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Determination of number-average aggregation numbers of bile salts micelles with a special emphasis on their oxo derivatives—The effect of the steroid skeleton

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ABSTRACT

Background: The special geometry of the steroid skeleton causes that bile acid anions, in contrast to aliphatic amphiphiles, form micelles with a small aggregation number.
Methods: The number-average aggregation numbers $(\bar{n})$ are determined using Moroi–Matsuoka–Sugioka thermodynamic method. Also, for analysed bile acid sodium salts functions between spin–lattice relaxation time (T_1) and concentration of monomers (c_{BA^-}) are determined.
Results: For 7-oxodeoxicholic (7-ODC) acid and hyodeoxicholic acid (HD) monomers, curve $T_1 = f(c_{BA^-})$ contains two inflexion points. Mentioned monomers and cholic acid anion (C) are influential observations in relation to a line of linear regression between $\bar{n}$ and para\meter of monomer hydrophobicity ($\ln k$, retention capacity from RPHPLC). This suggests that, in micelles of bile acid anions: 7-ODC, HD and C, beside main, hydrophobic interactions, hydrogen bonds are also possible between building units.
Conclusion: The increase in the number of oxo groups in the molecule is accompanied with a decrease in the hydrophobicity of the convex side of the steroid skeleton of the bile acid anion, resulting in a lower aggregation number. Obtained results indicate that C12 and C7 α-axial OH and oxo groups on the same C atoms of the investigated bile acid molecules have different spatial environment, which is confirmed by conformational analysis.
General significance: Deviation from the linear model: number-average aggregation numbers with hydrophobicity of monomers, suggests the existence of additional, intermolecular interactions beside hydrophobic in micelles.

1. Introduction

Bile acid salts are ionic amphiphilic compounds with a steroid skeleton. They represent a special group of amphiphilic compounds as they belong to biplanar amphiphiles. This is due to molecular geometry of cholanoic acid, of which all other bile acids of the 5β series can be derived. The concave (α) side of the steroid skeleton of bile acid molecules is hydrophilic (presence of OH groups), whereas the convex side with its angular methyl groups is hydrophobic. Bile acid salts in concentrations above critical micelle concentration form aggregates—micelles in water solutions [1–3]. According to the oldest, Smalls concept, primary micelles whose building units are connected over their steroid skeletons β sides (hydrophobic binding) are formed first. At higher concentrations, primary micelles form secondary micelles by hydrogen bonds [4,5]. Bile acid anions aggregation numbers range from 2 (Smalls primary micelles) to 15 (secondary micelles), so are far behind aggregation numbers of alkylsulfates (sodium-dodecylsulfat can have aggregation numbers up to 120) [6].

Primary bile acids (cholic and chenodeoxycholic) are synthesised in the liver of vertebrates, where they conjugate with glycine and taurine. The main physiological role of bile salts is emulsification of fats in the intestinal lumen, which enhances efficiency of the hydrolysis of triglycerides with the enzyme lipase. Bile salts form mixed micelles with monoacylglycerol, long-chain fatty acids and cholesterol, which facilitates transport of lipid components from the lumen to the intestinal epithelium. Bile acids also participate in the regulation of cholesterol homeostasis. Namely, in the gall bladder, salts of conjugated bile acids form mixed micelles with phospholipids and cholesterol, that are excreted to the duodenum [7,8]. Bile acids are endogen ligands for nuclear FXR receptor and G-protein coupled TGR5/Gpbrar1 receptor. By modulation of signal paths where higher receptors are included, bile acids modulate their biosynthesis *i.e.* participate in regulation of homeostasys of fat and glucose [9–12].

Sodium salts of chenodeoxycholic and ursocholic acids are of importance in the pharmacological industry, as their preparations are used for dissolution of cholesterol gallstone [8]. Generally, the more hydrophobic is the bile acid anion, the greater is its solubilisation effect. However, at the same time its membranotoxicity is increased [13–16]. Due to a lower hydrophobicity of the β (convex) side of the steroid skeleton, bile acid oxo derivatives exhibit a lower toxicity to membranes [17]. In the *in vitro* conditions, these compounds show a promotive action

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on the transport of some drugs (morphine, lidocaine, verapamil, etc.) through the hydrophobic barriers (depot effect) [18,19]. In the pharmacological experiments on animals, they lowered the glucose blood level and increased permeability of the blood–brain barrier, etc. [20–26]. Many pharmacological effects of bile oxo derivatives are due to interaction of their mixed micelles and the drug (biomolecule) [16,18,27–31]. Because of that, it is necessary to acquire detailed knowledge of micelle parameters such as critical micelle concentration (CMC) and aggregation number (n). CMC values of bile acid oxo derivatives have been determined [1,17]. However, data about their aggregation numbers lack in literature. The knowledge of these characteristics of bile acids oxo derivatives is of importance in view of their application in the pharmacological industry. Namely, a higher value of the aggregation number means a higher probability of being accepted of a hydrophobic guest species.

The objective of this work is determination of unknown number-average aggregation numbers ($\bar{n}$) [32] of bile salts oxo derivatives micelles (Fig. 1) using the thermodynamic Moroi–Matsuoka–Sugioka pH-metric method in a system consisting of the bile acid solid phase and the aqueous solution of its sodium salt (in monomer and micellar forms) [33,34]. The above mentioned method is chosen since it is non-invasive and can be applied for micelles with relatively small aggregation numbers as bile acid anion micelles. For example, static light scattering method for bile acid salts gives rough estimation of $\bar{n}$ [34] (which is not the case for conventional surfactants as alkylsulfates with micelles of 80 and more building units). The aim as well is to obtain the curve of dependence between spin–lattice relaxation time (T_1) and concentration of bile acid sodium salt (c_{BA^-}). The shape of the curve $T_1 = f(c_{BA^-})$ may help to shed light on aggregation processes of bile acid anions (number of jumps *i.e.* inflexion points on the curve, stretched or sharp changes) [35]. It is known that there is a good correlation between critical micelle concentration (CMC) of bile acid salts and reversed phase chromatographic parameters [36–41]. Also it is known that for aliphatic surface active molecules aggregation number grows with increase in the length of hydrocarbon series (on CMC values of sodium dodecylsulfat $n = 33$, while for sodium-octadecylsulfat $n = 78$ [42]). Thus, in order to examine dependence between $\bar{n}$ and hydrophobicity of bile acid sodium salts, capacity factors (k, measure for hydrophobicity of a molecule) in reversed phase HPLC are determined. The emphasis in the discussion is on the analysis of the effect of the structure of bile acid molecules steroid skeleton on the value of the micelle aggregation number (using new molecular referent system based on molecular graph [43]).

2. Materials and methods

2.1. Synthesis of oxo derivatives of cholic, deoxycholic and chenodeoxycholic acids

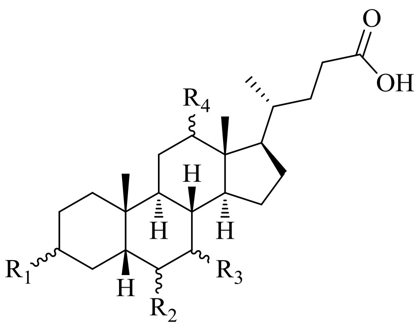
Cholic, deoxycholic and chenodeoxycholic acids (Sigma, New Zealand) were used as starting compounds for the synthesis of their oxo derivatives.

3α-Hydroxy-12-oxo-5β-cholanoic acid (12-OL) and 3α, 7α-dihydroxy-12-oxo-5β-cholanoic acid (12-OCD) were prepared according to the procedure of Miljković et al. [44], while 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (7-ODC) and 3α-hydroxy-7-oxo-5β-cholanoic acid (7-OL) were obtained according to Tullar [45]. 3α-Hydroxy-7, 12-dioxo-5β-cholanoic acid (7,12-DOC) was synthesised by a selective oxidation of the 7α-hydroxy group of 3α,7α-dihydroxy-12-oxo-5β-cholanoic acid following the procedure of the same author (Tullar). 3,12-dioxo-5β-cholanoic acid (3,12-DOC) and 3,7-dioxo-5β-cholanoic acid (3,7-DOC) were obtained according to Fieser and Rajagopalan [46] Hyodeoxycholic acid was purchased from Sigma, New Zealand. All bile acids were transformed to their sodium salts by known procedure [3].

