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Spectroscopic investigation of tolmetin interaction with human serum albumin

Silvia Neamtu*, Nicoleta Tosa, Mircea Bogdan

Department of Molecular and Biomolecular Physics, National Institute of Isotopic and Molecular Technology, 400293 Cluj-Napoca, Romania

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ABSTRACT

The interaction of tolmetin (TOL) with human serum albumin (HSA) in physiological buffer solution (pH 7.4) was studied by fluorescence and UV–vis absorption spectroscopy at different temperatures, combined with time-resolved fluorescence measurements. The experimental results showed that there was a strong fluorescence quenching of HSA by tolmetin. Using the continuous variation method, a single class of binding sites for TOL on HSA was put in evidence. The binding constants K_a were calculated at different temperatures, using a nonlinear fit to the experimental data, and the thermodynamic parameters ΔH^0, ΔS^0 and ΔG^0 were given. The obtained thermodynamic signature suggests that at least van der Waals and electrostatic type interactions are present. Quenching efficiency calculations, based on steady state and time-resolved spectroscopy, indicate that both static and dynamic quenching mechanisms are present.

1. Introduction

Tolmetin [1-methyl-5-(4-methylbenzoyl)-1H-pyrrol-2yl] acetic acid is a nonsteroidal anti-inflammatory drug (NSAID) belonging to the family of arylalkanoic acid. Tolmetin (TOL) exhibits anti-inflammatory, analgesic and antipyretic properties. In addition, this drug is used to decrease the level of hormones that cause pain, swelling, tenderness and stiffness that results from muscle skeletal and bone related diseases, such as rheumatoid arthritis, juvenile arthritis and osteoarthritis [1]. As many others NSAIDs, the role of TOL is to inhibit cyclooxigenases (COX) enzymes, which have an active role in the production of prostaglandins.

Human serum albumin (HSA), a major soluble protein, has many physiological functions, including regulation of colloid osmotic pressure and transport of various endogenous ligands throughout the circulatory system [2]. It plays an important role in the transport and deposition of many drugs molecules in the blood. Since the overall distribution, metabolism and efficacy of many drugs in the body are correlated with their affinities toward HSA, the investigation of drug – HSA binding is imperative. Studies on the interaction between TOL and HSA were reported using equilibrium dialysis [3,4], fluorescence and circular dichroism [4] and NMR [5]. The obtained results are quite different. This may be due to the method used and to the fact that tolmetin binding is dependent on temperature and HSA concentration [6]. Thus the analysis of 0.4% HSA equilibrium dialysis data at 37 °C [3] showed that tolmetin had 3 classes of binding sites ($n_1 = 1$, $K_1 = 8.3 \times 10^5\ M^{-1}$; $n_2 = 4$, $K_2 = 2.4 \times 10^4\ M^{-1}$; $n_3 = 44$, $K_3 = 79\ M^{-1}$), while at 22 °C with [HSA] = 0.25 mM and the constraint $n_1 = 1$ and $n_2 = 2$, the best fit of the dialysis data [4] was obtained with $K_1 = 3.84 \times 10^5\ M^{-1}$ and $K_2 = 1.24 \times 10^4\ M^{-1}$. NMR spectroscopy [5] was used to determine the number of low-affinity binding sites of tolmetin on HSA and the apparent association constant. It was assumed that all the binding sites are independent, that the binding reaction at different sites is a first order reversible fast process and that all binding interactions have the same equilibrium association constant K. The optimized values obtained at 25 °C were $n = 28 \pm 2$ and $K = 617 \pm 45\ M^{-1}$. Regarding the fluorescence quenching measurements [4], the relative fluorescence was plotted as a function of tolmetin concentration at fixed HSA concentration (15.5 μM). The final tolmetin concentration was around 18 μM. Extrapolation of the linear portions of the experimental titration curve gave the stoichiometric point from which the mole ratio of bound tolmetin to HSA was obtained, but its value was not reported. The association constant was found to be $7.7 \times 10^5\ M^{-1}$ using an Eq. [7], which assumes the equivalence and independence of interaction sites in HSA.

Due to the enormous popularity of using fluorescence quenching methodology to study ligand binding to HSA, the aim of this investigation is to use a correct data treatment, without extrapolations or using linearization methods, in order to obtain more accurate or at least more reliable results that describe the interaction between TOL and HSA. For this reason we used steady state

* Corresponding author. Tel.: +40 264 584037; fax: +40 264 420042.
E-mail address: silvia.neamtu@itim-cj.ro (S. Neamtu).

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fluorescence, time resolved fluorescence and UV–vis spectroscopy, in order to determine the binding sites, the binding constants, the binding mode and the binding thermodynamic parameters.

2. Materials and methods

2.1. Apparatus

A JASCO-6500 fluorescence spectrophotometer equipped with a Xenon lamp, 1.0 cm quartz cell and thermostatic bath was used to measure the fluorescence spectra of HSA. The fluorescence emission spectra were recorded in the wavelength range of 300–500 nm, upon excitation at λ_{ex} = 280 nm and λ_{ex} = 295 nm. Excitation and emission bandwidth was set to 3 nm for λ_{ex} = 280 nm and 3 nm/5 nm for λ_{ex} = 295 nm. The UV–vis absorption spectra were recorded in the range 250–500 nm, on a double beam JASCO-550 spectrophotometer equipped with 1.0 cm quartz cell. Fluorescence lifetime (τ) measurements were performed using a time-correlated single photon counting (TCSPC) setup (Light Conversion Co. Ltd.). The excitation source was a diode-pumped femtosecond Yb:KGW laser amplifier set at 280 nm, delivering 13.8 nJ energy per pulse at 170 fs (FWHM) duration and 80 kHz repetition rate. The HSA fluorescence decay was monitored between 300 and 400 nm in 1.0 mm quartz cell using a suitable cut-off filter for excitation wavelength. pH measurements were made on a Seven Multi pH Meter with a combined glass electrode.

