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The influence of displacement compounds on the binding of ochratoxin A to human serum albumin examined with fluorescence anisotropy methods

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The subject of the research is the application of the methods of fluorescence anisotropy measurements to study the interaction between human serum albumin (HSA) and toxins and selected medicines (ibuprofen, warfarin, flurbiprofen). Optical spectroscopic methods are useful tools for the study of biologically active compounds. Determining binding intensity of ochratoxin A (OTA) to albumin may be helpful in explaining the effects of toxic influence of OTA. The main factor influencing the distribution of OTA is its affinity for plasma proteins. It was shown that ochratoxin binds strongly to albumin. By the use of the method of fluorescence anisotropy it was proved that the unbound fraction of OTA is higher due to competing interactions with drugs. As a result of separating ochratoxin from protein by competitive compounds, a decrease in the fluorescence anisotropy of the HSA-OTA complex was observed. The largest increase in free fraction of OTA is caused by flurbiprofen, then ibuprofen and warfarin. It will accelerate OTA transport to target organs and shortening its half-life period, leading consequently to a decrease in chronic toxic effects.

Keywords: ochratoxin A, binding of drug to albumin, flurbiprofen, ibuprofen, warfarin.

1. Introduction

The biophysical properties of compounds of therapeutic value, determined by fluorescence spectroscopy methods, help to predict the behaviour of such compounds under physiological conditions [1, 2]. Fluorescence methods of determining the affinity of ligands for plasma proteins may be in many cases competitive in comparison with pre-

viously used methods. They eliminate the need for dissolution of the tested solution samples in mobile phase solvents, what enables the measurement under physiological conditions. Their main advantage is non-invasiveness, because the tested systems interact only with low intensity UV-VIS radiation. Compared to other methods, these provide a more accurate measurement of the protein-ligand affinity constant [3, 4]. It is relatively easy to analyse even a large group of samples. The most important advantage of fluorescence methods is their high sensitivity which enables to test compounds of low concentration. The disadvantage is the fact that only those compounds, the molecules of which have delocalized electrons, are subject to fluorescence.

Serum albumin is the basic protein of blood plasma. This protein serves a significant function in pharmacotherapy – it binds drugs and transports them to the tissue [5]. Albumin molecule has at least two important sites called Sudlow site I and Sudlow site II [6]. Drugs and toxins bind usually to one of this site with high affinity. The interaction between drugs and serum protein has an effect on biological half-life, metabolism and excretion of drugs. If the unbound fraction of drug diffuses across the wall of small arterioles, the equilibrium is disturbed and the bound fraction starts to separate from albumin. Drugs strongly bound to plasma proteins to a lesser extent penetrate into the organs. Drugs and toxins compete for a binding position in albumin [7]. If both ligands pretend to take the same position in albumin, there may be an increase in their free fractions. Drug–toxin interaction is pharmacologically important, because it can cause more or less toxicity of the toxin.

Ochratoxin A is a toxic chemical substance produced by certain species of moulds growing on some food products, particularly cereals. After penetrating into the circulation of blood, OTA is distributed in the whole volume of blood plasma in the course of several minutes and then it binds reversibly with albumins [8]. Determination of affinity of OTA to plasma proteins may be crucial for choosing the method of treating acute poisonings or dialysis application.

Flurbiprofen is used in the symptomatic treatment in rheumatoid arthritis, arthroses, in other chronic pains, as well as in the symptomatic treatment of respiratory tract infections with sore throat. Warfarin is an anticoagulant which belongs to the group of coumarins. It is a vitamin K antagonist. Ibuprofen is used as an anti-inflammatory and analgesic drug in diseases of locomotion system and connective tissue such as: rheumatoid arthritis, chronic polyarthritis and in other osteoarticular diseases of a degenerative origin [9].