2.2. ^{1}H NMR studies

Stock solution of bile acid salts (10, 20, 40, 100, 140 or 300 mM in D_2O; dependent of the studied bile acid salts solubility) was diluted with D_2O to cover the appropriate concentration range. Measurements were performed at 25 °C on a Bruker Spectrospin-500 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) [35,47]. Selected peak areas for nine different interpulse delays τ were determined.

The spin–lattice relaxation time T_1 was determined for the singlet of the CH_3-18 methyl group of sodium salt's of the following bile acids: C; CDC; 7-ODC; 7-OL; 3,7-DOC and HD at 0.68–0.80 ppm in D_2O, and at 1.00–1.13 ppm in D_2O for the two overlapping singlets belonging



$R_1 = R_3 = R_4 = OH$; $R_2 = H$	3α,7α,12α-trihydroxy-5β-cholanoic acid (cholic a.); **C**
$R_1 = R_3 = OH$; $R_2 = R_4 = H$	3α,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic a.); **CDC**
$R_1 = R_4 = OH$; $R_3 = {=O}$; $R_2 = H$	3α,12α-dihydroxy-7-oxo-5β cholanoic acid (7-oxodeoxycholic a.); **7-ODC**
$R_1 = R_3 = OH$; $R_4 = {=O}$; $R_2 = H$	3α,7α-dihydoxy-12-oxo-5β-cholanoic acid (12-oxochenodeoxycholic a.); **12-OCD**
$R_1 = OH$; $R_3 = R_4 = {=O}$; $R_2 = H$	3α-hydroxy-7,12-dioxo-5β-cholanoic acid; **7,12-DOC**
$R_1 = OH$; $R_4 = {=O}$; $R_2 = R_3 = H$	3α-hydroxy-12-oxo-5β-cholanoic acid (12-oxolithocholic a.); **12-OL**
$R_1 = OH$; $R_3 = {=O}$; $R_2 = R_4 = H$	3α-hydroxy-7-oxo-5β-cholanoic a. (7- oxolithocholic a.); **7-OL**
$R_1 = R_4 = {=O}$; $R_2 = R_3 = H$	3,12-dioxo-5β-cholanoic acid; **3,12-DOC**
$R_1 = R_3 = {=O}$; $R_2 = R_4 = H$	3,7-dioxo-5β-cholanoic acid; **3,7-DOC**
$R_1 = R_2 = R_4 = OH$; $R_3 = H$	3α,6α-dihydroxy-5β-cholanoic acid (hyodeoxycholic acid); **HD**

Fig. 1. Structures of tested bile acids.

to the CH_3-18 and CH_3-19 protons of sodium salt's of: 12-OCD; 12-OL; 7,12-DOC and 3,12-DOC.

2.3. Determination of average aggregation numbers by the Moroi–Matsuoka–Sugioka method

Solid bile acid was suspended in a distilled water by stirring on a magnetic stirrer, and an increment of NaOH solution was added with the aid of microsyringe. In this way the total concentration of the bile acid anion (monomer) is regulated. After the 24-hour equilibriation, the pH of the clear solution was measured (Boeco BT600) without separation of the solid phase, and taking care not to disturb it [33,34]. This gave one point on the titration curve and the procedure was repeated to obtain about 20 points, of which at least 5 in the micellar region. Measurements were performed at room temperature. For each point on the titration curve the standard deviation did not exceed 3% (n = 5).

2.4. Reverse phase HPLC method

The HPLC system Agilent 1100 Series, equipped with degasser, binary pump, automatic injector and DAD detector with software system for data processing AgilentChemStation was used and the analyses were performed on a reversed-phase C-18 column: Eclipse Plus C18 (250 mm × 3 mm, 5 μm, 250 Å) column (Zorbax SD). The mobile phase was 0.01 M phosphate buffer: methanol = 70: 130 v/v maintained at pH 7 and the injection loop was 10 μL. Solutions of bile acids and their derivatives in mobile phase were prepared in concentration of 1 mg/ml. All separations were performed isocratically at a flow rate of 1 mL/min and a column temperature changing from 25 ± 0.1 °C. The detection was performed at 210 nm.

The HPLC capacity factor (k) was calculated from the eluted peak retention time (t):

$$k = \frac{t_x - t_0}{t_0}, \tag{1}$$

where t_x and t_0 are the retention times of the bile acids and the unretained solvent front respectively. Trials were repeated ($n = 5$) for reproducibility, variance in data indicated ±3% error.

2.5. Data treatment

The data were treated using the Statistica 8.0. 3D models (energetically most favorable) of bile acids were generated according to the MOPAC protocol (ChemBio3D Ultra 11.0).

3. Results

3.1. ^{1}H NMR studies

For determination of CMC non-invasive NMR experimental technique was used in order to avoid usage of probe molecule which would, to some extent, disrupt the structure of the micelle, *i.e.* hydrophobic domain of the micelle. Spin–lattice relaxation time (T_1 = time it takes to get back 66% of equilibrium population of magnetic spins, after inversion—nonequilibrium distribution of magnetic spins) is connected to magnet dipole coupling between magnetic dipoles (spins), which depend on distance between magnet dipole (nuclear spins) and angle of radius vectors (that connects two dipoles) that form with the direction of external magnet moment. As the rotational movements (internal rotational movement around sigma bond) of molecule and rotational movement of the whole molecule occurs, local fluctuating magnetic field occurs from dipole coupling. Fluctuating magnetic field is characterised with average square of the local field strength ($\overline{H^2}$) and connected to T_1 over equation [48]:

$$\frac{1}{T_1} = \gamma^2 \overline{H^2} \frac{2\tau_c}{1 + (\omega_0 \tau_c)^2}, \tag{2}$$

where γ is giromagnetic coefficient, ω_0 circular NMR (resonant) frequency, while τ_c is rotational correlation time (the time required for rotation molecule of 1 rad). In Eq. (2) $2\tau_c/(1 + (\omega_0\tau_c)^2) \approx P(\omega_0)$ represents probability that local fluctuating magnetic field has component whose circle frequency corresponds to NMR resonance frequency ω_0. According to Eq. (2) maximum relaxation rate (*i.e.*the smallest value of T_1) is achieved if $\omega_0\tau_c = 1$. Then $P(\omega_0) \approx \tau_c$ *i.e.* there is the greatest probability that corresponding component of the local magnetic field has resonance angular frequency. If experiment is performed on 500 MHz (like in this paper) $\omega_0/2\pi = 5 \times 10^8 s^{-1}$ the maximum speed of longitudinal relaxation would be achieved if molecules have $\tau_c = 2$ ns (from condition $\omega_0\tau_c = 1$). According to Sanders and Hunter [49] rotational correlation time on room temperature can be estimated over equation $\tau_c = M$, whereby correlation time is in ps units and molecular mass (M) is in g mol^{-1}. For bile acid salt monomers it is $\tau_c \approx 0.4$ ns, while for micelle with 2 building units—which is the most probable size of the micelle on CMC—it is $\tau_c \approx 0.8$ ns, *i.e.* monomer and dimer micelle have the lower τ_c value than it is maximal correlation time for 500 MHz. Thus, $\omega_0\tau_c \ll 1$ so $P(\omega_0) \approx 2\tau_c$. This means that with the rise of τ_c (slower rotation of molecules) T_1 decreases (according to Eq. (2)) *i.e.* relaxation accelerats. This occurs when molecular weight of a particle increaes during micellisation—change in the freedom motion of the surfactants [35]—so T_1 decreases in function of $T_1 = f(c_{BA^-})$ during micellisation process.

