Biochimica et Biophysica Acta 1850 (2015) 1345–1353

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Micellization parameters (number average, aggregation number and critical micellar concentration) of bile salt 3 and 7 ethylidene derivatives: Role of the steroidal skeleton II

Mihalj Poša [a,*], Srđan Bjedov [b], Dušan Škorić [b], Marija Sakač [b]

[a] Department of Pharmacy, Faculty of Medicine, University of Novi Sad, Hajduk Veljka 3, 21000 Novi Sad, Serbia
[b] Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, Trg D. Obradovića 3, 21000 Novi Sad, Serbia

ARTICLE INFO

Article history:
Received 23 November 2014
Received in revised form 23 March 2015
Accepted 24 March 2015
Available online 1 April 2015

Keywords:
Micelle of bile salts
Aggregation number
Steroidal skeleton
Hydrophobic linear congeneric group
Excess Gibbs energy

ABSTRACT

Background: Bile salts are steroidal biosurfactants. Micellar systems of bile salts are not only important for solubilization of cholesterol, but they also interact with certain drugs thus changing their bioavailability.
Methods: The number-average aggregation numbers ($\overline{n}$) are determined using the Moroi–Matsuoka–Sugioka thermodynamic method. Critical micellar concentrations were determined by spectrofluorometric method using pyren and by surface tension measurements.
Results: Micelles of ethylidene derivatives possess the following values for $\overline{n}$: 7-Eth-D ($\overline{n}$ = 11 (50 mM)–$\overline{n}$ = 14.8 (100 mM)); 12-Ox-7-Eth-L ($\overline{n}\approx$8.8, without concentration dependence) and 7,12-diOx-3-Eth-Ch ($\overline{n}\approx$2.9, without concentration dependence). In the planes $\overline{n}$–ln *k* and ln CMC–ln *k* derivative 7-Eth-D is outlier in respect to hydrophobic linear congeneric groups.
Conclusion: Gibbs energy of formation for 7-Eth-D anion micelles in addition to the Gibbs energy of hydrophobic interactions consists excess Gibbs energy (G^E) from hydrogen bond formation between building blocks of micelles. Gibbs energy of formation for 7,12-diOx-3-Eth-Ch and 12-Ox-7-Eth-L anion micelle is determined by the Gibbs energy of hydrophobic interactions. Relative increase in hydrophobicity and aggregation number for ethylidene derivatives is larger when ethylidene group is introduced from the C7 lateral side of steroidal skeleton then it is when ethylidene group is on C3 carbon.
General significance: Position of outlier towards hydrophobic congeneric groups from $\overline{n}$–ln *k* and ln CMC–ln *k* planes indicates the existence of excess Gibbs energy (G^E) which is not of hydrophobic nature (formation of hydrogen bonds). For the bile salt micelles to have G^E (formation of secondary micelles) it is necessary that steroidal skeleton possesses C3-α-(*e*)-OH and C12-α-(*a*)-OH groups.

© 2015 Elsevier B.V. All rights reserved.

1. Introductions

Bile salts are steroidal amphiphilic compounds synthesized in liver of vertebrates [1–3]. Conventional surfactants possess hydrophobic conformationally flexible tail and hydrophilic head (ionic or with polar groups), while bile salts possess two different surfaces of steroidal skeleton [4–6]. Convex side (β side) of steroidal skeleton with angular methyl groups is hydrophobic side, concave side (α side), on the other hand, with axial OH groups is hydrophilic. Because of the cis fusion between A and B rings α equatorial OH group on C3 carbon is parallel to angular methyl groups as α axial OH groups from B and C rings are [5,7]. Bile salts above critical micellar concentration (CMC) form relatively small micelles with low aggregation number from 2 to 15 [8–10].

In biological systems, effects of bile salts are based on their surface activity (formation of micelles, mixed micelles with phospholipids, solubilization of cholesterol, solubilization and emulgation of lipid components of food) [11–13] or on their regulatory potential towards some enzymatic reactions and transport processes through binding to proteins (farnesoid X (FXR); G-protein-coupled receptors (GPCRs); TGR5 (GPBAR1, M-BAR and BG37); large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}), etc.) [1,14–16]. This gave rise to the increasing application of bile acid derivatives as therapeutics in metabolic disorders [17–29].

Usually more hydrophobic bile salts have higher promotional action on transport (generally on bioavailability) of a certain drug, however, with increasing hydrophobicity of steroidal skeleton grows the membrane toxicity [30–33]. The oxo derivatives of bile salts, which are

* Corresponding author. Tel./fax: +381 21 422 760.
E-mail address: mihaljp@uns.ac.rs (M. Poša).

http://dx.doi.org/10.1016/j.bbagen.2015.03.010
0304-4165/© 2015 Elsevier B.V. All rights reserved.

obtained by oxidation of OH groups with the usual position on the steroidal skeleton, are less membranotoxic [32,33]. Also, micelles of bile salts with oxo groups can bind appropriate drug in the micellar phase (e.g., morphine hydrochloride, which thus becomes more hydrophobic and stays longer in the cell), because of that in the past fifteen years pharmaceutical and pharmacological application of oxo derivatives of bile salts is investigated [1,12,34,35].

In general, the main problem with biopharmaceutical application of bile salts is that they form small micelles (only a few building blocks). Especially oxo bile acid anions form micelles consisting of 2 to 6 building blocks [9,10]. Therefore there is a need to increase hydrophobic phase of bile salt aggregate since this increases their capacity to accept hydrophobic guest molecules. This can be achieved by using binary mixtures of surfactants (bile salt and conventional surfactant) [4,36,37] or by subtle increase of steroidal skeleton hydrophobicity in oxo derivatives, whereby membrane toxicity should not be larger than membrane toxicity of deoxycholic and chenodeoxycholic acid salts [38].

The aim of this work is to determine the parameters of micellization (aggregation number and CMC) for sodium salts of new bile acid ethylidene derivatives (Fig. 1): 3(*EZ*)-ethylidene-7,12-dioxo-5β-cholanoic acid (7,12-diOx-3-Eth-Ch) (**1**); 3α-hydroxy-12-oxo-7(*E*)-ethylidene-5β-cholanoic acid (12-Ox-7-Eth-L) (**2**); and 3α,12α-dihydroxy-7(*E*)-ethylidene-5β-cholanoic acid (7-Eth-D) (**3**) [38]. Furthermore, the aim is to determine the impact of the relative position of an ethylidene group in terms of increasing the hydrophobicity and the aggregation number. In this paper ethylidene derivatives were examined in a set of bile acids with OH and oxo group in steroidal skeleton (7,12-dioxo-5β-cholanoic acid (7,12-diOx-Ch) (**4**); 3α-hydroxy-12-oxo-5β-cholanoic acid (12-oxolithocholic acid, 12-Ox-L) (**5**); chenodeoxycholic acid (CD) (**6**) and cholic acid (**7**); Fig. 1) in order to indirectly determine, on the basis of thermodynamical and chemometrical analysis (excess Gibbs energy to Gibbs energy of hydrophobic interactions, outlier and influential observations [39]), the presence of hydrogen bonding in their micelles relative to micelles of derivatives from hydrophobic linear congeneric group [10].

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-Ox-L) was prepared according to the procedure of Miljković et al. [40]. 7,12-Dioxo-5β-cholanoic acid (7,12-diOx-Ch) was prepared by selective oxidation (Ag_2CO_3/celite) of cholic acid methyl esters C3-OH group according to the method of Tsreng [41]. The obtained C3-oxo derivative was reduced in the Wolff–Kishner's reaction, and finally the obtained 7,12-dihydroxy derivative is oxidized according to Fieser and Rajagopalan [42]. Ethylidene derivatives (Fig. 1; 7,12-diOx-3-Eth-Ch; 12-Ox-7-Eth-L; 7-Eth-D) were obtained in the Wittig reaction of the suitable keto derivatives of cholic acid [38].

2.2. 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 equilibration, the pH of the clear solution was measured (Boeco BT600) without separation of the solid phase, and taking care not to disturb it [43,44]. 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% ($n = 5$).

