Scientific paper

Study of Association of 12-Monoketocholic Acid by ^{1}H NMR Relaxation Method

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Abstract

12-Monoketocholic acid (12-MKC) shows a promotive effect in the transport of some drugs (lidocaine, morphine, quinine, gliclazide, etc.). In the mechanism of pharmacological action of 12-MKC an important role plays its aggregation (self-association), as well as formation of mixed micelles. These phenomena were studied by the ^{1}H NMR spin-lattice relaxation method using solutions of sodium salt of 12-MKC in D_2O and in D_2O solution saturated with 1-octanol. The function describing the dependence of the spin-lattice relaxation time (T_1) on the concentration of Na-12-MKC (c_{BA}) in D_2O has one inflexion point (at the concentration of 48 mM), whereas the same function for the saturated solution of 1-octanol has two inflexion points (one at the concentration of 45 mM and the other at 87 mM). These differences in the curves $T_1 = f(c_{BA})$, as well as the shift of the solubilization curve of lecithin in the presence of 1-octanol towards higher concentrations of Na-12-MKC, indicate that sodium salt of this keto-bile acid forms mixed micelles with 1-ocatnol, so that the application of the micellar solution of Na-12-MKC in the presence of 1-octanol decreases membrane toxicity (membrane lysis) of this bile acid. The curves $T_1 = f(c_{BA})$ for methyl esters of 12-MKC and cholic acid in the $CDCl_3$ solution have one inflexion point at the concentration above 5 mM, indicating formation of the micelles of Small's type, whereas methyl ester of 3,7,12-triketocholic acid forms a gel-like structure.

Keywords: 12-monoketocholic acid, ^{1}H NMR relaxation method, aggregation

1. Introduction

Specific geometry of bile acid molecules, especially of those that on the α side of the steroid skeleton have two or more hydroxyl groups (deoxycholic, chenodeoxycholic and cholic acids), allows formation of molecular aggregates – micelles of ellipsoidal shape with a hydrophobic core, when the bile acid is present in an aqueous solution at a concentration exceeding the critical micellar concentration (CMC).[1,2]

The knowledge of how molecular aggregates of bile acids in aqueous media are formed is important because of the solubilization of liposoluble substances in biological systems.[3–6] The CMC value of a bile acid is an important characteristic since it determines the bile acid ability of self-association[7,8] and formation of mixed micelles, especially with hydrophobic cationic drugs.[9]

It is supposed that the promotive effect of bile acids on the transport of some drugs through cell membranes is due to the formation of reverse micelles in the membrane lipid phase[10]. However, one can also find in the literature the models in which bile acids form normal micelles in the membrane like those formed in an aqueous medium. This is explained by the fact that the increase in the hydrophobic area of a bile acid molecule enhances the transport of peptide drugs through the cell membrane.[11] In the mechanism of promotive action of bile acids on the transport of drugs through the cell membrane an important role plays their interaction with membrane phospholipids.[12–14] At the same time, bile acids damage cell membrane by withdra-

wing phospholipids from it.[11] Hence the CMC value is an important parameter for predicting of either promotive or membrane-damaging effects of bile acids.

Pharmacological studies of the promotive action of keto derivatives of cholic acid began in the middle of the nineties of the last century.[15–18] It has been shown that 7-monoketocholic acid (7-MKC)[18–19] and 12-monoketocholic acid (12-MKC) are especially active. Besides, recent investigations showed that 12-MKC exhibits a significant hypoglycemic effect.[20,21]

The objective of this work was to study the association (aggregation) of 12-MKC (Fig. 1) in a D_2O solution as well as in the D_2O solution saturated with 1-octanol (to examine protective action of this bile acid), that is in deuterated chloroform (model of the cell membrane). Also, the aim was to study lecithin solubilization by 12-MKC in the experiment involving an aqueous solution of lecithin or an aqueous solution saturated with 1-octanol (indirect examination of membrane toxicity).

2. Experimental

2. 1. Chemicals and Solutions

Cholic acid (Sigma, New Zealand, 98%) was used for the synthesis of 12-MKC by the procedure of Miljković *et al.*[22]. Methyl ester of 3,7,12-triketocholic acid was prepared according to Fieser and Rajagopalan[23]. Methyl ester of 12-MKC was obtained in the following way: 100 mg of the bile acid was stirred in methanol (25 mL) for 24 h in the presence of p-toluenesulfonic acid as a catalyst. After evaporation, the reaction mixture was dissolved in chloroform (25 mL) and washed with a 5% solution of $NaHCO_3$ (50 mL). D_2O (Aldrich, 99.9%); $CDCl_3$ (Aldrich, 99.8); Eggs lecithin (Sigma, 99.0%); 1-octanol (Fluka, 96.0%); 7-oxo-octanoic acid (Sigma, 98%).

Saturated 1-octanol solution in D_2O was prepared by mixing 10 mL of 1-octanol and 10 mL of D_2O on a magnetic stirrer (900 rpm).