2.2. Materials

Tolmetin-Na dihydrate and human serum albumin, fraction V (fatty acid free, 99%) were purchased from Sigma–Aldrich Chemie GmbH. 0.02 M Tris–HCl buffer solution was used to keep the pH of the solutions at 7.4. Double distilled water was used throughout the experiments.

2.3. Procedures

The solutions were prepared at room temperature using doubly distilled water and Tris–HCl as buffer. For fluorescence quenching measurements, the concentration of HSA was kept fixed at 4 μM and the TOL concentration was varied from 0 to 24 μM. The same set of samples was used to obtain the UV–vis spectra. The fluorescence and UV–vis spectra were then recorded at three temperatures: 298, 310 and 320 K, using thermostated chamber. Fluorescence lifetime measurements were performed for HSA 4 μM in Tris–HCl buffer and in the presence of tolmetin at two concentrations: 20 μM and 40 μM.

3. Results and discussion

3.1. Fluorescence quenching

Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecules. Fluorescence quenching can be caused by inner-filter effect, collisional quenching or binding-related changes in fluorescence [8]. The emission spectra of HSA in the presence of various concentrations of TOL at 25° C are shown in Fig. 1. It is observed that the fluorescence intensity of HSA regularly decreases with the concentration of TOL, but there is no significant emission wavelength shift with the addition of TOL.

As a first step, we checked if the fluorescence intensity is affected by the inner-filter effect. The inner-filter effect refers to the absorption of light at the excitation or emission wavelength by the compounds present in solution. That is, when the ligand is added

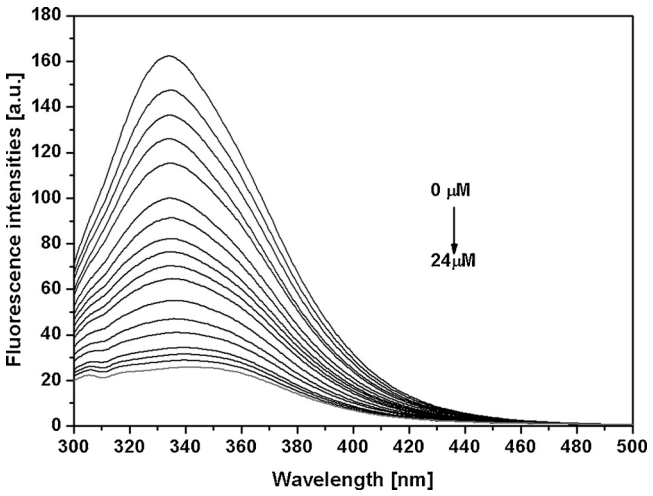

Fig. 1. Fluorescence emission spectra of HSA at 25° C, in the presence of various tolmetin concentrations: [TOL]/10^{-6} M: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24.

to a solution, it may reduce the amount of excitation radiation that reaches the fluorophore, or it may absorb some radiation emitted by the fluorophore. The easiest correction method is to determine the absorbance at the excitation and emission wavelength for each concentration of ligand, including the protein without ligand. The corrected fluorescence can be then estimated from [9].

$$F_{corr} = F_{obs} 10^{\frac{A_{exc}+A_{em}}{2}} \quad (1)$$

where F_{corr} is the corrected fluorescence value, F_{obs} the measured fluorescence value, A_{exc} and A_{em} the measured changes in absorbance value at the excitation and emission wavelength, respectively. This particular equation is an appropriate estimation only if the absorbance is measured with the same optical path length of the fluorescence quartz cell. As can be seen from Fig. 2, the increase of tolmetin concentration led to a gradual enhancement in UV–vis intensity. At the highest added tolmetin concentration (24 μM), the change in the absorbance measured at the excitation and emission wavelength has a value of 0.14 and 0.38 a.u., respectively (Fig. 2A).

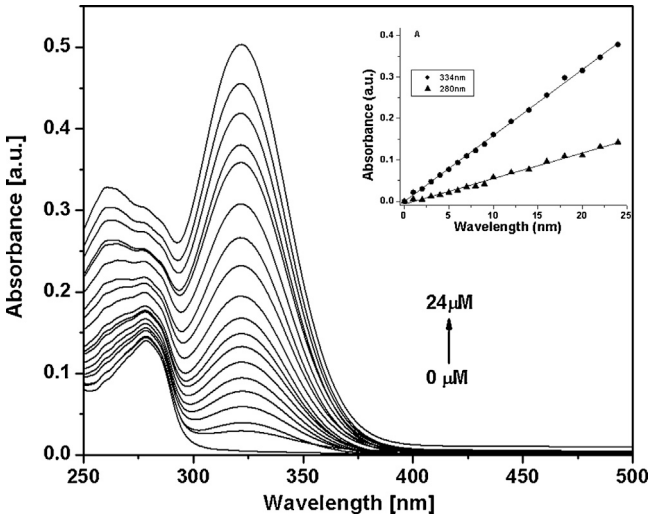

Fig. 2. Absorbance spectra of HSA in the presence of various tolmetin concentrations. (Inset) Change in UV–vis absorbance with increasing tolmetin concentration.