2.3. Spectrofluorimetric measurements of CMC

Fluorescence measurements were carried out using Agilent Cary Eclipse Fluorescence Spectrophotometer. Pyrene was used as

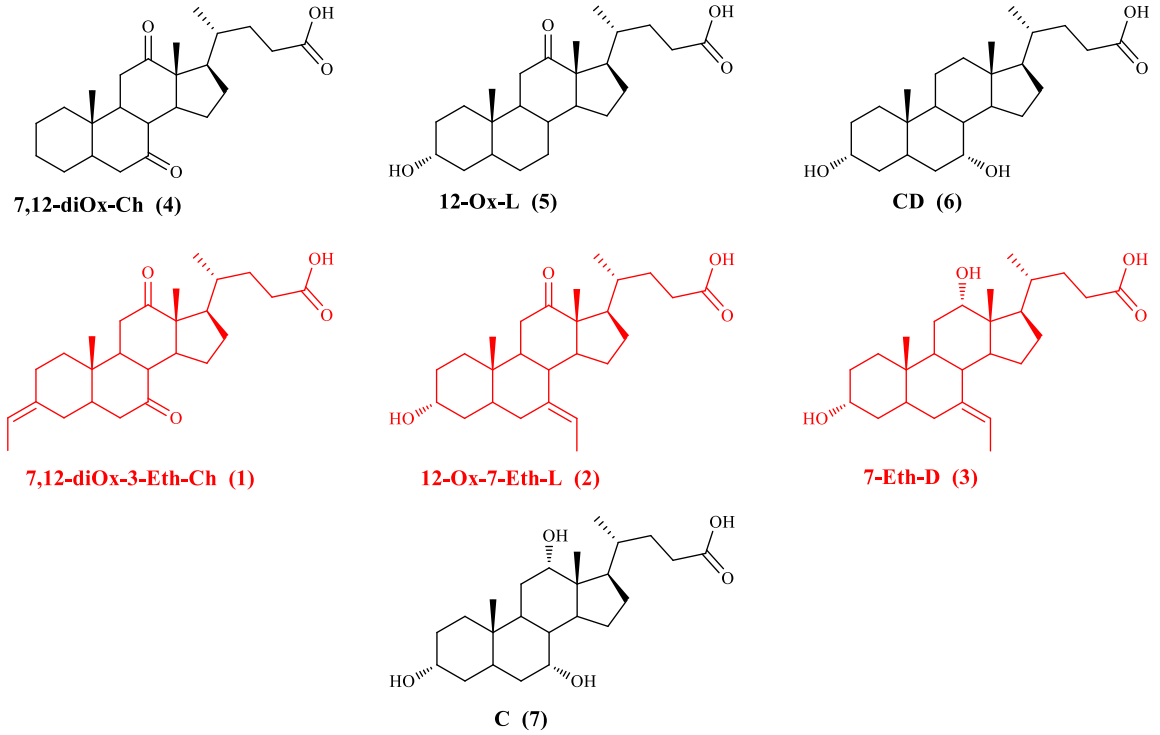

Fig. 1. Examined bile acids.

a fluorescence probe molecule. All solutions of bile salts were prepared using pyrene saturated water. Fluorescence emission spectra of these solutions were recorded employing an excitation wavelength of 334 nm.

The intensities of first (I_1) and third (I_3) vibrational bands of pyrene emission spectrum were measured at 373 and 384 nm respectively. The I_1/I_3 ratio was monitored as a function of total bile salts concentration at 25 °C. The errors in determination of the CMC values are estimated to be less than 5% ($n = 5$).

2.4. Surface tension measurements of CMC

The CMC values of the studied surfactants were also determined by surface tension measurements on a Sigma 703D tensiometer using the du Nouy ring method. All measurements were repeated three times at 25 ± 0.1 °C. The CMC determination error did not exceed 3%.

2.5. 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 = 70:125 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.

3. Results and discussions

In the literature, for the association of bile salts — formation of micelles — is widely accepted multiple association model [13,43–46] which can be modeled by a single-phase process if the micelles appear to the average aggregation number. So the building blocks bile acid salts are connected via convex plane of the molecule, according to Small–Kavamura model these are primary micelles with relatively small aggregation number (2–10) [3,48]. At higher concentrations than the CMC, Small's model predicts formation of secondary micelles by hydrogen bonding between primary micelles [3,8–10]. Molecular dynamic (MD) simulations confirm the importance of hydrophobic interactions in the association of bile salts, and the formation of secondary micelles. However, unlike the classical Small's primary micelle arising solely as a result of hydrophobic interactions, according to the MD simulations hydrogen bonds are possible in small aggregates but hydrophobic interaction remains a main driving force [49–52]. Modeling of critical micellar concentration for bile salts with retention parameters of reverse phase high performance liquid chromatography also confirms the importance of hydrophobic interactions in bile salts micelles [53–56].

If we considered that bile acids (ions) bind to the hydrophobic stationary phase over their β side of steroid skeleton [6,57], then standard Gibbs energy of micelle formation ($\Delta G_M^{\odot}$) (determined by hydrophobic interactions as main driving force) and standard Gibbs energy of bile acid adsorption on hydrophobic stationary phase ($\Delta G_A^{\odot}$) can be connected in equation: $\Delta G_M^{\odot} = b\Delta G_A^{\odot}$, where b is constant of proportionality. For constant of proportionality b in certain congeneric group (LKG − j) next relation should be satisfied (Appendix A):

$$\left.\begin{array}{l}\Delta G_M^{\odot}(i)_{LKG-j} = b_i \Delta G_A^{\odot}(R)_{LKG-j} \\ A_{hf}(i)_{LKG-j} = r_i A_{hf}(R)_{LKG-j}\end{array}\right\} b_i = \xi_{LKG-j} r_i + \beta_{LKG-j} \Big| : \quad \forall i \in \mathrm{LKG}-j \tag{2}$$

where R means referent molecule that could be whatever molecule from the linear congeneric group LKG − j, A_{hf} is hydrophobic surface of the molecule. Taking into account the relation: $\Delta G_M^{\odot}(i)_{LKG-j} = b_i \Delta G_A^{\odot}(R)_{LKG-j}$ and the thermodynamic condition for equilibrium in the formation of bile salts (BS) micelles with average aggregation number $\overline{n}$: $\overline{n}\mathrm{BS} \rightleftarrows^{K} (\mathrm{BS})_{\overline{n}}$, following equations, that are describing hydrophobic linear congeneric groups in $\overline{n}$–ln k and ln CMC–ln k planes, can be obtained [10] (Appendices A and B):

$$\ln k = const. - \frac{\overline{\ln \mathrm{CMC}}}{\overline{b}}\overline{n} \tag{3}$$

$$\ln k = const. - \frac{\overline{\overline{n}}}{\overline{b}} \ln \mathrm{CMC}, \tag{4}$$

where $\overline{\ln \mathrm{CMC}}$ presents the mean value of critical micelle concentration in LKG − j, and $\overline{\overline{n}}$ represents the mean value of number average aggregation number also in LKG − j. Values of critical micellar concentration (CMC), number average aggregation numbers ($\overline{n}$) and logarithmic values of retention coefficients (ln k) which were used to determine the affiliation of examined compounds to the linear congeneric groups (Eqs. (3) and (4)) are shown in Table 1. Based on our previous work (principal component analysis (PCA) and biplot of retention coefficients on different temperatures) ethylidene derivatives 7,12-diOx-3-Eth-Ch (**1**); 12-Ox-7-Eth-L (**2**); and 7-Eth-D (**3**), form a joint congeneric group with OH and oxo bile acid derivatives [38]. Therefore, for analysis of bile acid affiliation to some linear congeneric groups (3 and 4) retention coefficient can be used according to Eq. (1).