The determination of the relaxation times for angular methyl (C18 and C19 or C18+C19) protons was chosen (Fig. 2) in view of the assumption that, according to the Kawamura and Small model [4,5,50], bile acids in the primary micelle bind *via* their hydrophobic surfaces, that is over the β side of the steroid skeleton, which yields to a change in the environment around the angular methyl groups. Since $\overline{H^2}$ origins from dipol coupling of nuclear spins, relaxation speed is proportonal to distance between magnet dipol over r^{-6}. During micelle formation protons from angular methyl group of one building units approach to protons of angular methyl groups of the other methyl group, which strengtens local fluctuating magnetic field *i.e.* accelerates relaxation process. Since there is no growth of $\overline{H^2}$ in other parts of the micelle, especially from the α side of the building units steroid skeletons, the change of T_1 with c_{BA^-} is much smaller. The change of T_1 values for H atoms of angular methyl groups in inflexion point of function $T_1 = f(c_{BA^-})$ is 20 ms, while fluctuations of T_1 value before and after CMC value are about 4 ms (Fig. 2). If we observe function $T_1 = f(c_{BA^-})$ where T_1 corresponds to interval of sum of protons of ^{1}H NMR spectar from 1.5 to 2.5 ppm of chemical shifting, the change of T_1 value on CMC is 5 ms, while fluctuations of T_1 values in analysed range of concentrations (5 mM–80 mM) are 3 ms. This suggests that, if correlation time corresponds to rotation movement of a whole molecule, the change of T_1 for 20 ms is a consequence of the rise in $\overline{H^2}$. Naimely if rotation correlaton time would determine the change in T_1 value on CMC, inflexion point would be obtained in function $T_1 = f(c_{BA^-})$ where T_1 values correspond to protons from the spectra area 1.5–2.5 ppm.

Fig. 2(A) shows the ^{1}H NMR spectra of sodium salts of 3,7-DOC in D_2O at a concentration of 80 mM, which are used for the determination of the relaxation time T_1 by the 180°-τ-90°-AQC method [47]. Each spectrum was recorded after a certain time τ upon applying a pulse of 180°: the first spectrum in Fig. 2 was recorded after 0.010 s, the second after 0.025 s, and so on. To study self-association of bile acids it is necessary to record a series of spectra for each concentration tested, as shown in Fig. 2, that is to apply the 180°-τ-90°-AQC method. In Fig. 4(A) denote the signals of the C18 protons, whose intensities (areas) are measured as a function of time. On the other hand, Fig. 2(B) shows the change of

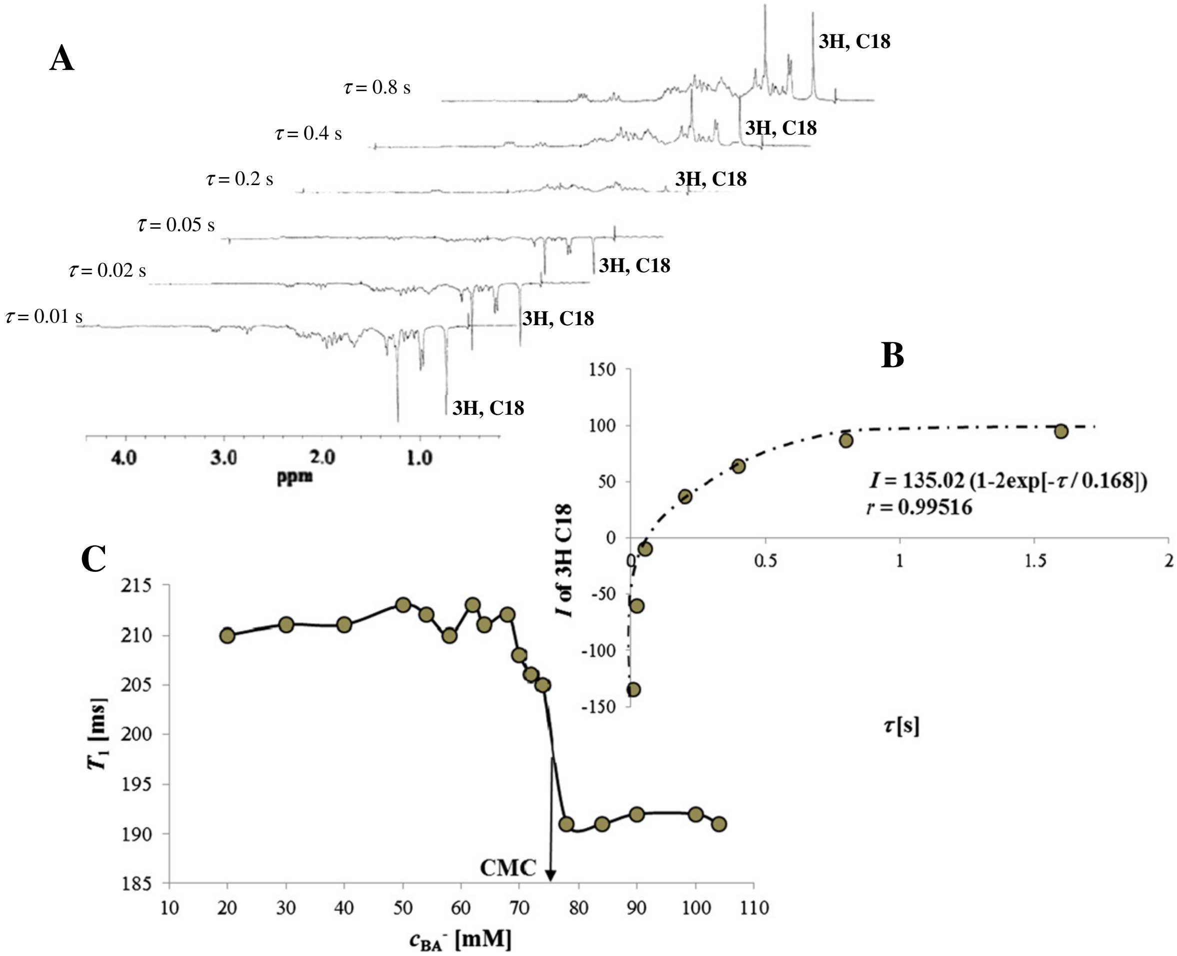


Fig. 2. (A) Representation of ^{1}H-NMR spectra of sodium-3,7-DOC (c_{BA^-} = 80 mM) obtained by the inversion recovery experiments (180°-τ-90°-AQC); (B) selected peak areas for different pulse delays (data obtained by fitting to Eq. (1)); (C) spin–lattice relaxation time (T_1) as a function of the concentration (c_{BA^-}).

the signal area of the C18 protons as a function of time that elapsed from the application of the 180° pulse, the time τ being measured from the termination of the action of the 180° signal. To each point in the graph (Fig. 2(B)) 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 instants: $\tau = 0.010$ s; $\tau = 0.025$ s; $\tau = 0.050$ s; $\tau = 0.10$ s, etc. The relaxation time from the graph (Fig. 2(B)) was determined by fitting experimental data to the following equation (*I*—signal surface):

$$I = I_0\left(1-2\exp\left[\frac{-\tau}{T_1}\right]\right) \tag{3}$$

To determine the relaxation time, a graph of the signal area of the C18 proton *vs.* time τ, was constructed for each tested bile salt concentration. The relaxation times obtained in such way, T_1, are presented as a function of the concentration c_{BA^-} of the tested bile acid (Fig. 2(C)). If the function $T_1 = f(c_{BA^-})$ has an inflexion point (abrupt jump) this means that micelle formation happend and c_{BA^-} = CMC (Figs. 3, 4).

CMC values of analysed bile acid salts are presented in Table 1. Generally, CMC values determined according to NMR experiment have a bit higher value than CMC values measures with the probe molecule (for example: C = 8 mM [1], HD = 12 mM [1]). However, they are very similar to CMC values obtained by measuring surface tension (for example: C = 13 mM [3], HD = 14 mM [3]). For anions of bile acids 7-ODC and HD there are two inflexion points on curves $T_1 = f(c_{BA^-})$. First, with the higher jump of T_1 value which probably corresponds to Smalls primary micelles (CMC-I, Figs. 3, 4), and the second inlexion point, with the smaller jump of T_1 values (CMC-II, Figs. 3, 4). Also, if T_1 value of hydrogen from C21 methyl group is observed, function $T_1 = f(c_{BA^-})$ has three inflexion points. However, by using T_1 values of sum of H atoms in 1.5–2.5 ppm interval of NMR spectar in function of monomers concentration we cannot get significant inflexion points. This suggests that acceleration of relaxation process in the second inlexion point, according to Eq. (2), is a consequence of the rise of $\overline{H^2}$ due to probable deceasing of the distance between hydrophobic surfaces of

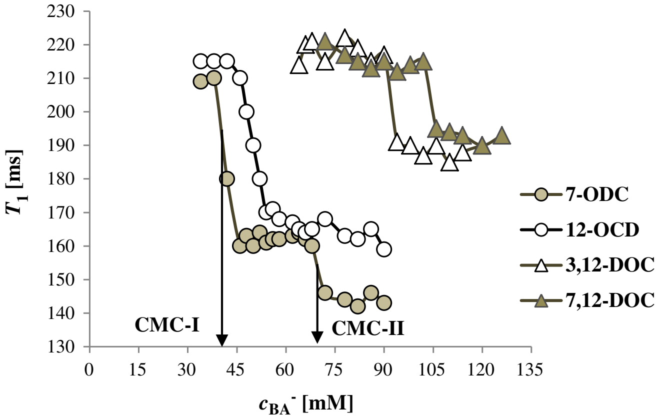


Fig. 3. Dependence between spin–lattice relaxation time (T_1) from concentration of analysed bile acid sodium salts.