2. Materials and methods

Flurbiprofen, warfarin, ibuprofen, ochratoxin A (OTA) and human serum albumin (HSA) were received from Sigma-Aldrich. Stock solutions of drugs were prepared in ethanol. For fluorescence anisotropy measurements, concentration of OTA in final samples was equal to 1 μM. The albumin was diluted in phosphate-buffered saline (PBS) at pH 7.4. A PTI (Photon Technology International, Birmingham, NJ, USA) spectrofluorometer

was used for the measurement of steady-state fluorescence anisotropy. Measurements of fluorescence anisotropy of OTA in HSA solution containing competing drugs were performed with the instrument in the "L-format" using excitation at 370 and 420 nm long-pass filters on the emission channel. Using of long-pass filters on the emission channel ensures the separation of fluorescence from scattering light. The single fluorescence anisotropy measurements for each albumin concentration were recorded. The temperature of the sample was kept constant (37°C) using the ultrathermostat TW2.03 (ELMI). Coefficients of determination and standard errors of slopes and intercepts were calculated with the use of ORIGIN 7.0 software application. Then, the standard error of the binding constant K was estimated.

Fluorescence anisotropy of the mixture of bound and unbound drug molecules is given by [10]

$$r = r_F f_F + r_B f_B \tag{1}$$

where r_F and r_B are the anisotropies of the free and bound drugs, respectively, f_F and f_B are the fractions of fluorescence intensity of the free and bound drugs, respectively. By rearranging Eq. (1) and assuming that the quantum yield of the drug is not changed by binding to HSA, one can obtain

$$F_B = f_B = \frac{r - r_F}{r_B - r_F} \tag{2}$$

where F_B is the fraction of drug bound to human serum albumin. Equation (2) permits on the basis of measurements of fluorescence anisotropy to calculate the fraction of bound drug. For determining the binding constant of quercetin to HSA, the following formula was used [11]:

$$K = \frac{\left[\mathrm{HSA}_B\right]}{\left[C_F\right]\left[\mathrm{HSA}_F\right]} \tag{3}$$

where HSA_B is the concentration of bound protein, while C_F and HSA_F are concentrations of free drug and free protein, respectively. By substituting $\mathrm{HSA}_F = \mathrm{HSA}_T - \mathrm{HSA}_B$ into Eq. (3), the following formula can be obtained:

$$\frac{\mathrm{HSA}_B}{\mathrm{HSA}_T} = \frac{KC_F}{1 + KC_F} \tag{4}$$

where HSA_T is the total concentration of HSA. Because $C_F = F_F C_T$ and $C_B = F_B C_T$, Eq. (4) can be rearranged yielding the modified Scatchard equation

$$\frac{F_B}{\mathrm{HSA}_T (1 - F_B)} = -\frac{F_B C_T}{\mathrm{HSA}_T} K + K \tag{5}$$

where C_T is total concentration of ochratoxin. The affinity constant can be calculated by determining a slope or intercept of a line fitted to the points ($F_B C_T / \mathrm{HSA}_T$, $F_B / [\mathrm{HSA}_T(1 - F_B)]$). By rearranging Eq. (4) to the quadratic equation, one can obtain

$$KC_F^2 + C_F(K\,\mathrm{HSA}_T - KC_T + 1) - C_T = 0 \tag{6}$$

On the basis of Eq. (6) one can estimate free and bound fractions of drugs in blood.

3. Results and discussion

The measurement of fluorescence anisotropy provides useful information about the behaviour of molecules in the presence of albumin. As a result of combining the fluorescent molecules and albumin, the possibility of rotational motion of molecules significantly decreases and the value of anisotropy will increase greatly in a function of albumin concentration. After the introduction of a ligand, there are 2 types of fluorophores in the albumin solution – bound and free. Fluorescence anisotropy of bound fluorophores is high (close to 0.3) and of free (not bound) fluorophores – low (close to 0) [10]. Figure 1 shows the changes in fluorescence anisotropy of ochratoxin depending on the concentration of albumin in the presence of ibuprofen at different concentrations. To measure the fluorescence anisotropy, ochratoxin at a concentration of 1 μM and albumin – at 0–3 μM, were used.

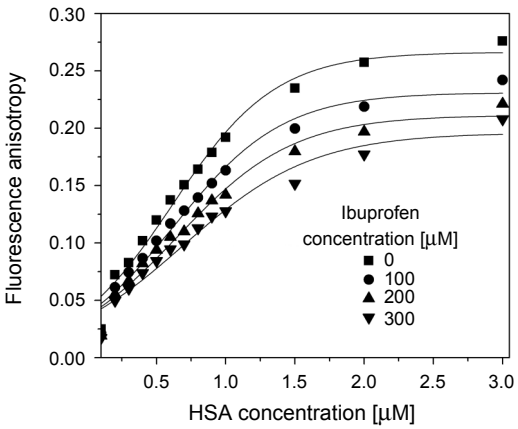

Fig. 1. Fluorescence anisotropy of OTA depending on HSA concentration in presence of ibuprofen.

