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THE STUDY OF INTERACTIONS BETWEEN DICLOFENAC AND BOVINE SERUM ALBUMIN (BSA)

Review: Professor Stanisław Wysocki, Ph. D., D. Sc.

Investigation of the interactions between diclofenac and BSA was performed using UV-Vis absorption and fluorescence methods. There was a quenching effect found between these two molecules in the fluorescence measurement methods; steady-state and time-resolved. The fluorescence decay time enables to determine the quenching mechanism and thus conclude that the complex is formed in ground-state but also collisional quenching -when the excited-state fluorophore of BSA is deactivated- occurs. Computer modeling was applied to observe and optimize the formation of the complex between N-acetyl-L-tryptophanamide and diclofenac.

1. Introduction

Diclofenac is a non-steroidal-anti-inflammatory-drug (NSAID) that is used as an active compound in analgesic, antipyretic, anti-rheumatic medicaments (Fig. 1). It belongs to the group of the NSAID that are derivatives of the phenylacetic acid and its chemical name is 2-(2,6- dichloroanilino) phenylacetic acid. It is used in the form of sodium or potassium salt. Diclofenac is a weak acid with a pKa 4.0, it has a protein-binding ability of more than 99% and it is light sensitive [1].

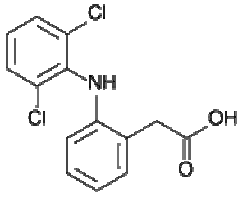

Fig. 1. The chemical structure of diclofenac

The exact mechanism of its actions is not entirely known, but it is thought that the primary mechanism is inhibition of prostaglandin synthesis by inhibition of cyclooxygenase (COX). COX1 and 2 are the most explored COX isoenzymes and COX1 is a constitutive enzyme involved i.e. in the protection of the gastrointestinal mucosa and COX2 is an inducible enzyme that upregulates the inflammatory response. Inhibition of COX also decreases prostaglandins in the epithelium of the stomach, making it more sensitive to corrosion by gastric acid. This is also the main side effect of diclofenac. Diclofenac has a low to moderate preference to block the COX2-isoenzyme (approximately 10-fold) and therefore it is said to have a somewhat lower incidence of gastrointestinal complications than noted with other NSAIDs i.e. aspirin [2].

Human serum albumin (HSA) is a carrier for several drugs i.e. diclofenac and by this it plays an important role in the regulation of plasma concentrations of these, including both endogenous and exogenous compounds. HSA shares 76% homology with Bovine serum albumin (BSA) and as this is easily accessable, of low cost and of medical importance it can therefore be used in this project [3, 4]. BSA consists of amino acid chains forming a single polypeptide with a well-known sequence, which contains three homologous α-helixes domains (I–III). Each domain contains 10 helixes which are divided into anti-parallel six-helixes and four subdomains. HSA contains 585 amino acid residues with only one tryptophan located at position 214 along the chain. The BSA molecule is formed by 582 amino acid residues; with tryptophan at position 134 and 212 [5]. The aim of the experiment is to explore and verify the molecular interactions between diclofenac and BSA in the ground and exited state. The interactions were explored by fluorescence techniques as they are very sensitive, rapid and easily implemented [3].

2. Experimental details

2.1. Chemicals

Diclofenac, BSA, N-Acetyl-L-tryptophanamide (NATA) and tripeptide lysine-tyrosine-lysine (L-Y-L) used in this project were commercial products of the best available quality purchased at Sigma-Aldrich and were used as received. All chemicals were dissolved in 0.1 M phosphate buffer (pH = 7.0).

2.2. Experimental Equipment

Absorption measurements were made by using a Nicolet Evolution 300 UV- Vis spectrophotometer (resolution 1nm, range 0-6) from Thermo Electron Corporation using Vision/Pro program. Steady-state fluorescence measurements were made using spectrofluorometer Generic Fluoromax-2 (2000 signals/noise) from Jobin Yvon-Spex with the resolution of 13 nm. Solutions were placed into a 10 mm quartz cuvette and temperature was kept constant at 21°C. Titrations were made with a constant BSA concentration while varying the concentration of diclofenac. Kinetics of the fluorescence decay was measured using a time-resolved fluorometer (Edinburgh Instruments OB-920) with a pulsed nanosecond hydrogen lamp as excitation source and temperature was again constant at 21°C. Time resolution was 100 ps.

2.3. Software-analysis

The data collected in the experiments was analyzed by the use of Microsoft Excel. This program was applied to obtain calculations and display the results in graphic form.

HyperChem was used as molecular modeling and simulation program to draw both molecules from atoms and convert them into 3-D models.

To show the interaction between BSA and diclofenac the program Gaussian with AM1 semi empirical methods was used. This computer program calculates the amount of energy released when two molecules interact.

3. Results and discussion

3.1. Experimental results

3.1.1. UV-Vis spectra of pure diclofenac, BSA, NATA and L-Y-L

In Fig. 3.1 the spectrum for diclofenac shows a maximum at 275 nm which corresponds with the literature value [6].