In the plane $\overline{n}$–ln k (Fig. 2) ethylidene derivatives 7,12-diOx-3-Eth-Ch (**1**) and 12-Ox-7-Eth-L (**2**) form hydrophobic linear congeneric group with CD (**6**); 7,12-diOx-Ch (**4**) and 12-Ox-L (**5**) molecules:

$$\overline{n} = -3.319(\pm 0.636) + 6.244(\pm 0.404) \ln k, \tag{5}$$

$$N = 5;\ \ R = 0.994;\ \ F = 238.58;\ \text{ and }\ sd = 0.426.$$

This indicates that the formation of micelles of a bile acid anions 7,12-diOx-3-Eth-Ch (**1**); 12-Ox-7-Eth-L (**2**); CD (**6**); 7,12-diOx-Ch (**4**) and 12-Ox-L (**5**) is determined by hydrophobic interactions, i.e., the building blocks of micelles are in contact with each other through the β-sides of steroidal skeleton. Between the elements of the linear congeneric group (5) (Fig. 2) derivatives 7,12-diOx-Ch (**4**) and 7,12-diOx-3-

Table 1
Hydrophobicity (ln k), number average aggregation number at 80 mM of total bile salt concentration and critical micellar concentration.

Bile acids	ln k	n	CMC	
			a	b
12-Ox-L	1.61	6.8	21.5	20.1
12-Ox-7-Eth-L	1.87	8.9	8.5	7.8
7,12-diOx-Ch	0.91	2.4	68.0	61.0
7,12-diOx-3-Eth-Ch	1.01	2.8	52.0	49.0
7-Eth-D	2.18	13.2	3.2	2.9
CD	2.11	9.4	5.8	5.5
C	1.74	12	9.1	8.8

a = spectrofluorimetric measurements of CMC.
b = surface tension measurements of CMC.

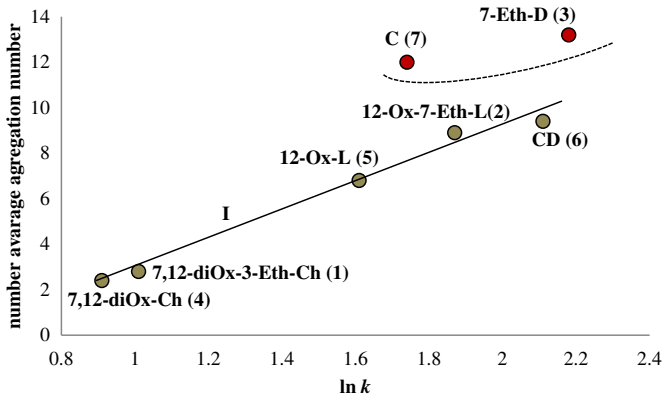

Fig. 2. Linear dependence between $\overline{\pi}$ and ln k (**I**), molecules: C (**7**) and 7-Eth-D (**3**) (outliers) have higher values of $\overline{\pi}$ than it would be expected from their hydrophobicity.

Eth-Ch (**1**) are closest to each other. Low aggregation number of their micelles and high CMC values (Table 1) can be explained by the position and orientation of the oxo group in steroidal skeleton. Namely, oxidation converts α axial (*a*) OH group to an pseudo-α oxo group which is moved for 60° to the β-side of steroidal skeleton relative to the orientation of the α-(*a*)-OH group in corresponding Newman projection formula (i.e., obtained oxo group makes 30° angle with steroid skeleton mean plane (SSMP)) [5,32,33]. Oxo group shift towards convex plane of the steroidal skeleton (β-side, i.e., hydrophobic surface of the molecule) enables hydrogen bond formation with water molecules from the hydration layer on the steroidal skeleton β-side. Therefore, tendency of dehydration of the β-side of the steroidal skeleton is reduced in the micelles formation. Namely, during micelle formation, water molecules, which are not hydrogen bonded to the polar groups of the surfactant molecule, are moved into the bulk solution. Thus, with the increase of the number of water molecules which are hydrogen-bonded to the oxo group/groups, tendency of bile acid anions towards the formation of micelles decreases [6]. However, as the two oxo groups on C7 and C12 carbon atoms are in mutual transposition (opposite orientation) continuance of hydrophobic surfaces on the β-side of steroidal skeleton is disrupted on 2/3 of the surface (starting from A ring). Because of that the compact hydrophobic surface of the steroidal skeleton convex plane is divided into two parts. Reference element for determination of cis/trans relation is $(C_2)^1$ axis of the **SG** subgraph (Fig. 3). The line that connects nuclei of two oxygen atoms from C7 and C12 oxo groups makes angle with $(C_2)^1$ axis that is closest to the right angle taking into account another possible trans-orientations of oxo groups from A, B and C rings of steroidal skeleton. In the same time on the α-side of steroidal skeleton the distance between oxygen atoms of two pseudo-equatorial oxo groups is longer than the distance between axial oriented oxygen atoms (Fig. 3). The result of this is a "hydrophobic

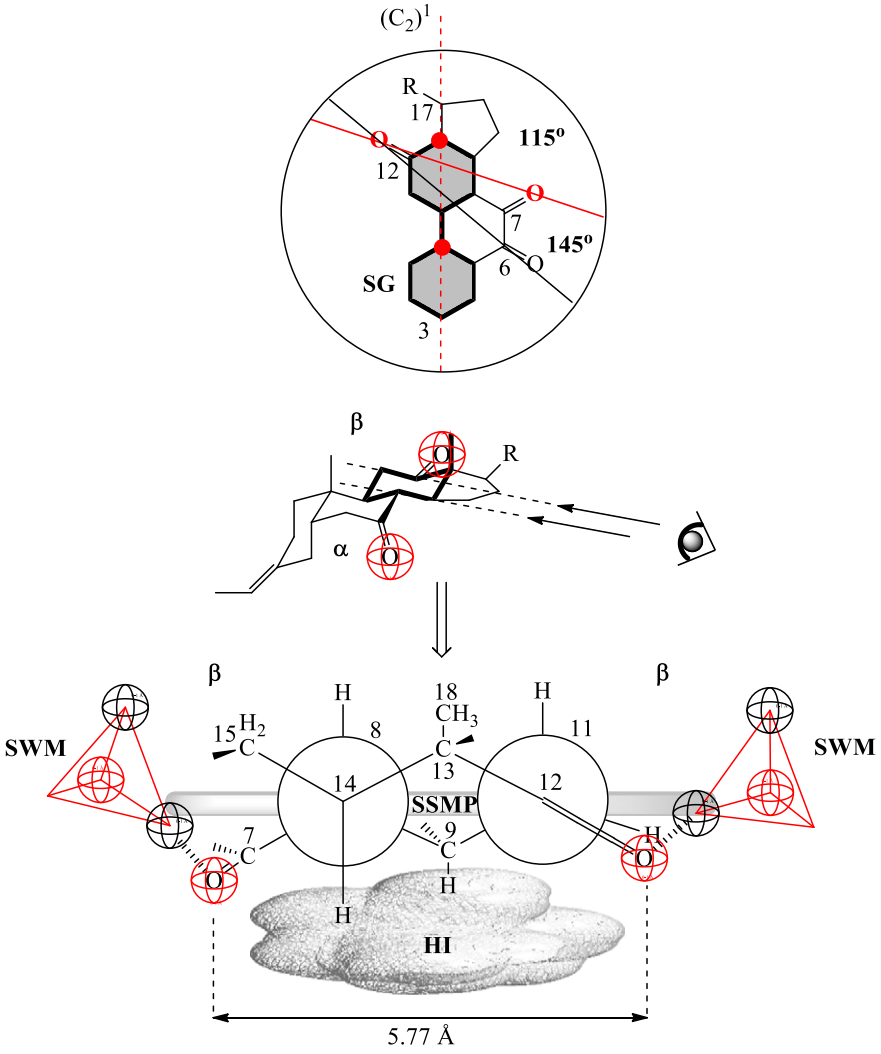

Fig. 3. Trans relationship between oxo groups on C7 and C12 in respect to the $(C_2)^1$ axis of subgraph **SG** and α equatorial position are resulting in disruption of continuance in hydrophobic and hydrophilic surface of the steroidal skeleton — decrease of the biplanar amphiphility (SWM = stabilized water molecules, SSMP = steroid skeleton mean plane, HI = hydrophobic island).

island" — continuance of hydrophilic surface on concave side of the molecule is disrupted [12]. According to Roda et al. existence of continuous hydrophobic and hydrophilic surfaces is the key structural feature of the bile salt aggregation [2]. So in 7,12-diOx-Ch (**4**) and 7,12-diOx-3-Eth-Ch (**1**) anions ability to form micelles and biplanar amphiphility is decreased due to disruption in the continuity of hydrophobic and hydrophilic surfaces. Micelles of 3,12-dioxo-5β-cholanoic acid and 3,7-dioxo-5β-cholanoic acid anions have a number average aggregation numbers 4.16 and 4.72 [10], which are almost 2 times higher than the number average numbers of 7,12-diOx-Ch (**4**) (constitutional isomer) and ethylidene derivative 7,12-diOx-3-Eth-Ch (**1**) micelles. However, oxo groups of 3,12-dioxo-5β-cholanoic acid and 3,7-dioxo-5β-cholanoic acid are not in trans-position in respect to $(C_2)^1$ axis of the **SG** subgraph (Fig. 3), therefore the continuance of the hydrophobic surface on the β-side of the steroidal skeleton is to a lesser extent disturbed — it is not halved.

