Research article

Binding of harmane to human and bovine serum albumin: fluorescence and phosphorescence study

Krystian Gałęcki,[1*] Biljana Despotović,[2] Céline Galloway,[3] Angelos-Gerasimos Ioannidis,[4] Tahereh Janani[5], Yuki Nakamura,[6] Grace Oluyinka[7]

[1] Institute of General Food Chemistry, Lodz University of Technology, 90-924 Lodz, Poland
[2] University of Sarajevo, 71000, Sarajevo, Bosnia and Hercegowina
[3] University of Newcastle Upon Tyne, NE1 7RU, Newcastle Upon Tyne, England
[4] Agricultural University of Athens, 11855, Athens, Greece
[5] Norwegian University of Science and Technology, 7491, Trondheim, Norway
[6] Kyushu University, 8120053, Fukuoka, Japan
[7] University of Greenwich, SE10 9LS, London, England

*kgalecki87@gmail.com

Abstract: *The binding of harmane with human serum albumin (HSA) and bovine serum albumin (BSA) were studied by fluorescence and phosphorescence spectroscopic methods. Quenching of fluorescence of serum albumins by harmane was found to be a static quenching process. The equilibrium constant (K) of complex formation was found to be equal to* $(5.16\pm0.28)x10^4$ M^{-1} *and* $(4.32\pm0.30)x10^4$ M^{-1} *for HSA and BSA, respectively. It was found that the interactions of harmane with HSA and BSA were also in the excited triplet state. The determined bimolecular constant or triplet state quenching* (k_q^T)*of the proteins studied by harmane was* $(1.15\pm 0.10)x10^7$ M^{-1} s^{-1} *and* $(2.88\pm0.22)x10^7$ M^{-1} s^{-1} *for HSA and BSA, respectively. Based on the similar value of K and* k_q^T *for HSA and BSA, a possible suggestion is that, most probably, the binding site of harmane is located in the drug site 1 in the subdomain IIa.*

Keywords: *human and bovine serum albumin, harmane, fluorescence, phosphorescence.*

Introduction

Harmane (1-methyl-β-carboline) (Figure 1) is one of the derivatives of alkaloids which are produced naturally by plants. It has been found in alcoholic beverages, well-cooked foods and tobacco smoke. According to previous researches, β-carboline has been reported as a normal constituent of human tissues and body fluids [1]. Also β-carboline affects a variety of biochemical, psychopharmacological, and behaviors in animals and humans [2].

The applications of alkaloids involve in biological control of herbivores; they also have pharmacological veterinary and medical interest [3]. For instance,

a wide range of pharmacological properties including antimicrobial [4], anti-HIV [5] and antiparasitic properties [6,7] have been found in some alkaloids belonging to the β-carboline group. Harmane has some biochemical action including inhibition of monoamine oxidase [8] and cytochrome P-450 [9], and binding to benzodiazepine [10] and serotonin receptors [11]. Funayama et al. [12] demonstrated that the relaxation activity of DNA topoisomerase I and II is inhibited by this compound.

For instance, harmane binds with high affinity to benzodiazepine receptors and tryptamine sites in the brain of rats [13] and will evoke an anxiety-like state [14] which is attenuated by a benzodiazepine [15]. Furthermore, harmane is a monoamine-oxidase (MAO) inhibitor (inhibitor of MAO-A and MAO-B, respectively) which acts to modify the addictive effects of nicotine and effects on motor activity. Harmane binds with several bio-molecules such as serum albumins.

Serum albumins are proteins located within the plasma and, are present in vast quantities [16]. These proteins have numerous functions including carrier proteins, maintenance of pH and regulation of osmotic pressure within the extra-vascular fluids [17]. Serum albumins contain three homologous helical domains (I–III), each divided into A and B subdomains. The chemical structures of human serum albumin (HSA) and bovine serum albumin (BSA) are very similar, they share 76% homology [18], and both contain tryptophanyl residues (Trp). BSA has two of these residues; Trp 134 and Trp 213, whereas HSA contains only one; Trp 214 [19]. Serum albumins can bind to a large number of substances including endogenous and exogenous molecules thus the binding of drugs is of key interest [17]. In HSA and BSA structure two binding sites of drugs (1 and 2), located in subdomains IIA and IIIA, respectively can be distinguished. Both the drug binding sites are topologically similar but they differ in shape, size and polarity. Tryptophanyl residues Trp 213 and Trp 214 in HSA and BSA, respectively are located in the site 1 [20, 21].