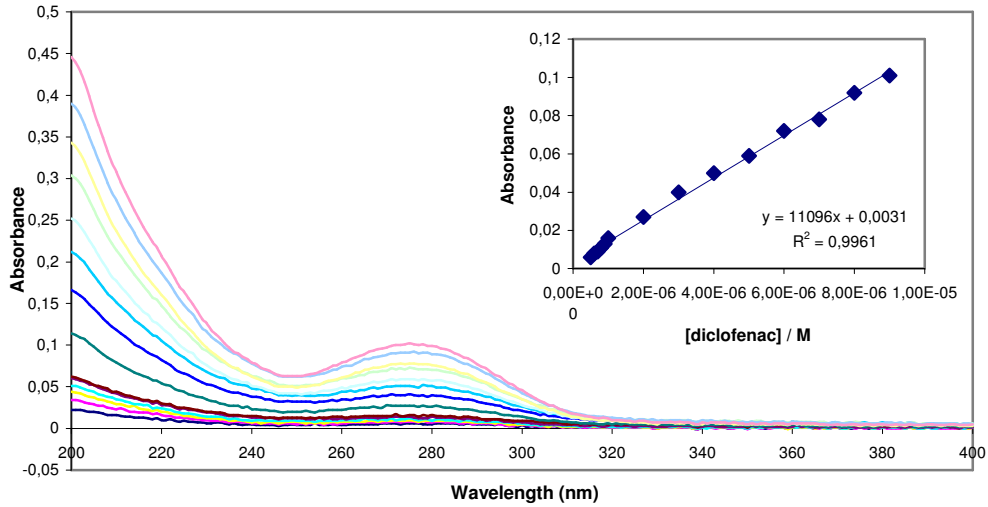

Fig. 3.1. UV-Vis spectrum of diclofenac ($5x10^{-7}$ – $9x10^{-6}$M) and its dependence on concentration at maximum 275 nm (insert)

From the Beer-Lambert law we can easily find the molar extinction coefficient ε:

$$A = \varepsilon \cdot l \cdot c \tag{1}$$

where: A – absorbance
ε – molar extinction coefficient with units of L $mol^{-1}cm^{-1}$ or $M^{-1}cm^{-1}$
l – path length of the sample - that is the path length of the cuvette in which the sample is contained (in our case 1 cm)
c – concentration of the compound in solution, expressed in mol L^{-1}

From the equation describing the straight line in insert in Fig. 3.1 we can determine the experimental molar extinction coefficient to $1.1x10^4$ $M^{-1}cm^{-1}$ at 275 nm.

BSA spectrum in Fig. 3.2 shows two peaks with maxima at 210 nm and 279 nm. The first peak (210 nm) differs from literature values which is 200 nm [7]. The experimental extinction coefficient is $4.1x10^4$ M^{-1} cm^{-1} at 279 nm which reasonably good corresponds with the literature value of $4.4x10^4$ M^{-1} cm^{-1} at 280 nm [8].

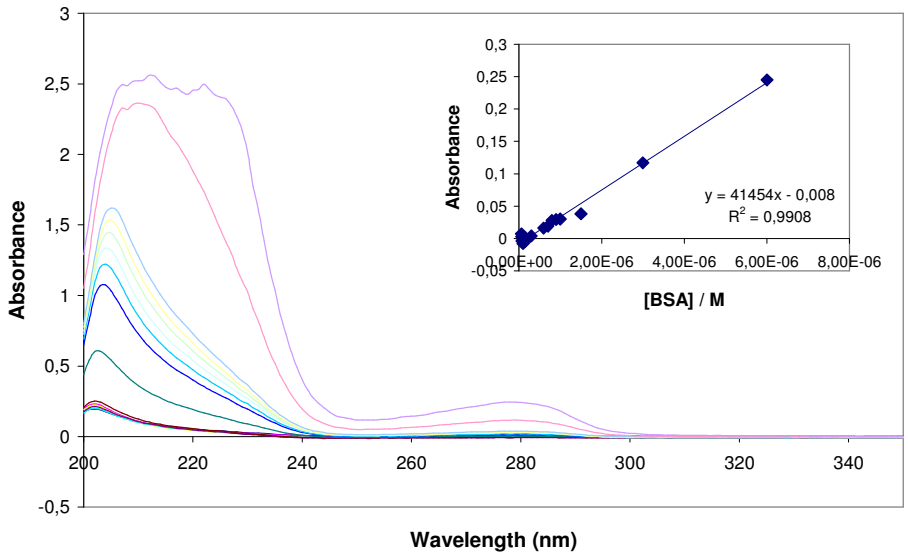

Fig. 3.2. UV-Vis spectrum of BSA ($6x10^{-8}$ – $6x10^{-6}$) and its dependence on concentration at maximum 279 nm (insert)

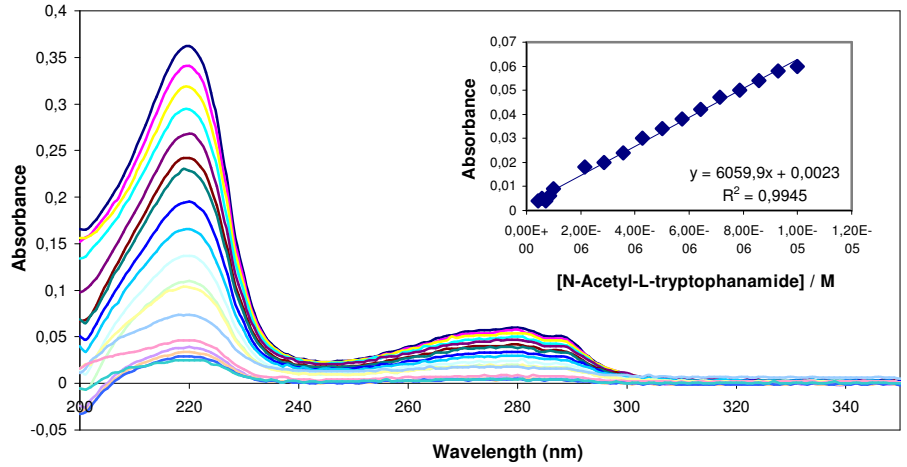

Fig. 3.3. UV-Vis spectrum of NATA ($4.29x10^{-7}$ – $1x10^{-5}$M) and its dependence of the concentration at maximum 280 nm (insert)