2. 2. ^{1}H NMR Experiments

Measurement in D_2O solution: For this purpose, stock solution of Na-12-MKC (c = 120 mM in D_2O and the same concentration in D_2O saturated with 1-octanol) was diluted with D_2O or with saturated 1-octanol solution in D_2O respectively, to cover the concentration range of 10–110 mM.

Determination in $CDCl_3$ solution: Stock solution of methyl esters of either 12-MKC, cholic or 3,7,12-triketocholic acid (c = 60 mM in $CDCl_3$) was diluted with $CDCl_3$ to cover the concentration range 5–55 mM. Methyl esters of these acids were used because of their solubility in $CDCl_3$.

Measurements were performed at 23 °C on a Bruker AC-250 instrument with standard Bruker software. The ^{1}H NMR spectra were recorded using a spectral window of 3200 Hz. Spin-lattice relaxation times T_1 were determined by the inversion recovery experiments (180°-τ-90°-AQC). Selected peak areas for nine different interpulse delays τ (0.001, 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6) were determined.

2. 3. Spectrophotometric Determination of Lecithin Solubilization

Lecithin solubilization with bile acids was investigated by the modified Sewell's method.[24] Namely, the original Sewell's method with visual detection was replaced by spectrophotometric determination of lecithin via phosphate.

Suspensions of lecithin (100 mg) and sodium salt of bile acid (10 mL, 0.15 mM NaCl, pH 7.4 $H_2PO_4^-$/HPO_4^{2-}) were prepared so that bile acid concentration was in the range from 2 mM to 10 mM. If, however, no lecithin solubilization was observed in this concentration range, a new series of solutions was prepared in the range from 10 mM to 20 mM, etc. The suspension of lecithin and bile acid was kept in a water bath at 37 °C for 24 h at the constant stirring on a magnetic stirrer (300 rpm). After that the suspension (if it was necessary) was filtered through a previously warmed filter (0.22 μm). The clear filtrate (600 μL) was evaporated in vacuo and then concentrated perchloric acid (1.0 mL) was added to hydrolyze lecithin ester. The hydrolysis was carried out in an oil bath (in a microvial equipped with a water condenser) at 180 °C for 45 min. After the hydrolysis, the solution was cooled to room temperature and distilled water (3 ml), ammonium-molybdate reagent (1.0 mL, 2.5% ammonium molybdate, 10% sulfuric acid) and reducing reagent (1.0 mL, 1% ascorbic acid and 0.016% copper(II)-sulfate) were successively added to it. The mixture was shaken manually and left for 10 min. Absorbance was measured at 630 nm on an Agilent 8453 spectrophotometer, using the calibration plot made for inorganic phosphate.[25] The blank was prepared in the identical way using distilled water (600 μL) instead of the filtrate of bile acid and lecithin.

2. 4. Spectral Shift of 7-oxo-octanoic Acid

A series of solutions consisting of Na-12-monoketocholate of the following concentrations (mM): 20, 30, 40, 50, 60, 70, 80, 90, 100, each being 3 mM in 7-oxo-octanoic acid, were prepared in the phosphate buffer pH 7.4. Another series of the same solutions but without 7-oxo-octanoic acid served as the blanks in the measurement of the absorbance of the keto group of 7-oxo-octanoic acid at 280 nm.

2. 5. Data Treatment

Data treatment (curve fitting, hierarchical clustering) was performed using SPSS 10.0 for Windows.

3. Results and Discussion

Figure 1 shows the ^{1}H NMR spectrum of Na-12-MKC (D_2O), in which the signal belonging to the overlapped CH_3-18 and CH_3-19 protons (marked with asterisk), served as the basis for determining the relaxation time T_1 by the 180°-τ-90°-AQC method. These protons were chosen for monitoring in view of the assumption that, according to Small's model,[26,27] bile acid molecules associate via their hydrophobic surfaces, that is the β-sides of the steroid skeleton, and hence, the change of the environment is most pronounced with the angular methyl groups. If the relaxation time is calculated on the basis of the signals of the protons, CH_3-21 the obtained values do not differ statistically from the magnitudes of relaxation times obtained with respect to the protons CH_3-18 and CH_3-19. The protons with the α orientation were not taken into account since they are on the hydrophilic side of the steroid skeleton, and hence do not influence the aggregation of bile acids.