Until now the interactions between harmane and serum albumins were analyzed by the use of UV-Vis and FTIR spectroscopy. Recent studies suggest that BSA can be considered a good carrier for transportation of β-carboline alkaloids [22].

The aim of this work is to study the interactions between harmane and serum albumins by the use of fluorescence and phosphorescence spectroscopy. This should give information about the mechanism in the ground and excited state interaction. A precise knowledge of drug-biomolecular interactions is crucial for a rational design of pharmaceuticals [17].

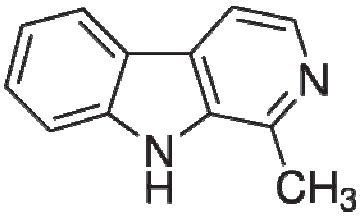

Figure 1. Structure of harmane

Experimental

Materials

Harmane, BSA, HSA, potassium iodide and sodium sulphite used in this study were commercial products of Sigma-Aldrich and were used as received. All chemicals were dissolved in 0.05 M phosphate buffer (pH = 7.0). Also high-purity water from Milli-Q system was used in the performed.

Methods

Steady-state fluorescence measurements were made using spectrofluoremeter Fluoromax-4 (3000 signals/noise) from Jobnon Yvon-spex. Fluorescence lifetime measurements were carried out with a FL900CDT time-correlated single photon counting fluorimeter from Edinburgh Analytical Instruments. Solutions were placed into a 10 mm quartz cuvette which were kept at room temperature.

Phosphorescence measurements were made on a homemade system. The heart of this system consists of 800 MHz gated photon counters with 32 bit counters and down to 250 ns per channel (PMS-400A, Becker&Hickl GmbH). Emission was excited by UV xenon flash lamp with 400 ns pulses, pulse energy 7.8 μJ, light output stability 1.9% p-p and repetition rate up to 100 Hz. The system was calibrated on an aqueous solution of $TbCl_3$. The lifetime was 426.5 microseconds and consistent with literature data (427 microseconds). O_2 removal was achieved by the application of a moderate vacuum and inlet of ultrapure nitrogen. The pre-purified nitrogen gas (0.1 ppm of O_2) was further purified by passing through an oxygen-trapping filter. This degassing procedure was further accompanied by the addition of 0.3 ml of 0.1 M Na_2SO_3 as an O_2 scavenger [23]. The sample was placed in a quartz cuvette, which was connected to the N_2/vacuum line by tubing. Five cycles of deoxygenation were performed. After deoxygenation, the cuvette was moved into the phosphorimeter.

Time-resolved fluorescence and phosphorescence data results were analyzed according to the multi-exponential decay law:

$$I(t) = \sum_{i=1}^{n} \alpha_i \exp\left(-\frac{t}{\tau_i}\right) \qquad \text{(eq 1)}$$

where α_i and τ_i are preexponential factor and decay lifetime of component i, respectively.

Results and discussion

Steady-state fluorescence measurements

Useful information can be provided by fluorescence measurements about the molecular environment in the region of the fluorophore. The effect of harmane on the fluorescence emission spectrum of HSA and BSA, excited at 290 nm are presented in Figure 2.

The emission spectrum showed two maxima, the first one at about 345 nm (which is characteristic for tryptophan in serum albumins) and the second peak with very high fluorescence intensity at about 430 nm (which can be assigned to harmane).