NATA spectrum in Fig. 3.3 shows two peaks, the first one at 220 nm and the second one is at 280 nm. The experimental extinction coefficient is $6.1x10^{3}$ M^{-1} cm^{-1} at 280 nm which corresponds to the literature value of $5.7x10^{3}$ M^{-1} cm^{-1} measured at the same wavelength [9].

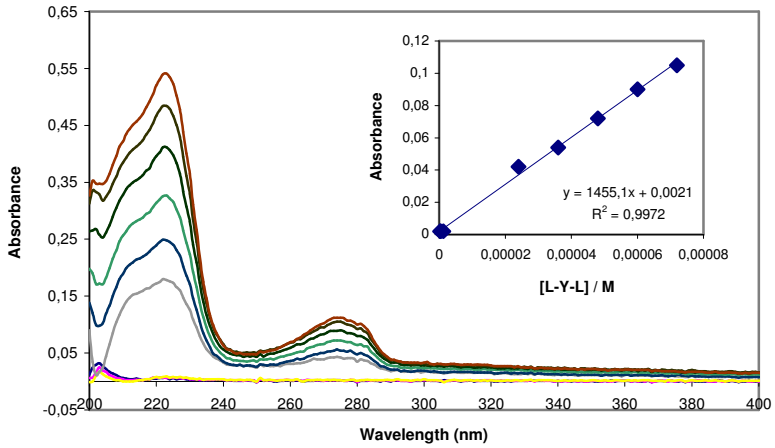

Fig. 3.4. UV-Vis spectrum of a tripeptide solution containing lysine – tyrosine - lysine (L-Y-L) ($2.5x10^{-7}$ – $8.4x10^{-5}$M) and its dependence on the concentration at maximum 275 nm (insert)

L-Y-L spectrum in Fig. 3.4 shows three peaks, the first one at 223, the second one at 275 nm while the third one is at 283 nm. The experimental extinction coefficient is $1.5x10^3$ M^{-1} cm^{-1} at 275 nm which is comparable to the literature value of $1.3x10^3$ M^{-1} cm^{-1} at 280 nm for model tripeptide glycyl-L-tyrosylglycine [9].

The calibration curves in Figures 3.1, 3.2, 3.3 and 3.4 show a linear relationship between concentration and absorbance.

3.1.2. Fluorescence spectra of pure diclofenac, BSA, NATA and L-Y-L

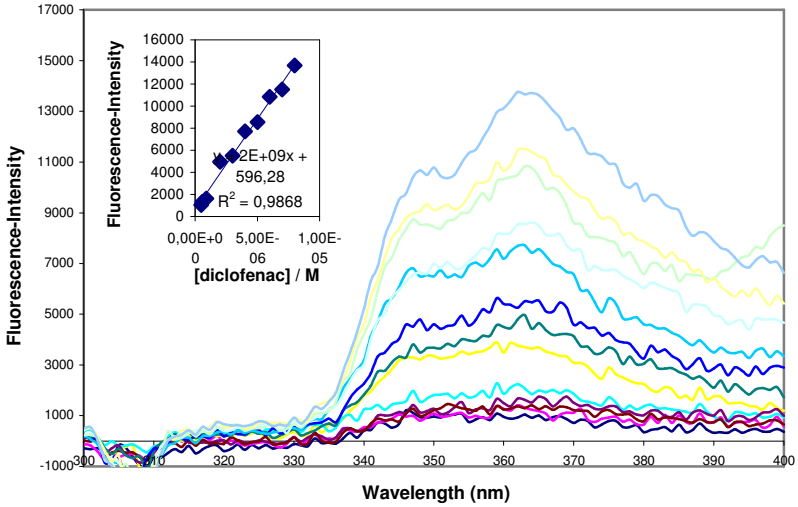

Fig. 3.5. Fluorescence spectrum of diclofenac ($5x10^{-7}$ – $9x10^{-6}$M; exc. 279 nm) and its dependence on the concentration at maximum 363 nm (insert)

Fig. 3.5 shows that diclofenac fluorescence has two peaks at 348 nm and at 363 nm.

