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Conductometric and ^{1}H NMR Determination of the Critical Micellar Concentrations of Two Oxo-Derivatives of Cholic Acid. Which of the Two Is More Hydrophobic?

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Abstract: Critical micellar concentrations (CMCs) were determined for two novel promoters of membrane permeability – 7-monoketocholic acid (7-MKC) and 12-monoketocholic acid (12-MKC), using two non-invasive (conductometry and ^{1}H NMR relaxation experiment) methods. Studies by these methods suggest the different aggregation abilities of the investigated bile acid derivatives. In an aqueous solution, 7-MKC has a somewhat lower CMC value (43 mM) than 12-MKC (50 mM). Further, it was found that, in addition to the primary micelles, 7-MKC forms also secondary micelles. Based on the CMC value 7-MKC is more hydrophobic than 12-MKC, although the opposite data can be found in the literature. The apparent hydrophobicity of 7-MKC is a consequence of the formation of secondary micelles, shifting the monomer equilibrium to the direction of first micelles, which is manifested as a decrease in the CMC value.

Keywords: Cholic acid monoketo derivatives; Critical micellar concentration, Primary and secondary micelles

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Introduction

Bile acid salts are compounds with two different surfaces in their molecules. The convex surface of the steroidal skeleton (β side) is hydrophobic, whereas the concave surface (α side) is hydrophilic because of the presence of OH groups and carboxylic group of the side chain. Simultaneous presence of both polar and non-polar surfaces in bile acid molecules (amphiphilic molecules) has as a consequence their self-association, as well as a number of physicochemical properties on their boundary surfaces. Bile acid salts belong to the class of anionic surface active molecules [1-6].

Formation of micelles is characterized by the value of critical micellar concentration (CMC), which represents the concentration of the amphiphilic molecule at which begin significant changes in physicochemical properties of the solution [2]. The knowledge of CMCs of bile acids is also important in view of their pharmacological applications. Namely, it is known that the hydrophilic-hydrophobic balance (which is in correlation with the CMC) of bile acids determines their binding to large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, which results in the relaxation of smooth muscles of the endothelium[7]. Also, the CMC value indicates the promotive properties of bile acids in the transport of polar drugs through biological membranes. However, the membrano-toxic properties of bile acids depend also on the CMC. The lower the CMC value of the bile acid, the greater the possibility of the cell membrane damage [8,9].

Pharmacological studies have especially pointed out the significance of keto (oxo) derivatives of bile acids [10-13]. Namely, the introduction of an oxo group into the bile acid molecule increases the CMC value, lowering thus its membrano-toxic effect [14,15]. Therefore, the ratio of the pharmacological and toxic effects can be adjusted by varying the number and position of the keto groups in their steroid skeleton.

There are two groups of experimental methods for determining CMC values. To one group belong non-invasive methods (light dispersion, potentiometry, conductometry, NMR, isothermal calorimetric titration, etc.), in which one measures the changes in a certain physicochemical property of the solution of surface active substance (SAS) that occur as a consequence of the aggregation of SAS monomer units. The other group of methods are the invasive methods, in which use is made of a probe hydrophobic molecule which builds in into the hydrophobic interior (cage) of the SAS micelle, which then causes changes in the probe molecule's surroundings.

Therefore, in the non-invasive methods one measures the change in physicochemical characteristics associated with the probe molecule (fluorescence, changes in the UV-VIS spectrum, spin labeling, etc.). By comparing these two groups of methods of CMC determination it can be concluded that a shortcoming of the noninvasive methods is the necessity of formation of a larger number of aggregates; hence, the CMC value thus determined is higher than the real value. A shortcoming of the invasive methods is in that that the probe molecule and SAS form mixed micelles, which then lowers the CMC value. Besides, the probe molecule disturbs the real structure of the micelle [16].

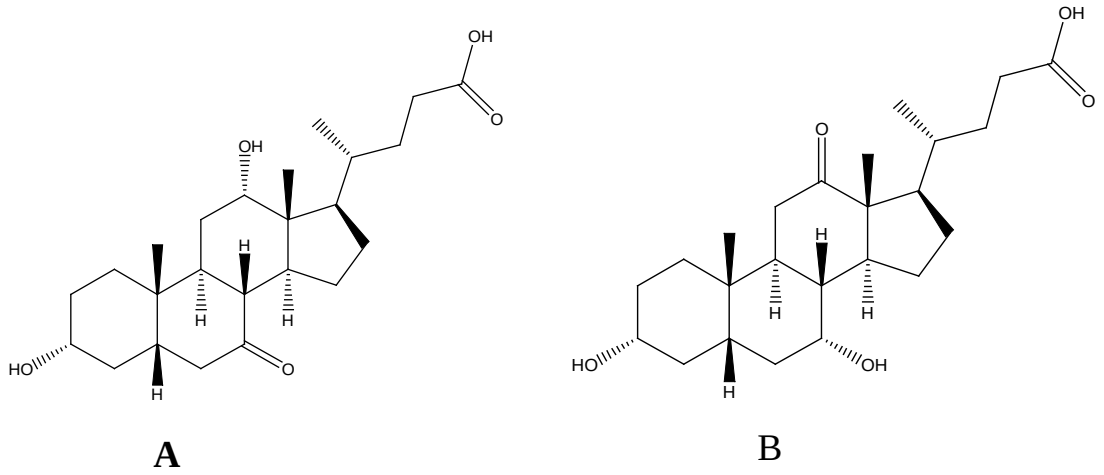

Figure 1. **A**) 3α,12α-dihydroxy-7-oxo-5β cholanoic acid (7-MKC);
B) 3α,7α-dihydoxy-12-oxo-5β-cholanoic acid (12-MKC).