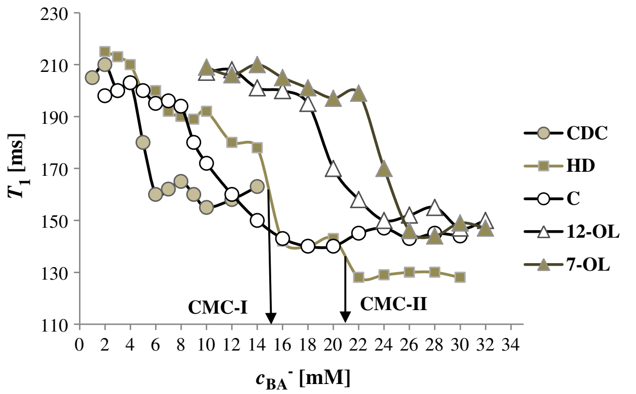


Fig. 4. Dependence between spin–lattice relaxation time (T_1) from concentration of analysed bile acid sodium salts.

building units steroid skeletons. Mutual approaching of monomers in aggregate can be a consequence of the fact that with the increase in aggregation number (in the second inflexion point: CMC-II) beside hydrophobic interactions, additional stabilisation of micelle is possible due to the formation of hydrogen bonds between building units. By using pyrene method as fluorescence probe molecule in investigation of micellisation HD Matsuoka et al. [51] realised that there are two concentration regions. First one appears on concentration of 5 mM where small aggregates are formed. The second region appears around concentration of 14 mM. Then small stabile micelles are formed. By measuring relaxation times *i.e.* $T_1 = f(c_{\mathrm{BA}^-})$ first Matsuok region is not separated from the second one and change of T_1 time in the first region suits most to linear decrease of T_1 (with concentration). Thus, inflexion point cannot be determined there, while in the second region inflexion point can be determined (Fig. 4). For HD in function $T_1 = f(c_{\mathrm{BA}^-})$ on concentration 21 mM second inflexion point appears (Fig. 4). Of all examined bile acid sodium salts cholic acid (C) have the least severe change of T_1 value in inflexion point (Fig. 4).

3.2. Number-average aggregation numbers

According to Moroi–Matsuoka–Sugioka method, solid phase of the bile acid is always present in the experimental system [33,34]. Below the micellar region, the bile acid solubilisation (BAH) can be presented by the following equations:

$$\mathrm{BAH}_{(s)} \rightleftarrows \mathrm{BAH}_{(aq)} + \mathrm{BA}^-_{(aq)} \tag{R1}$$

$$\mathrm{BAH}_{(aq)} + \mathrm{H_2O}_{(aq)} \overset{K_a}{\rightleftarrows} \mathrm{BA}^-_{(aq)} + \mathrm{H_3O}^+_{(aq)}. \tag{R2}$$

The total concentration of dissolved bile acid is: $c^t_{\mathrm{BAH}} = c_{\mathrm{BAH}} + (c_{\mathrm{BA}^-})_e$. The concentration of the dissociated bile acid can be expressed through the corresponding acidic constant $K_a : c^t_{\mathrm{BAH}} = c_{\mathrm{BAH}} + (K_a \cdot c_{\mathrm{BAH}})/\left(\gamma_{\mathrm{BA}^-} \cdot a_{H_3O^+}\right) = c_{\mathrm{BAH}}\left[1 + K_a/\left(\gamma_{\mathrm{BA}^-} \cdot a_{H_3O^+}\right)\right]$ [33,34]. Hence, the solubilisation of the bile acid up to the CMC value depends linearly on $1/\left(\gamma_{\mathrm{BA}^-} \cdot a_{H_3O^+}\right)$ (Fig. 5). In the Matsuoka–Moroi–Sugioka method, the concentration of non-dissociated bile acid (BAH) in the aqueous solution can be neglected; hence: $c^t_{\mathrm{BAH}} = (c_{\mathrm{BA}^-})_e$, and below the CMC: $c^t_{\mathrm{BAH}} = (c_{\mathrm{BA}^-})_e = c_{\mathrm{BA}^-}$, where c_{BA^-} corresponds to the total concentration of bile acid sodium salt. Above the CMC, the total concentration of the bile acid anion is: $c^t_{\mathrm{BA}^-} = (c_{\mathrm{BA}^-})_e + \sum_i n_i c_{M_i}$, where M_i stands for the micelle with the aggregation number n_i. The concentration of free bile acid anion $(c_{\mathrm{BA}^-})_e$ in the micellar region at a total concentration c_{BA^-} can be determined by extrapolating the straight line (A) of the region below the CMC to the region above the CMC (Fig. 5). Formation of mixed micelles with non-ionised micelle can be neglected. The average aggregation number $\bar{n}$ is determined by the following equation:

Table 1
Critical micellar concentration (primary micells), number-average aggregation number (et the concentration of 125 mM of bile acid salts) and capacity factor.

Bile acids	CMC [mM]	$\bar{n}$	*k*
C	12	12.60	5.71
CDC	5	8.38	10.87
7-ODC	42	7.38	1.89
12-OCD	50	5.48	2.08
7,12-DOC	105	3.28	0.47
12-OL	24	6.92	5.03
7-OL	20	7.10	4.02
3,12-DOC	75	4.16	2.81
3,7-DOC	70	4.72	2.38
HD	15	7.5	3.17

$$\bar{n} = \frac{c_{\mathrm{BA}^-} - (c_{\mathrm{BA}^-})_e}{C_m - (c_{\mathrm{BA}^-})_e}, \tag{4}$$

C_m is the sum of the molar concentrations of all particle types (free bile acid anion and micelles of different aggregation numbers), which is determined by the numerical integration

$$C_m = \int_0^{c_{\mathrm{BA}^-}} \frac{c_{\mathrm{BA}^-}}{(c_{\mathrm{BA}^-})_e} dc_{\mathrm{BA}^-} \frac{1}{n}. \tag{5}$$

The $(c_{\mathrm{BA}^-})_e$ value that corresponds to a c_{BA^-} value in the micellar region is determined by extrapolation of the straight line (A) (Fig. 5). The dependence of $(c_{\mathrm{BA}^-})_e$ on c_{BA^-} in the region above the CMC corresponds to $(c_{\mathrm{BA}^-})_e \approx$ *const.* or shows a slight increase, whereas in the vicinity of CMC, $(c_{\mathrm{BA}^-})_e$increases significantly (Fig. 6).

The average aggregation numbers of the investigated Na salts of bile acids are shown in Fig. 7 and Table 1. For Na cholate (C) $\bar{n} = 12.60$ and for Na chenodeoxycholate (CD) $\bar{n} = 8.38$. The literature values for the aggregation numbers of these bile acids determined by pH-metric method are 13.5 and 7.5 respectively (above 50 mM, at 298 K). Above concentration of 100 mM for C and 7-ODC $\bar{n}$ slightly grows with the increase in concentration, while for other bile acid sodium salts value of $\bar{n}$ is constant (Fig. 7). Bile acid sodium salts that form only primary micelles, due to relatively small hydrophobic surface of the steroid skeleton β side, probably posses border package, which is achieved on high concentrations.

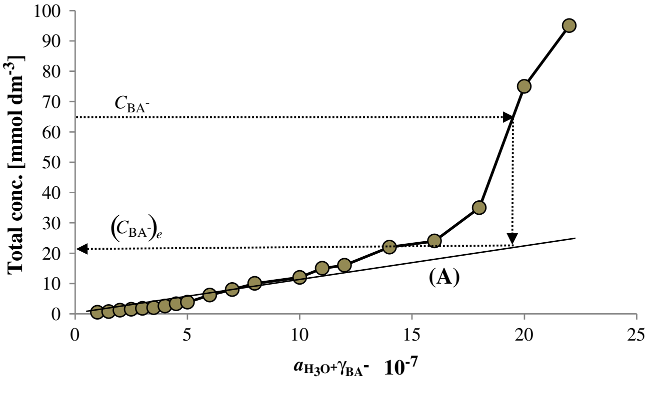


Fig. 5. The pH dependence of the total concentration of bile acid anion (example of 12-OCD).