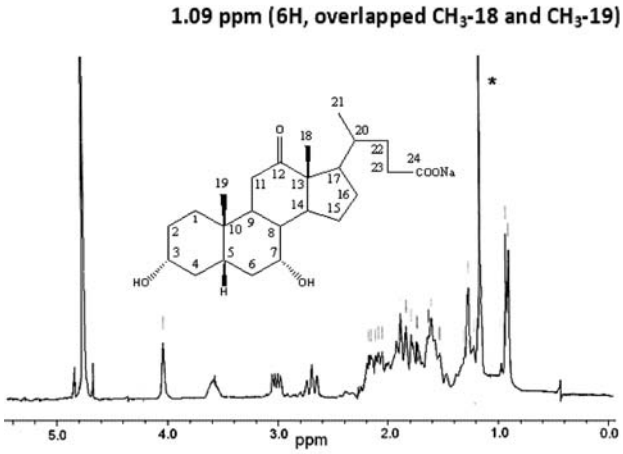

Figure 1. Structure of Na-12-MKC and its ^{1}H NMR spectrum in D_2O.

Figure 2A shows the ^{1}H NMR spectra of sodium-12-MKC in D_2O at the concentration of 60 mM that were used to determine the relaxation times T_1 according to the 180°-τ-90°-AQC method[28]. Each spectrum was recorded after a time τ upon applying the 180° pulse. To study self-association of the bile acid it was necessary to record a series of spectra like those in Fig. 2A for each concentration tested. The signals in Fig. 2A, marked by arrows, belong to the protons at the C18 and C19, whose areas are measured as a function of time. On the other hand, Fig. 2B shows the dependence of the area of the same proton signals as a function of the time after the 180° pulse, the time τ being measured from the moment of termination of the pulse action. The relaxation time is determined by fitting the experimental data to the following equation (*SA*= signal area):

$$SA = SA_0(1\text{-}2e^{-\tau/T_1}) \quad (1)$$

The relaxation time was also determined by the so-called nul method, using the following equation:

$$T_1 = \tau_{null}/\ln 2 \quad (2)$$

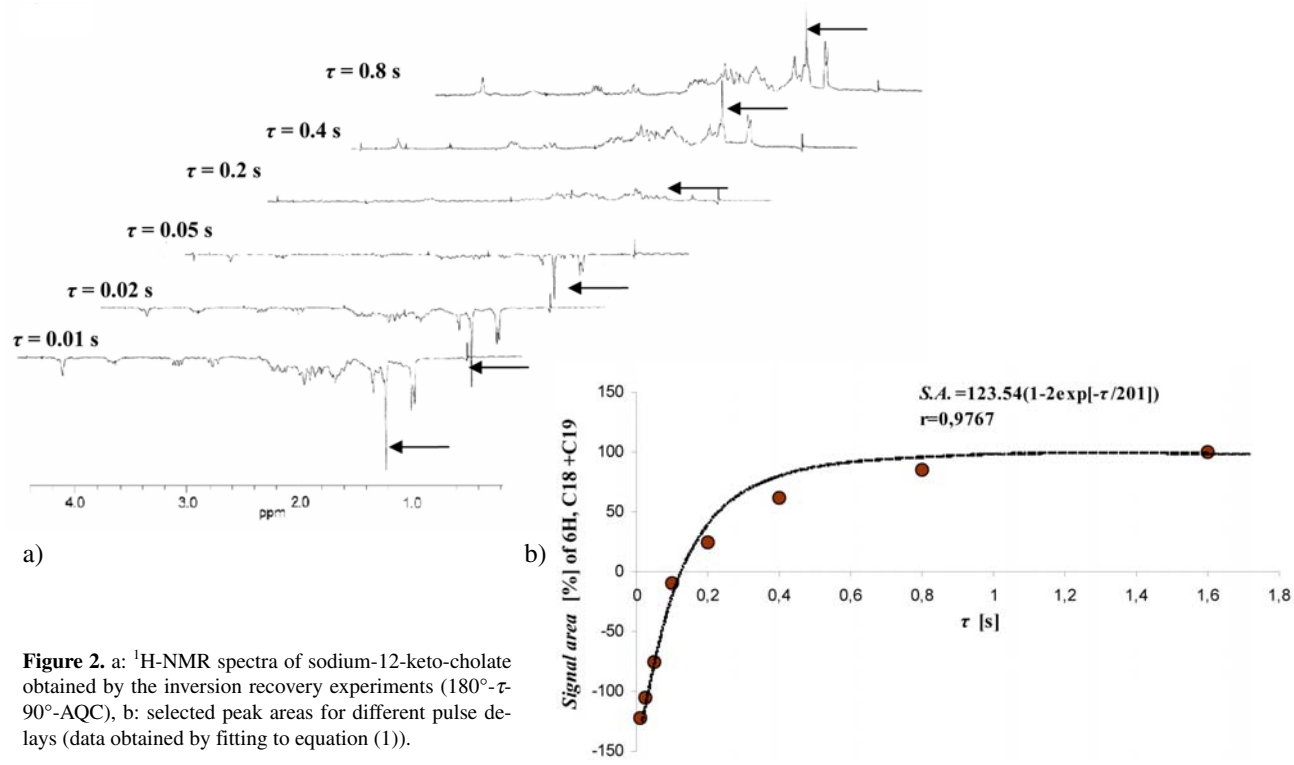

Figure 2. a: ^{1}H-NMR spectra of sodium-12-keto-cholate obtained by the inversion recovery experiments (180°-τ-90°-AQC), b: selected peak areas for different pulse delays (data obtained by fitting to equation (1)).