Relative increase in hydrophobicity (ln *k*) and aggregation number of bile acid anion micelle is larger when ethylidene group is introduced at C7 position of steroidal skeleton than it is when ethylidene group is introduced at C3 position (Fig. 4). In primary micelle (Fig. 5A) building blocks are oriented so that the hydrophobic β-side of the steroidal skeleton is hydrated as little as possible, and that the carboxylate groups are as far as possible from each other. Due to the curvature of β-side of the steroidal skeleton part of the A ring is still hydrated in micellar phase (Fig. 5A). This is proven by measurements of demicellization heat capacity for bile salts [47]. If the ethylidene group is introduced in C7 position of the steroidal skeleton (B ring) then in bile acid anions primary micelle the building units hydrophobic surface (β-sides) is greater sheltered from the hydration, since B ring fully participates in the overlapping of convex surfaces (Fig. 5B). While an ethylidene group at position C3 does not provide a significant contribution to the increase in the area that is sheltered from the hydration (screened) in dimeric primary micelles (Fig. 5C), a certain increase of the screened surface of the convex side of molecules is possible when the aggregation number of micelles is 3 or 4.

Molecules 7-Eth-D (**3**) and C (**7**) in the plane $\overline{n}$–ln *k* to the hydrophobic linear congeneric group **I** (Fig. 2) are outliers, which means that in 7-Eth-D (**3**) and C (**7**) micelles there is, in addition to hydrophobic interactions, an additional interaction — excess Gibbs energy (G^E)

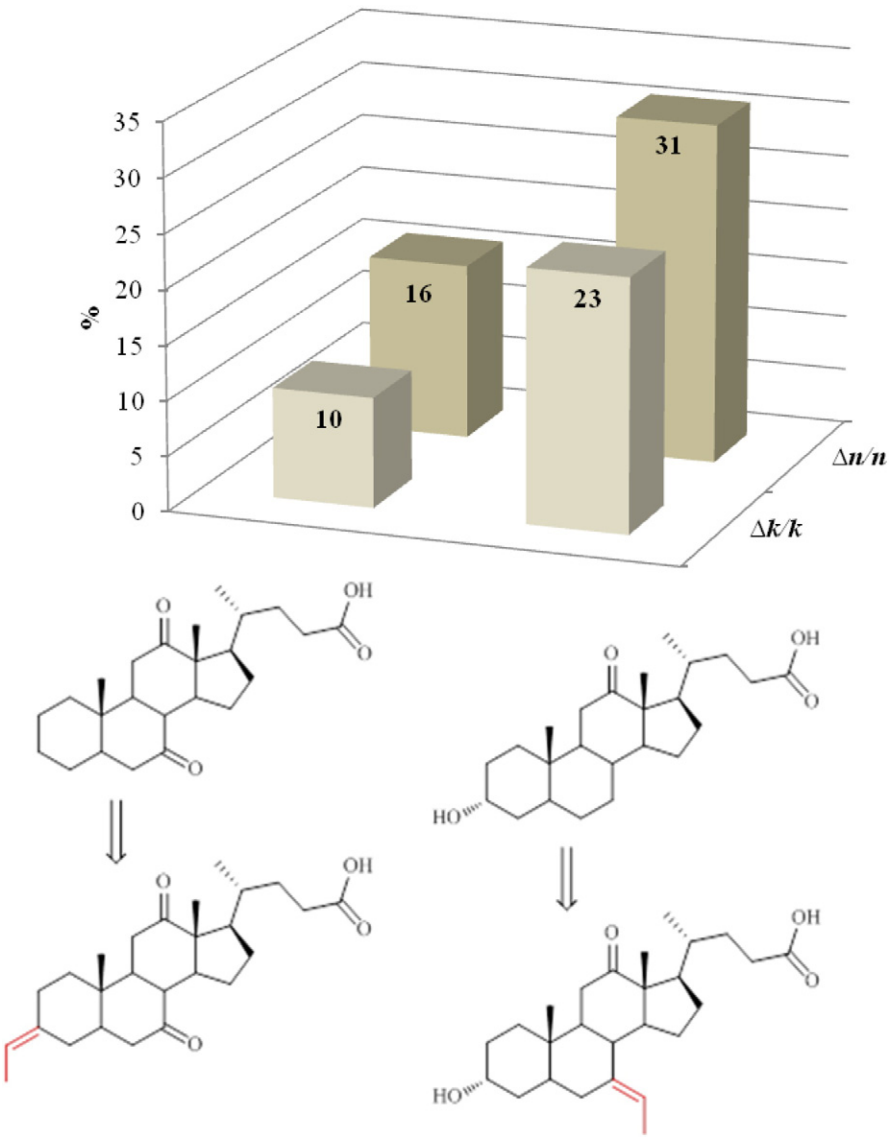

Fig. 4. Relative change in hydrophobicity (ln *k*) and aggregation number.

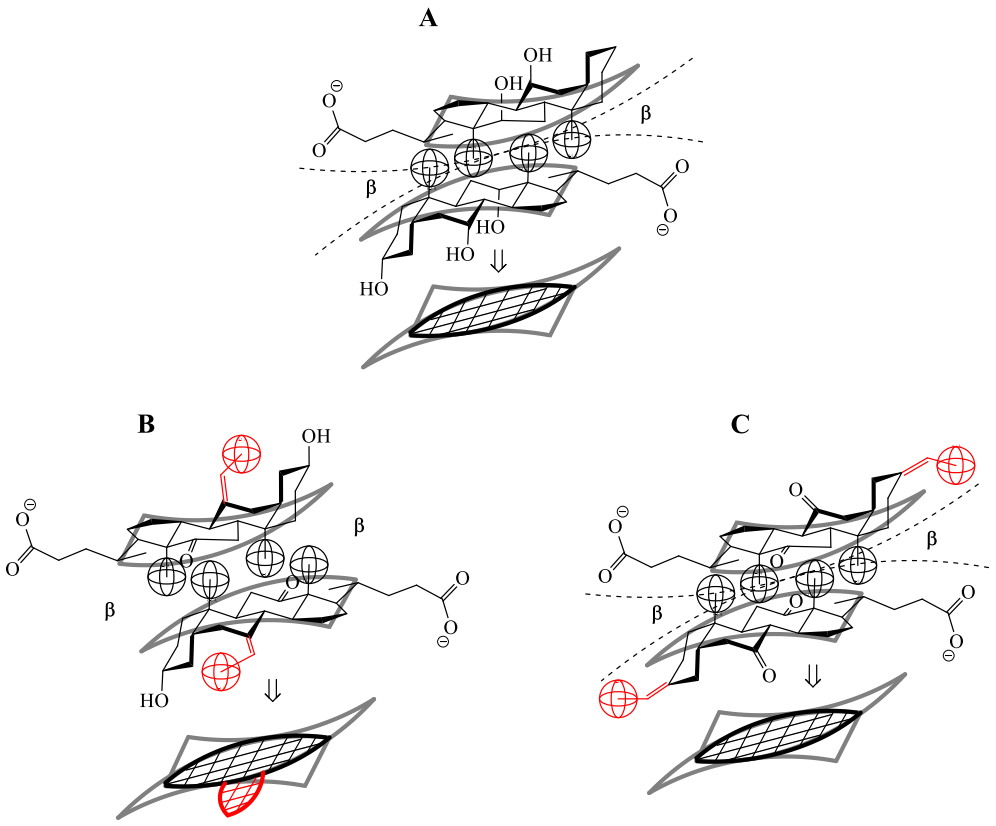

Fig. 5. Overlapping of the convex planes (β-sides) in formation of dimeric primary micelles of the bile acid anions (lattice shows effective overlapping of the convex surfaces i. e. surfaces that are screened from hydration).

