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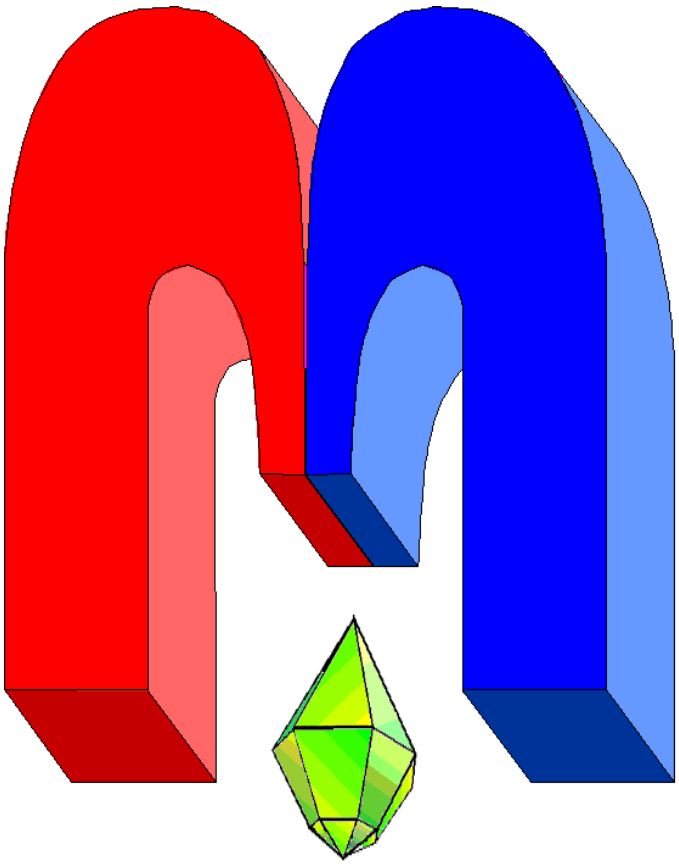

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* In Kazan University the Electron Paramagnetic Resonance (EPR) was discovered by Zavoisky E.K. in 1944.

NMR study of cholesterol complexes with glycyrrhizic acid

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Nuclear magnetic resonance (NMR) relaxation technique was applied to study the interaction of cholesterol with natural complexant – glycyrrhizic acid. Stoichiometry, stability constants and thermodynamic parameters of the complex have been measured. We propose that the complexation with glycyrrhizic acid could be an effective approach to regulate the cholesterol level inside and outside of cage membranes.

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Keywords: NMR, relaxation, cholesterol, glycyrrhizic acid, atherosclerosis, complex of cholesterol.

1. Introduction

Cholesterol (Fig. 1) is a natural lipophilic alcohol contained in the cell membranes of most living organisms. It enters the body from two sources: from food and from endogenous synthesis, with the majority (80%) is synthesized by the body. Cholesterol plays an important role in many biochemical processes, in particular, ensures the stability of cell membranes in a wide range of temperatures, and is needed to produce vitamin D and various steroid hormones. In addition, cholesterol participates in the synthesis of bile acids, and according to some sources - affects the activity of the synapses of the brain and the immune system, including protection against cancer. At the same time, cholesterol earned him notoriety for his involvement in the formation of atherosclerotic plaques. To date, it is believed that increased blood levels of cholesterol and low density lipoprotein (LDL) cholesterol are the main factors for risk of atherosclerosis. But there is another insight into the problems of cholesterol: it is like a "repair material" accumulated in the field of microdamage of blood vessels and blocking these lesions. In addition, the oxidation products of cholesterol were found in atherosclerotic plaques, which may play a key role in the pathogenesis of atherosclerosis and other diseases [1-4].

At the moment there are several ways of treatment the atherosclerosis disease. All of them use the drugs which are inhibitors of enzymes responsible for the different stages of cholesterol biosynthesis. They terminate the chain of cholesterol formation, but block also the formation of some other important products of biosynthesis. In addition, these drugs often demonstrate toxic properties. This is why the search for alternative methods of regulation of cholesterol level is in progress. One such approach might be to use natural complexants which are able to bind to cholesterol molecules and affect their properties. At now, only one successful example of such approach is known. It is a complex of cholesterol with cyclodextrins (CDs) which effectively remove cholesterol from membranes [5, 6]. But in this case some negative effects occur. First, this disturbs the structure of membranes, and second, the possibility of crystallization of both the CD and their complexes was detected.