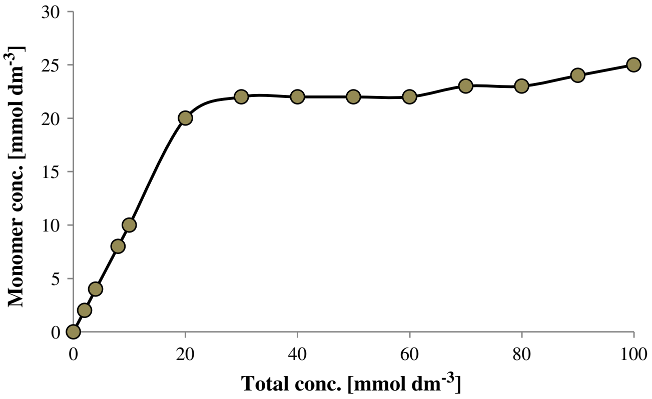


Fig. 6. Dependence of the monomer concentration $(c_{BA^-})_e$ on the total concentration of bile acid anion c_{BA^-} (example of 12-OCD).

3.3. Hydrophobicity and retention capacity (RPHPLC)

Bile acid bind for hydrophobic stationary phase over their β sides of the steroid skeleton [30–32]. Thus, by replacing α axial OH groups with α equatorial OH or oxo groups (whose appropriate oxygen atoms are switched toward steroid skeleton mean plain *i.e.* to β side of the steroid skeleton) derivatives with lower values of retention factors (k) are obtained (molecules hydrophobic surface on the β side of the steroid skeleton). So, k is adequate measure of hydrophobicity of steroid skeleton convex side (Table 1).

4. Results and discussion

4.1. Micellisation: dependence of CMC and number-average aggregation number from parameter of hydrophobicity (k)

For classic amphiphiles of type: hydrophobic tail—polar head, micelle have the higher aggregation number if amphiphile molecule (ion) is more hydrophobic. [42]. There is a discussion in literature whether hydrogen bonds between bile acid anions are formed already in primary micelles or hydrogen bonds are formed only in primary micelles while seconadry micelles are formed (mutual binding of primary micelles over hydrogen bonds) [6]. Molecular dynamic simulations show that in primary micelles, beside leading (main) hydrophobic interactions, hydrogen bonds between building units are possible as well [52–54]. Presence of hydrogen bond between monomers of primary micelles lowers critical micelle concentration and rises aggregation number, *i.e.* micelle have aggregation parameters that would suit to more hydrophobic building units than building units are in reality (good example is 7 and 12 glycosilated cholic acid [55]). On the other side two-dimensional NMR study shows that in environment of CMC bile acid primary micelles are usually dimmers, whose building units are connected over their β hydrophobic side of steroid skeleton, *i.e.* structure of Small primary micelle is confirmed [56].

According to original Smalls model, formation of primary micelles of bile acid salts with hydrophobic interactions is a single phase process [5,6], *i.e.* on the CMC only micelles with certain aggregation numbers are formed—monodisperse system (R3) (the counterion binding to the micelle is assumed neglible, since it does not influence thermodynamic analysis in this paper, *i.e.* experiments show that for majority of bile acids binding of counterion can be neglected [33,34]):

$$n\mathrm{BA} \overset{K}{\rightleftarrows} (\mathrm{BA})_n \tag{R3}$$

However, the change lengthiness of physico-chemical parameters of the system in vicinity of CMC suggests that formation of primary micelles is due to appropriate multiple association model (R4).

$$\left\{\begin{array}{c} \mathrm{BA}+\mathrm{BA} \overset{K_{12}}{\rightleftarrows} (\mathrm{BA})_2 \\ \mathrm{BA}+(\mathrm{BA})_2 \overset{K_{23}}{\rightleftarrows} (\mathrm{BA})_3 \\ \cdots \\ \mathrm{BA}+(\mathrm{BA})_{\mathrm{i}-1} \overset{K_{\text{i-1i}}}{\rightleftarrows} (\mathrm{BA})_{\mathrm{i}} \\ \mathrm{BA}+(\mathrm{BA})_{\mathrm{i}} \overset{K_{\mathrm{ii+1}}}{\rightleftarrows} (\mathrm{BA})_{\mathrm{i+1}} \end{array}\right\} \Rightarrow \overline{n}\mathrm{BA} \overset{K}{\rightleftarrows} (\mathrm{BA})_{\overline{n}} \tag{R4}$$

In literature, multiple association of bile acid anions is widely accepted model for formation of primary micelles [2,32,33,51].

In reaction scheme (R4) multiple association of bile acid anions (monomers) is substituted with the process in which formed micelle is characterised with number-average aggregation number $\overline{n}$. Using thermodynamic condition for equilibrium of process (R4) equation can be obtained that connects retention coefficient (k), critical micelle concentration (CMC) and number-average aggregation number ($\overline{n}$) (Supporting materials):

$$\ln k = \underbrace{\frac{a-\overline{b}c}{\overline{b}RT}}_{const.} - \frac{1}{\overline{b}}\overline{n}\ln \mathrm{CMC}. \tag{6}$$

According to Eq. (6) linear dependence between ln$k$ and lnCMC is expected, likewise linear dependence between ln$k$ and $\overline{n}$. Since examined bile acids belong to congeneric group of bile acids with 24C atoms (conjugated bile acids and those with introduced additional C atoms

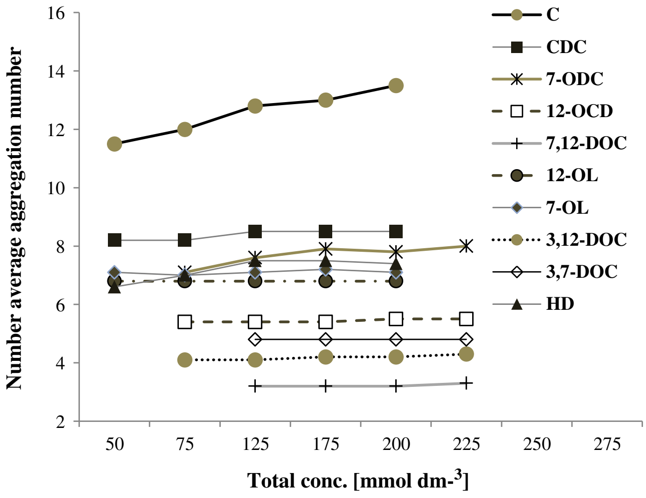


Fig. 7. Dependence of the number-average aggregation number $\overline{n}$ on the total concentration of bile acid anion c_{BA^-} at 298 K.

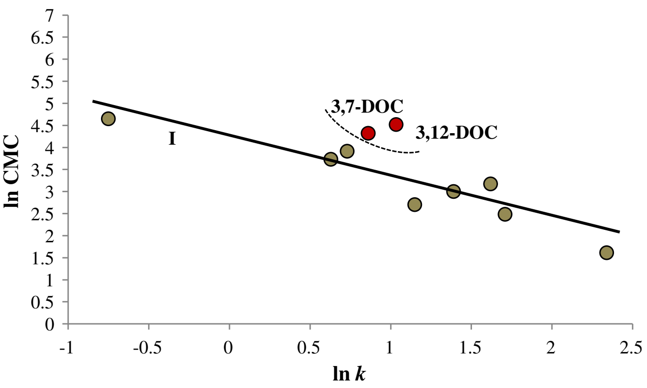


Fig. 8. Linear dependence between ln CMC and ln k (**I**), molecules 3,7-DOC and 3,12-DOC are su influential observation related to **I**.

