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Fluorescence spectroscopic investigation of competitive interactions between ochratoxin A and 13 drug molecules for binding to human serum albumin

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ABSTRACT: Ochratoxin A (OTA) is a highly toxic mycotoxin found worldwide in cereals, foods, animal feeds and different drinks. Based on previous studies, OTA is one of the major causes of the chronic tubulointerstitial nephropathy known as Balkan endemic nephropathy (BEN) and exerts several other adverse effects shown by cell and/or animal models. It is a well-known fact that OTA binds to various albumins with very high affinity. Recently, a few studies suggested that reducing the bound fraction of OTA might reduce its toxicity. Hypothetically, certain drugs can be effective competitors displacing OTA from its albumin complex. Therefore, we examined 13 different drug molecules to determine their competing abilities to displace OTA from human serum albumin (HSA). Competitors and ineffective chemicals were identified with a steady-state fluorescence polarization-based method. After characterization the competitive abilities of individual drugs, drug pairs were formed and their displacing activity were tested in OTA-HSA system. Indometacin, phenylbutazone, warfarin and furosemide showed the highest competing capacity but ibuprofen, glipizide and simvastatin represented detectable interaction too. Investigations of drug pairs raised the possibility of the presence of diverse binding sites of competing drugs. Apart from the chemical information obtained in our model, this explorative research might initiate future designs for epidemiologic studies to gain further *in vivo* evidence of long-term (potentially protective) effects of competing drugs administered to human patients. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: competitive interaction; drug molecules; fluorescence polarization; human serum albumin; ochratoxin A

Introduction

The highly toxic mycotoxin ochratoxin A (OTA) is produced mainly by *Aspergillus* and *Penicillium* fungi and is found worldwide in cereals, plant and animal products (1–3). Its structure consists of a dihydroisocoumarin moiety linked with L-phenylalanine (Fig. 1); therefore, the molecule shows strong fluorescence. Long-term exposure to OTA results in its accumulation in human and animal body fluids and tissues as well (2,4,5). Food contamination might be reduced by different methods but the eradication of OTA from the food chain is practically impossible (6). Based on previous studies OTA is considered one of the major causes of a chronic tubulointerstitial nephropathy commonly known as Balkan endemic nephropathy (BEN) (7,8). Besides nephrotoxicity, in animal models and in cell experiments it also showed hepatotoxic, neurotoxic, immunotoxic and carcinogenic effects (9–12). The mode of action has not been fully understood. Possible mechanisms are lipid peroxidation, direct or indirect DNA damage, inhibition of protein synthesis and mitochondrial energy production, disruption of calcium homeostasis, inducing G_2 phase cell cycle arrest and provoking apoptosis and necrosis (13–17). Antioxidants (vitamin E, catechins, plant extracts, etc.) and aspartame supplementation, cholestyramine, non-steroid anti-inflammatory drugs (NSAIDs), etc. have shown positive effects in cellular models and/or in animal experiments (18–22); however, to date we still lack any preventive agents or antidotes that could be used on a long-term basis with satisfying results and without serious side effects.

It is widely known that OTA binds to human serum albumin (HSA) with very high affinity, the bound toxin exists in a dianion form (23), and the primary binding site on albumin is located in subdomain IIA (24). Plasma half-life of OTA is about 1 month in humans (corresponding approximately to the half-life of albumin). Because of the high-binding capacity and long half-life

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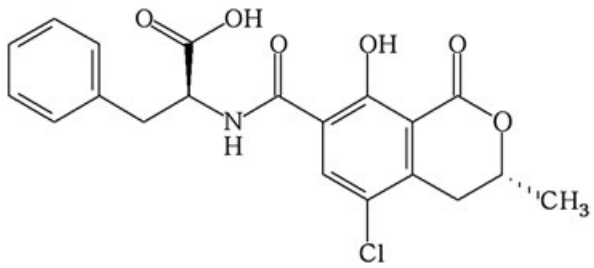

Figure 1. Chemical structure of the mycotoxin, ochratoxin A.

of the toxin (more than 99.8% of circulating OTA is bound to albumin in the human body), it possesses a high bioavailability (about 93%) (25,26). In healthy populations plasma levels are very low (0.1–2.0 ng/mL), but in endemic areas it could reach even the concentrations of up to hundred times more (50 ng/mL or even higher values) (27–29). Previous data from animal experiments suggest that reducing the bound fraction of the toxin results in its increased elimination rate, thereby shortening the half-life of circulating OTA, which in turn might alleviate its toxicity (30–32). Desorbing of OTA from albumin increases the concentration of free toxin both in blood and in urine but we hypothesize that this does not lead to an increased risk of acute toxicity (no available clinical evidence yet).