Physicochemical properties of keto cholic acid derivatives have not been studied sufficiently. The knowledge of the CMCs of 7-MKC and 12-MKC is important in view of the fact that their values indicate both promotive and membrane toxicity properties of these bile acids. According to Poša *et al.* both of these monoketocholic acids have CMC values which do not differ statistically from each other [14]. However, the experiments carried out by Sârbu *et al*. [17] (reversed phase high performance thin layer chomatography, RPTLC) indicated that 12-MKC is by 4% more hydrophobic than 7-MKC. The entropy factor of micelle formation at 30°C ($T\Delta S_m$) for 12-MKC is +4.57 kJmol^{-1}, whereas for 7-MKC it is +4.15 kJmol^{-1}, which should also imply that 12-MKC is more hydrophobic, that is it should have a lower CMC value [11]. Because of this, the aim of the present work was to examine which of these two monoketocholic acids (Fig. 1) is more hydrophobic. To answer this question we undertook measurements of CMCs of these acids (studies of self-aggregation) by two noninvasive techniques (conductometry and NMR).

Experimental

Chemicals

Cholic acid (Sigma, New Zealand, 98%) was used for the synthesis of 7-MKC according to the procedure by Tullar [18], and of 12-MKC by the procedure of Miljković *et al.* [19]. D_2O (Aldrich, 99.99). Urea and NaCl were from Merck (analytical reagent grade).

Apparatus

Conductivities were measured using a conductivity meter (model "OK–102"; Radelkis, Budapest, Hungary). NMR experiments were carried out on a PC-controlled spectrometer (model "AC-250"; Bruker, USA).

Procedures

Conductometric Measurements

The cell containing the solution to be analyzed was immersed in a water bath keeping the temperature at 25 ±0.1 °C. The stock solution of bile salts (120 mM in double distilled water) was diluted for measurements with double distilled water, covering the concentration range of 10-100 mM. Measurements were also carried out in the presence of NaCl at the concentrations of 50 mM and 150 mM as well as 2 M urea. In this case the stock solution (120 mM bile acid, 50 or 150 mM NaCl or 2 M urea in double distilled water) was diluted to a desired concentration with 50 mM or 150 mM solutions of NaCl in one case or with 2 M solution of urea in the other.

^{1}H NMR Studies

Stock solution of bile acid salts (120 mM in D_2O) was diluted with D_2O to cover the concentration range of 10-100 mM. 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 were determined.

Results and Discussion

The dependence of the conductivity κ on the concentration of bile acids tested is presented in Fig. 2. The CMC value is obtained as the point at the curve break. Below the CMC, bile acid monomers are present, whereas above the CMC the monomer concentration is constant, which is seen as a smaller slope of the curve $\kappa = f(c_{BA})$ [20,21].