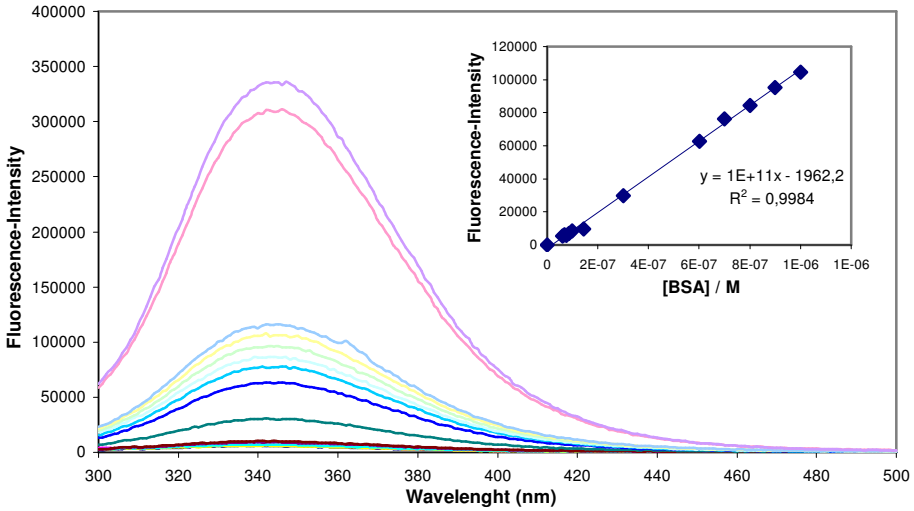

Fig. 3.6. Fluorescence spectrum of BSA ($3x10^{-6}$ – $1.5x10^{-6}$; exc. 279 nm) and its dependence on the concentration at maximum 344 nm (insert)

The fluorescence spectrum of BSA shown in Figure 3.6 has the maximum peak at 344 nm. The value obtained correlates with literature values [10].

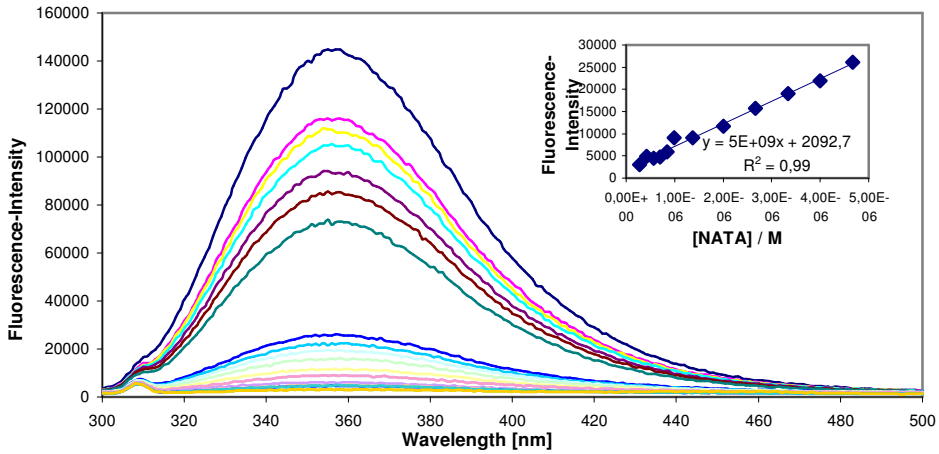

Fig. 3.7. Fluorescence spectrum of NATA ($4.29x10^{-7}$ – $5.6x10^{-6}$M; exc. 279 nm) and its dependence on the concentration at maximum 358 nm (insert)

Fluorescence spectrum of NATA has the maximum peak at 358 nm as shown in Fig. 3.7.

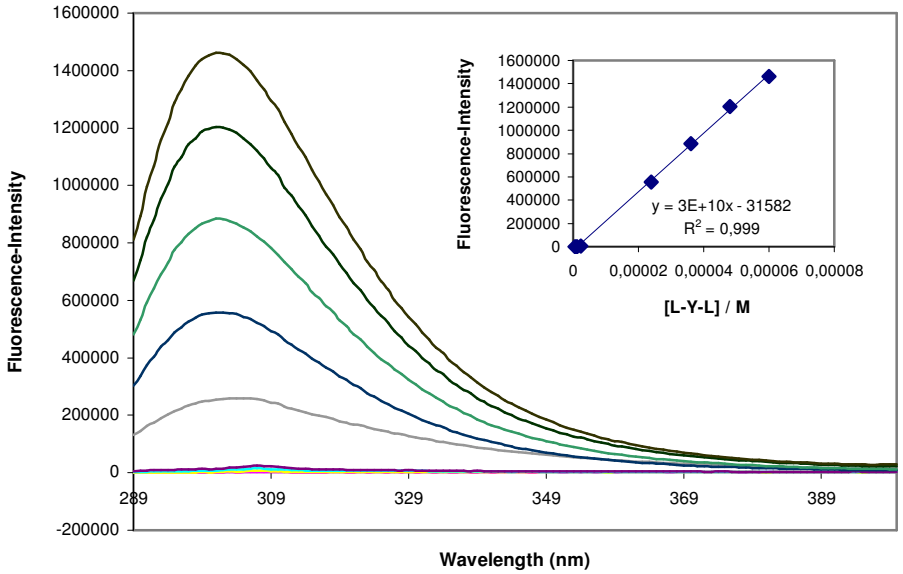

Fig. 3.8. Fluorescence spectrum of L-Y-L ($2.5x10^{-7}$ – $8.4x10^{-5}$M; exc. 279 nm) and its dependence on the concentration at maximum 301 nm (insert)

Fluorescence spectrum of L-Y-L has the maximum peak at 301 nm as shown in Fig. 3.8.

The calibration curves in figures 3.5, 3.6, 3.7 and 3.8 show a linear relationship between concentration and fluorescence.