Namely, because of the molecular structure and dynamics, it may happen that the determination of relaxation time by fitting to equation (1), does not give good results.[29]

For each concentration of the bile acid tested a graph is plotted showing the dependence of the area of the C18 proton signal on time τ, from which is then determined the relaxation time. Therefore, for each bile acid concentration a relaxation time T_1 is obtained, which is then plotted as a function of the bile acid concentration c_{BA} (Fig. 3).

The values of relaxation times determined according to (1) and (2) differ in average by 5%.

If the function $T_1 = f(c_{BA})$ has one inflexion point (occurring between the upper pseudo CMC(a) and the lower CMC(b)), this indicates that micelles are formed.[28] In this work the CMC is determined as the arithmetic mean of the values of the upper pseudo CMC(a) and the lower pseudo CMC(b), to avoid the error of determining the inflexion point with the aid of auxiliary lines.[30]

In view of $T_1 = f(c_{BA})$, the mean CMC value for Na-12-MKC (D_2O) is 45 mM, so that this curve has one inflexion point between 30 mM and 60 mM (Fig. 3). On the other hand, the same curve for Na-12-MKC in D_2O solution saturated wih 1-octanol (0.3 mM) has two inflexion points, so that the mean CMC at the first inflexion point is 45 mM (occuring between 40 and 50 mM), whereas the second inflexion point, CMC-2 is 85 mM (the mean of 80 and 90 mM). Figure 3 shows the dendrograms for curves I and II. The dendrograms were obtained on the basis of the Euclid matrix of distances of T_1 values, using the Ward method of clustering (the objects concentrate in the one-dimensional space T_1). On the dendrogram corresponding to the curve I there are two clusters (the significance of forming two clusters is at the level $p < 0.01$) of concentrations of Na -12-MKC, one encompassing the concentrations 20, 30 and 40 mM, and the other the concentrations between 50 and 110 mM, which confirms that the mean CMC value is between 40 and 50 mM. On the dendrogram corresponding to curve II, to the first of cluster enter the same concentrations as in the case of the dendrogram I. However, in contrast to the previous dendrogram there also appear a third dendrogram (the level of significance $p < 0.5$), whose elements are the concentrations 90, 100 and 110 mM, and hence the mean CMC-II is between 80 and 90 mM.

Also, as can be seen from Fig. 3, the function $T_1 = f(c_{BA})$ for 12-MKC in D_2O is broadened around the inflexion point, whereas in the presence of 1-octanol the curve

CMC [mM]