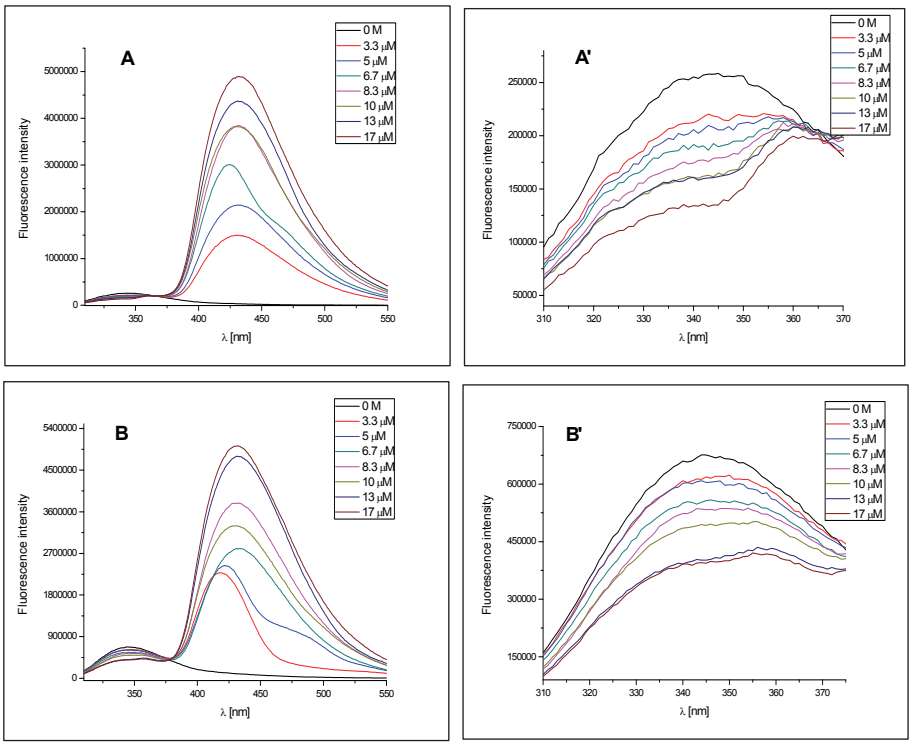

Figure 2. Emission spectra of (A) HSA and (B) BSA in the presence of various concentrations of harmane. The characteristic emission region for HSA and BSA, are presented in fig A' and B', respectively. The total concentration of proteins was 3 μM. The wavelength excitation was 290 nm

Fluorescence quenching can be related to any process which reduces the fluorescence intensity of a given sample. There are two main types of quenching that can describe the mechanism of the binding between quencher and

macromolecules: static and dynamic (which is also known as collision quenching) [24]. The mechanism of harmane binding to HSA and BSA is described using the Stern-Volmer equation:

$$\frac{F_0}{F} = \left(1 + K[Q]_0\right) \cdot \left(1 + k_q \tau_0 [Q]_i\right) \qquad \text{(eq 2)}$$

where F_0 and F are the fluorescence intensities in the absence and the presence of the quencher, respectively, K is the equilibrium constant for complex formation in ground state, in the presence of quencher; $[Q]_0$ is the analytical concentration of the quencher; k_q is the rate constant for quenching; τ_0 is the fluorescence lifetime of the chromophore in the absence of quencher; $[Q]_i$ is the concentration of a free quencher. The first bracket describes static quenching and the second describes dynamic quenching. The Stern-Volmer plot of the studied molecules are presented in Figure 3.

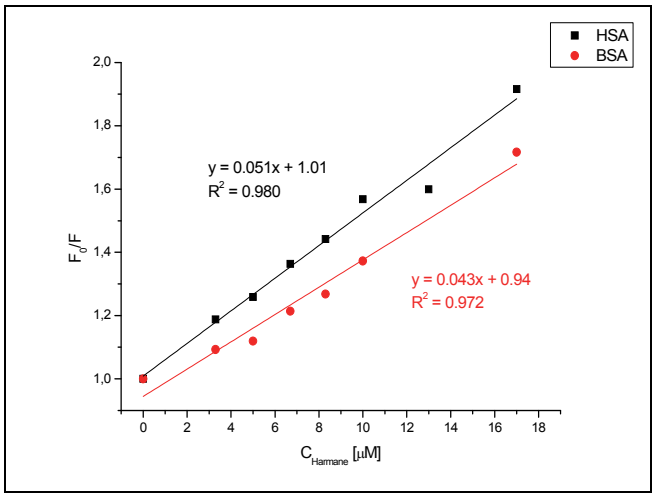

Figure 3. The Stern-Volmer plots for harmane quenching of the proteins studied at room temperature. The concentration of proteins was 3 μM. The wavelength excitation was 290 nm and the wavelength emission was 340 nm

The straight line obtained in the Stern-Volmer plot suggests the presence of either purely static or dynamic quenching, but identification of one unequivocal quenching mechanism at this stage is not possible.

Time-resolved fluorescence measurements

Therefore, time resolved fluorescence spectroscopy was used to determine the decay times, thus enabling the differentiation between the presence of static and dynamic quenching. The kinetics of the fluorescence decay of the molecules studied are presented in Figure 4 and the decay parameters are listed in Table 1.