The fact that OTA is a highly fluorescent molecule enables us to follow the competition between OTA and drug molecules by a steady-state fluorescence polarization-based method. In our present research competitive interactions with 13 different drug molecules were investigated: acetylsalicylic acid (ASA), furosemide (FUR), glipizide (GLIP), ibuprofen (IBU), indometacin (IND), nifedipine (NIF), phenobarbital (PHENOB), phenylbutazone (PHENBU), phenytoin (PHENYT), simvastatin (SIM), theophylline (THEO), verapamil (VER), and warfarin (WAR). All examined drugs bind to HSA (Table 1) (33,34). Based on previous studies the binding site for several of them was also identified as subdomain IIA on albumin (35–45). Competition of WAR and NSAIDs (IBU, naproxen, PHENBU and piroxicam) with OTA for binding to HSA were published previously by Il'ichev *et al*. (24,46). In our experiments, an extensive study was designed to explore the OTA-competing ability of different drug molecules possessing various therapeutic effects and molecular structures. We are aware that these medications are incapable of preventing chronic toxicity of OTA because long-term application of them is related to numerous adverse effects. Nevertheless, the experimental results might give useful data for a better understanding of molecular behavior and competitive properties of OTA-HSA interaction. At the same time, it also gives a good starting point and an opportunity in the future to apply epidemiologic methods for investigating the long-term

Table 1. Important properties of the examined drug molecules: therapeutic effect, plasma protein binding, and normal serum levels

Drug name	Therapeutic effect	Protein-bound in plasma (%)	Peak concentration (μg/mL)
ASA	NSAID	49	24.4 ± 4.0 [a]
Ibuprofen	NSAID	>99	61.1 ± 5.5 [b]
Indometacin	NSAID	90	≈2.4 [c]
Furosemide	Diuretic	96–99	1.7 ± 0.9 [d]
Glipizide	Oral antidiabetic	98.4	0.465 ± 0.139 [e]
Nifedipine	Antihypertensive	96	0.079 ± 0.044 [f]
Phenobarbital	Antiepileptic	51	13.1 ± 4.5 [g]
Phenylbutazone	NSAID	96–99	50–100 [h]
Phenytoin	Antiepileptic	89	5–10 [i]
Simvastatin	Antihyperlipidemic	94	0.056 ± 0.025 [j]
Theophylline	Antiasthmatic	56	15 ± 2.8 [k]
Verapamil	Antihypertensive	90	≈0.272 [l]
Warfarin	Anticoagulant	99	R: 0.9 ± 0.4 [m] S: 0.5 ± 0.2 [m]

NSAID, non-steroidal anti-inflammatory drug.
[a]Following a single 1.2-g oral dose given to adults.
[b]Following a single 800-mg dose of racemate.
[c]Following a single 50-mg oral dose, given after a standard breakfast.
[d]Following a single 40-mg oral dose (tablet).
[e]Following a single 5-mg oral dose (immediate-release tablet) given to healthy young adults.
[f]Mean value following a single 10-mg immediate-release capsule given to healthy male adults.
[g]Mean steady-state concentration following a 90-mg oral dose, given daily for 12 weeks to patients with epilepsy.
[h]Steady-state plasma concentration following a 200-mg oral dose, given daily for 2 weeks to 56–65-year-old adults.
[i]Population frequency of total phenytoin concentrations following a 300-mg oral dose (capsule), given daily to steady state.
[j]Data for total inhibitors following a 40-mg oral dose, given once daily for 17 days to healthy adults.
[k]Following a 400-mg oral dose given twice a day for 5 days.
[l]Mean data following a 120-mg oral conventional tablet given twice a day.
[m]Mean steady-state, 12-h postdose concentrations of warfarin enantiomers following a daily oral dose of 6.1 ± 2.3 mg of racemic warfarin, given to patients with stabilized (1–5 months) anticoagulant therapy.

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(potentially protective) effects of competing drugs administered to human patients who use them therapeutically. Characterization of the biological behavior of these competitors in the human body is very important because recent studies strongly support the phenomenon that OTA can show very different kinetic parameters in the case of several species (25). For example, the half-life of albumin in different species or the bound fraction of Ochratoxin A and binding affinity of OTA to albumins might be very diverse. Therefore, we assume that, for example, data from rat experiments can not be directly extrapolated to humans in this case. In our study with human albumin, we will show that IND, PHENBU, WAR and FUR are the most potent competitors; however, IBU, GLIP and SIM also exerted considerable interaction. One has to bear in mind that OTA represents only a few nmol/L concentration in plasma while the above drugs are circulating in blood at several hundreds of nmol/L or more (μmol/L) (Table 1) reaching a much higher concentration of drug–OTA ratio than used in our experiments.