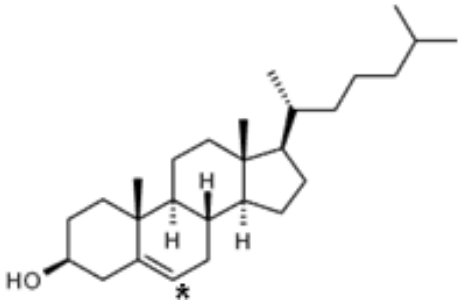

Figure 1. Cholesterol

There are several reasons why the interaction of cholesterol with glycyrrhizic acid (GA, Fig. 2) may be of interest. First, the GA - the natural complexants that exhibits a wide spectrum of biological activity. It forms inclusion compounds with many drugs and is widely used in medicine [7]. Secondly,

unlike the CD, there is no evidence indicating toxicity of GA. Thirdly, there are some data on the influence of GA on the biosynthesis and properties of cholesterol [8]. In experiments on animals with atherosclerosis, GA and its salts reduce cholesterol, LDL and triglycerides level [8]. Fourthly, there are data that indicate the ability of GA to reduce the oxidation of cholesterol [9]. However, the molecular mechanism of these effects is currently unknown. Complex formation of cholesterol with GA may shed light on these facts and open a new way to treatment of atherosclerosis. The purpose of this study is to investigate the possibility of complexation of cholesterol with GA by NMR relaxation technique.

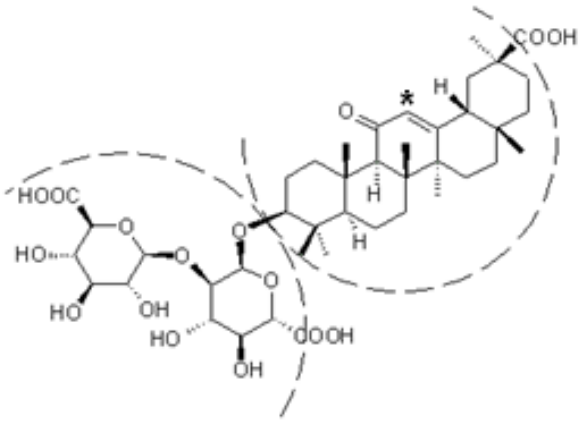

Figure 2. Glycyrrhizic acid

2. Results and Discussion

In this study the complex formation between cholesterol and glycyrrhizic acid was observed for a first time. The complexes were prepared by mixing the starting materials in deuterated methanol. For the calculation of stability constants and thermodynamic parameters of the complex the samples with different ratios of cholesterol and GA: 5 mM / 5 mM, 10 mM / 5 mM and 5 mM / 10 mM were analyzed at two temperatures: 300 K and 320 K. The formation of complexes was studied by NMR relaxation technique. All NMR experiments were performed using DPX-200 Bruker NMR spectrometer (200 MHz ^{1}H operating frequency) equipped with temperature control. T_2 relaxation time of cholesterol and GA (the corresponding protons are marked with ∗ in Fig. 1 and Fig. 2) was measured by means of Carr-Purcell-Meiboom-Gill sequence: $p(90°) - (\tau - p(180°) - \tau)\, n$ – acquisition, where $\tau = 0.6$ ms and n was varied from 0 to 2000.

It is known that the relaxation times of protons are very sensitive to molecular mobility. The formation of complex decreases the mobility of molecules, and this leads to a significant decrease in the relaxation time of protons. In general, the complex formation results in bi-exponential or even three-exponential relaxation kinetics, if there are several different types of aggregates in solution. Measurement of the pre-exponential factors allows to determine the fraction of molecules located in the complex and hence to calculate the stability constants and stoichiometry of the complex. In general the stability constant of the reaction: $n\text{Chol} + m\text{GA} \leftrightarrow \text{Chol}_n\text{GA}_m$ is determined as:

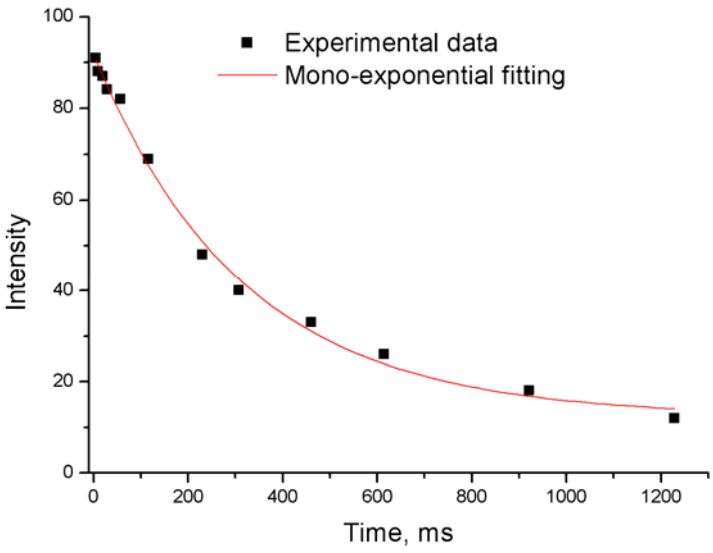

Figure 3. Decay kinetics of echo signal for pure cholesterol in methanol.

$$K = \frac{[\mathrm{Chol}_n \mathrm{GA}_m]}{[\mathrm{Chol}]^n\,[\mathrm{GA}]^m}, \qquad (1)$$

where [Chol] is the free cholesterol concentration, and [GA] is the concentration of free GA. The values of *m* and *n* were calculated using the optimization program of experiments with different ratios of $[\mathrm{Chol}]_0$ and $[\mathrm{GA}]_0$.

The T_2 relaxation kinetics for both precursors in the free state is mono-exponential with characteristic times of $T_2 \sim 300$ ms for cholesterol and $T_2 \sim 400$ ms for GA protons. As an example, Fig. 3 shows the kinetic of relaxation of proton at the double bond (marked by ∗ in Fig. 1) of pure cholesterol. After mixing the cholesterol with GA three-exponential kinetics for the protons of both substances appear: the fastest component has a characteristic decay time in order of several milliseconds, the second component in the order of several tens of milliseconds and the third component - the order of several hundred milliseconds (Fig. 4). Over time, the shortest component disappears. While the process of establishing the equilibrium takes a few days, it accelerates when the temperature rises to 60° C. Fig. 5 shows the kinetic of the echo signal decay for the sample stored for one day at room temperature.

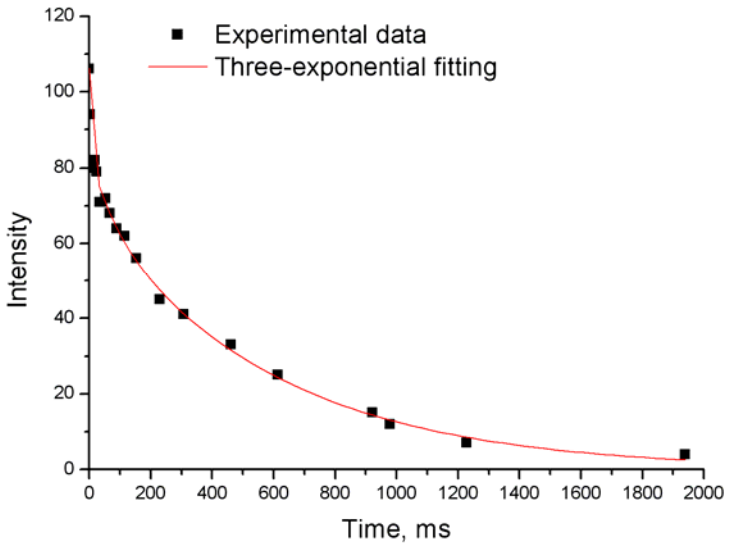

Figure 4. Decay kinetics of echo signal of cholesterol in the presence of GA in methanol.