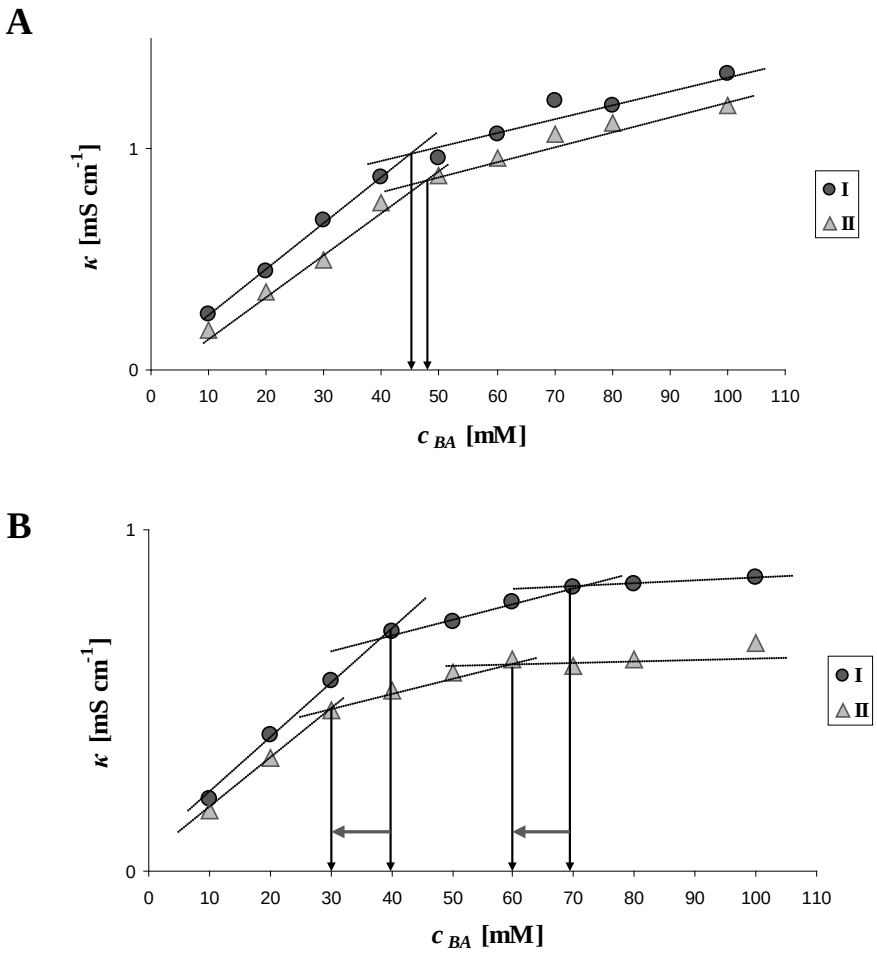

Figure 2. *Dependence of conductivity κ on concentration of sodium salts of bile acids*
A: sodium 12-MKC; **B**: sodium 7-MKC; I: water, II solution of 2 M urea.

Solutions of the monoketo derivatives of cholic acid were also investigated in the presence of 2 M urea and 50 and 150 mM NaCl. Namely, it is known that urea influences formation of bile acid micelles by lowering their aggregation number [23, 24]. In the graph $\kappa = f(c_{BA})$ for 12-MKC, there is a break point at the concentration of 46 mM, whereas in the presence of 2 M urea the break point is at 48 mM (Fig. 2A). These two CMC values do not differ statistically, which means that urea does not affect aggregation of 12-MKC.

In the graph $\kappa = f(c_{BA})$ for 7-MKC there are two break points, one at the concentration of 39 mM and the other at 69 mM. In the presence of urea the both points shift in the direction of lower concentrations. The occurrence of two break points and the influence of urea on the CMC value of 7-MKC suggests that the aggregation of this bile acid is different from that of 12-MKC. The presence of NaCl lowered the CMC of both acids (Table 1), exhibiting in the case of 7-MKC an effect similar to that of urea.

Table 1. *Effect of NaCl and urea on the CMC [mM] values.*

Bile salt	Water		50 mM NaCl		150 mM NaCl		2 M urea	
	Tp1	Tp2	Tp1	Tp2	Tp1	Tp2	Tp1	Tp2
7-MKC	39±3	69±2	35±2	65±4	28±2	58±3	30±3	60±2
12-MKC	46±4	--	41±3	--	35±3	--	48±2	--

Legend: n = 5; Tp1 ... break point at lower c_{BA} concentration; Tp2 ... break point at higher c_{BA} concentration in the curve; $\kappa = f(c_{BA})$; -- ... not defined.

The difference in the functions $\kappa = f(c_{BA})$ for the two bile acids tested is a consequence of the formation of different micelles. Namely, in the 7-MKC molecule, the oxygen atom of the C7 oxo group is shifted towards the mean plane of the steroid skeleton compared to the oxygen of the C7 hydroxyl group of 12-MKC (Fig. 3A). However, the C12 oxo group of the 12-MKC molecule, forms, via water molecules, hydrogen bonds with the hydroxyl group of the side chain, since the oxo group is shifted towards the β side (Fig. 3B). This is in contrast to the C12 hydroxyl group in the molecule of 7-MKC, so that according to Bertolasi *et al.*, the C12 oxo group can form hydrogen bond with the side chain [25]. Hence 7-MKC has a smaller hydrophobic surface area than 12-MKC since the oxo group of the molecule of the latter is screened by the hydrophobic C23, C22 methylene and C21 methine groups of the side chain, and this just explains the higher hydrophobicity of 12-MKC obtained on the basis of the retention index (RPTLC) [17]. However, the lower CMC value for 7-MKC obtained by conductometric measurements suggests that 7-MKC is more hydrophobic. This contradiction can be explained in terms of the existence of two break points in the curve $\kappa = f(c_{BA})$ (Figs. 2) if, apart from primary micelles, secondary ones are formed too.

According to the Small model [22,25], primary micelles are formed by binding of bile acid hydrophobic areas, and, if the bile acid molecule has a larger hydrophobic area, then primary micelles of a larger aggregation number are formed.