Experimental

Reagents

A 5000 μmol/L stock solution of Ochratoxin A (Sigma-Aldrich, Budapest, Hungary) was prepared in 96% ethanol (Reanal, Budapest, Hungary, spectroscopic grade) and kept at 4°C protected from light. HSA, GLIP, FUR, NIF, SIM, VER and WAR (all Sigma-Aldrich), acetylsalicylic acid, IBU, IND, PHENOB, PHENBU, PHENYT, THEO (all Hungaropharma, Budapest, Hungary) were used as received. Stock solutions (2500 μmol/L) of drugs were prepared in 96% ethanol. Phosphate-buffered saline (PBS) contained NaCl (137 mmol/L), KCl (2.7 mmol/L), NaH_2PO_4 (8 mmol/L), K_2HPO_4 (1.5 mmol/L) in tridistilled water (pH 7.4). Ammonium acetate buffer contained acetic acid (16 mM) and the amount of 2.5% ammonium hydroxide solution needed to reach the desired pH value (pH 4.7).

Instrumentation

Hitachi F-4500 fluorescence spectrophotometer was used to determine spectra and steady-state fluorescence polarization data. Spectra applied for calculation the binding constants were measured by Fluorolog τ3 spectrofluorimetric system (Jobin-Yvon/SPEX, Longjumeau, France). In this case, 5 nm spectral bandwidths for excitation and emission with right angle detection have been applied. All analyses were performed in the presence of air at +25°C.

Data analyses

Steady-state fluorescence polarization data were used to quantify the interaction of OTA with drugs. The degree of fluorescence polarization was calculated as

$$P = \frac{(I_{VV} - G * I_{VH})}{(I_{VV} + G * I_{VH})} \quad (1)$$

where I_{vv} means the fluorescence intensities measured in vertical excitation polarizer and vertical emission polarizer settings, while I_{vh} is the intensities measured at vertical excitation and crossed emission polarizer settings. G is the actual measured instrument factor. For calculating the degree of polarization, 30 measuring points were averaged.

OriginPro8 software (OriginLab Corp., Northampton, MA, USA) was used for background correction of fluorescence emission spectra and to evaluate fluorescence intensities.

Spectral analyses of Ochratoxin A in the absence and presence of human serum albumin

Excitation maxima of free monoanionic and free dianionic form of OTA were at 334 and 380 nm, respectively, while at saturating conditions with HSA (100% bound toxin) its maximum was at 393 nm (Figs 2 and 3). The emission maxima of both the free and bound forms were very similar: 444 nm (free dianionic OTA) and 446 nm (OTA + HSA), respectively. HSA did not show any excitability or emission within these wavelength intervals. When increasing concentrations of HSA were added to standard 1 μmol/L OTA, our experiments demonstrated that complete saturation was reached at a 1.6-fold or higher concentration of HSA vs. OTA (data not shown).

Comparing the fluorescence polarization-based method (method A) with two intensity-based approaches (methods B and C).

Method A (phosphate-buffered saline, pH 7.4). In this part of the study, increasing amounts of HSA was added to 1 μmol/L OTA solutions and fluorescence polarization values were measured at 393 and 446 nm wavelengths. PBS was used to approximate physiological conditions. Polarization values of albumin-free OTA solution in PBS ($P = 0.011–0.015$) were provided as the data corresponding to no bound toxin (0% saturation)

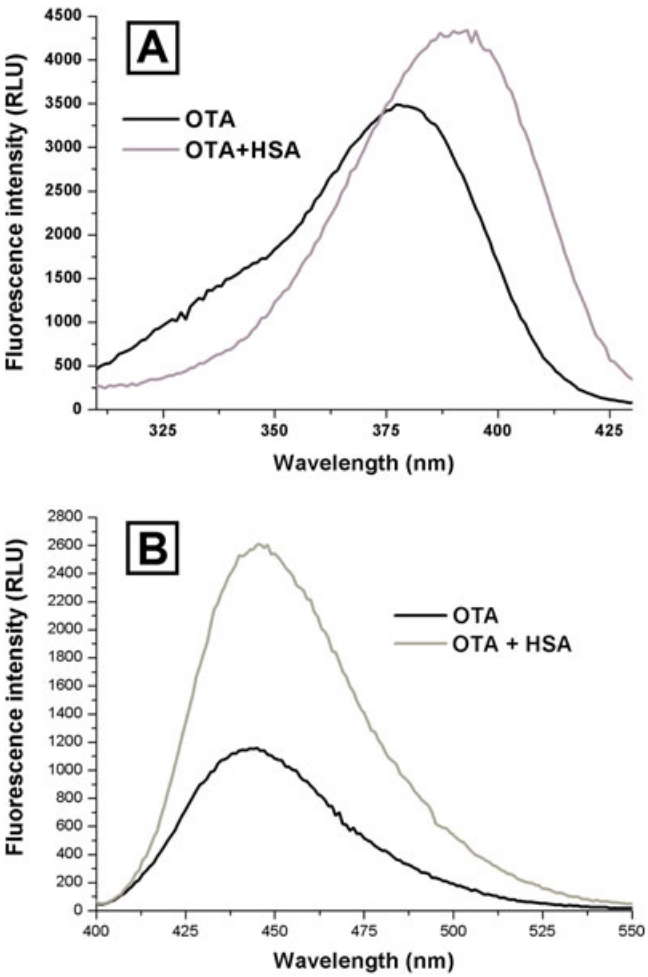

Figure 2. Fluorescence excitation (a) and emission (b) spectra of free (black) and albumin-bound (gray) OTA in phosphate-buffered saline (pH7.4) [OTA (1 μmol/L): $\lambda_{exc} = 380$ nm, $\lambda_{em} = 444$ nm; OTA (1 μmol/L) + HSA (2 μmol/L): $\lambda_{exc} = 393$ nm, $\lambda_{em} = 446$ nm]. HSA, human serum albumin; OTA, ochratoxin A.