which may occur due to hydrogen bond formation i.e., building of secondary micelles. Specifically for the molecules 7-Eth-D (**3**) and C (**7**) is valid (Appendix A and B):

$$\left.\begin{array}{l}\Delta G_M^{\ominus}(u)_{LKG-j} = b_u \Delta G_A^{\ominus}(R)_{LKG-j} + G^E \\ A_{hf}(u)_{LKG-j} = r_u A_{hf}(R)_{LKG-j}\end{array}\right\} b_u \neq \xi_{LKG-j} r_u + \beta_{LKG-j}, \qquad (6)$$

i.e., the Gibbs energy of micelle formation is larger (more negative) than it would be expected based on the hydrophobic surfaces of 7-Eth-D (**3**) and C (**7**) molecules.

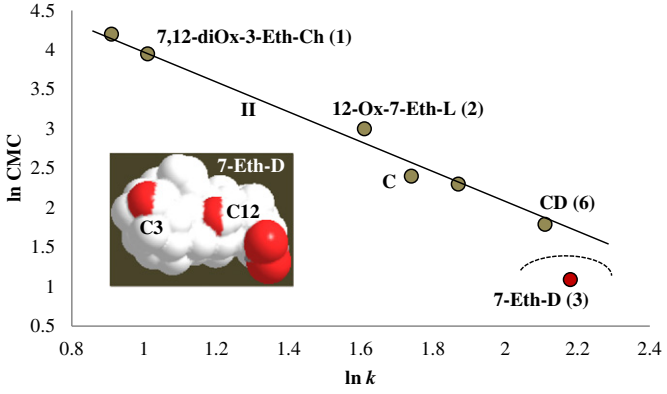

Fig. 6. Linear dependence between ln CMC and ln *k* (**II**), molecule 7-Eth-D (**3**) (outlier) have higher values of CMC than it would be expected from their hydrophobicity.

In the plane ln CMC–ln *k* (Fig. 6) bile acid anions 7,12-diOx-3-Eth-Ch (**1**); 12-Ox-7-Eth-L (**2**); CD (**6**); C (**7**); 7,12-diOx-Ch (**4**) and 12-Ox-L (**5**) are forming hydrophobic linear congeneric group:

$$\ln \mathrm{CMC} = -1.988(\pm 0.116) + 6.006(\pm 0.186)\ln k, \qquad (7)$$

$$N = 6;\ \ R = 0.993;\ \ F = 293.216;\ \ \text{and}\ \ sd = 0.125$$

while derivative 7-Eth-D (**3**) is outlier. Near critical micellar concentration micelles with small aggregation number are formed. It was found that dimeric micelles (Fig. 5) play a key role during the micellization [58]. Accordingly, in the vicinity of the CMC resulting micelles are determined by hydrophobic interactions, hence holds the relation (2). In derivative 7-Eth-D (**3**) biplanar amphiphilicity is disrupted because there are C3-OH, C12-OH and carboxylate group on the molecules common edge — hydrophilic edge (Fig. 6). This structure increases the affinity of 7-Eth-D molecules towards micelle formation resulting in low CMC value (Table 1). Because of that 7-Eth-D anions probably build micelles of Kawamura type — hydrophobic parts of the molecule i.e., β-side and most of the α-side of the steroidal skeleton are forming interior of the cylindrical micelle, while hydrophilic edge of the steroidal skeleton is on the mantle of cylinder, and the carboxylate groups are located alternately on the two bases of the cylinder [48]. The behavior of 7-Eth-D (**3**) molecules according to relation (6) in the plane ln CMC–ln *k* (Fig. 6) means that even at the critical micellar concentration there is an excess Gibbs energy relative to the Gibbs energy of hydrophobic interactions in micelles of 7-Eth-D (**3**) anions. G^E exists probably due to the formation of hydrogen bonds between the cylindrical Kawamura-type micelles.

Pyrene (probe molecule) enters the hydrophobic domain of the micelles resulting in a decrease in the fluorescence intensity of its first and third vibrational band ratio (I_1/I_3) values. Zana and Guveli [59] concluded, by comparing magnitude of (I_1/I_3) values decrease and half-life

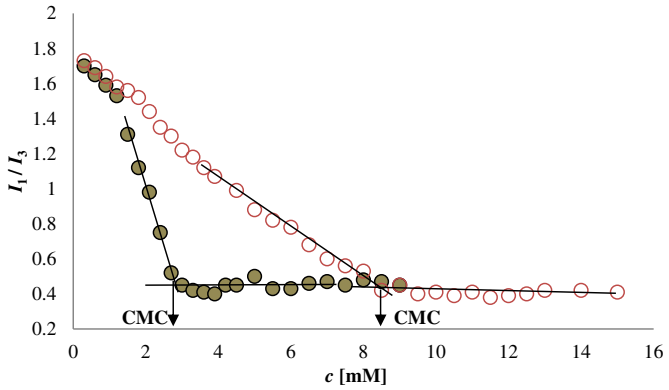

Fig. 7. Change in the (I_1/I_3) ratio depending on the total concentration of the bile acid salt (filled dots: 7-Eth-D (**3**); empty dots: 12-Ox-7-Eth-L (**2**)).

of micellary solubilized pyren for conventional surfactant micelles and for bile salt micelles, that in bile acid anion micelles pyren is placed between the convex planes of steroidal skeleton. Also, pyren is not staying inside micelle for a long time, but in the course of its micellar phase half-life (order of a microsecond) it enters inside many micelles and because of that pyren does not disturb the structure of the micelles much. That makes pyren a good probe molecule [60,61]. Curve of change in (I_1/I_3) values as a function of total bile salt concentration (c) (Fig. 7) for 7-Eth-D (**3**) possess sharp change below critical micellar concentration, while the same function ($I_1/I_3 = f(c)$) for 12-Ox-7-Eth-L (**2**) (Fig. 7) and 7,12-diOx-3-Eth-Ch (**1**) (Fig. 8) micellization has a stretched change. This means that in 12-Ox-7-Eth-L (**2**) and 7,12-diOx-3-Eth-Ch (**1**) micelles aggregate size gradually increases in a relatively wide concentration range, while in 7-Eth-D (**3**) micelles association is finished in relatively short concentration interval — characteristics of $I_1/I_3 = f(c)$ function near the formation of thermodynamically stable micelles [3,44,46]. From this data (Figs. 7 and 8) it could not be determined whether the thermodynamically stable micelles are formed due to hydrophobic interactions or by hydrogen bonds. However, exclusion the 7-Eth-D (**3**) derivative from hydrophobic linear congeneric groups in planes $\overline{n}$–ln k and ln CMC–ln k (Figs. 2 and 6) indicates that standard Gibbs energy of formation for micelles is not determined only with hydrophobic interactions but also with excess Gibbs energy which is not of hydrophobic nature. This excess Gibbs energy most likely originates from the formation of hydrogen bond bridges between the micelle building units.

Critical micellar concentrations determined by spectrofluorimetric method (probe molecule: pyrene) are showing good correlation ($R^2 = 0.9989$; Table 1) with CMC values which are determined by surface tension measurement. This means that identical patterns of objects (molecules) are obtained whether the hydrophobic linear congeneric group in ln CMC–ln k plane is described by spectrofluorimetric CMC value or by tensiometric CMC value. Tensiometric CMC values are somewhat lower than the spectrofluorimetric CMC values. This is probably due to the fact that when the surface of water solution–air interface is saturated (tensiometric CMC value) aggregates in the bulk solution are still not formed. They are formed at slightly higher total concentration of the surfactant (4–5%) (Table 1).