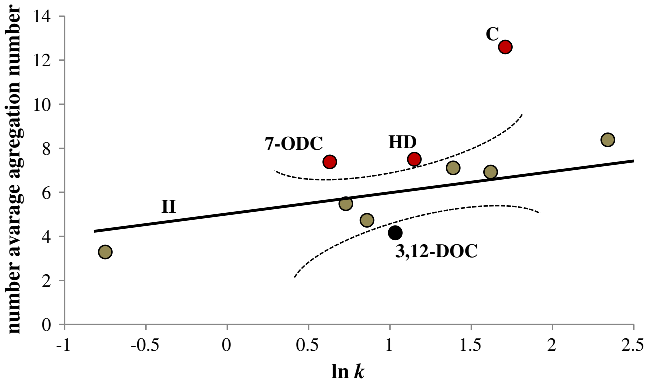


Fig. 9. Linear dependence between $\bar{n}$ and ln$k$ (**II**), molecules: C (outlier), 7-ODC and HD (influential observations) have higher values of $\bar{n}$ than it would be expected from their hydrophobicity; 3,12-DOC (influential observation) related to **II** due to overestimated hydrophobicity over ln$k$.

in the steroid skeletons are not examined)—they can be observed as one congeneric group. For structurally similar molecules, hydrophobicity can be adequately described with retention coefficient, while for structurally different compounds for describing hydrophobicity chromatographic parameters as: index of hydrophobicity (φ_0), gradient retention time (t_R) and chromatographic hydrophobicity index parameter (CHI) are used [41].

If during the micelle formation process (formation of primary or of secondary micelles) between building units hydrogen bonds are formed as well then, beside $\Delta G^{\odot}_{M}$ (only hydrophobic interactions are taken into account) there is additional term in total Gibbs energy of micelle formation ($\Delta G^{\odot}_{corr.}$ correction due to formation of H bond), *i.e.* exists $\Delta G^{\odot}_{M}+\Delta G^{\odot}_{corr.}$. If $\Delta G^{\odot}_{corr.}$ is taken into account Eq. (6) is:

$$\ln k = \underbrace{\frac{a-\bar{b}c}{\bar{b}RT}}_{const.} - \frac{1}{\bar{b}}\bar{n}\ln \mathrm{CMC} + \frac{\Delta G^{\odot}_{corr.}}{bRT}. \tag{7}$$

According to Eqs. (6) and (7) if beside hydrophobic interactions between building units in micelle there are hydrogen bonds as well, micelle have aggregation parameters that suit for more hydrophobic building units than building units really are.

In the group of analyzed molecules, there is the next linear dependence (Fig. 8):

$$\ln \mathrm{CMC} = 4.46(\pm 0.32) - 0.98(\pm 0.24)\ \ln k$$
$$N = 10; R = 0.8197; F = 16.86; sd = 0.60. \tag{8}$$

Related to linear regression (8) molecules 3,12-DOC and 3,7-DOC are so called influential observations [57] because they have only oxo groups in their steroid skeleton and their biplanar amphifilicity is destroyed [17]. Thus, these molecules can bond to hydrophobic stationary phase or to already adsorbed molecules over α side of the steroid skeleton as well as for molecules 3,12-DOC and 3,7-DOC. Thus, by using k their

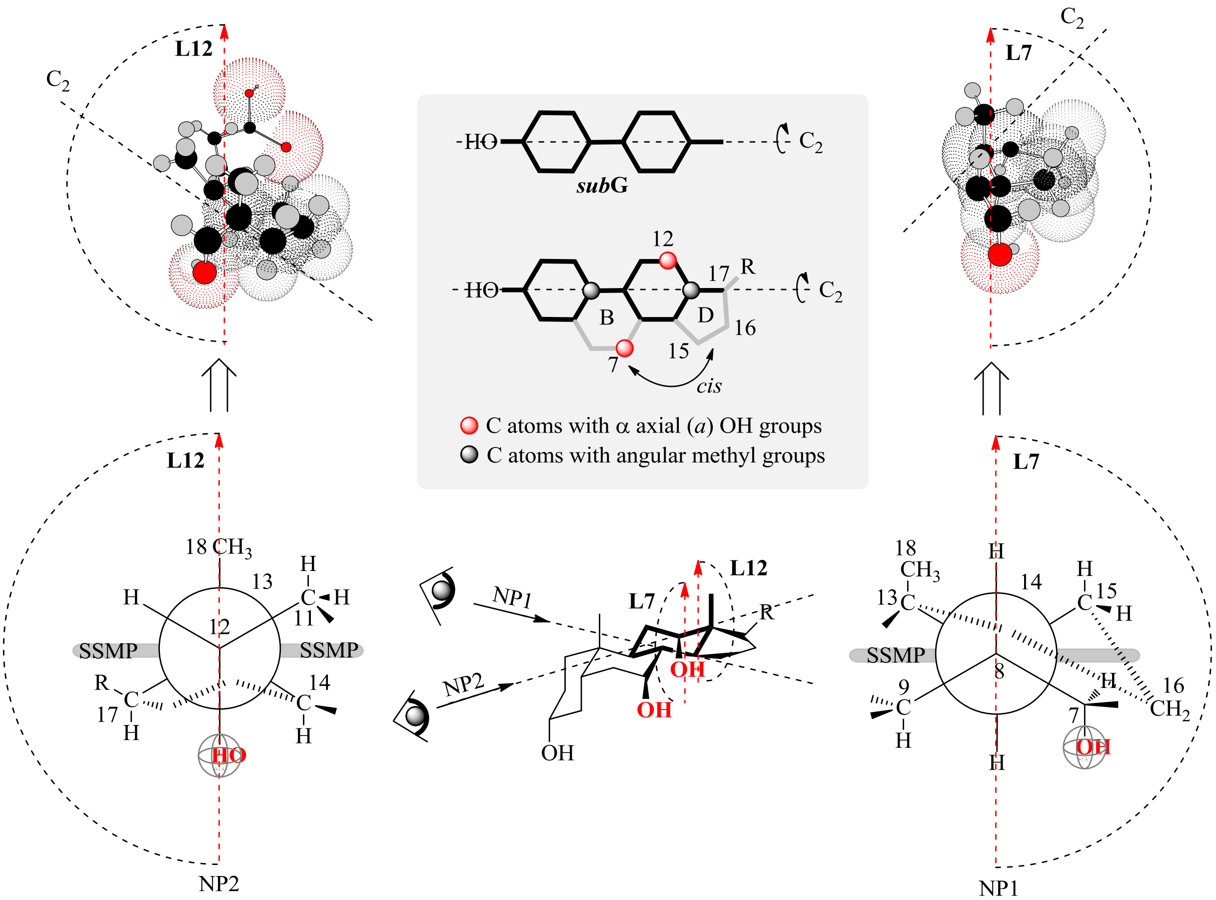


Fig. 10. Geometrical isomery of the graph of cholic acid with respect to the C_2 axis of the subgraph (*sub*G). Carbon atoms C15, C16 and C17 of the steroid skeleton ring D form different steric environments of the C12 and C7 α-(*a*)-OH groups.

hydrophobicity is overestimated [58]. Molecule 3,12-DOC behaves similarly as in reversed phase thin layer chromatography [59]. Without molecules 3,12-DOC and 3,7-DOC statistical parameters of linear regression are improved (Eq. (9)).

$$\begin{aligned}&\ln \mathrm{CMC} = 4.92(\pm 0.21) - 0.94(\pm 0.15)\ \ln k\\ &N = 8; R = 0.9399; F = 38.0; sd = 0.37\end{aligned} \tag{9}$$

If bile acid anions micelles are formed over hydrophobic interactions and total standard Gibbs energy of micelle formation is equal to $\Delta G^{\odot}_{M}$, it means that, according to Eq. (6) there is a linear dependence between $\overline{n}$ and ln$k$. However, for bile acid sodium salts which form H bonds in the micelles (primary or secondary) $\overline{n}$ will have higher value then it would be expected according to hydrophobicity (ln$k$) of steroid skeleton (Eq. (7)), *i.e.* these bile acid anions will deviate more or less (depending on the number of H bonds) from the linear dependence between $\overline{n}$ and ln$k$ (they behave as outliers or influential observations). In order to determine this deviation for further analysis values of $\overline{n}$ on concentration of bile acid sodium salts of 125 mM are taken (Table 1). According to molecule dynamic simulations the probability of formation of hydrogen bonds in micelles is higher if there is a higher concentration of building units in a solution [52,53]. In the whole group of bile acid sodium salts there is the next linear dependence between $\overline{n}$ and ln$k$:

$$\begin{aligned}&\overline{n} = 4.45(\pm 1.14) + 2.14(\pm 0.84)\ \ln k\\ &N = 10; R = 0.6695; F = 6.5; sd = 2.07.\end{aligned} \tag{10}$$