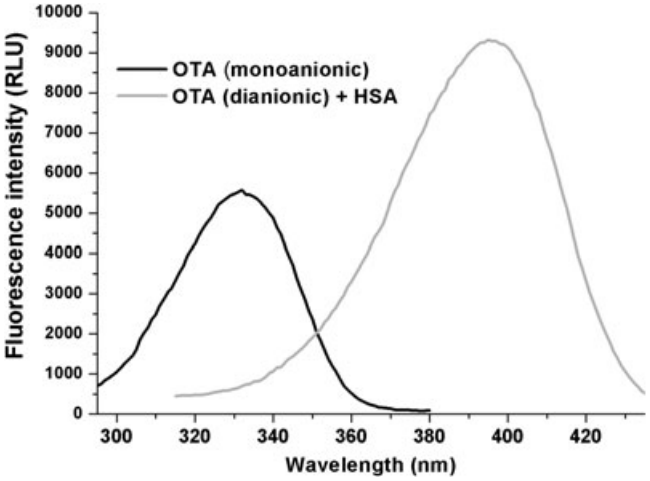

Figure 3. Fluorescence excitation spectra of monoanionic (black) and bound dianionic (gray) OTA in ammonium acetate buffer (pH4.7) [OTA (1 μmol/L): $\lambda_{exc} = 334$ nm, $\lambda_{em} = 451$ nm; OTA (1 μmol/L) + HSA (2 μmol/L): $\lambda_{exc} = 393$ nm, $\lambda_{em} = 446$ nm]. HSA, human serum albumin; OTA, ochratoxin A.

in the samples. Complete saturation of albumin with OTA (100%) was defined when polarization values ($P = 0.326$–0.333) did not increase any more upon addition of further amounts of HSA to the solution. A polarization-based approach was performed and binding parameters determined as described earlier by Il'ichev *et al*. (46):

$$\alpha = \frac{(P - P_f)}{[\theta * (P_b - P) + P - P_f]} \quad (2)$$

where α is the bound fraction of the toxin, P is the measured polarization, P_f and P_b are fluorescence polarization values of free and bound OTA, respectively. Furthermore:

$$\theta = \frac{\varepsilon_b * \Phi_b}{\varepsilon_f * \Phi_f} \quad (3)$$

where ε_b and ε_f are the molar absorptivities of bound and free toxin, Φ_b and Φ_f are the fluorescent quantum yields of bound and free OTA.

Method B (phosphate-buffered saline, pH 7.4). Using intensity-based experimentation, increasing amounts of HSA was added to 1 μmol/L of OTA solutions in PBS. Fluorescence emission spectra were recorded ($\lambda_{exc} = 393$ nm; $\lambda_{em} = 446$ nm). Fluorescence emission intensity of 1 μmol/L OTA in the absence of albumin was defined as the value for zero saturation (no bound toxin in the sample). On the other hand, fluorescence intensity for complete saturation of albumin with OTA (100%) was defined in albumin concentrations when emission intensities did not increase any more by further addition of HSA. Our data showed that about 1.6 times higher molar concentration of HSA vs. toxin was needed for the total saturation. The emission spectra of free OTA were determined first at five different concentrations (0.5–2.5 μmol/L) and intensities were plotted against OTA concentrations. Then all of these OTA concentrations were measured in the presence of HSA at above the saturating concentration (two times the molarities of OTA). The emission spectra were recorded and intensities were plotted as the function of OTA concentration. The linear equations are:

$$I_f = a_1 * c_f + b_1 \quad (4)$$

$$I_b = a_2 * c_b + b_2 \quad (5)$$

where I_f and I_b are the measured intensities of the free and bound toxin respectively, c_f and c_b are the concentrations of free and bound OTA, a_1 and a_2 are the slopes of the lines, b_1 and b_2 are the intercepts. Using eqns (4) and (5), we can define the following:

$$\Sigma I = I_f + I_b = (a_1 * c_f + b_1) + (a_2 * c_b + b_2) \quad (6)$$

$$\Sigma c = c_f + c_b \quad (7)$$

where ΣI is the measured intensity of the samples, Σc is the weighed OTA concentration. From these two equations, we can define the concentration of bound toxin, and the percentage of binding as follows:

$$c_b = [\Sigma I - a_1 * \Sigma c - (b_1 + b_2)]/(a_2 - a_1) \quad (8)$$

$$binding(\%) = 100 * c_b/\Sigma c \quad (9)$$

Method C (ammonium acetate buffer, pH 4.7). Unfortunately, bound and free dianionic OTA show the same emission maxima; therefore, fluorescence emission intensities of the two forms cannot be measured separately. To overcome this difficulty ammonium acetate buffer was used (pH 4.7). Under these circumstances, OTA is represented only in monoanionic form, but it is well known that OTA can bind to albumin only in the dianionic form (23). Separate detection of free monoanionic and bound dianionic OTA is possible as there is a large difference between the two excitation maxima (334 and 393 nm; Fig. 3). Linearity of the fluorescence emission of free OTA and OTA-HSA system was studied at six different OTA concentrations (0.5–2.5 μmol/L) in ammonium acetate buffer, at pH 4.7 without albumin ($\lambda_{exc} = 334$ nm, $\lambda_{em} = 451$ nm) and with HSA ($\lambda_{exc} = 393$ nm, $\lambda_{em} = 446$ nm). The applied

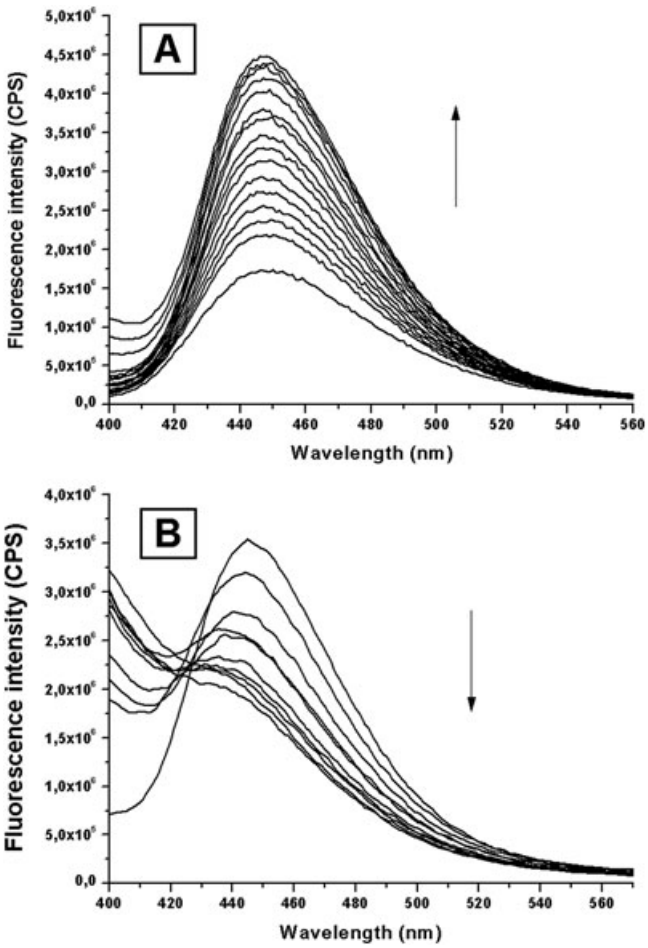

Figure 4. Emission spectra of OTA (1 μmol/L) upon the addition of increasing amounts of HSA (0–3 μmol/L) (a). Emission spectra of 1 μmol/L OTA in presence of 1.7 μmol/L HSA upon the addition of indometacin (0–100 μmol/L) (b). All measurements were done in phosphate-buffered saline, pH 7.4 ($\lambda_{exc} = 380$ nm, $\lambda_{em} = 446$ nm). HSA, human serum albumin; OTA, ochratoxin A.

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albumin concentrations were above the saturation level (two times the molarities of OTA). The fluorescence emission intensities of OTA showed concentration-dependent linear behavior ($R^2 = 0.999$) both for the free (monoanionic) and the albumin bound (dianionic) forms. Saturation characteristics of OTA–albumin systems were followed by adding increasing concentrations of HSA to standard amounts of OTA (1 μmol/L). Using 393 nm excitation and 446 nm emission wavelengths fluorescence emission intensities were analyzed; hence, the amount of bound dianionic OTA was selectively measured. Our data showed that under these conditions complete saturation of HSA with OTA could also be reached when the albumin–OTA molar ratio exceeded a value of approximately 1.6. The fluorescence properties, including polarization values of OTA–albumin complexes and saturating albumin concentrations were practically identical in both ammonium acetate buffer (pH 4.7) and in PBS (pH 7.4). Therefore, we hypothesize that the same saturation characteristics of the OTA-HSA complex could be observed in the case of the two buffers. Thereafter, fluorescence emission intensities corresponding to 100% bound OTA were determined by using OTA-HSA complexes at the minimal saturating concentration of albumin and four more concentrations above it. Emission intensities were averaged ($n = 5$) and accepted as I_{max} (100% OTA is bound). As the linearity between concentration and fluorescence emission intensity was previously verified, the percentage of bound OTA could be determined for each different albumin concentration as follows:

$$\text{Binding } (\%) = 100 * I/I_{max} \tag{10}$$

Table 2. Binding characteristics of OTA (1 μmol/L) to HSA in phosphate-buffered saline (pH 7.4; methods A and B) and in ammonium acetate buffer (pH 4.7; method C): The percentage of bound fraction of OTA was expressed in the presence of increasing HSA concentrations (0.10–1.63 μmol/L). Three independent experimental data were averaged for all three methods (explanation in the text). SD values are represented in parentheses