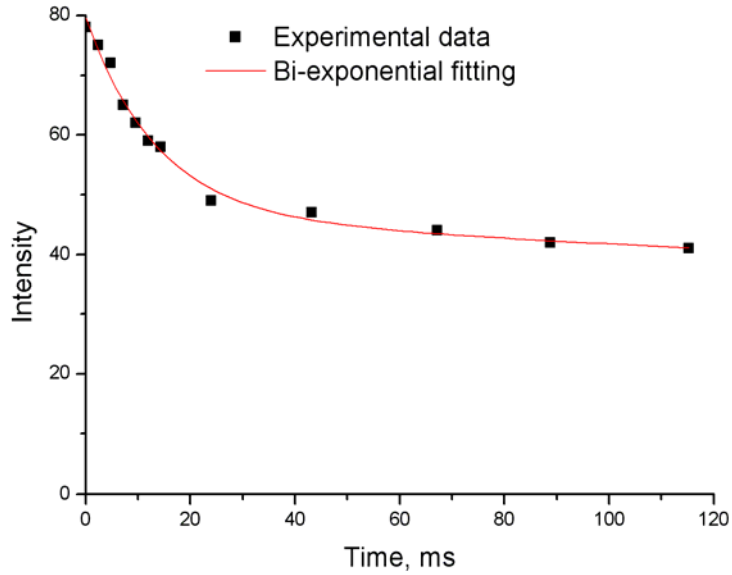

Figure 5. Decay kinetics of echo signal of cholesterol in the presence of GA in methanol after store of one day at RT.

We assume that the component with shortest relaxation time corresponds to large aggregates consisting of several molecules of cholesterol and GA. The same order of magnitude of relaxation time is observed, in particular, for micelles solution. Over time, they are likely to disintegrate into smaller complexes, which correspond to the relaxation time of several tens of milliseconds. Long component of several hundred milliseconds corresponds to the free states of the substances involved in the complex.

The analysis of the experimental data was performed using simple bi-exponential model:

$$A(t) = P_1 \exp(-t / T_{21}) + P_2 \exp(-t / T_{22}). \qquad (2)$$

Measurement of the pre-exponential factors allows to determine the fraction of molecules located in the complex and hence to calculate the stability constants and stoichiometry of the complex using equation (1). Usually bi-exponential behavior is assigned to the difference in the relaxation rates of the protons belonging to the same type and located in an associate and in non-associate states. Note that the necessary condition of the observation of bi-exponential relaxation kinetics is slow exchange rate between the associate and non-associate states: $1/\tau < 1/T_2$. In these experiments we have not observed significant changes the chemical shifts of the protons investigated in the free and bound forms. Taking into account our previous studies, we assume that this is common feature of GA complexes [10-12].

The stoichiometry and stability constants of the complex were determined by the fitting of decay kinetics at different concentrations of cholesterol and GA after the establishment of equilibrium using equations (1) and (2) for concentrations of the complex and free cholesterol: $n \times [\mathrm{Chol}_n\mathrm{GA}_m] = P_1$ and $[\mathrm{Chol}] = P_2$. The resulting stoichiometry is 1:2 (one molecule of cholesterol on the two molecules of GA). This result is consistent with existing data on the complexation of GA with other substances [10-12]. The calculated stability constant for this stoichiometry is $K_{12} = (3 \pm 0.6) \times 10^3\ \mathrm{M}^{-2}$.

The thermodynamic parameters of the complex were calculated from the temperature dependence of the stability constant using equations:

$$\ln\left(\frac{K_2}{K_1}\right) = \frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right),$$

$$\Delta G = -RT\ln K, \quad \Delta G = \Delta H - T\Delta S, \quad \Delta S = (\Delta H - \Delta G)/T.$$

As a result, the following values were obtained: $\Delta G(300\ \mathrm{K}) = (-20 \pm 4)$ kJ/mol × K; $\Delta H = (0.4 \pm 0.08)$ kJ/mol; and $\Delta S(300\ \mathrm{K}) = (68 \pm 14)$ J/mol × K. It is seen that the entropy factor makes the main contribution to the complex stability. This could mean that the desolvation of the cholesterol molecule occurs during the complex formation. It should be kept in mind that all measurements were made in methanol solution. One can expect that in aqueous solution the binding of cholesterol with GA will be significantly enhanced by the hydrophobic interaction.

3. Conclusion

Thus, the formation of stable aggregates of cholesterol with glycyrrhizic acid was observed for a first time. The measurement of T_2 relaxation time of the protons of cholesterol and GA shows the existence of several types of aggregates: complexes with stoichiometry 1:2 and more large aggregates which disappear at higher temperature. Taking into account also the ability of GA to gain the permeability of cage membranes, where near 90% of cholesterol is located, this study might be the beginning of new approach development to the regulation of the cholesterol level.

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