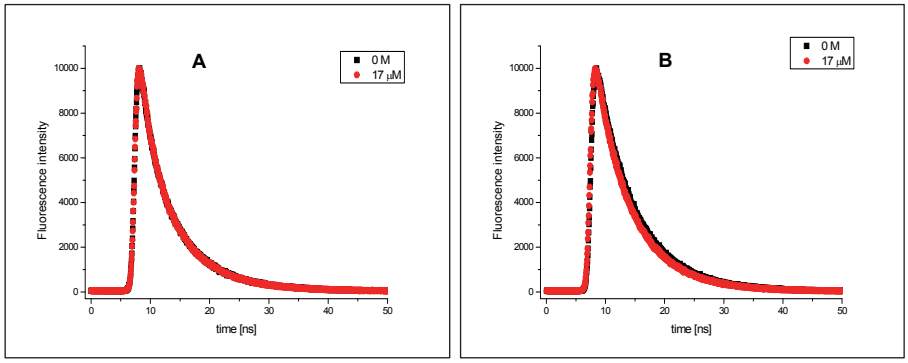

Figure 4. The kinetics of the fluorescence decay of (A) HSA and (B) BSA in the absence and presence of harmane. The total concentration of proteins was 3 μM. The wavelength excitation was 295 nm and the wavelength emission was 345 nm

Table 1. Parameters of the fluorescence decay fits, using the eq 1

	$C_{harmane}$ [μM]	τ_1 [ns]	τ_2 [ns]	τ_{av} [ns]
HSA	0	2.23±0.04	6.80±0.03	5.90±0.05
	3.3	2.25±0.04	6.77±0.03	5.85±0.05
	10	2.26±0.04	6.76±0.03	5.84±0.04
	17	2.28±0.04	6.76±0.03	5.83±0.04
BSA	0	3.01±0.23	6.69±0.04	6.44±0.08
	3.3	2.98±0.19	6.62±0.04	6.39±0.07
	10	2.96±0.15	6.60±0.04	6.35±0.06
	17	2.92±0.13	6.59±0.03	6.34±0.08

Despite the addition of harmane, the τ_{av} values for HSA and BSA have not been changed within the experimental error limits. This indicated that there is no interactions between the studied proteins and harmane in the excited state and suggest that there is only static quenching. Therefore, the slope in Figure 3 may be considered as the equilibrium constant for complex formation in the ground state with values $(5.16\pm0.28)\times10^4$ M^{-1} and $(4.32\pm0.30)\times10^4$ M^{-1} for HSA and BSA, respectively. The determined value for BSA is not consistent with the values obtained by Nafisi et al. (1.41×10^3 M^{-1}) [22].

Phosphorescence measurements

Phosphorescence can provide useful information about the nature of the interactions between the proteins and the quencher and its affect on the lifetime of the triplet state, because it observed drastic dependence of the tryptophan phosphorescence lifetime on the differences in the rigidity of the indole environment [25]. As shown in Figure 5, the maximum of phosphorescence emission was 460 nm and 450 nm for HSA and BSA, respectively and this values

are typical for tryptophan in protein. The phosphorescence signal for harmane was not registered in the region of 480-600 nm.

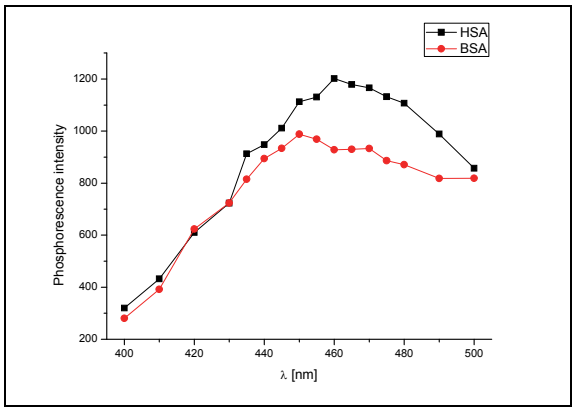

Figure 5. Phosphorescence spectra for HSA and BSA. The concentration of proteins was 30 μM. The wavelength excitation was 290 nm

We observed a decrease in phosphorescence lifetime with an increased concentration of harmane. The bimolecular constant rate for quenching of the triplet states k_q^T was calculated using the following equation:

$$\frac{1}{\tau_{ph}} = \frac{1}{\tau_{ph}^0} + k_q^T[Q] \qquad \text{(eq 3)}$$

where τ_{ph}^0 is the phosphorescence lifetime in the absence of quencher; [Q] is the concentration of harmane. A plot of $1/\tau_{ph}$ vs [Q] should yield a straight line with an intercept of $1/\tau_{ph}$ and a slope equal to the bimolecular triplet state quenching rate constant k_q^T (Figure 6).