Statistical parameters of linear regression between $\overline{n}$ and ln$k$ are considerably improved if molecules: C (outlier), 7-ODC and HD (influential observations) are left out [57]. (Fig. 9):

$$\begin{aligned}&\overline{n} = 3.97(\pm 0.53) + 1.69(\pm 0.39)\ \ln k\\ &N = 7; R = 0.8883; F = 18.72; sd = 0.91\end{aligned} \tag{11}$$

According to that, micelles of C, 7-ODC and HD have higher values of number-average aggregation number than it would be expected from hydrophobicity of their building units. On the basis of hydrophobicity (expressed as ln$k$) monomers C, 7-ODC and HD enter linear hydrophobic congeneric group that forms linear regression Eqs. (8) or (9). Since CMC values are also determined by hydrophobic interactions formation of micelle on CMC and temperature of 25 °C is entropy driven process [60]. Hydrogen bonds are formed only at higher concentrations than CMC is. Increase in aggregation number for micelles of C, 7-ODC and HD suggest that in these aggregates hydrogen bonds are possible as well. Of course, above mentioned thermodynamic analysis cannot say if hydrogen bonds are present in primary or secondary micelles, only that they are possible in aggregates of C, 7-ODC and HD monomers. For molecules 7-ODC and HD in function $T_1 = f(c_{\mathrm{BA}^-})$ (Figs. 3 and 4) two inflexion points existed which can indicate that they are perhaps capable for formation of secondary micelles. For C, in function $T_1 = f(c_{\mathrm{BA}^-})$ there is only one inflexion point. However, the change of function around CMC is very stretched. Other bile acid sodium salts whose functions $T_1 = f(c_{\mathrm{BA}^-})$ have one inflexion point and whose values of $\overline{n}$ are covered with linear regression Eq. (11) form micelles with hydrophobic interactions. By

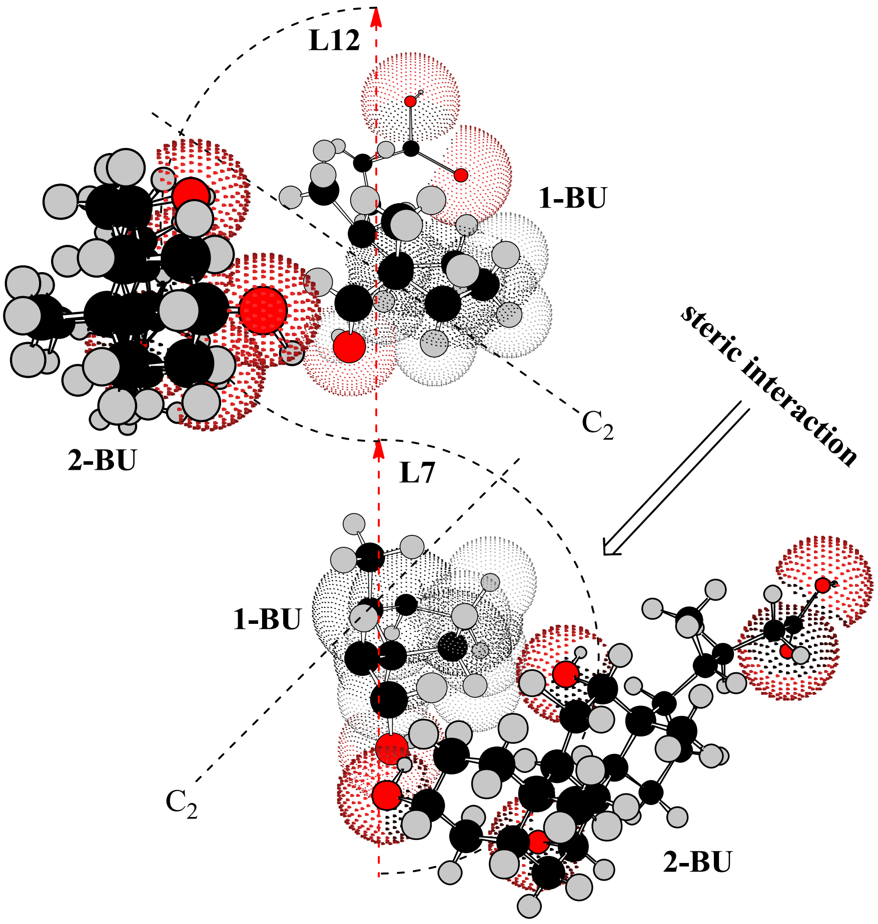


Fig. 11. Steric interactions in the vicinity of the C7-α-(*a*)-OH and C12-α-(*a*)-OH groups of one building unit (1-BU) in the formation of hydrogen bonds with the another builiding unit of the same or another micelle (2-BU) (in the formation of the hydrogen bond from 2-BU participates the least screened C3-α-(*e*)-OH group). For a better orientation, the figure also shows the C$_2$ axis of the *sub*G.

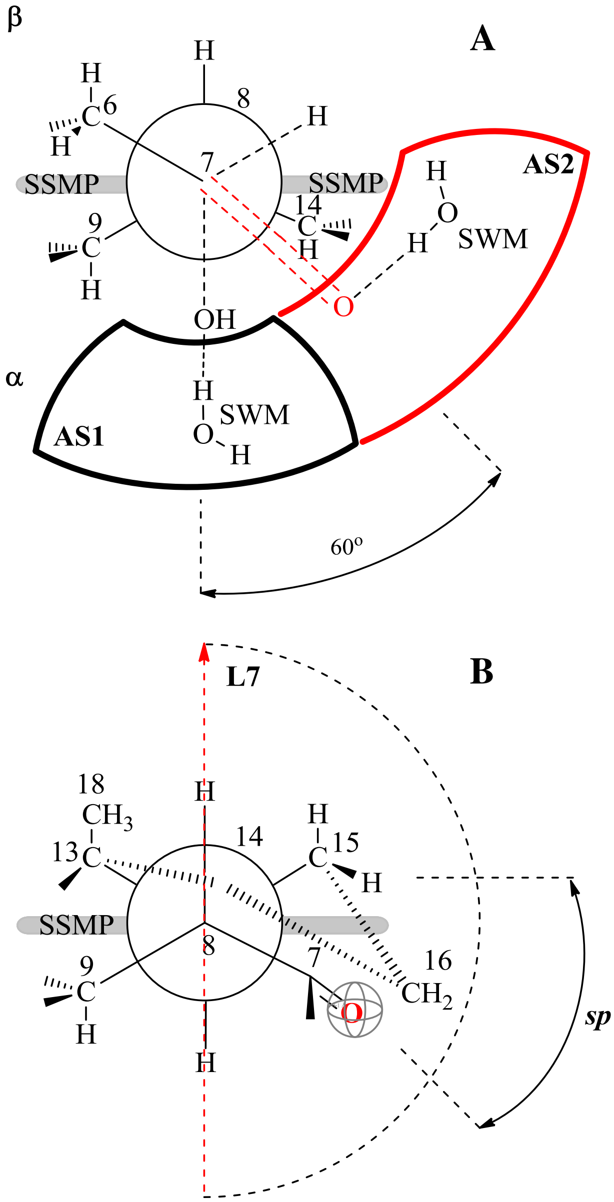


Fig. 12. Displacement of the area of stabilization (AS) toward the β side of the steroid skeleton in the oxidation of the α-(*a*)-OH group to oxo group (example of oxidation of the C7-OH group), SSMP = steroid skeleton mean plane, SWM = stabilized water molecules (A). The C7 oxo group, whose O atom is equatorially oriented with respect to the mean plane of the ring D of the steroid skeleton, is in a synperiplanar position (B).

leaving out 3,12-DOC molecule (influential observation) from further linear regression analysis (since for 3,12-DOC hydrophobicity over ln*k* is overestimated) statistical parameters of linear dependence between $\bar{n}$ and ln*k* (Eq. (12)) are improved:

$$\bar{n} = 4.23(\pm 0.34) + 1.69(\pm 0.24)\ln k \qquad (12)$$

$$N = 6; R = 0.9598; F = 46.79; sd = 0.57.$$

4.2. Number-average aggregation number and the structure of bile acids steroid skeleton

Judging from the findings of reverse-phase high-performance liquid chromatography (RPHPLC), CDC is more hydrophobic than C (Table 1), and hence, it could be expected that CDC forms micelles of larger aggregation number. This difference in the $\bar{n}$ values can be explained by the assumption that, in addition to hydrophobic interactions, hydrogen bonds are also involved in the micelles formed by C monomers. Similarly, according to the RPHPLC retention parameters, 12-OCD is more hydrophobic than 7-ODC (Table 1), whereas micelles of the 7-ODC derivative have larger average aggregation numbers.