3.1.3. Titration experiments

BSA-diclofenac

Fluorescence of BSA is quenched by the diclofenac as shown in Fig. 3.9.

Emission of light can be expressed by the following equation:

$$F_i = \alpha \cdot \sum_i \cdot \phi_i \cdot \varepsilon_{i\lambda_{ex}} \cdot c_i \qquad (2)$$

where: F_i – fluorescence intensity of i
α – apparatus factors
Φ_i – quantum yield of i
$\varepsilon_{i\,\lambda ex}$ – molar extinction coefficient of i for proper excitation wavelength
c_i – concentration of the i compound in solution

Fluorescence intensity is proportional to the concentration provided that $A \leq 0.1$.

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. In this study the focus is on dynamic and ground state quenching. However, quenching may also result from energy transfer and excited state reactions.

Static quenching is due to ground-state complex formation between the fluorophore and a quencher. This complex is nonfluorescent and when it absorbs light, it immediately returns to the ground state without emission of a photon.

Dynamic quenching results from collisions between the fluorophore and a quencher in the excited-state [12].

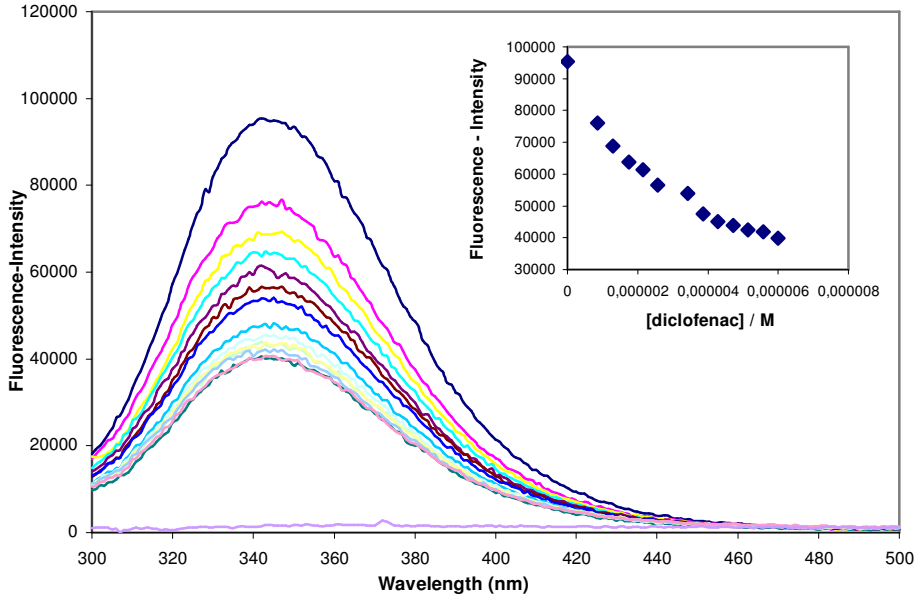

Fig. 3.9. Quenching of BSA fluorescence with increasing diclofenac concentrations (exc. 279 nm); fluorescence intensity dependence on the concentration of diclofenac at maximum 344 nm (insert)

In the absence of the quencher (diclofenac) fluorescence of the chromophore (BSA) can be expressed as:

$$F_0 = \alpha \cdot \Phi_0 \cdot \varepsilon_{\lambda_{ex}} \cdot c_0 \tag{3}$$

where: F_0 – fluorescence intensity in the absence of the quencher

Φ_0 – quantum yield of the pure chromophore

c_0 – initial concentration of the chromophore in the absence of the quencher

When concentrations of the quencher are different, the fluorescence intensity changes, therefore:

$$F = \alpha \cdot \Phi \cdot \varepsilon_{\lambda_{ex}} \cdot c \tag{4}$$

Dividing eq.(3) and eq.(4) we obtain the following relationship:

$$\frac{F_0}{F} = \left(\frac{\Phi_0}{\Phi}\right) \cdot \left(\frac{c_0}{c}\right) \tag{5}$$

It can be seen that the first part of the formula (first brackets) refers to dynamic quenching and the second part (second bracket) refers to static quenching.

Difference in the fluorophore's quantum yield in the absence and in the presence of the quencher can be described as:

$$\frac{\Phi_0}{\Phi} = \frac{\tau_0}{\tau} \tag{6}$$

where: τ_0 – lifetime of fluorescence decay of fluorophore (BSA) in the absence of quencher

τ – lifetime of fluorescence decay of fluorophore in the presence of quencher

Therefore:

$$\frac{F_0}{F} = \left(\frac{\tau_0}{\tau}\right) \cdot \left(\frac{c_0}{c}\right) \tag{7}$$

Dynamic quenching is described by the Stern–Volmer equation, which gives the ratio between fluorescence intensities in the absence or the presence of a quencher as a function of its concentration, taking into account the fluorophore's lifetime in the quencher's absence.

$$\frac{\tau_0}{\tau} = \left(1 + k_q \cdot \tau_0 \cdot [Q]_i\right) = \left(1 + K_D \cdot [Q]\right) \tag{8}$$

where: k_q – bimolecular rate constant for quenching
$[Q]_i$ – concentration of the free quencher
K_S – equilibrium constant for complex formation in ground state, in presence of the quencher
$[Q]_o$ – analytical concentration of the quencher

The kinetics of the fluorescence decay of BSA and mixture (BSA + diclofenac) are di-exponential and its parameters are listed in table 1.