For OTA alone, the value of anisotropy increased significantly with the rise in albumin concentration. At the albumin concentration of 3 μM, the value of fluorescence anisotropy is about 0.28. The increase in anisotropy proves the attachment of the toxin to the protein as the albumin concentration increases. As a result of being attached to the protein, the toxin becomes a less mobile molecule and its anisotropy increases. Adding a competing compound, ibuprofen, to the OTA-HSA complex causes a smaller anisotropy increase in the function of albumin concentration. This increase becomes

smaller with the increase in the amount of ibuprofen. At the albumin concentration of 3 μM, fluorescence anisotropy values equal 0.24, 0.22, 0.2, respectively, for ibuprofen concentrations of 100, 200 and 300 μM. It proves that ibuprofen detaches ochratoxin from the protein. OTA free molecules move faster, thus the solution anisotropy decreases. By plotting the values $F_B / [\mathrm{HSA}_T(1 - F_B)]$ *versus* $F_B C_T / \mathrm{HSA}_T$, a straight line was obtained (see Fig. 2).

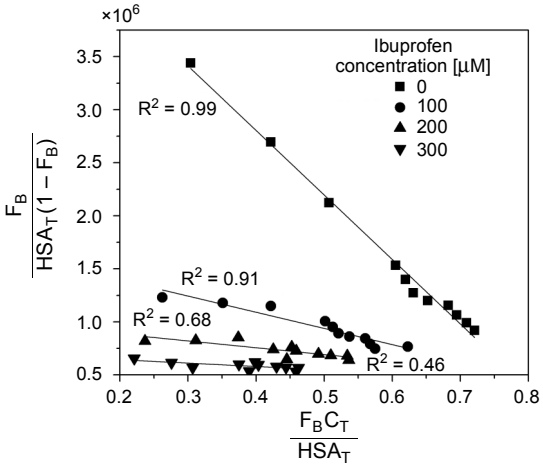

Fig. 2. The Scatchard plot obtained according to Eq. (5).

Its intersection with the axis of ordinates is, according to Eq. (5), the value of an affinity constant. Thus determined affinity constant of OTA to albumin equals 5271 mM^{-1}. On the basis of the obtained values it can be concluded that ochratoxin presents very high affinity for albumin. Assuming 640 μM as the average HSA concentration in the human blood, it can be calculated that about 99.97% of OTA is bound with HSA. In the view of the fact that the half-life of albumin in plasma is approximately 19 days and over 99% of the toxin binds with albumins, a long-term exposure to the toxin after its consumption is to be expected. OTA is still present even 2 months after the exposure [12]. Moreover, it is released from bindings with proteins mainly as a result of catabolism. Ones of the main organs involved in this process are the kidneys. This process strongly exposes kidneys to the toxin, which causes kidney failure. The toxic potential of ochratoxin A has been extensively documented in experimental studies which have revealed that a high content of the toxin in fodder resulted in nephropathy of pigs and rats [12–14]. The protein-bound part of the toxin is released with a decrease in free fraction of OTA in the body. It can be concluded that as long as OTA is albumin-bound, it does not cause any toxic effects. Only after disconnection due to protein catabolism or decreasing the free fraction, it goes into tissue and causes harmful effects.

In HSA-containing solutions to which ibuprofen was added, a significant reduction in the affinity constant of OTA to HSA can be seen. At ibuprofen concentration of 100,

200 and 300 μM, affinity constants of OTA to HSA are, respectively, 1830, 1000 and 705 mM^{-1}. It is interesting to note a larger spread of values with the increase in ibuprofen concentration. The adjustment of trendline to all test points, not as good as in the case of OTA itself, shows the changes in the binding positions in albumin under the influence of ibuprofen. The coefficient of determination drops with the increase in ibuprofen concentration. This may be due to the fact that in the presence of ibuprofen some OTA molecules bind non-specifically to HSA. Some authors claim that displaced drug rebounds to its low affinity binding site on albumin especially with large concentration of competing drug [7].