	CMC (a)	CMC (b)	CMC	CMC-2 (a)	CMC-2 (b)	CMC-2
I	30	60	45			
II	40	50	45	80	90	84

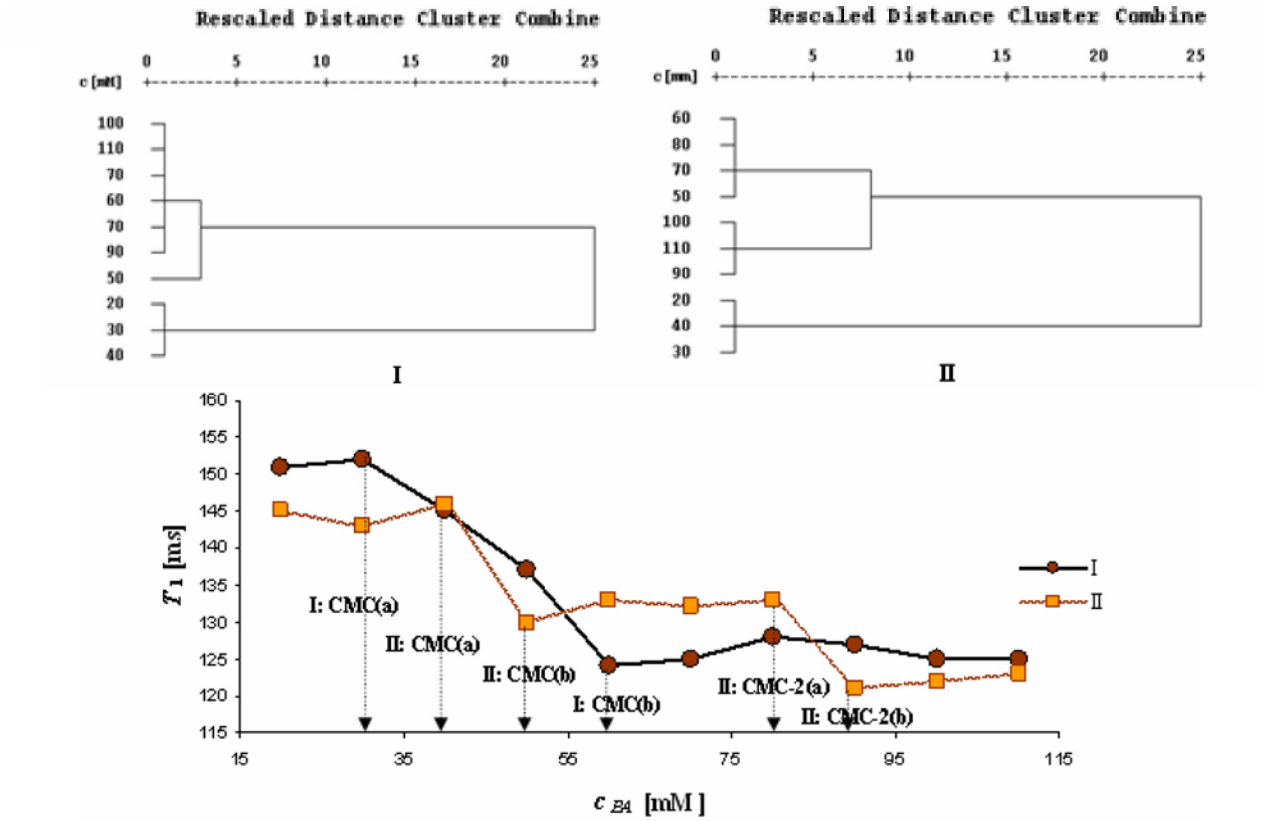

Figure 3. Spin-lattice relaxation time (T_1) as a function of the concentration (c_{BA}) of Na-12-MKC: I – in D_2O solution, II – in D_2O solution saturated with 1-octanol.

is significantly sharper in the neighborhood of this point. This difference in the characteristics of the function $T_1 = f(c_{BA})$ in the presence of 1-octanol suggests that there occurred a change in the mode of Na-12-MKC aggregation. The slowly-changing curve $T_1 = f(c_{BA})$ for 12-MKC (D_2O) between the two pseudo CMCs (between which the inflexion point is located) and the absence of a second inflexion point indicate that Na-12-MKC in D_2O forms primary micelles of Small's type.[26,27] Namely, according to the Small model, the aggregation number of bile acid aggregates formed at lower concentration (around the CMC) is 2 (M_2), with the carboxylate groups on the opposite sides of the longitudinal micelle (Fig. 4A), so that the repulsive interactions are minimal.[1,26,27] A consequence of the increase in bile acid concentration is the change in the structure of their primary micelles, so that the aggregation number of the most probable micelle amounts to 4. In a primary micelle consisting of four molecules of bile acid (M_4), a core consisting of two bile acid molecules is formed first, the carboxylate groups being on the same side of the aggregate (the system's energy enhanced due to repulsive interactions) (Fig. 4 B1). This core is of a longitudinal shape on the two opposite sides, of which one is with carboxyl group and the other with *cis*-bonded A-ring of the steroid skeleton. Two such cores then associate forming a micelle with an aggregation number equal 4 (Fig. 4 B2). However, since M_4 is less stable, the equilibrium is shifted in the direction of M_2, so that no abrupt jump is observed at the inflexion point.

The existence of two inflexion points of the function $T_1 = f(c_{BA})$ in the case of the presence of 1-octanol (Fig. 4) may mean that there are two kinds of micelles present in the system (solution), and these are the micelles formed only of Na-12-MKC molecules and mixed micelles of the molecules of Na-12-MKC and 1-octanol (after the second inflexion point) (Fig. 5). Namely, the micelles formed at lower concentrations of Na-12-MKC have a smaller aggregation number (lower energy content) – first inflexion point, whereas the micelles formed at higher concentrations have an aggregation number 4 (higher energy content); 1-octanol with its octyl group entering the hydrophobic core of the M_4 micelle, while the OH group of 1-octanol is bonded by hydrogen bonds to the carboxylate group of Na-12-MKC from M_4, and thus stabilizes the mixed micelle – second inflexion point.

The assumption of forming mixed micelles between 1-octanol and Na-12-MKC is also supported by the experiment of measuring the absorbance of the keto group of

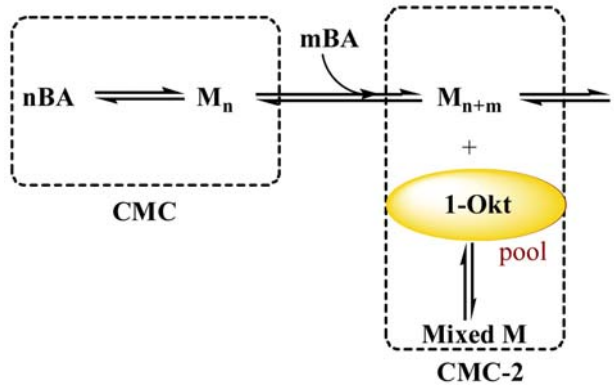

Figure 5. Proces of aggregation of Na-12-MKC in D_2O solution saturated with 1-octanol. BA – monomer of Na-12-MKC, M – micelle of Na-12-MKC, 1-Okt – 1-octanol, Mixed M – mixed micelle of Na-12-MKC and 1-octanol.

