ vs. [Th]/[HSA] were plotted and the molar ratio relating to the saturation of drug–albumin complex was obtained. Eq. (4) allowed us to calculate the dissociation constant which finally was used to determine the association constant (Eq. (5)) for all studied systems. The parameters are collected in Table 2.

For the drug concentration range which is associated with the complex formation i.e. complex is not saturated and albumin is able to bind new molecules of the drug (Fig. 6A), it was noted that the slope of the curve on the plot $1/(\delta - \delta_f)$ vs. [Th] changes (Fig. 6B). This phenomenon can arise from the fact that theophylline molecules

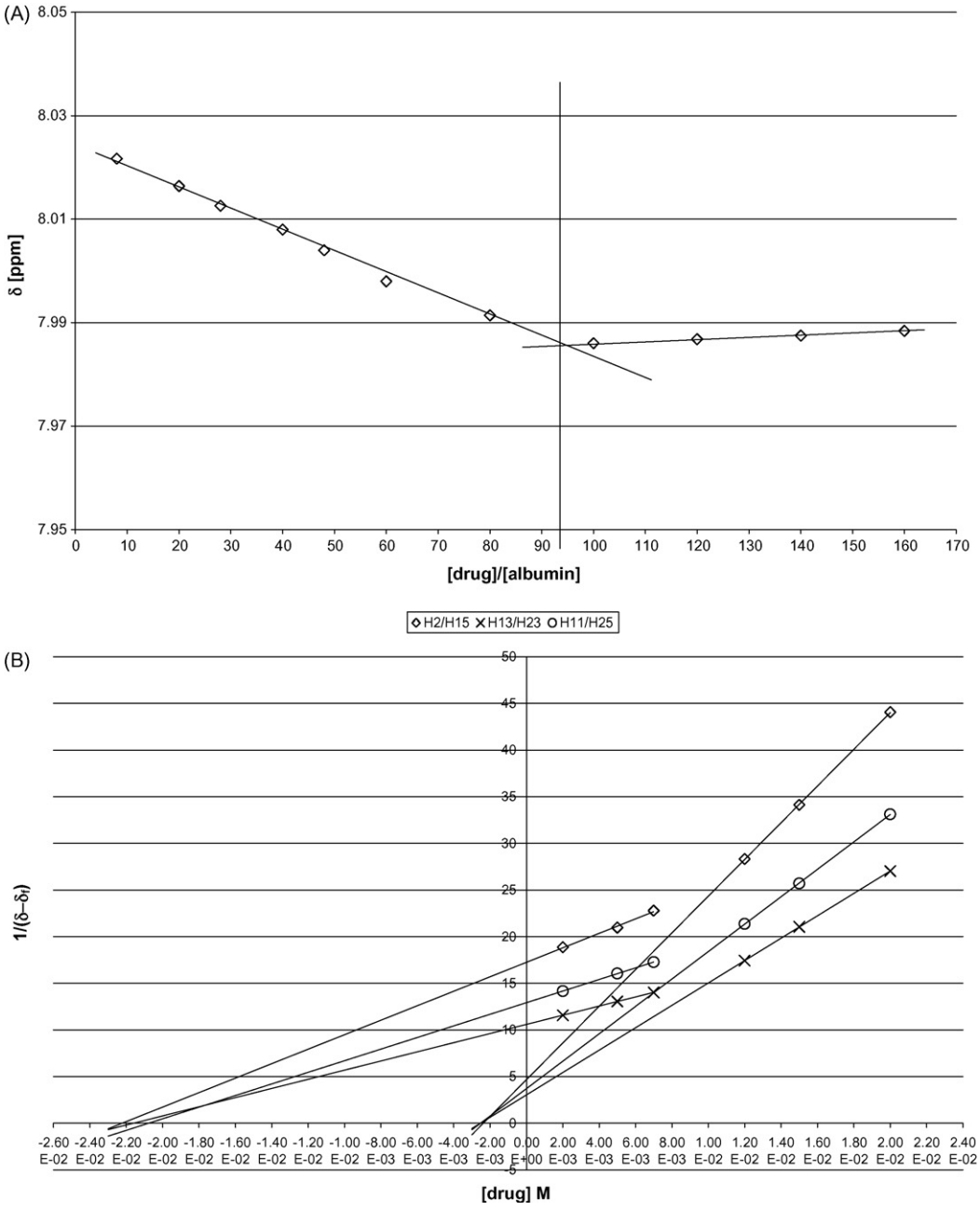

Fig. 6. Chemical shifts of proton H2/H15 resonance of Th at increasing [Th]/[HSA] molar ratio (A) and $1/(\delta - \delta_f)$ vs. [Th] dependence (B) for Th–HSA complex; HSA 2.5×10^{-4} M and Th 2×10^{-3} to 4×10^{-2} M.

locate in two classes of the low affinity binding site. For each of these classes association constant was determined (Table 2).