HSA concentration (μmol/L)	Method A (bound fraction of OTA in %)	Method B (bound fraction of OTA in %)	Method C (bound fraction of OTA in %)
0.10	8.4 (±0.8)	7.5 (±0.8)	7.7 (±0.4)
0.25	19.6 (±0.6)	18.9 (±0.4)	18.9 (±0.6)
0.38	29.0 (±2.0)	28.2 (±1.5)	28.3 (±0.4)
0.50	37.1 (±4.0)	37.1 (±1.2)	37.4 (±0.7)
0.63	48.0 (±0.3)	48.1 (±1.8)	47.1 (±0.8)
0.75	60.9 (±2.0)	58.5 (±1.0)	57.0 (±0.2)
0.88	67.7 (±2.4)	68.6 (±3.5)	66.1 (±0.9)
1.00	76.3 (±3.4)	78.1 (±1.5)	76.3 (±0.6)
1.13	85.6 (±0.8)	86.8 (±4.0)	85.9 (±0.3)
1.25	92.2 (±2.1)	94.7 (±2.2)	95.0 (±0.2)
1.38	97.1 (±1.9)	97.8 (±1.9)	98.0 (±0.3)
1.50	98.7 (±0.9)	98.8 (±1.3)	99.4 (±0.4)
1.63	100.0 (±0.0)	99.9 (±2.8)	100.1 (±0.3)

HSA, human serum albumin; OTA, Ochratoxin A.

Table 3. Measured fluorescence polarization (*P*) data of OTA-HSA system (OTA: 1 μmol/L, HSA: 1.7 μmol/L) in phosphate-buffered saline (pH7.4) in the absence and in the presence of 40, 70 and 100 μmol/L drug concentrations. The decrease of *P* values indicates the molecular displacement of OTA from HSA. Results and SD values were calculated from seven independent measurements

	Fluorescence polarization (40 μmol/L drug)	Fluorescence polarization (70 μmol/L drug)	Fluorescence polarization (100 μmol/L drug)
Control	0.332 ± 0.003	0.328 ± 0.003	0.327 ± 0.006
ASA	0.329 ± 0.005	0.328 ± 0.004	0.321 ± 0.005
Ibuprofen	0.314 ± 0.006	0.297 ± 0.005	0.279 ± 0.007
Indometacin	0.289 ± 0.006	0.244 ± 0.006	0.203 ± 0.006
Furosemide	0.306 ± 0.002	0.283 ± 0.005	0.266 ± 0.003
Glipizide	0.315 ± 0.006	0.306 ± 0.005	0.292 ± 0.008
Nifedipine	0.324 ± 0.002	0.321 ± 0.002	0.314 ± 0.004
Phenobarbital	0.326 ± 0.003	0.326 ± 0.004	0.322 ± 0.004
Phenylbutazone	0.286 ± 0.002	0.260 ± 0.005	0.237 ± 0.007
Phenytoin	0.326 ± 0.004	0.322 ± 0.003	0.315 ± 0.003
Simvastatin	0.312 ± 0.003	0.301 ± 0.004	0.296 ± 0.003
Theophylline	0.330 ± 0.003	0.327 ± 0.002	0.323 ± 0.002
Verapamil	0.328 ± 0.004	0.325 ± 0.004	0.321 ± 0.003
Warfarin	0.295 ± 0.005	0.274 ± 0.002	0.253 ± 0.007

HSA, human serum albumin; OTA, ochratoxin A.

where I is the actual measured emission intensity, I_{max} is the intensity of completely bound OTA.

Spectral properties of drugs in the absence and in the presence of Ochratoxin A and/or human serum albumin

All measurements were done in PBS at pH 7.4. Fluorescence excitation and emission spectra of drug solutions (100 μmol/L) were determined in the absence and presence of OTA (1 μmol/L), HSA (1.7 μmol/L) and OTA + HSA at saturating conditions. Our results showed that at 100 μmol/L concentration of drugs (the highest concentration used) and within the wavelength range of interest ($\lambda_{exc} = 393$ nm, $\lambda_{em} = 446$ nm) there were no detectable fluorescent signals (data not shown). Fluorescence spectra of OTA in the presence of drug molecules were not amended indicating zero interactions between drugs and OTA. Drugs and HSA alone in the absence of OTA ($\lambda_{exc} = 393$ nm, $\lambda_{em} = 446$ nm) showed no fluorescent signal. Nevertheless, the drug-OTA-HSA system gave slightly diverse excitation spectra in those cases where notable competitive interaction was detected (because of the different excitation spectral properties of free and protein-bound OTA); while in all cases the emission maxima were practically unchanged.