In derivatives 7,12-diOx-3-Eth-Ch (**1**); 12-Ox-7-Eth-L (**2**); CD (**6**); 7,12-diOx-Ch (**4**) and 12-Ox-L (**5**) the number average aggregation number shows no dependence on the total bile salt concentration (Fig. 9). That means that the maximum number of building blocks which gives maximum screening from hydration of hydrophobic surface of the molecule for a given packing has been reached. Further introduction of building blocks (increasing of the aggregation number) would lead to partial hydration of the convex surface of the steroidal skeleton. Micelles of derivatives C (**7**) and 7-Eth-D (**3**) above the critical micellar concentration showed an increase in the aggregation number with increasing total concentration of bile salts (Fig. 9), which is probably due to the formation of secondary micelles by hydrogen bond connecting of primary micelles. Santhanalakshmi et al. using the small-angle neutron scattering experiment, made a conclusion that enlargement of rod-like primary micelle of sodium cholate above CMC is possible in both y axis direction (hydrophobic direction) and x axis direction (formation of secondary micelles by hydrogen bonding) (Fig. 10A) [62]. In our previous work it was found that anions of cholic acid, 7-oxo-deoxycholic acid and hyodeoxycholic acid are excluded from hydrophobic linear congeneric group in $\overline{n}$–ln k plane [10]. While in this study it was found that a derivative 7-Eth-D (**3**) also builds micelles with an excess Gibbs energy to the hydrophobic Gibbs energy of micellization — building of secondary micelles. The presence of OH groups in C3 and C12 positions of the steroidal skeleton is common for these bile acids, therefore, association of primary micelles to secondary micelles likely occurs via the C3-α-(e)-OH, and C12-α-(a)-OH group (Fig. 10A). Presence of the OH group in position 7 of the steroidal skeleton seems to give no contribution in primary micelles association. Namely, in subgraph **SG** (Fig. 3) C7 carbon and D ring are *cis* to each other, therefore C7-α-(a)-OH group is sterically hindered by D ring [10], because of that association from the α-side of steroidal skeleton is difficult via this OH group (Fig. 10B). Probably hydrogen bond formation via C3-OH group is not sufficient for the formation of secondary micelles, two hydrogen bonds are needed between primary micelles.

4. Conclusion

For ethylidene derivative 7-Eth-D (**3**) function $I_1/I_3 = f(c)$ possesses sharp change when concentration (c) is near CMC value. The number average aggregation number of 7-Eth-D (**3**) micelle above CMC (concentration range 50 mM–100 mM) increases from 11 to 14.8. Respective

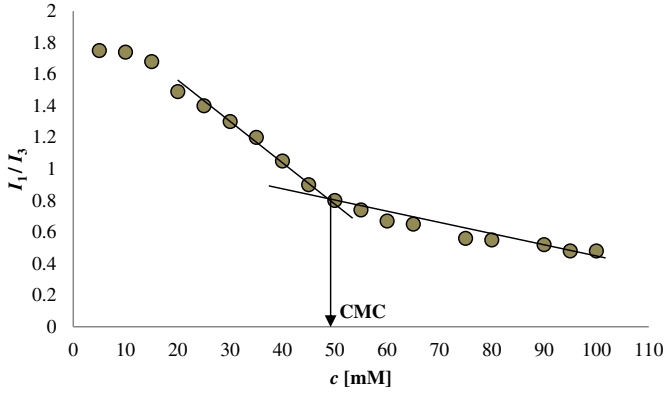

Fig. 8. Change in the (I_1/I_3) ratio depending on the total concentration of the bile acid salt 7,12-diOx-3-Eth-Ch (**1**).

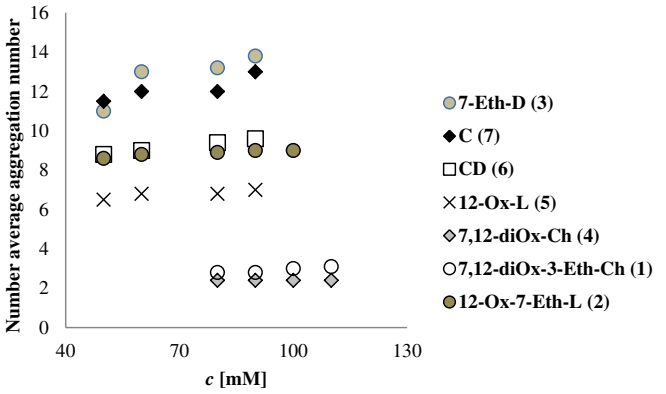

Fig. 9. Dependence of the number average aggregation number on the total concentration of bile acid anion at 298 K.

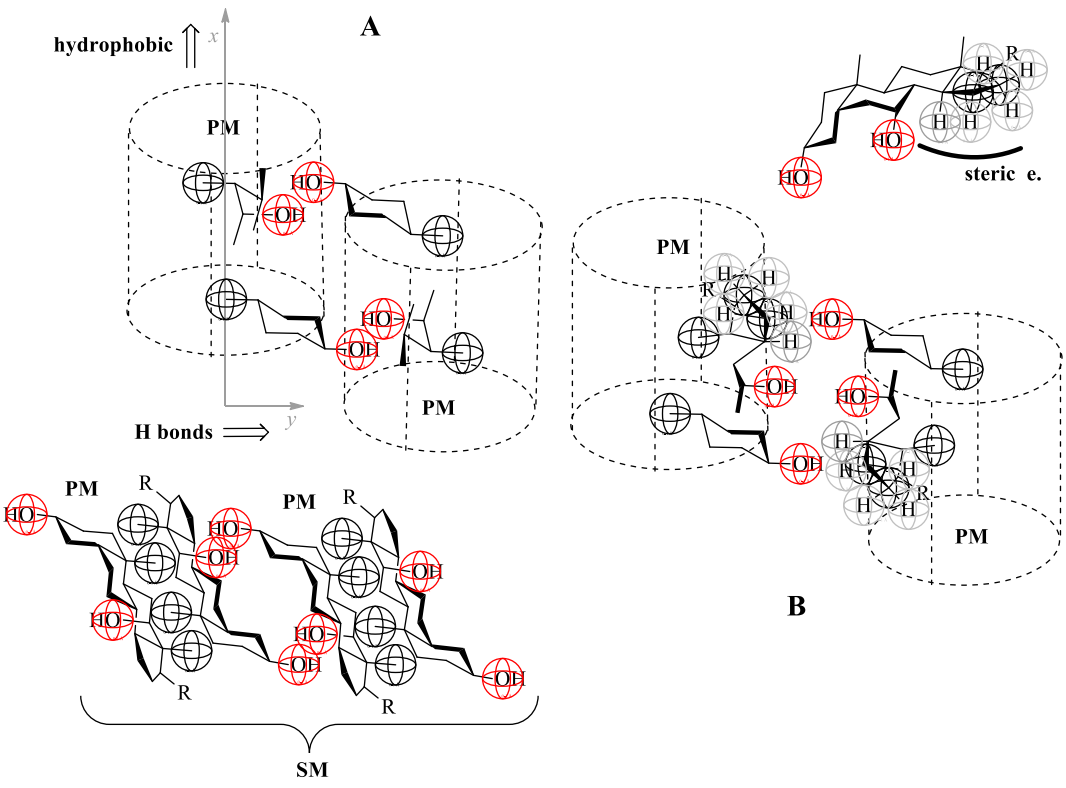

Fig. 10. Secondary micelle formation (PM = primary micelle, SM = secondary micelle, steric e. = steric ecraning).