Binding constants for Th–HSA complex in the absence of FA are 4×10^2 and 0.5×10^2 M^{-1} for two classes, respectively. The constants are one and two orders of magnitude lower than those estimated on the basis of the fluorescence measurements. Binding sites of theophylline observed with the use of NMR technique are probably located on the surface of the albumin, whereas hydropho-

Table 2
Binding constants K_a for the independent classes of binding sites of the drug in albumin molecule and [Th]/[HSA] molar ratio corresponding to complex saturation determined for Th–HSA complex in the absence and presence of various amounts of saturated (SFA) and unsaturated (USFA) fatty acids.

	K_{aI} ($\times 10^{-2}$ M^{-1})			K_{aII} ($\times 10^{-2}$ M^{-1})			[Th]/[HSA] molar ratio complex saturation
	H2/H15	H13/H23	H11/H25	H2/H15	H13/H23	H11/H25	
Th–HSA	4.0	4.0	3.7	0.5	0.5	0.5	95
Th–HSA–FA_{phys}	2.6	2.0	2.0	0.5	0.5	0.6	95
Th–HSA–FA_{4S}	1.2	1.1	1.1	0.4	0.4	0.4	115
Th–HSA–FA_{8S}	1.1	1.0	1.0	0.3	0.3	0.3	80
Th–HSA–FA_{4US}	1.4	1.4	1.4	0.3	0.3	0.3	95
Th–HSA–FA_{8US}	0.8	0.8	0.6	0.4	0.5	0.5	75

bic pockets containing Trp 214 and Tyr 401 and 411 are the inner binding sites. The association constants calculated on the basis of chemical shifts of all observed proton resonances of theophylline have very similar or identical values for a particular class of binding sites (Table 2). This evidences that an equal participation of both rings of the drug occurs in the interaction with albumin.

The addition of FA at the concentration which corresponds to the physiological condition *in vivo* results in a 2-fold decrease of the affinity in the first class of binding site, while no changes in K_a for the second class as well as in [Th]/[HSA] molar ratio were noted (Table 2). The 4-fold increase of saturated FA concentration causes a further drop in K_a value at the first class of binding sites. Similarly as in the above studied complex, no significant changes in drug–albumin interaction at the second class of binding sites was observed. Further increase of SFA in the solution did not affect the affinity between Th and albumin in reference to the previous system (Table 2).

The unsaturated fatty acids at concentration 4-fold higher then in the semi-physiological conditions influence drug–protein interaction in the same manner as saturated FA. The association constant decreases by about 30% in comparison to Th–HSA–FA_{phys} system and by about 65% in comparison to Th–HSA system.

For the Th–HSA–FA_{8NN} system the association constant calculated for the first class of binding sites was 0.6-, 5- and 3.2-fold lower in comparison to Th–HSA–FA_{8NN}, Th–HSA and Th–HSA–FA_{phys}, respectively (Table 2). No significant changes of affinity in second class of binding sites were noted.

On the basis of ^{1}H NMR data we can conclude that the presence of fatty acid decreases the affinity of albumin towards theophylline in the low affinity binding site. The effect is not dependent on the saturation of the chain of FA, but only on the total concentration of FA in the solution. The value of the association constant estimated for the second class of binding sites indicates very weak interactions between Th and HSA reflecting a very fast exchange between the free and bound drug molecules. Additionally, the lack of a significant change of K_a in the presence of increased concentration of FA suggests that the molecules of theophylline may approach the surface of the protein and form weak interactions despite of the increasing number of FA molecules.

The equilibrium dialysis studies done by Shum and Jusko [6] showed that individual and mixed fatty acids have a minimal effect on Th binding to albumin over a normal physiological range. They also found that the binding significantly decreases with the rise of FA concentration. Changes of affinity recorded by Shum and Jusko [6] observed simultaneously for all existing binding sites can be explained by the results of our studies. The presence of fatty acids affects the binding of theophylline in its outer low affinity sites whereas interactions between the drug and albumin in Th inner site are stable and relatively resistant to FA influence.

4. Conclusions

On the basis of spectrofluorescence studies we indicated that theophylline binds in subdomain IIA of HSA with a high affinity to tryptophan and tyrosines residue. The comparison of the interaction between Th and these two amino acids allowed us to investigate the changes of the location of drug molecules within this binding site, induced by the presence of fatty acids. The influence on fatty acids on the theophylline binding to human serum albumin is dependent on the fatty acid molecules bound to the protein while the chemical structure of fatty acids contained in the solution plays a minor role. However, the impact of fatty acids on the affinity in Th–HSA complex seems to be negligible in the high affinity binding sites. We found that cooperativeness between a bound ligand in particular binding sites enhances the affinity between Th and albumin and contributes to the total stability of the complex.

It was noted that theophylline has a relatively low affinity to albumin in comparison to other drugs. The use of ^{1}H NMR spectroscopy allowed us to investigate the effect of FA on Th binding in low affinity sites. The results showed that the presence of fatty acids significantly decreases the affinity between Th and HSA. Moreover, the elevated level of FA enhances the negative effect. Similarly to the influence on binding in high affinity sites, the kind of fatty acids does not substantially influence the observed changes. We also found that theophylline may form very weak interactions with the albumin on the surface of the protein which are not affected by the presence of FA. The proved decrease of stability in Th–albumin complex may foster the replacement of theophylline by other drugs co-administered in multidrug therapy.

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