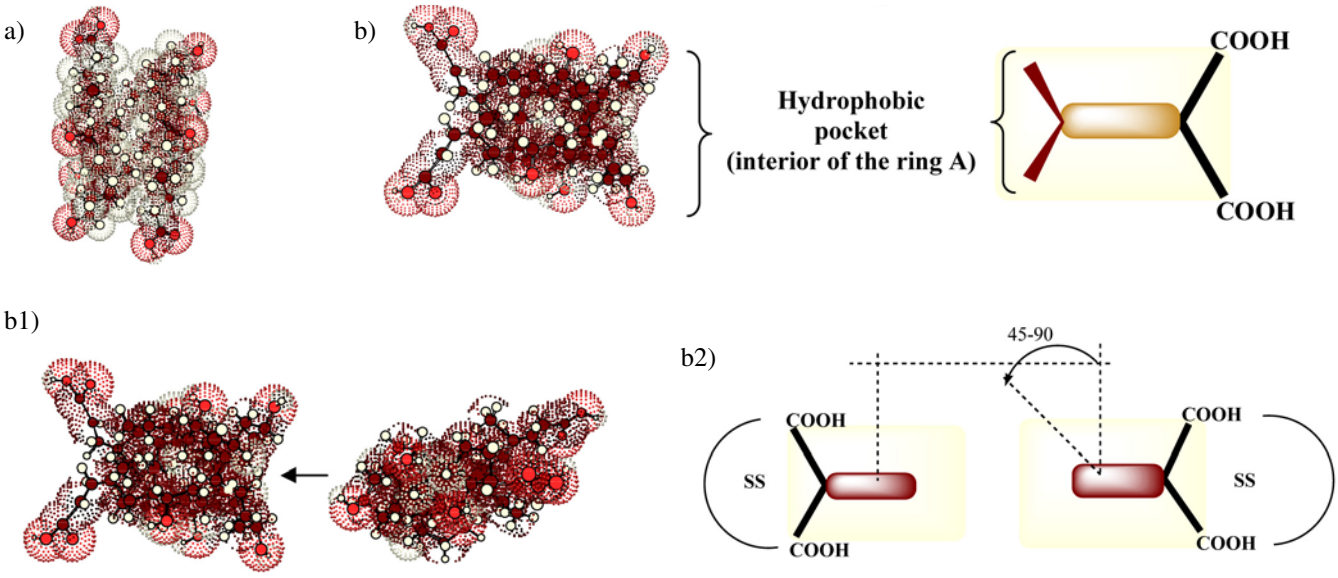

Figure 4. Aggregation of Na-12-MKC acid. a: Micelle with the aggregation number 2 (Small's model). b: Formation of primary micelle of Na-12-MKC with the aggregation number 4. b1: Formation of primary core. b2: In bonding, two cores make an angle from 45° to 90°, SS-site of stabilization with 1-octanol.

Table 1. Dependence of the absorption of the keto group of 7-oxo-octanoic acid in the presence of different concentrations of Na-12-monoketocholate.

c_{BA} [mM]	20	30	40	50	60	70	80	90	100
$A_{280\ nm}$	0.25	0.26	0.23	0.25	0.24	0.08	0.04	0.001	DL

7-oxo-octanoic acid (probe molecule) as a function of the Na-12-MKC concentration. As can be seen from Table 1, the decrease of absorbance occurs starting from the concentration of 70 mM, which can be explained by the assumption that the probe molecule entering the hydrophobic core changes the environment of the keto group (screening effect), lowering thus the absorbance.

In our previous study[7], using the method of solubilization of water-insoluble dye, we obtained a value of 65 mM for the CMC of Na-12-MKC. The difference from 45 mM can be explained on the basis of the work of Subudhi and Mishra[31]. According to these authors, the CMC value depends on the nature of the probe molecule. Namely, a probe molecule that fits less effectively the micelle's hydrophobic core causes its distortion, so that the hydrophobic interior of the micelle is exposed to water molecules, and the monomers that form micelle look like more polar than they really are, which then lowers their tendency to aggregation, that is, increases the CMC value. Further, it has been reported that CMCs of bile acids depend markedly on the measuring method, for example, the surface tension method gave for Na-12-MKC 92 mM and solubilization of rhodamine 6G 35 mM[1].