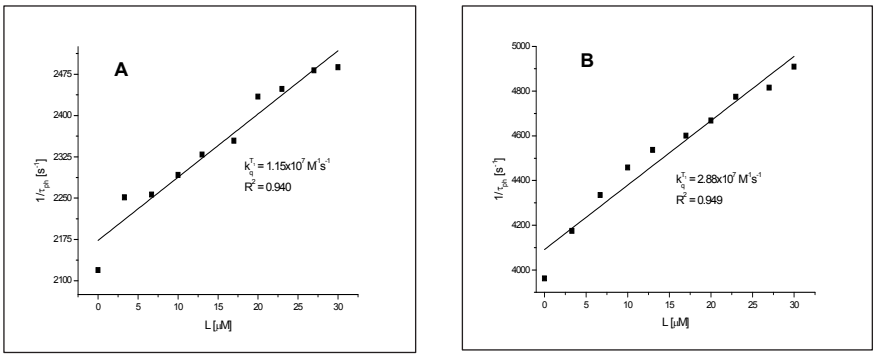

Figure 6. Harmane quenching of the triplet state of HSA (A) and BSA (B). Concentration of the studied protein and KI was 30 μM and 0.2 M, respectively. The wavelength excitation was 290 nm and the emission was observed with the filter 440-460 nm

The determined bimolecular constant for triplet state quenching of the proteins studied by harmane was $(1.15\pm0.10)x10^7$ M^{-1} s^{-1} and $(2.88\pm0.22)x10^7$ $M^{-1}s^{-1}$ for HSA and BSA, respectively. The phosphorescence lifetime in the absence of the quencher was calculated to be 0.472 ms for HSA and 0.252 ms for BSA. Potassium iodide (KI, heavy atom) was used to enhance intersystem crossing by ensuring that the triplet state will be the major pathway of tryptophan S_1 decay. The concentration of KI was kept constant so it did not affect the phosphorescence lifetime.

Conclusions

The aim of this research was to study the interactions between the serum albumins and harmane by the use of fluorescence and phosphorescence spectroscopy. The fluorescence measurements revealed that harmane quenched fluorescence of tryptophan in HSA and BSA and, most probably, the mechanism involved is static quenching. Also the interactions between the studied compounds have been observed in the excited triplet state. The decrease in both the fluorescence and phosphorescence intensity and similar value of the equilibrium constant for complex formation in ground state for HSA and BSA indicate that, most probably, the binding site of harmane is located in the drug site 1 in the subdomain IIa. This study suggests that albumin, as a drug carrier, plays an important role in the pharmacological effects of harmane and gives us further insight into the pharmacokinetics.

Acknowledgements

The authors would like to thank Prof. S. Wysocki, head of the Institute of General Food Chemistry, who founded the project and provided the equipment.

References

1. Yu AM, Idle JR, Krausz KW, Küpfer A, Gonzalez FJ. Psychedelic 5-Methoxy-*N,N*-dimethyltryptamine: Metabolism, Pharmacokinetics, Drug Interactions, and Pharmacological Actions. J Pharmacol Exp Ther **2003**, 305:315–322.
2. Airaksinen MM, Kari I. 3-Carbolines, psychoactive compounds in the mammalian body. Med Biol **1981**, 59:190–211.
3. T. Aniszewski. Alkaloids – Secrets of Life: Alkaloid Chemistry, Biological Significance, Applications and Ecological Role. Elsevier Science, Amsterdam-Oxford, The Netherlands-UK, **2007**, p 181.
4. Aassila H, Bourguet-Kondracki ML, Rifai S, Fassouane A, Guyot M. Identification of harman as the antibiotic compound produced by a tunicate-associated bacterium. Mar Biotechnol **2003**, 5:163–166.
5. Ishida J, Wang HK, Masayoshi O, Cosentino CL, Hu CQ, Lee KH. Anti-AIDS Agents 46. Anti-HIV Activity of Harman, an Anti-HIV Principle from Symplocos setchuensis, and its Derivatives. J Nat Prod **2001**, 64:958–960.