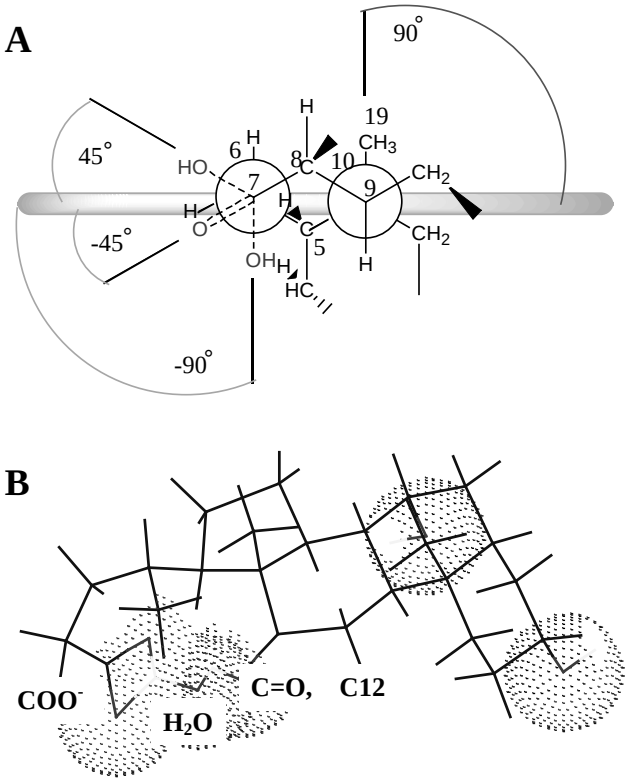

Figure 3. A) Shift of the C7 oxo-group oxygen towards steroid skeleton mean plane, **B**) formation of the hydrogen bond via water molecule between the C12 oxo group and carboxylic function of the side chain of 12-MKC (conformations were optimized by the MOPAK protocol).

Since 12-MKC molecule has a larger hydrophobic area than 7-MKC, the increase in the concentration of the former bile acid will yield formation of primary micelles with successively larger aggregation number (Fig. 4. II-IV). On the other hand, the second break point in the graph $\kappa = f(c_{BA})$ for 7-MKC indicates the formation of secondary micelles (primary micelles bonded by hydrogen bonds, Fig. 4 V-VI). Formation of secondary micelles means the withdrawing of primary micelles from the system of monomer aggregation, which then shifts the equilibrium of association of 7-MKC monomers in the direction of primary micelles. Hence the CMC value is smaller than it would be expected on the basis of the molecule hydrophobicity.

Fig. 5 illustrates the hydrogen bonds formed between 7-MKC molecules that also exist in secondary micelles. The decrease in the slope after the second break on the curve of the function $\kappa = f(c_{BA})$ (Fig. 2B) can be also explained by the presence of secondary micelles.

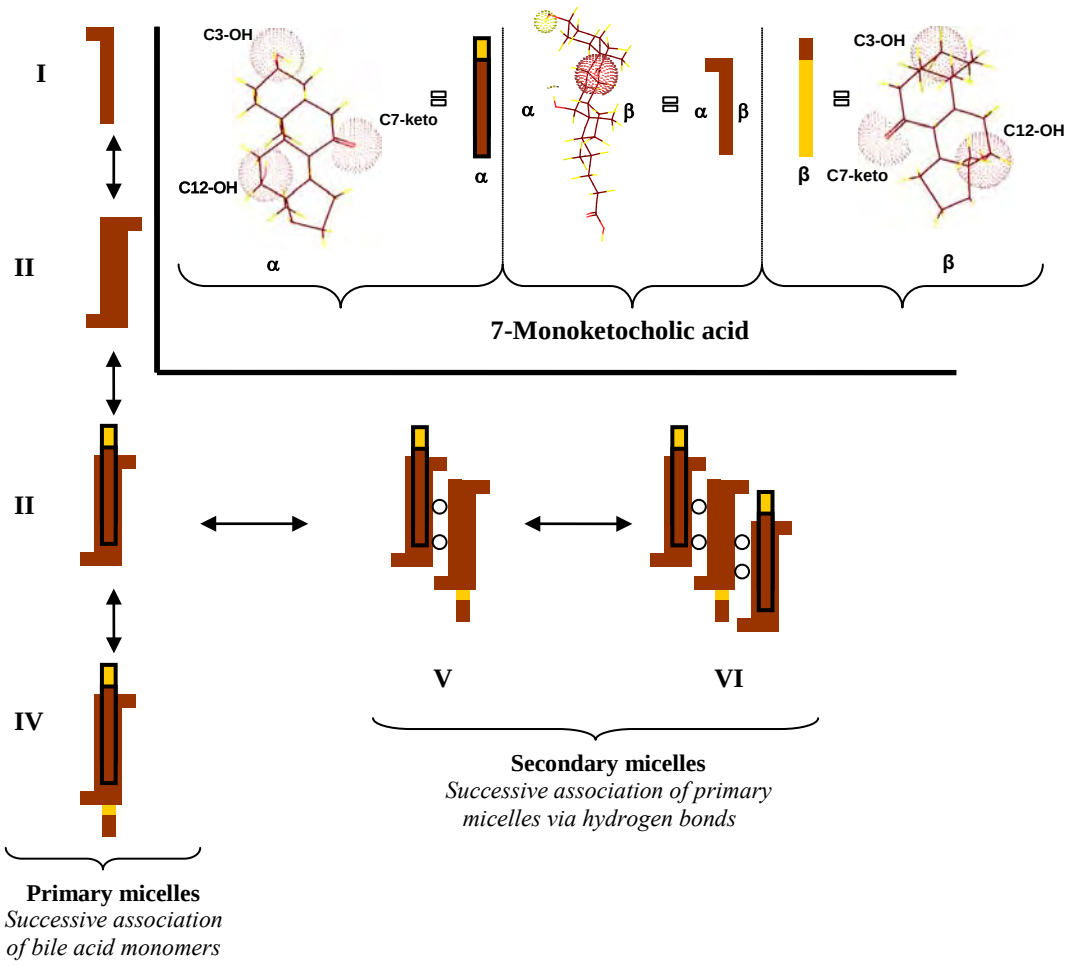

Figure 4. *Formation of primary micelles* of bile acid monomers **I** by hydrophobic interactions **I-IV**; formation of secondary micelles by successive association of primary micelles via hydrogen bonds **V-VI** (○ denotes hydrogen bond).