Competitive interaction between Ochratoxin A and drugs for human serum albumin

The fluorescence polarization-based method (46) was used to investigate the competitive interaction between drugs and OTA for HSA. Standard concentrations of OTA (1 μmol/L) and HSA above the saturating concentration (1.7 μmol/L) at three different drug levels were studied (40, 70, 100 μmol/L, respectively). Ethanol (final concentrations were a maximum of 4

Table 4. Calculated bound fractions of OTA in percentage from the polarization data based on method A (OTA: 1 μmol/L; HSA: 1.7 μmol/L; in phosphate-buffered saline) in case of 40, 70 and 100 μmol/L drug concentrations. Results show that some of the drug molecules are able to significantly decrease the amount of albumin-bound OTA. Results and SD were averaged from seven independent measurements

	Bound fraction of OTA in % (40 μmol/L drug)	Bound fraction of OTA in % (70 μmol/L drug)	Bound fraction of OTA in % (100 μmol/L drug)
Control	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
ASA	98.5 ± 1.5	99.8 ± 2.3	97.2 ± 2.7
Ibuprofen	89.2 ± 3.4	82.6 ± 2.1	74.6 ± 2.7
Indometacin	76.4 ± 2.8	58.8 ± 2.7	45.0 ± 2.2
Furosemide	84.4 ± 1.8	76.1 ± 2.4	69.9 ± 2.1
Glipizide	90.3 ± 2.4	87.9 ± 1.7	82.5 ± 2.4
Nifedipine	95.0 ± 2.4	95.9 ± 1.1	93.6 ± 2.5
Phenobarbital	96.7 ± 2.3	98.8 ± 1.8	97.6 ± 3.8
Phenylbutazone	74.4 ± 1.2	65.6 ± 1.8	59.0 ± 1.8
Phenytoin	97.1 ± 2.1	96.4 ± 0.8	92.6 ± 2.8
Simvastatin	88.7 ± 1.8	84.5 ± 1.8	83.6 ± 2.8
Theophylline	98.8 ± 1.8	99.5 ± 1.4	96.7 ± 2.3
Verapamil	97.6 ± 3.2	98.3 ± 1.3	95.7 ± 2.9
Warfarin	78.6 ± 2.7	71.7 ± 0.9	65.0 ± 2.7

HSA, human serum albumin; OTA, ochratoxin A.

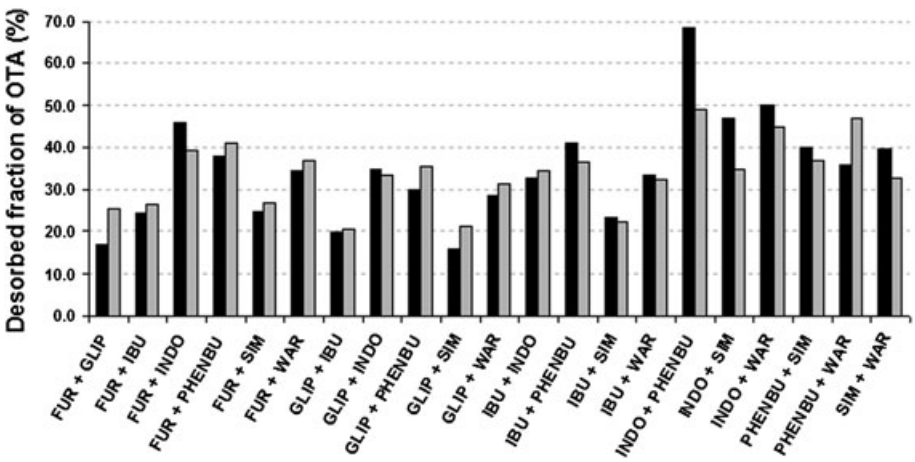

Figure 5. Desorbed fraction of OTA (%): measured (black) and calculated (gray) values (OTA: 1 μmol/L, HSA: 1.7 μmol/L, drugs: 40–40 μmol/L) in phosphate-buffered saline, pH 7.4. ASA, acetylsalicylic acid; FUR, furosemide; GLIP, glipizide; HSA, human serum albumin; IBU, ibuprofen; IND, indometacin; NIF, nifedipine; OTA, ochratoxin A; PHENBU, phenylbutazone; PHENOB, phenobarbital; PHENYT, phenytoin; SIM, simvastatin; THEO, theophylline; VER, verapamil; WAR, warfarin.