6. Rivas P, Cassels BK, Morillo A, Repetto Y. Effects of some beta-carboline alkaloids on intact Trypanosoma cruzi epimastigotes. Comp Biochem Physiol C **1999**, 122:27-31.
7. Mishra BB, Kale RR, Singh RK, Tiwari VK. Alkaloids: future prospective to combat leishmaniasis. Fitoterapia **2009**, 80:81–90.
8. Kim H, Sablin SO, Ramsay RR. Inhibition of monoamine oxidase A by beta-carboline derivatives. Arch Biochem Biophys **1997**, 337:137–142.
9. Tweedie DJ, Prough RA, Burke MD. Effects of induction on the metabolism and cytochrome P-450 binding of harman and other beta-carbolines. Xenobiotica **1988**, 18:785–796.
10. Airaksinen MM, Mikkonen E. Affinity of beta-carbolines on rat brain benzodiazepine and opiate binding sites. Med Biol **1980**, 58:341–344.
11. Glennon RA, Dukat M, Brella B, Hong SS, Costantino L, Teitler M, Smith C, Egan C, Davis K, Mattson MV. Binding of β-carbolines and related agents at serotonin (5-HT2 and 5-HT1A), dopamine (D2) and benzodiazepines receptors. Drug Alcohol Depend **2000**, 60:121-132.
12. Funayama Y, Nishio K, Wakabayashi K, Nagao M, Shimoi K, Ohira T, Hasegawa S, Saijo N. Effects of beta- and gamma-carboline derivatives of DNA topoisomerase activities. Mutat Res **1996**, 349:183–191.
13. Airaksinen MM, Lecklin A, Saano V, Tuomisto L, Gynther J. Tremorigenic effect and inhibition of tryptamine and serotonin receptor binding by beta-carbolines. Pharmacol Toxicol **1987**, 60:5-8.
14. Brüning G, Rommelspacher H. Solubilization of high-affinity [^{3}H]tryptamine-binding sites from rat brain. Life Sci **1984**, 34:1441-1446.
15. Rommelspacher H, Brüning G, Schulze G, Hill R. Neuroreceptors. De Gruyter, Berlin, Germany, **1982,** p. 2741.
16. Ghuman J, Zunszain PA, Petitpas I, Bhattacharya AA, Otagiri M, Curry S. Structural basis of the drug-binding specificity of human serum albumin. J Mol Biol **2005**, 353:38-52.
17. Vayá I, Pérez-Ruiz R, Lhiaubet-Vallet V, Jiménez MC, Miranda MA. Drug-protein interactions assessed by fluorescence measurements in the real complexes and in model dyads. Chem Phys Lett **2010**, 486:147-153.
18. Jabłońska J, Casares D, Fünfer J, Habenschuss T, Jimenez G, Klemm AL, Machado-Junior AM, Miloshevska S, Papišta I, Ulrich-Lynge SL. The study of the interactions between diclofenac and bovine serum albumin (BSA). Food Chemistry and Biotechnology **2008**, 72:46-61.
19. Silva D, Cortez CM, Louro SRW. Chlorpromazine interactions to sera albumins. A study by the quenching of fluorescence. Spectrochim Acta A **2004**, 60:1215–1223.
20. Ghuman J, Zunszain PA, Petitpas I, Bhattacharya AA, Otagirl M, Curry S. Structural basis of the drug-binding specificity of human serum albumin. J Mol Biol **2005**, 353:38-52.
21. Bhattacharya B, Nakka S, Guruprasad L, Samanta A. Interaction of bovine serum albumin with dipolar molecules: fluorescence and molecular docking studies. J Phys Chem B **2009**, 113:2143-2150.
22. Nafisi S, Panahyab A, Sadeghi GB. Interactions between β-carboline alkaloids and bovine serum albumin: Investigation by spectroscopic approach. J Lumin **2012**, 132:2361-2366.

23. Kuijt J, Ariese F, Brinkman UAT, Gooijer C. Room temperature phosphorescence in the liquid state as a tool in analytical chemistry. Anal Chim Acta **2003**, 488:135-171.
24. Sobczuk D, Craig R, Nogueroles M, Radišić S, Shen B, Simon S. The study of the interactions between naproxen and N-acetyl-L-tyrosine ethyl ester. Zesz Nauk PŁ Chem Spoż Biotechnol **2006**, 70:23-36.
25. Cioni P, Gabellieri E, Gonnelli M, Strambini GB. Heterogeneity of protein conformation in solution from the lifetime of tryptophan phosphorescence. Biophys Chem **1994**, 52:25-34.