Table 1

Parameters of fluorescence decay fits, using the equation:

$$F = A + B_1 e^{-t/\tau_1} + B_1 e^{-t/\tau_2} \quad (9)$$

	τ_1		τ_2		Average τ
	(ns)	B_1	(ns)	B_2	(ns)
BSA	4.280	0.019	7.074	0.022	6.115
BSA + diclofenac ($8.57*10^{-7}$)	4.455	0.027	7.309	0.015	5.816
BSA + diclofenac ($2.14*10^{-6}$)	3.525	0.028	6.871	0.014	5.177
BSA + diclofenac ($3.00*10^{-6}$)	3.058	0.027	6.046	0.014	4.571
BSA + diclofenac ($3.86*10^{-6}$)	3.176	0.031	6.062	0.012	4.402
BSA + diclofenac ($4.71*10^{-6}$)	3.159	0.033	5.931	0.012	4.284

The average lifetime τ was calculated by the following equation:

$$\langle \tau \rangle = \frac{(\tau_1^2 \cdot B_1) + (\tau_2^2 \cdot B_2)}{(\tau_1 \cdot B_1) + (\tau_2 \cdot B_2)} \quad (10)$$

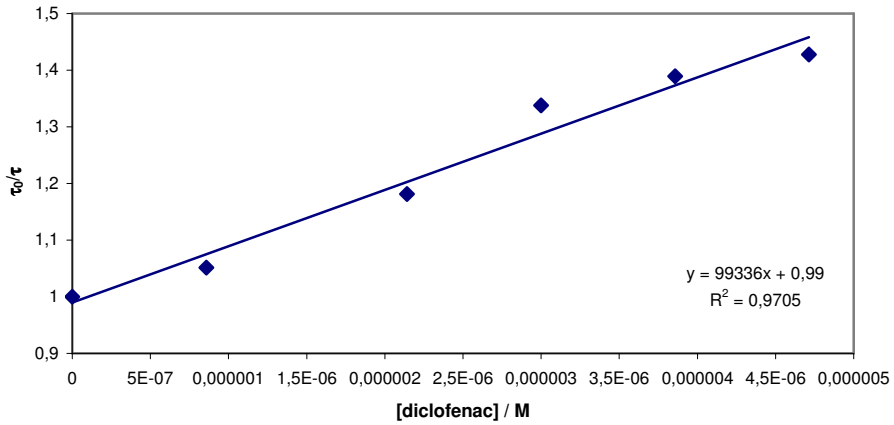

Fig. 3.10. Stern-Volmer plot (eq.(2)) for the quenching of BSA lifetime by diclofenac (exc. 279 nm; em. 348 nm)

The time-resolved fluorescence results show dynamic quenching and the constant of this dynamic quenching, as shown in Fig. 3.10, is 9.93 x 10^4 M^{-1}.

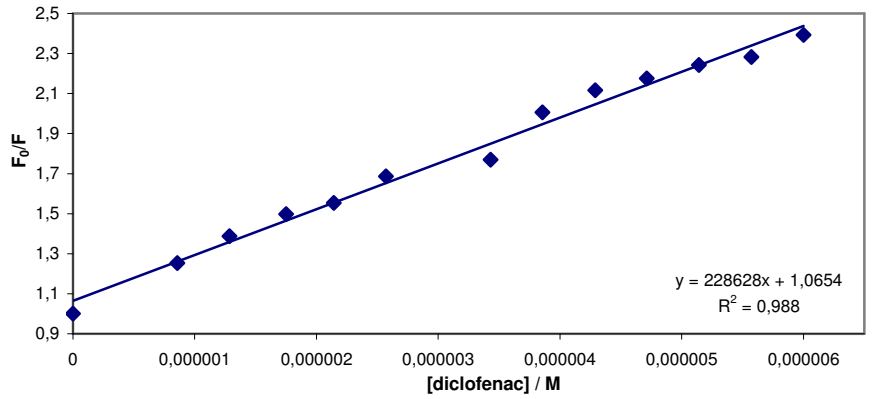

Fig. 3.11. Stern-Volmer plot (eq. (7)) for the quenching of BSA fluorescence by diclofenac

By comparing Fig. 3.10 with 3.11 it can be seen that the quenching constants have different values. This indicates that BSA could be quenched by collisions or complex formation with diclofenac, respectively.

In order to distinguish static and dynamic quenching constants eq. (7) was rearranged:

$$\frac{(F_0/F)}{(\tau_0/\tau)} = \frac{c_0}{c} \tag{11}$$

Because:

$$c = c_0 - c_k \tag{12}$$

where: c_0 – concentration of BSA
c_k – concentration of the BSA-diclofenac complex

The following formula was used to calculate the interaction at ground state:

$$\frac{(F_0/F)}{(\tau_0/\tau)} = \frac{c_0}{c_0 - c_K} \tag{13}$$

In order to calculate the association constant only at ground state the formula involving C_K was used [11].

$$c_k = \frac{1}{2}\left\{\left(c_N^0 + c_0 + \frac{1}{K_S}\right) - \sqrt{\left(c_N^0 + c_0 + \frac{1}{K_S}\right)^2 - 4c_N^0 c_0}\right\} \tag{14}$$

c_N^0 – initial concentration of diclofenac
K_S – equilibrium constant for complex formation in ground state, in presence of the quencher