Namely, the conductivity of the micellar solution of bile salts is mainly due to the existence of monomeric anions and, to a lesser extent, to the involvement of micelles of different types of aggregation, bearing a negative charge (the micelle charge is smaller than the sum of the charges of the monomeric anions that participate in the micelle formation since counter-ions are adsorbed on the micelle surface).

However, since the size of secondary micelles is 2 to 4 times larger than that of primary micelles, their mobility decreases because of the increased resistance to their motion towards the electrodes. In other words, after the second break point in the curve $\kappa = f(c_{BA})$ the concentration of free monomer anions in the solution decreases compared to the concentration at the first break point since the equilibrium of aggregate formation is shifted toward formation of primary micelles (primary micelles form secondary micelles).

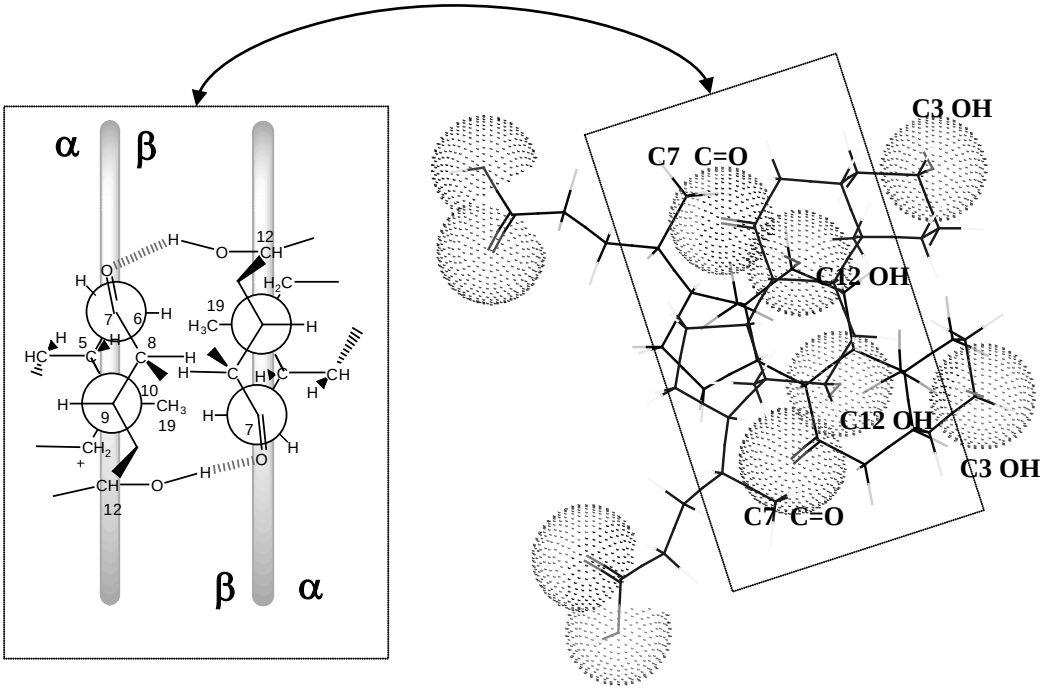

Figure 5. *Hydrogen bonds formed between two monomers of 7-MKC in the aggregation of secondary micelles* (configurations were optimized by MOPAK protocol).

In our recent work, spin-lattice relaxation time T_1 was determined for the singlet of the CH_3-18 methyl group of sodium-7-monoketocholate at 0.74 ppm in D_2O, and at 1.09 ppm in D_2O for the two overlapping singlets belonging to the CH_3-18 and CH_3-19 protons of sodium-12-monoketocholate [21,22]. The determination of the relaxation times for protons of the CH_3-18 methyl group was chosen in view of the assumption that according to the Small model [23,26-28] bile acids in the micelle bind via their hydrophobic surfaces, that is via the β side of the steroid skeleton, which yields a change in the environment of the angular CH_3-18 methyl group. If the association of primary micelles would not occur by binding the bile acid monomers via their β sides (Small's model), then the lattice environment of angular protons would not change, and, by the same token, there would be no change in the $T_{1.}$ value.