The assumption of the formation of mixed micelles between Na-12-MKC and 1-octanol is supported by the experiment of lecithin solubilization (Fig. 6). Namely, the solubilization of 100 mg of lecithin requires a larger amount of Na-12-MKC if the process takes place in the D_2O solution saturated with 1-octanol. This can be explained by the assumption of forming mixed micelles between the bile acid and 1-octanol, which stabilizes the mixed micelles. On the other hand, lecithin is solubilized only at higher concentration of the bile acid, while 1-octanol

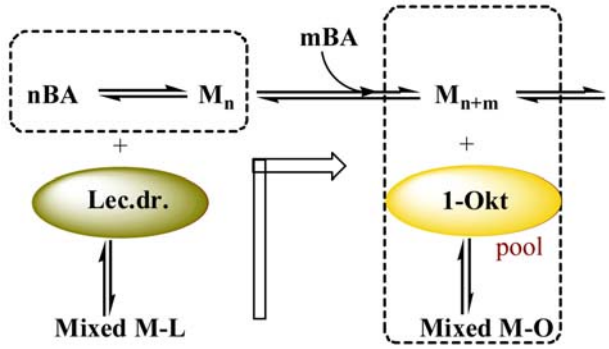

Figure 7. Mixed micelles between bile acid and 1-octanol, Mixed M-O, shifts formation of the mixed micelle of bile acid and lecithin, Mixed M-L, towards bile acid monomer, Lec.dr. = lecithin droplet.

is also incorporated into the lecithin core of the mixed micelle (Na-12-MKC plus lecithin).

Bile acids and lecithin form large mixed micelles whose cores consist of more than 20 lecithin molecules, and it is generally known that such a process leads to lower CMC values. In contrast to this, with 1-octanol and 7-oxo-octanoic acid, as well as with the majority of probe molecules, bile acids form small mixed micelles with one probe molecule in their core. Hence, in the system consisting of bile acid, lecithin, and 1-octanol, mixed micelles formed between bile acid and 1-octanol shift the equilibrium of formation of mixed micelles between bile acid and lecithin to the direction of monomers (Fig. 7).

The shift of the solubilization curve towards higher concentrations of Na-12-MKC indicates that 1-otanol exhibits a membrane-protecting activity. Namely, it has been

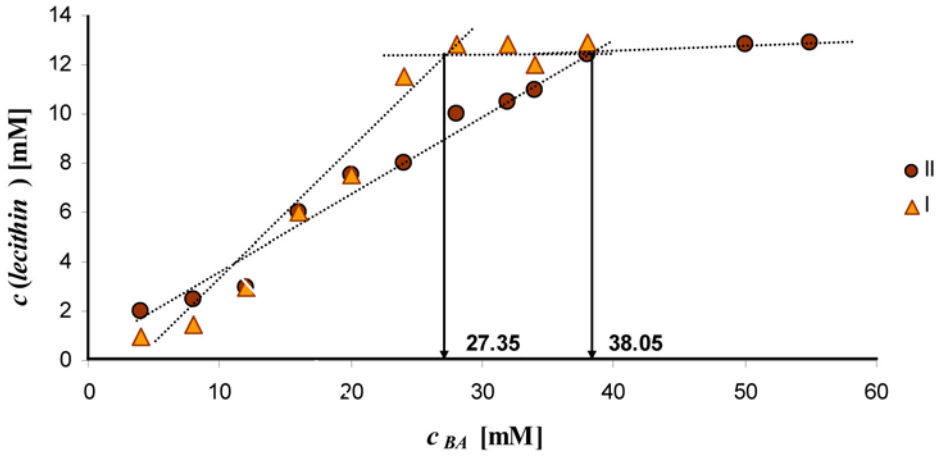

Figure 6. Solubilization of lecithin with Na-12-MKC. I: D_2O solution, II: D2O solution saturated with 1-octanol.

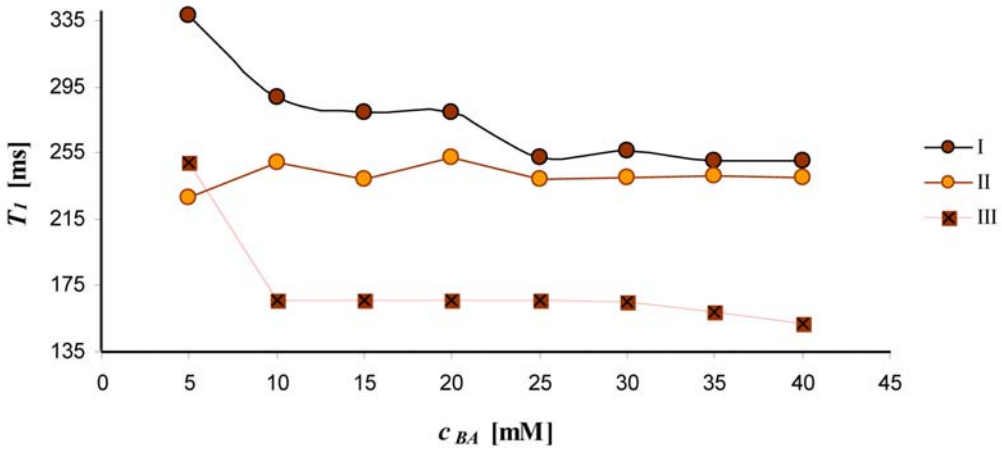

Figure 8. Aggregation of methyl esters of bile acid derivatives in $CDCl_3$, I – methyl ester of 12-MKC, II – methyl ester of 3,7,12-triketocholic acid, III – methyl ester of cholic acid.