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v/v %) was used to facilitate dissolution of drugs. Control samples were spiked with the same ethanol concentrations as were used for the dissolution of drugs. Binding parameters were determined at the optimal excitation and emission wavelengths of bound OTA (393 and 446 nm, respectively). Seven independent measurements were used to calculate the means and the standard deviations (SD).

To confirm our results the displacing capacity of the most potent competitor, IND was quantified. Fluorescence emission spectra were determined using 380 nm excitation wavelength for free OTA (in PBS; OTA: 1 μmol/L, HSA: 1.7 μmol/L). Figure 4 shows the emission spectra of the OTA-HSA system alone (Fig. 4a) and in the presence of 20, 30, 40, 50, 60, 70, 80, 90 and 100 μmol/L IND (Fig. 4b). Using these spectra the binding constants (logK) were determined by the Hyperquad program package (47). A simplified 1:2 stoichiometry of both HSA-OTA and HSA-IND complexes was used as the model according to Perry *et al*. (48).

Competition between Ochratoxin A and drug pairs for human serum albumin

In spite of the fact that the accurate binding sites of tested drugs are not precisely characterized on HSA in the next series of experiments seven chemicals exerting visible competitive interaction with OTA for HSA (FUR, GLIP, IBU, IND, PHENBU, SIM and WAR) were examined in pairs. Besides the standard amount of OTA (1 μmol/L) and HSA (1.7 μmol/L) equal amounts of the drug pairs were added to the system (40–40 μmol/L, using as a rule). Measurements were done under the same conditions as for single competitors above; five independent data were used for evaluation. After determination of the actual binding parameters in the samples, hypothetical additive effects (desorbed fraction of OTA in percentage) were also calculated (using the results of Table 3) as follows: desorbed fractions of OTA at 40 μmol/L level of the individual drugs used for pair formation were summed.

Results and discussion

The results of the three saturation methods (A–C) are shown in Table 2 where the calculated bound fraction of OTA (%) is expressed as the function of HSA concentration. Our intensity-based experimental methods verified the suitability of the known fluorescence polarization method.

Based on our experiments, six drug molecules showed no or almost no interaction: ASA, NIF, PHENOB, PHENYT, THEO and VER. The other seven substances showed detectable competition and the ability to displace OTA from HSA (results are shown in Tables 3 and 4 where the measured polarization data were illustrated and the bound fraction of OTA is expressed in percentage of the complete saturation). The most conspicuous effect was represented by two NSAIDs: IND and PHENBU. At drug concentrations of 40 μmol/L, both IND and PHENBU showed similar displacing abilities. However, at higher levels (70 and 100 μmol/L, respectively) IND proved to be a stronger competitor than PHENBU. In competing efficiency the two former NSAIDs were followed by WAR, FUR and IBU (in this order). Finally, GLIP and SIM can be mentioned with a lower but measurable effect. It is noteworthy that WAR, which contains a coumarin structure too (OTA presents a dihydroisocoumarin moiety), showed weaker competitive properties than IND and PHENBU.

The logK values of HSA-OTA and HSA-IND complexes are 11.84 ± 0.14 and 9.51 ± 0.04, respectively. As lower logK value is associated to the HSA-IND complex formation, to get effective competition a much higher IND than OTA concentration is needed. However, considering the complex protein–ligand binding mechanism further study is essential to give detailed description about the exchange reaction of OTA by IND on the HSA surface.

When drug pairs were analyzed, we compared the measured and calculated data as well. Data are shown in Fig. 5 where, unlike in Table 4, the desorbed fractions of OTA are expressed in percentages. Drug molecules in pairs were applied to test the hypothesis that additive or less effect could be expected in competing ability knowing that the applied drugs had the same or at least nearby binding sites on albumin. This assumption proved to be true in most of the cases; nevertheless, considerable positive differences (calculated value subtracted from measured value) were observed in the case of IND-PHENBU (19%) and IND-SIM (12%) pairs. It was clearly seen that the 40–40 μmol/L concentrations of IND and PHENBU together gave a higher effect than the 100 μmol/L IND in the absence of PHENBU. Furthermore, similar but lower differences were shown for FUR-IND (7%), SIM-WAR (7%), IBU-PHENBU (5%) and IND-WAR (5%) pairs. This phenomenon can be explained by the possibility of the presence of distinct binding sites within subdomain IIA (it may also be feasible that some of the studied drugs are not competitive antagonists but rather allosteric modulators). This assumption has not been verified and is not available for us yet.

In conclusion, the interaction between OTA and drug molecules to compete for HSA was examined with a steady-state fluorescence polarization-based method. IND, PHENBU, WAR and FUR showed the highest competing capacity but IBU, GLIP and SIM represented considerable interaction too. Data obtained for the combined addition of drugs in pairs suggest that some of these drugs may occupy different binding sites on albumin. We would like to apply this explorative investigation in the future to examine the effect of these drugs to OTA toxicity in humans using epidemiologic approaches.

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