Fig. 6A shows the ^{1}H NMR spectra of 7-MKC in D_2O at a concentration of 70 mM, which are used for the determination of the relaxation time T_1 by the 180˚-τ-90˚-AQC method [22]. Each spectrum was recorded after a certain time τ upon applying a pulse of 180˚ the first spectrum in Fig. 6 was recorded after 0.010 s, the second one after 0.025 s, and *etc.* To study the self-association of bile acids it is necessary to record a series of spectra for each concentration tested, as shown in Fig. 6, that is to apply the 180˚-τ-90˚-AQC method.

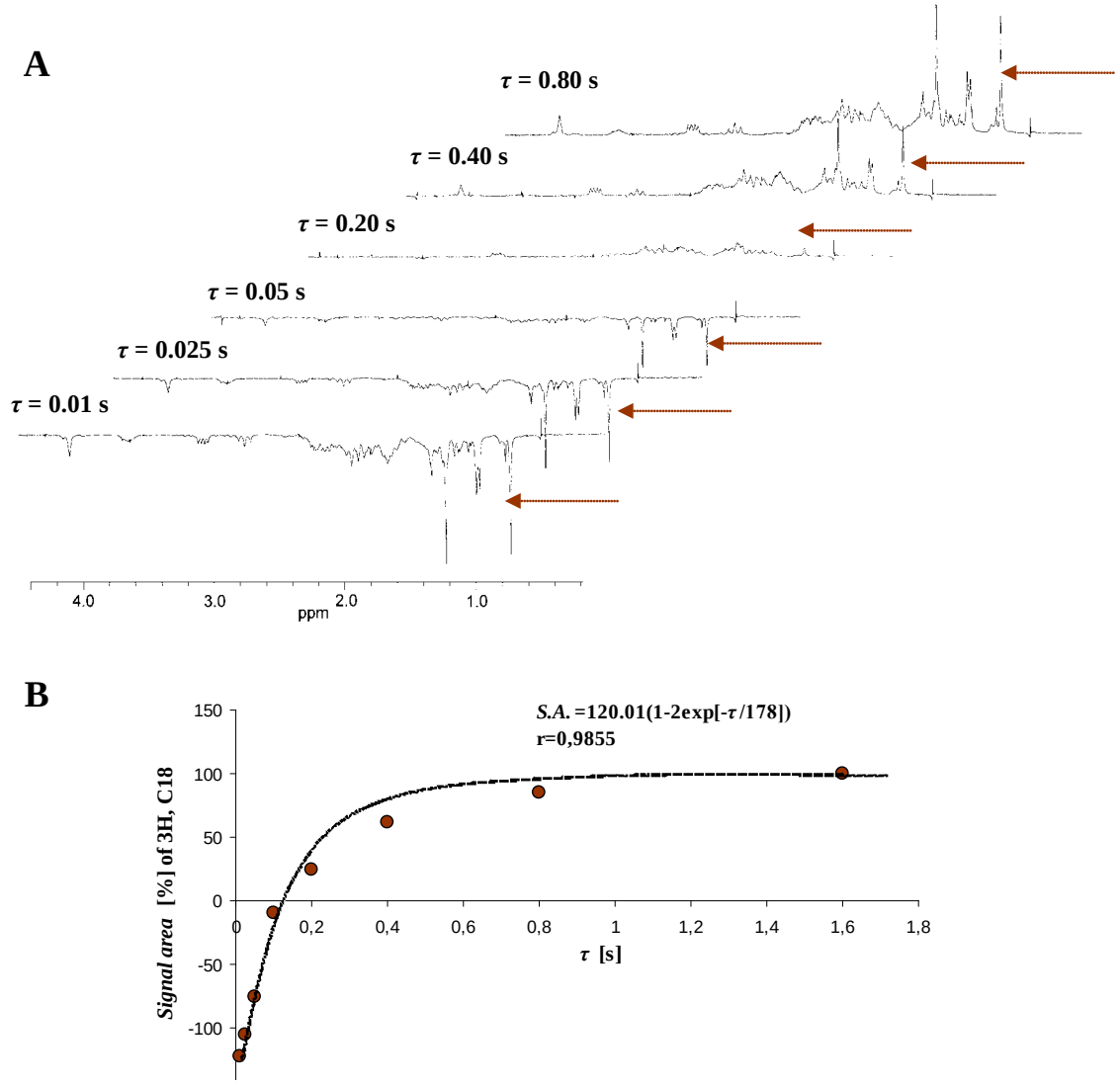

Figure 6. A) *^{1}H-NMR spectra of sodium 7-MKC obtained by the inversion recovery experiments* (180˚-*τ*-90˚-AQC). **B**) *Selected peak areas for different pulse delays*; the line represents the best fit of equation, obtained by non-linear regression.