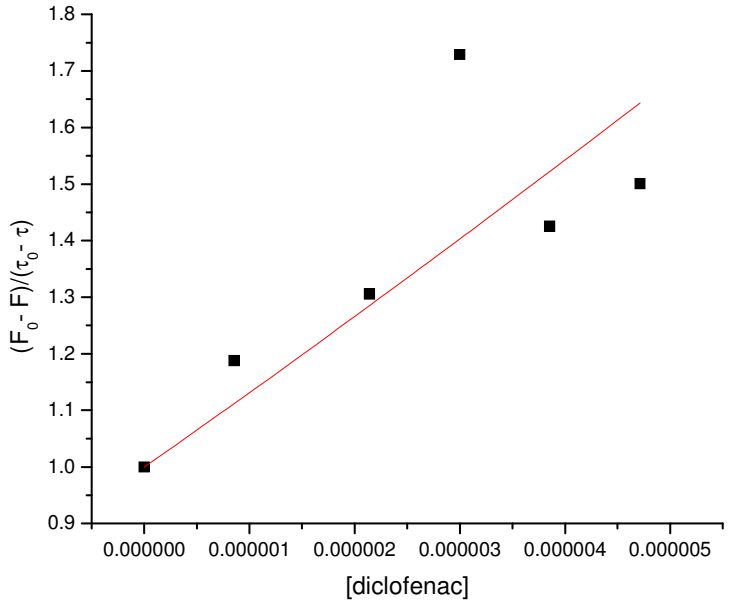

Fig. 3.12. Distinction of static and dynamic quenching using equation (11)

For calculating the association constant at ground state, C_k from equation (14) can be substituted into equation (13). By the use of *OriginLab* program, the fitting of formula 14 into the spectra in fig. 3.12 was possible.

By the use of Fig. 3.12 the K_s value can be obtained. In the graph the value represents $1/K_s$, so $K_s = 1.50 \times 10^5\ M^{-1}$.

NATA-diclofenac

To get deeper insight into the nature of quenching BSA fluorescence by diclofenac, an experiment on the interaction of *N*–Acetyl–L–tryptophanamide (NATA) and the tripeptide lysine-tyrosine-lysine (L-Y-L) with diclofenac was carried out.

As mentioned before BSA fluorescence occurs mostly by its tryptophan residue so we expected that quenching BSA fluorescence by diclofenac is caused mainly due to quenching tryptophan fluorescence as suggested for apo-hCox-2 protein and diclofenac [13]. To confirm our assumptions we carried out a titration experiment with NATA and diclofenac.

In opposite to our expectations NATA fluorescence was not quenched by diclofenac as can be seen in fig. 3.13. The emission intensity rises with the increasing amount of diclofenac, so quenching of BSA fluorescence is not caused simply by quenching the tryptophan residue.

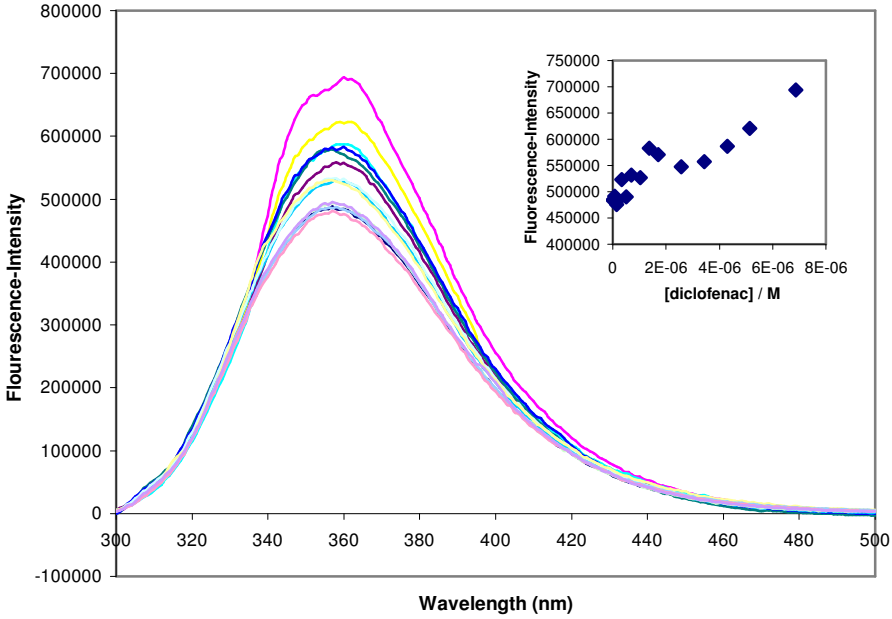

Fig. 3.13. Changes of NATA fluorescence with the increase of diclofenac (exc. 279 nm); fluorescence intensity dependence on the concentration at maximum 360 nm (insert)

L-Y-L-diclofenac

Total fluorescence of BSA consists of fluorescence of tryptophan residue and fluorescence of tyrosine residue. After it had turned out that fluorescence of NATA is not quenched by diclofenac, we carried out another titration experiment with a tripeptide containing tyrosine and diclofenac.