shown that for some mechanisms of the promotive action of monoketo derivatives of cholic acid the presence of cholic acid in the membrane lipid phase is of essential importance.[18,19] However, the transport of the bile acid to the cell membrane in a micellar solution may affect the integrity of the cell membrane. On the other hand, if the transport takes place in the presence of mixed micelles involving higher alcohols or fatty acids, this reduces the ability of the bile acid (in this case Na-12-MKC) to withdraw phospholipids from the cell membrane, that is, decreases the membrane damaging (cell membrane lysis) caused by the bile acid.

For comparison, we also investigated the aggregation of methyl esters of 12-MKC, cholic and 3,7,12-triketocholic acids (Fig. 8). On the curve $T_1 = f(c_{BA})$ for methyl esters of 12-MKC and cholic acids the inflexion points occur after the concentration of 5 mM, which indicates that the aggregates formed in $CDCl_3$ are of the Small's type. The observation that the association of bile acid derivatives takes place in $CDCl_3$ at lower concentrations than in D_2O can be explained by the phenomenon that the $CDCl_3$ molecules that are not stabilized by hydrogen bonds from the β-side of the steroid skeleton in the aggregate of the bile acid derivative return to the solution, and thus increase the system's entropy. However, $CDCl_3$ molecules enter more easily the chloroform bulk, since the interactions between the $CDCl_3$ molecules from the interior are weaker than of those in D_2O (larger number of hydrogen bonds).

The importance of the phenomenon of association of the bile acids tested in $CDCl_3$ arises from the evidence that association of Small's type can also take place in the lipid phase, which yields formation of a pool of bile acid in the membrane. Since the aggregate dimensions are larger than of the monomers, the interior structure of the membrane is disturbed to a greater extent, forming thus water channels that participate in the transport of hydrosoluble drugs through the membrane (faciltated diffusion).

Based on the curve $T_1 = f(c_{BA})$, it can be concluded that methyl ester of 3,7,12-triketocholic acid forms probably a gel-like structure, which can also be observed visually.

4. Conclusions

The method of ^{1}H NMR spin-lattice relaxation was used to study the aggregation of sodium salt of 12-monoketocholic acid (Na-12-MKC) in D_2O solution and D_2O solution saturated with 1-octanol. The function describing the interdependence between the spin-lattice relaxation time and concentration of Na-12-MKC determined for the D_2O solution has one inflexion point, whereas the one for the saturated 1-octanol solution in D_2O has two inflexion points. This suggests formation of mixed micelles between Na-12-MKC and 1-octanol, which was also confirmed by the shift of the solubilization curve of lecithin with Na-12-MKC in the presence of 1-octanol toward higher concentration of the bile acid.

5. Acknowledgement

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Povzetek

12-Monoketoholna kislina (12-MKC) ima pospeševalni učinek pri transportu nekaterih zdravilnih učinkovin, kot so lidokain, morfin, kinin in glikolazid. Pri mehanizmu farmakološkega delovanja 12-MKC igrata agregacija (samo-asociacija) in tvorba mešanih micel pomembno vlogo. Ta pojav so avtorji študirali s pomočjo ^{1}H NMR spin-mrežne relaksacijske metode z uporabo raztopin žolčnih kislin v čisti D_2O in z 1-oktanolom nasičenih raztopin D_2O. Funkcija, ki opisuje odvisnost relaksacijskega časa (T_1) od koncentracije 12-MKC (c_{BA}) v D_2O ima eno upogibno točko (pri koncentraciji 48 mM), medtem, ko ima ista funkcija za nasičeno raztopino 1-oktanola v D_2O dve upogibni točki (eno pri koncentraciji 45 mM in drugo pri 87 mM). Razlike v krivuljah $T_1 = f(c_{BA})$ kot tudi premik topnostne krivulje za lecitin v prisotnosti 1-oktanola proti višjim koncentracijam 12-MKC kažejo na to, da proučevane žolčne kisline tvorijo mešane micele z 1-oktanolom. Zaradi tega uporaba micelnih raztopin 12-MKC v prisotnosti 1-oktanola zmanjša toksičnost membrane (razpad membrane) za obravnavane žolčne kisline. Krivulje $T_1 = f(c_{BA})$ za raztopine metilnih estrov 12-MKC in holne kisline v $CDCl_3$ imajo eno upogibno točko pri koncetraciji nad 5 mM, kar kaže na tvorbo micelov Small-ovega tipa, medtem ko metilni ester 3,7,12-triketoholne kisline tvori strukturo, ki je podobna gelu.