The arrows in Fig. 6A denote the signals of the C18 protons, whose intensities (areas) are measured as a function of time. On the other hand, Fig. 6B shows the change of the signal area of the C18 proton as a function of time that elapsed after applying the 180° pulse, the time τ being measured since the termination of the action of the 180° signal. To each point in the graph (Fig. 6B) corresponds a spectrum (integral of the signal of the C18 methyl group proton), that is the area under the signal of the C18 methyl group at the time delay: τ = 0.010 s; τ = 0.025 s; τ = 0.050 s; τ = 0.10 s, etc. The relaxation time from the graph (Fig. 6B) is determined by fitting experimental data to the following equation (S.A. – signal area):

$$S.A. = S.A._0\,(1-2e^{-\tau/T_1}) \qquad (1)$$

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

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

Namely, it is known from the literature that the determination of relaxation times by fitting to equation (1) does not give in some cases good results (which depends on the molecular structure and dynamics) [21]. The values of relaxation times determined by equation (1), that is by equation (2), do not differ from each other by more than 5% ($p<0.01$). Variation in T_1 values calculated by equations (1) and (2) is below 4%. The reproducibility condition is that the signal-to-noise (S/N) ratio for the protons for which relaxation times are measured should be equal to or larger than 8 ($S/N \geq 8$).

To determine the relaxation time a graph of the signal area of the C18 proton vs. time τ, was constructed for each tested bile acid concentration. The relaxation times thus obtained, T_1, are presented as a function of the concentration c_{BA} of the bile acid tested (Fig. 7). If the function $T_1 = f(c_{BA})$ has an inflexion point (abrupt jump) this means that micelle formation took place (micelle formation is accompanied by a change in the environment of the angular protons, which then leads to a change in the T_1 values; at the concentrations below the CMC the protons of angular methyl groups of the monomer have one value for T_1 and another after the micelle formation) [21].

In the case of 7-MKC the function $T_1 = f(c_{BA})$ has two inflexion points, one at the concentration of 42 mM (bigger change, Ip-1, Fig. 7A) and the other one at 75 mM (smaller change, Ip-2, Fig. 7A), in contrast to 12-MKC, for which there is only one inflexion point at the concentration of 50 mM (Ip-1, Fig. 7B). This difference in the function $T_1 = f(c_{BA})$ may indicate the difference in the aggregation of the tested monoketo derivatives of cholic acid.

Formation of primary micelles is also confirmed by broadening of the $T_1 = f(c_{BA})$ curve for 12-MKC around its inflexion point (Fig. 7B). Namely, with this bile acid, because of the relatively large hydrophobic surface area in its molecule, the equilibrium of formation of primary micelles at increased concentrations is shifted in the direction of formation of the aggregates with a larger aggregation number. In contrast to this, the curve for 7-MKC (Fig. 7A), with the sharper changes around the inflexion point, suggests that this bile acid forms primary micelles with smaller aggregation numbers. On the other hand, the second inflexion point in the graph $T_1 = f(c_{BA})$ for 7-MKC indicates the formation of secondary micelles (primary micelles bonded by hydrogen bonds; see Fig. 4).

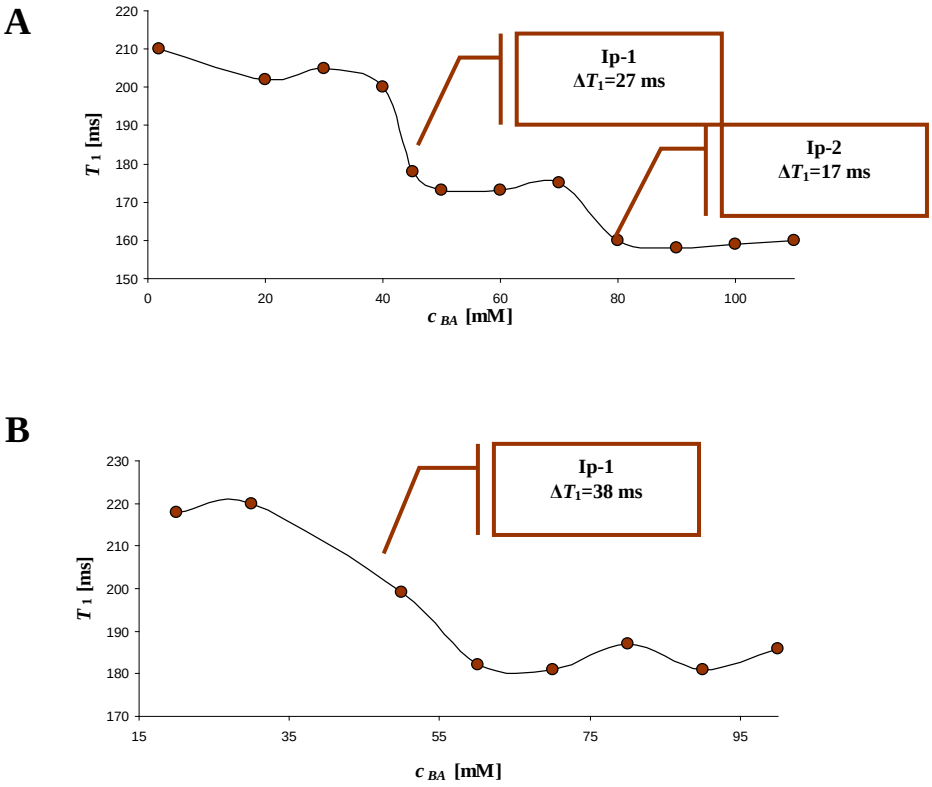

Figure 7. NMR relaxation times (T_1); **A**) sodium 7-MKC; **B**) sodium 12-MKC

Conclusions

When critical micellar concentration (CMC) is determined by non-invasive methods (^{1}H NMR spectroscopy and conductometry) based on the measurement of the change in physicochemical characteristics of the system that is a consequence of the aggregation of detergent molecules, the CMC value obtained for 7-MKC is smaller than that for 12-MKC. The ^{1}H NMR relaxation curves ($T_1 = f(c_{BA})$) and the conductometry results ($\kappa = f(c_{BA})$) suggest that the aggregation of 7-MKC is different from that of 12-MKC.