As shown in Fig. 3.14 diclofenac quenches L-Y-L fluorescence, but the quenching is not as significant as for BSA. In this experiment BSA was excited at 279 nm. The absorbance spectra show that at this wavelength both the tryptophan and the tyrosine residue are excited. We can assume that the difference between the decreasing fluorescence intensity of BSA and L-Y-L with increasing concentrations of the quencher is caused by some kind of energy transfer between the two residue. This might intensify the interaction with diclofenac.

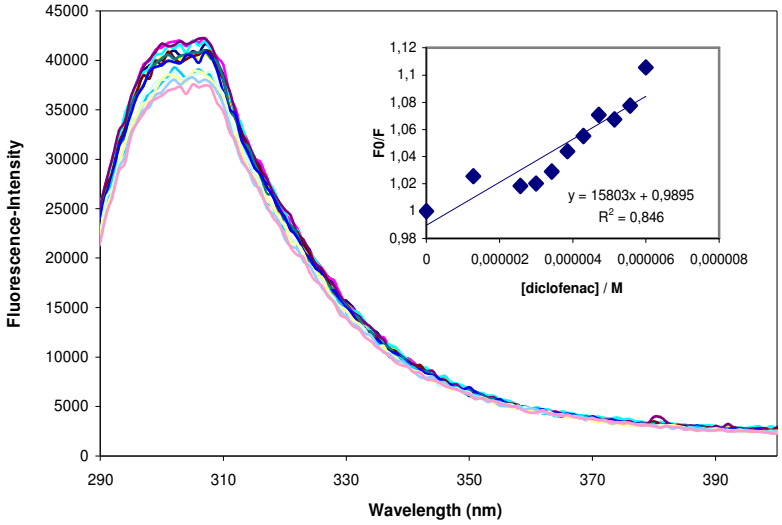

Fig. 3.14. Quenching of L-Y-L fluorescence with the increase of the diclofenac concentration (exc. 279 nm); Stern-Volmer plot for the quenching of L-Y-L fluorescence at maximum 302 nm (insert)

3.2. Modeling Results

As for the interaction between diclofenac and NATA, computer modeling was performed. The structure of the complex after optimization is shown in Fig. 3.15. Computer calculations for the free energy ΔG of the reaction provided a value of – 45,7 kcal/mol.

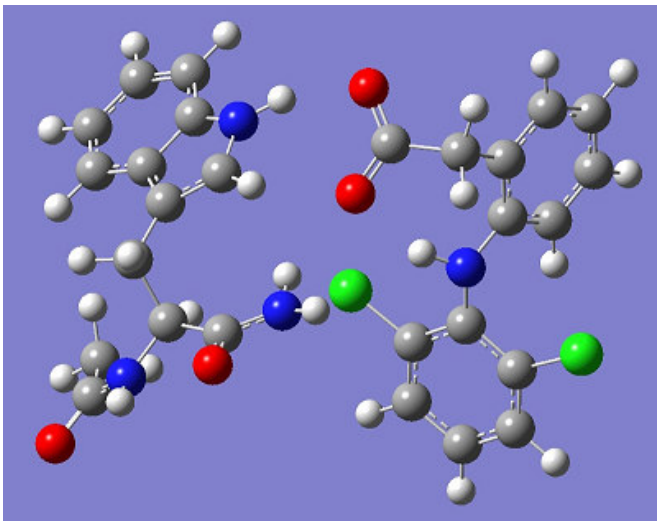

Fig. 3.15. Gaussian optimization of the interaction between NATA and diclofenac

4. Conclusions

In view of the given results there is one thing that catches the eye. The BSA protein is apparently quenched by diclofenac with high efficiency, but on the other hand, NATA is not significantly quenched. The tryptophan residue in BSA shows higher fluorescence intensity than the tyrosine residue, therefore it was expected that the tryptophan residue would be a site of quenching. The experimental result is supported by the computational result which shows a hydrogen bond between the chlorine atom at position 11 of the diclofenac molecule and a hydrogen atom of the amide group of NATA. Since the fluorophore of tryptophan is the indole ring, the computational results explain the lack of quenching by diclofenac.

In consideration of the observed quenching of tyrosine by diclofenac, we can assume that the tyrosine residue is the binding site for the drug within the protein. This, however, arises the question of the declining fluorescence intensity. A possible explanation would be resonance energy transfer from tyrosine to tryptophan upon diclofenac binding. A second explanation could be a conformational change during complex formation, causing reactions between the tryptophan residue and surrounding amino acids.

In general our experiments' results suggest existing interactions between BSA and diclofenac in ground state and excited state respectively.

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BADANIA ODDZIAŁYWAŃ POMIĘDZY DIKLOFENAKIEM I SUROWICZĄ ALBUMINĄ WOŁOWĄ (BSA)

Streszczenie

W pracy zbadano oddziaływania pomiędzy diklofenakiem i surowiczą albuminą wołową przy użyciu metod spektroskopii absorpcjyjnej UV-Vis oraz metod fluorescencyjnych. Wyniki wskazują na istnienie interakcji pomiędzy cząsteczkami zarówno w stanie wzbudzonym (wygaszanie dynamiczne) jak i podstawowym (powstawanie niefluoryzującego kompleksu). Za pomocą modelowania molekularnego zdołano zoptymalizować kompleks pomiędzy diklofenakiem a N-acetylo-L-tryptofanamidem.

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