A comparison of the steroid skeletons of C, CDC, 7-ODC and 12-OCD shows that all of them have a C3 equatorial (*e*) OH group, and anions which in the corresponding pairs (C-CDC, and 7-ODC–12-OCD) are characterised by a larger $\bar{n}$ value, have C12 axial (*a*) OH groups. Therefore, it seems that the C12-(*a*)-OH group behaves in the micelle formation in a different way than the C7-(*a*)-OH group (CDC and 12-ODC).

The higher $\bar{n}$ values for the micelles whose constituent units have a C12-α-(*a*)-OH group can be explained by observing the largest subgraph (*sub*G) [43] of the molecular graph of 3α-hydroxy-5β-cholanoic acid, with the C_2 symmetry axis, and considering the geometrical isomery in the graph of cholic acid with respect to this subgraph (Fig. 10). With respect to the C_2 symmetry axis of the *sub*G, the C7 atom with the α-(*a*)-OH group is in the *cis* position with respect to atoms C15 and C16 of the ring D of the steroid skeleton, whereas the C12 atom, also with the α-(*a*)-OH group, is in a *trans* position (Fig. 10).

As a result of the *cis* position of the C7 atom (with the α-(*a*)-OH group) and of the D ring in the corresponding Newman projection formula (Fig. 10, **NP1**), the C7-α-(*a*)-OH is in a position which is between synclinal (*sc*) and synperiplanar (*sp*) with C15 and C16 atoms of the D ring. This results in the steric screening of the C7-α-(*a*)-OH group with the D ring in the lateral projection plane L7 of the lateral side of the steroid skeleton and in hindering the formation of the hydrogen bond with the another constituent unit of the same or another micelle. Spatial accumulation of C atoms from the D ring in the L7 projection plane can be also observed in the 3D molecular model (fragment of

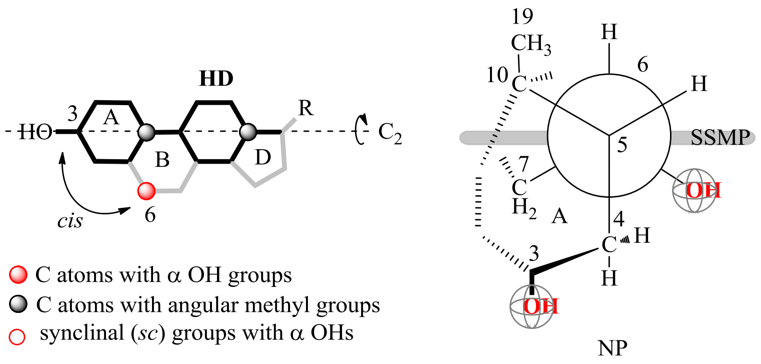


Fig. 13. C6-α-(*e*)-OH group of molecule HD is sterically ecraned with A ring of the steroid skeleton.

the rings C and D, Fig. 10). In the Newman projection formula NP2 (Fig. 10), α-(*a*)-OH group from C12 atom is in the *sc* position with respect to C17 and C14 atoms. However, other C atoms of the ring D are not localised in the L12 projection plane of the lateral side of the steroid skeleton, hence the D ring does not screen sterically the C12-OH group. This is also confirmed by the 3D model of the environment of the C12 atom and ring D (Fig. 10). However, it has been reported that the C12-α-(*a*)-OH group and the side chain at C17 interact. RPHPLC experiments also showed that because of the formation of a potential hydrogen bridge between the C12-α-(*a*)-OH group and the carboxylic function of the lateral chain, the environment of the C12-α-(*a*)-OH group is more hydrophobic than the environment of the C7-α-(*a*)-OH group [61]. Further, it is known that aliphatic chains are well flexible, so that there are a lot of conformations of the C17 side chain in which the C12-α-(*a*)-OH group is not sterically screened. This means that approaching of another constituent units' OH group (2-BU) to the C17 side chain may assume a conformation which does not hinder the access of the OH group from 2-BU to the C12-α-(*a*)-OH group of 1-BU, *i.e.* it does not hinder formation of hydrogen bonds in the L12 projection plane (Fig. 11). Therefore, the presence of the C12-α-(*a*)-OH group in monomer contributes to formation of micelle with a larger aggregation number. Because of the rigidity of the steroid skeleton, the ring D is permanently screening the C7-α-(*a*)-OH group in the L7 projection plane of the lateral side of the steroid skeleton. Hence, the formation of hydrogen bond between the C7-α-(*a*)-OH group and the OH group of the another constituent units' (2-BU) is accompanied by a steric repulsive interaction (Fig. 11). In view of this, formation of a hydrogen bond between steroid skeletons of bile acid anions *via* the C7-α-(*a*)-OH groups is unlikely to happen.

It can be seen from Table 1 that rise in number of substituted OH groups with oxo group for trihydroxy and dihydroxy derivatives of cholanoic acid results in lowering of the value of number-average aggregation number. Namely, the oxidation of the α-(*a*)-OH group gives an oxo group whose O atom, in the corresponding Newman projection formula (Fig. 12(A)), is shifted for 60° towards the steroid skeleton mean plane (SSMP) [1,61]. This switching of the oxygen atom leads to decrease of the molecules hydrophobic surface from β side of steroid skeleton [1,17,61], which leads to lowering of CMC and $\overline{n}$ values.

With the pairs of mono-oxo (7-OL and 12-OL) and di-oxo (3,12-DOC and 3,7-DOC) derivatives, monomer having an oxo group at the position C7 has a larger aggregation number compared to the derivative with oxo group at the C12 and C3 atoms of the steroid skeleton. Namely, in the molecules of derivatives 7-OL and 3,7-DOC the C7 oxygen atom of the oxo group is in a synperiplanar (*sp*) position with respect to the mean plane of the ring D (the Newman projection formula, Fig. 12(B)), which sterically hinders stabilisation of water molecules (by hydrogen bonds) in the L7 projection plane of the hydratation sheath. Therefore, the steric environment of the C7 oxo group is more hydrophobic than that of the C12 oxo group. In 3D space, during the formation of micelle, due to conformational flexibility of side chain C12 oxo group is stericaly less protected than C7 oxo group of steroid skeletons D ring.

In the anion of hyocholic acid, the C6 OH group has an α-(*e*) orientation as well as the α oxo group of studied derivatives [61]. Because of that, in the HD monomer the β side of the steroid skeleton also shows a lower hydrophobicity (Table 1), which should lead to a decrease in the aggregation number of HD micelles (it should have $\overline{n}$ as 7-OL and 12-OL). However, HD behaves as influential observation regarded to linear regression Eq. (10) (dependence between $\overline{n}$ and ln*k*), which suggests that HD can form micelles in which, beside hydrophobic interactions between monomers, H bonds may also exist. It can be seen from presented molecule graph of HD on Fig. 13. that C6-OH group is in *cis* position with certain C atoms of the steroid skeleton ring A. However, bond between C3 carbon of hydroxymethine group and C4 carbon of methylene group is oriented toward C_2 axis of symmetry in graph HD (Fig. 13). Thus, ring A of the steroid skeleton sterically does not ecranes C6-α-(*e*)-OH group (Fig. 13**, NP**), and so it can form hydrogen bonds.

5. Conclusion

Aggregation numbers of micelles of bile acid anions depend mostly on hydrophobicity of the β side of the steroid skeleton. Replacement of OH group with an oxo group results in a decrease in the average aggregation number. Increase in the aggregation number is observed with micelles in which the steroid skeleton of the monomer has the C12-α-(*a*)-OH group or C6-α-(*e*)-OH group. According to conformational analysis these OH groups can form hydrogen bond with OH group of the another monomer from the same or another micelle. The D ring of the steroid skeleton inhibits sterically the C7-α-(*a*)-OH group to form hydrogen bonds. In addition, the D ring increases the hydrophobicity of the C7 oxo group compared to the C12 oxo group.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.11.008.

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