Acknowledgements

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References

1. M. J. Armstrong, M. C. Carey: *J. Lipid Res*. ***23*** (1982) 70.
2. A. Roda, A. F. Hofmann, K. J. Mysels: J. *Biol. Chem.* ***258*** (1983) 6362.
3 K. Miyajima, K. Machida, T. Taga, H. Komatsu, M. Nakagaki: *J. Chem. Soc. Faraday Trans.* ***84*** (1988) 2537.
4. A. M. Dopico, J. V. Walsh, J. J. Singer: *J. Gen. Physiol*. ***119*** (2002) 251.
5. P. Mukerjee, K. J. Mysels: *Critical Micelle Concentration of Aqueous Surfactant System*. NSR-DS, NBS; Washington (1971).
6. J. E. Desnoyers, G. Caron, R. de Lisi, D. Roberts, A. Roux, G. Perron*: J. Phys. Chem.* ***87*** (1983) 1397.
7. M. A. Dopico, V. J. Walsh, J. Singer: *J. Gen. Physiol.* ***119*** (2007) 251.
8. C. Bowe, L. Mokhtarzadeh, P. Venkatesen, S. Babu, H. Axelrod, M.J. Sofia, R. Kakarla, T.-Y. Chan, J.-S. Kim, H.-J. Lee, G.L. Amidon, S.-Y. Choe, S. Walker, D. Kahne: *Proc. Natl. Acad. Sci. U.S.A*. ***94*** (1997) 12218.
9. S. Gordon, A. C. Moses, R. D. Silver, J. R. Flier, M. C. Carey: *Proc. Nat. Acad. Sci. U.S.A*. ***82*** (1985) 7419.
10. M. Mikov, J. P. Fawcett: *Bile Acids*. Medishet Publ., Geneva (2007).
11. M. Poša, S. Kevrešan, M. Mikov, V. Ćirin-Novta, K. Kuhajda: *Eur. J. Drug. Metabolism Pharmacokin.* ***32*** (2007) 109.
12 M. Poša, V. Guzsvány, J. Csanádi, S. Kevrešan, K. Kuhajda: *Eur. J. Pharm. Sci.* ***34*** (2008) 281.
13 M. Mikov, S. Kevrešan, K. Kuhajda, V. Jakovljević, V. Vasović: *Pol. J. Pharmacol.*, ***56*** (2004) 367.
14. M. Poša, S. Kevrešan, M. Mikov, V. Ćirin-Novta, C. Sârbu, K. Kuhajda: *Colloids & Surfaces B* ***59*** (2007) 179.
15. M. Poša, S. Kevrešan, M. Mikov, V. Ćirin-Novta, C. Sârbu, K. Kuhajda: *Colloids & Surfaces B* ***64*** (2008) 151.
16. M. Calabresi, P. Andreozzi, C. La Mesa: *Molecules* ***12*** (2007) 1731.
17. C. Sârbu, C. Onişor, M. Poša, K. Kuhajda, S. Kevrešan: *Talanta* ***75*** (2008) 651.
18. B. F. Tullar: *U.S. Patent* ***2***, 549, 947 (1951).

19. D. Miljković, K. Kuhajda, J. Hranisavljević: *J. Chem. Res. (S)*, ***106*** (1996).
20. Kabir-ud-Din, M. D. A. Al-Ahmadi, A. Z. Naqvi, M. Akram: *Colloids & Surfaces B* ***64*** (2008) 65.
21. M. Poša, V. Guzsvány, J. Csanádi: *Colloids Surfaces B* **74** (2009) 84.
22. M. Poša, V. Guzsvány, J. Csanádi, J. Borbás, F. Gaál: *Acta Chim. Slov.*, **56** (2009) 807.
23. D. M. Small: *Advan. Chem. Ser.* ***84*** (1964) 31.
24. W. S. Bennett, G. Eglinton, S. Kovac: *Nature* ***217*** (1967) 776.
25. V. Bertolasi, V. Ferretti, L. Pretto, G. Fantin: *Acta Crystallograph. B Struct. Sci.* ***61*** (2005) 346.
26. D. M. Small: *Adv. Chem. Ser.* ***84*** (1968) 31.
27. S. Gouin, X.-X. Zhu: *Langmuir* ***14*** (1998) 4025.
28. H. Kawamura. Y. Murata, T. Yamaguchi, H. Igimi, M. Tanaka, G. Sugihara, J. P. Kratohvil: *J. Phys. Chem.* ***93*** (1989) 3321.