$I_1/I_3 = f(c)$ functions of ethylidene derivatives 12-Ox-7-Eth-L (**2**) and 7,12-diOx-3-Eth-Ch (**1**) near CMC have stretched changes. Above critical micellar concentration aggregation number (concentration range 50 mM–140 mM) of 12-Ox-7-Eth-L (**2**) ($\overline{n}\approx 8.8$) and 7,12-diOx-3-Eth-Ch (**1**) ($\overline{n}\approx 2.9$) does not show concentration dependence. In the planes $\overline{n}$–ln k and ln CMC–ln k molecules 12-Ox-7-Eth-L (**2**) and 7,12-diOx-3-Eth-Ch (**1**) are included into the hydrophobic linear congeneric group, while 7-Eth-D (3) molecule is an outlier. This means that Gibbs energy of formation for 7-Eth-D (**3**) anion micelles is determined in addition to the Gibbs energy of hydrophobic interactions by excess Gibbs energy which probably originates from hydrogen bond energy in secondary micelle formation. C (**7**) is outlier in the $\overline{n}$–ln k plane, however it is included in to the hydrophobic linear congeneric group in ln CMC–ln k plane — micelles of anion C (**7**) are determined by hydrophobic interactions on critical micellar concentration, only when concentration is above CMC anions C (**7**) are forming micelles with excess Gibbs energy i.e., with hydrogen bonds. The fact that 7-Eth-D (**3**) is an outlier (unlike C) to the hydrophobic linear congeneric group in the plane ln CMC–ln k suggests that already at its critical micellar concentration in addition to the hydrophobic interaction 7-Eth-D (**3**) micelle includes a hydrogen bond.

Transparency documents

The Transparency documents associated with this article can be found, in online version.

Acknowledgements

This study was financially supported by the Domus Hungarian Scholarship (Hungarian Academy of Sciences, No. 110/6864/HTMT). The Ministry of Science and Technological Development of the Republic of Serbia (Project No. 172021) is acknowledged as well.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2015.03.010.

References

[1] M. Mikov, J.P. Fawcett, Bile Acids, Medishet Publisher, Geneva, 2007.
[2] A. Roda, A.F. Hofmann, K.J. Mysels, The influence of bile salt structure on self-association in aqueous solutions, J. Biol. Chem. 258 (1983) 6362–6370.
[3] D.M. Small, The physical chemistry of cholanic acids, in: P.P. Nair, D. Kritchevsky (Eds.),The Bile Acids: Chemistry, Physiology and Metabolism, vol. 1, Plenum Press, New York, 1971 (Chapter 8).
[4] M. Poša, Mixed micelles of binary surfactant mixtures: Tween 40–Na-3,12-dioxo-5β-cholanate; Tween 80–Na-3,12-dioxo-5β-cholanate and their thermodynamic description-characterisation, Chem. Eng. Res. Des. 92 (2014) 1826–1839.
[5] M. Poša, QSPR study of the effect of steroidal hydroxy and oxo substituents on the critical micellar concentrations of bile acids, Steroids 76 (2011) 85–93.
[6] M. Poša, Heuman indices of hydrophobicity in bile acids and compared with new developed and conventional molecular descriptors, Biochimie 97 (2014) 28–38.
[7] M. Poša, A. Sebenji, J. Trifunović, Influence of temperature on retention parameter of bile acids in normal phase thin-layer chromatography: the role of steroid skeleton, Acta Chim. Slov. 60 (2013) 151–158.
[8] D.M. Small, Studies on the size and structure of bile salt micelles, influences of structure, concentration, counterion concentration, pH and temperature, Adv. Chem. Ser. 84 (1968) 31–52.
[9] D. Madenci, S.U. Egelhaaf, Self-assembly in aqueous bile salts solution, Curr. Opin. Colloid Interface 15 (2010) 109–115.
[10] M. Poša, A. Sebenji, Determination of the number avarage aggregation numbers of bile salt micelles with a special emphasis on their oxo derivatives — the effect of the steroid skeleton, BBA-Gen. Subj. 1840 (2014) 1072–1082.
[11] A.F. Hofmann, A. Roda, Physicochemical properities of bile acids and their relationship to biological properties: an overview of the problem, J. Lipid Res. 25 (1984) 1477–1489.
[12] M. Poša, Hydrophobicity and self-association of bile acids with a special emphasis on oxo derivatives of 5-β-cholanic acid, Curr. Org. Chem. 16 (2012) 1876–1904.
[13] M. Calabresi, P. Andreozzi, C. La Mesa, Supra-molecular association and polymorphic behaviour in systems containing bile acid salts, Molecules 12 (8) (2007) 1731–1754.
[14] J.Y. Chiang, Bile acid regulation of hepatic physiology III. Bile acids and nuclear receptors, Am. J. Physiol. 284 (2003) G349–G356.
[15] C. Thomas, R. Pellicciari, M. Pruzanski, J. Auwerx, K. Schoonjans, Targeting bile-acid signalling or metabolic diseases, Nat. Rev. Drug Discov. 7 (2008) 678–693.