In the same manner, the affinity of OTA to albumin was determined in the presence of flurbiprofen and warfarin. The calculated values are presented in Table 1. The largest decrease in affinity constant of OTA to HSA is caused by flurbiprofen, and the smallest – warfarin. This is connected with the fact that flurbiprofen has larger affinity for albumin. It is notable that despite about seven times lower warfarin affinity to albumin as compared with ibuprofen [2], warfarin displaces ochratoxin only about 2–2.5 times weaker.

T a b l e 1. HSA affinity constants of OTA [mM^{-1}] in presence of different concentrations of flurbiprofen, ibuprofen and warfarin.

Drug	Competing drug concentration [μM]		
	100	200	300
Ibuprofen	1830 ± 190	1000 ± 130	710 ± 100
Flurbiprofen	1160 ± 130	640 ± 100	500 ± 80
Warfarin	3220 ± 270	2600 ± 190	1750 ± 170

This is connected with the fact that ochratoxin binds mainly at site I, where also warfarin is built in. However, based on the measured values of affinity constants of OTA to HSA in the presence of competing compounds, it can be concluded that they can be used to displace OTA from protein binding. It will result in an increase in the free fraction of ochratoxin in blood and cause its faster removal from the body.

Knowing the affinity constant and albumin concentration in the blood, one can solve Eq. (6), and estimate the percentage of drug bound and not bound with HSA (Fig. 3). For the concentration of ibuprofen equal to 300 μM, about a 7-fold increase in concentration of OTA free fraction will occur. Such a big increase in free fraction concentration results in serious pharmacological consequences. Since only the free fraction is pharmacologically active, it may be stated that at the concentration of flurbiprofen equal to 300 μM, OTA will induce an 11 times higher toxic effect (Fig. 3). However it will be also much faster expelled from the organism.

It seems that the influence of displacement compounds is important in the case of kidney diseases. Ochratoxin in a human body probably has a weak mutagenic effect,

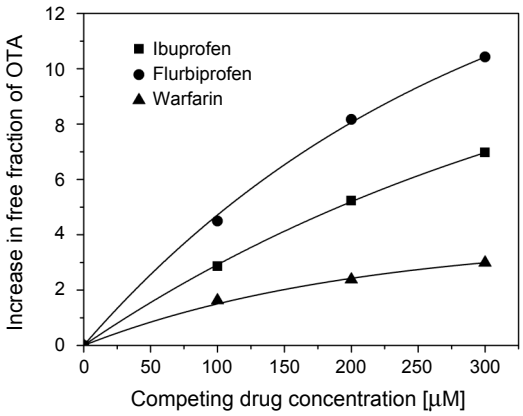

Fig. 3. Increase in free fraction of OTA in dependence on competing drug concentration.

which is caused by the induction of oxidative damage in the DNA structure [15]. The use of displacement compounds may contribute to increased short-term oxidation, but this process can be reduced by using antioxidants [16]. However, these analyses should be considered along with personal observation of the particular patient. Taking into account numerous, often very complex interactions in a body, only clinical trials can answer the question of whether the tested displacement compounds can help to reduce the side effects caused by ochratoxin.

4. Conclusion

The obtained results indicate the usefulness of a fluorescence anisotropy measurement method under *in vitro* conditions in determining the affinity constant of ochratoxin A to albumin. The compatibility of the our results with literature values can prove the correctness of the methods developed and applied in this work. It can be concluded that OTA shows a very high affinity for albumin. The data obtained from the measurements of fluorescence anisotropy of OTA-HSA complexes in the presence of competing compounds show that these compounds largely contribute to the weakening of OTA binding to albumin. It was shown that adding of drugs to the solution of OTA and HSA reduces the rate of an increase in OTA fluorescence anisotropy in dependence on HSA concentration. The degree of displacement of drugs depends on binding the affinity constant of competitive drugs to albumin and their concentration. However, the decrease in binding constant of OTA to albumin is much lower than expected. Probably OTA being displaced from a high affinity site rebinds to another site. The matter of using the competing compounds examined in this work to reduce toxicity of OTA needs further research. Displacement compounds and their possibly protective effect against the toxic effect of OTA require clinical trials of their application as an antidote. Moreover, the research shows that in pathological states with the patient

taking several drugs at the same time, parallel to the exposure to the effect of ochratoxin, a rapid release of the toxin may take place, which may lead to developing strong symptoms of poisoning.

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