[16] A.M. Dopico, J.V. Walsh, J.J. Singer, Natural bile acids and synthetic analogues modulate large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel activity in smooth muscle cells, J. Gen. Physiol. 119 (2002) 251–273.
[17] H. Sato, A. Macchiarulo, C. Thomas, A. Gioiello, M. Une, A.F. Hofmann, R. Saladin, K. Schoonjans, R. Pellicciari, J. Auwerx, Novel potent and selective bile acid derivatives as TGR5 agonists: biological screening, structure–activity relationships, and molecular modeling studies, J. Med. Chem. 51 (2008) 1831–1841.
[18] C. Thomas, A. Gioiello, L. Noriega, A. Strehle, J. Oury, G. Rizzo, A. Macchiarulo, H. Yamamoto, C. Mataki, M. Pruzanski, R. Pellicciari, J. Auwerx, K. Schoonjans, TGR5-mediated bile acid sensing controls glucose homeostasis, Cell Metab. 10 (2009) 167–177.
[19] G.S. Gordon, A.C. Moses, R.D. Silver, J.R. Flier, M.C. Carey, Nasal absorption of insulin: enhancement by hydrophobic bile salts, Proc. Natl. Acad. Sci. U. S. A. 82 (1985) 7419–7423.
[20] M. Mikov, S. Kevresan, K. Kuhajda, V. Jakovljevic, V. Vasovic, 3α,7α-Dihydoxy-12-oxo-5β-cholanate as blood–brain barrier permeator, Pol. J. Pharmacol. 56 (2004) 367–371.
[21] H. Al-Salami, G. Butt, I.G. Tucker, M. Mikov, Influence of the semisynthetic bile acid MKC on the ileal permeation of gliclazide *in vitro* in healthy and diabetic rats treated with probiotics, Methods Find. Exp. Clin. Pharmacol. 30 (2) (2008) 107–113.
[22] L. Yang, H. Zhang, M. Mikov, I.G. Tucker, Physicochemical and biological characterization of monoketocholic acid, a novel permeability enhancer, Mol. Pharm. 6 (2) (2009) 448–456.
[23] L. Yang, I.G. Tucker, J. Ostergaard, Effects of bile salts on propranolol distribution into liposomes studied by capillary electrophoresis, J. Pharm. Biomed. Anal. 56 (2011) 553–559.
[24] L. Yang, J.P. Fawcett, J. Østergaard, H. Zhang, I.G. Tucker, Mechanistic studies of the effect of bile salts on rhodamine 123 uptake into RBE4 cells, Mol. Pharm. 9 (2012) 29–36.
[25] L. Yang, J.P. Fawcett, H. Zhang, I.G. Tucker, Effect of 12-oxochenodeoxycholate on the pharmacokinetics and pharmacodynamics of morphine 6-glucuronide in Wistar rats, J. Pharm. Pharmacol. 65 (2013) 561–566.
[26] G. Chen, J.P. Fawcett, M. Mikov, I.G. Tucker, Monoketocholate can decrease transcellular permeation of methotrexate across Caco-2 cell monolayers and reduce its intestinal absorption in rat, J. Pharm. Pharmacol. 61 (2009) 953–959.
[27] L. Mrózek, L. Coufalová, L. Rárová, L. Plaček, R. Opatřilová, J. Dohnal, K. Král'Ová, O. Paleta, V. Král, P. Drašar, J. Jampílek, New polyfluorothiopropanoyloxy derivatives of 5β-cholan-24-oic acid designed as drug absorption modifiers, Steroids 78 (2013) 832–844.
[28] L. Coufalová, L. Mrózek, L. Rárová, L. Plaček, R. Opatřilová, J. Dohnal, K. Král'Ová, O. Paleta, V. Král, P. Drašar, J. Jampílek, New propanoyloxy derivatives of 5β-cholan-24-oic acid as drug absorption modifiers, Steroids 78 (2013) 435–453.
[29] I. Kuhajda, M. Poša, V. Jakovljević, V. Ivetić, M. Mikov, Effect of 12-monoketocholic acid on modulation of the analgesic action of morphine and tramadol, Eur. J. Drug Metab. Pharmacokinet. 34 (2009) 73–78.
[30] P. Garidel, A. Hildebrand, K. Knauf, A. Blume, Membranolytic activity of bile salts: influence of biological membrane properities and composition, Molecules 12 (2007) 2292–2326.
[31] 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, Design of compounds that increase the absorption of polar molecules, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 12218–12223.
[32] M. Poša, K. Kuhajda, Hydrophobicity and haemolytic potential of oxo derivatives of cholic, deoxycholic and chenodeoxycholic acids, Steroids 75 (2010) 424–431.
[33] M. Poša, Z. Farkaš, Cholesterol solubilization by oxo derivatives of selected bile acids and their osmotic resistance, Collect. Czechoslov. Chem. Commun. 75 (8) (2010) 767–784.
[34] M. Poša, J. Csanádi, E.K. Kövér, V. Guzsvány, Gy. Batta, Biophysico-chemical study of molecular interactions between selected sodium salts of bile acids and morphine hydrochloride, Colloids Surf. Biointerfaces 94 (2012) 317–323.
[35] M. Poša, V. Guzsvány, J. Csanádi, S. Kevrešan, K. Kuhajda, Formation of hydrogen-bonded complexes between bile acids and lidocaine in the lidocaine transfer from an aqueous phase to chloroform, Eur. J. Pharm. Sci. 34 (2008) 281–292.
[36] B. Jójárt, M. Poša, B. Fisher, M. Szöri, Z. Farkaš, B. Viskolcz, Mixed micelles of sodium cholate and sodium dodecylsulphate 1:1 binary mixture at different temperatures — experimental and theoretical investigations, PLoS One 9 (2014) e102114.
[37] M. Poša, D. Ćirin, Mixed micelles of sodium salts of bile acids and Tween 40: effect of the steroid skeleton on the coefficient of interaction in mixed micelles, Ind. Eng. Chem. Res. 51 (2012) 14722–14728.
[38] M. Poša, S. Bjedov, A. Sebenji, M. Sakač, Wittig reaction (with ethylidene triphenylphosphorane) of oxo-hydroxy derivatives of 5β-cholanic acid: hydrophobicity and haemolytic potential of derived ethylidene derivatives, Steroids 86 (2014) 16–25.
[39] M. Otto, Chemometrics, Second ed. Wiley-VCH, Weinheim, 2007.
[40] D. Miljković, K. Kuhajda, J. Hranisavljević, Selective C-12 oxidation of cholic acid, J. Chem. Res. Synop. (1996) 106–107.
[41] K.Y. Tserng, A convenient synthesis of 3-keto bile acids by selective oxidation of bile acids with silver carbonate—celite, J. Lipid Res. 19 (1978) 501–504.
[42] L.F. Fieser, S. Rajagopalan, Oxidation of steroids III. Selective oxidations and acylations in bile acid series, J. Am. Chem. Soc. 72 (1950) 5530–5536.
[43] H. Sugioka, Y. Moroi, Micelle formation of sodium cholate and solubilization into the micelle, Biochim. Biophys. Acta 1394 (1998) 99–110.
[44] K. Matsuoka, Y. Moroi, Micelle formation of sodium deoxycholate and sodium ursodeoxycholate, Biochim. Biophys. Acta 1580 (2002) 189–199.
[45] R. Nagarajan, Comments on interpreting fluorescence measurements: what does thermodynamics have to say about change in micellar aggregation number versus change in size distribution induced by increasing concentration of the surfactant in solution? Langmuir 10 (1994) 2028–2034.
[46] K. Matsuoka, K. Takagi, C. Honda, Micelle formation of sodium hydroxycholate, Chem. Phys. Lipids 172–173 (2013) 6–13.
[47] P. Garidel, A. Hildebrand, A. Neubert, A. Blume, Thermodynamic characterization of bile salt aggregation as a function of temperature and ionic strength using isothermal titration calorimetry, Langmuir 16 (2000) 5267–5275.
[48] H. Kawamura, Y. Murata, T. Yamaguchi, H. Igimi, M. Tanaka, G. Sugihara, J.P. Kratohvil, Spin label studies bile salt micelles, J. Phys. Chem. 93 (1989) 3321–3326.
[49] L.B. Pártai, P. Jedlovszky, M. Sega, Molecular aggregates in aqueous solutions of bile acid salts. Molecular dynamics simulation study, J. Phys. Chem. B 111 (2007) 9886–9896.
[50] L.B. Pártai, M. Sega, P. Jedlovszky, Counterion binding in the aqueous solutions of bile acid salts, as studied by computer simulation method, Langmuir 24 (2008) 10729–10736.
[51] L.B. Pártai, M. Sega, P. Jedlovszky, Morphology of bile salts micelles as studied by computer simulation methods, Langmuir 23 (2007) 12322–12328.
[52] B. Jójárt, B. Viskolc, M. Poša, Sz. Fejer, Global optimization of cholic acid aggregates, J. Chem. Phys. 140 (2014) 1443021–1443027.
[53] R. Shaw, W.H. Elliot, B.G. Barisas, Estimation of critical micelle concentrations of bile acids by reversed-phase high performance liquid chromatography, Microchim. Acta 105 (1991) 137–145.
[54] B. Natalini, R. Sardella, A. Gioiello, F. Ianni, A. Michele, M. Marinozzi, Determination of bile salt critical micellization concentration on the road to drug discovery, J. Pharm. Biomed. Anal. 87 (2014) 62–81.
[55] B. Natalini, R. Sardella, E. Camaioni, A. Gioiello, R. Pellicciari, Correlation between CMC and chromatographic index: simple and effective evaluation of the hydrophobic/hydrophilic balance of bile acids, Anal. Bioanal. Chem. 388 (2007) 1681–1688.
[56] B. Natalini, R. Sardella, E. Camaioni, A. Macchiarulo, A. Gioiello, G. Carbone, R. Pellicciari, Derived chromatographic indices as effective tools to study the self-aggregation process of bile acids, J. Pharm. Biomed. Anal. 50 (2009) 613–621.
[57] D.M. Heuman, Quantitative estimation of the hydrophilic–hydrophobic balance of mixed bile salt solutions, J. Lipid Res. 30 (1989) 719–730.
[58] S. Gouin, X.X. Zhu, Fluorescence and NMR studies of the effect of a bile acid dimer on the micellization of bile salts, Langmuir 14 (1998) 4025–4029.
[59] R. Zana, D. Guveli, Fluorescence probing study of the association of bile salts in aqueous solutions, J. Phys. Chem. 89 (1985) 1687–1690.
[60] J.H. Kim, M.M. Domach, R.D. Tilton, Pyrene micropartitioning and solubilization by sodium dodecyl sulfate complexes with poly(ethylene glycol), J. Phys. Chem. B 103 (1999) 10582–10590.
[61] N. Yoshida, M. Takechi, T. Asano, Y. Moroi, R. Humphry-Baker, M. Grätzel, Chem. Phys. Lett. 332 (2000) 265–270.
[62] J. Santhanalakshmi, G. Shantha Lakshmi, V.K. Aswal, P.S. Goyal, Small-angle neutron scattering study of sodium cholate and sodium deoxycholate interacting micelles in aqueous medium, Proc. Indian Acad. Sci. 113 (2001) 55–62.