Determination of Aggregation Numbers of Bile Salt Micelles with the Depression of the Solution Freezing Point

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Abstract: The special geometry of the steroid skeleton causes formation of bile acid anion micelles with small aggregation numbers, in contrast to aliphatic amphiphiles. Relative to the tendency to reduce membrane toxicity, pharmacological investigations of bile acids are mainly concerned with their oxo derivatives. Since micelles of these bile acids have been insufficiently studied, the objective of this work is the determination of aggregation numbers of corresponding micelle monomers. The aggregation numbers were determined using the freezing point depression of the solutions by applying the appropriate equations of Debye-Hückel, Guggenheim and Gibbs-Duhems, and using pNa data measured with a Na-selective electrode. Depending on the structure of the bile acid anion, the values obtained for the aggregation numbers were in the range from 2.09 to 3.44. The increase in number of oxo groups in the molecule is accompanied by a decrease in hydrophobicity of the convex side of the steroid skeleton of the bile acid anion, resulting in a lower aggregation number.

Keywords: Micelles, bile salts, aggregation number, steroid skeleton, critical micellar concentration.

1. INTRODUCTION

Bile acid salts are steroid amphiphilic compounds. Bile acid salts or conjugate salts in vertebrates act as biosurfactants. They form mixed micelles with phospholipides (mostly lecithine) which gives them considerable capacity to solubilize cholesterol. Bile salt micelles have an important function in lipid digestion in the small intestine (emulgation and micellar transport) [1, 2].

Bile acid salts and their synthesized derivatives have a positive effect on transport of certain medicaments trough cellular lipid barriers, and they are also used as co-surfactants or drug carriers. In the last 20 years biomedical experiments extensively researched sodium salts of oxo derivatives of bile acids, becuse they cause less mambrane toxicity than sodium doxycholate or sodium chenodeoxycholate [3-7].

Critical micellar concentration (CMC) and aggregation number are important parameters of the micellization process. The less the value of CMC a certain compound has, the more easily it forms micelles, and it has a negative shift in the Gibbs energy. Relative to the application of micelles as a means to enhance solubility of hydrophobic molecules, it is recommended that the micelle has a large aggregation number value [1, 8].

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Bile acid salt micelles have aggregation numbers ranging from 2 to 16. It should be considered that overall value of aggregation numbers of most surfactants (alkyl sulfates for example) can be over 100 [1, 8].

Aim of this publication is determination of the aggregation numbers of bile acids' oxo derivative sodium salts, since the aggregation numbers for these bile acid salts are not yet known (Figure **1**).

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 by method used by Miljković *et al*. [9], while 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (7-ODC) and 3α-hydroxy-7-oxo-5β-cholanoic acid (7-OL) were obtained by method according to Tullar [10]. 3α-Hydroxy-7, 12-dioxo-5β-cholanoic acid (7,12-DOC) was synthesized 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 by method according to Fieser and Rajagopalan [11] Hyodeoxycholic acid was purchased from Sigma, New Zealand.

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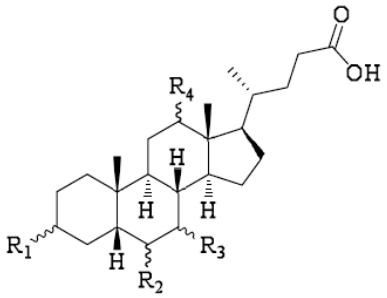

$R_1 = R_3 = R_4 = OH$; $R_2 = H$	3α,7α,12α-trihydroxy-5β-cholanoic acid (cholic a.); **C**
$R_1 = R_3 = OH$; $R_2 = R_4 = H$	3α,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic a.); **CDC**
$R_1 = R_4 = OH$; $R_3 = =O$; $R_2 = H$	3α,12α-dihydroxy-7-oxo-5β cholanoic acid (7-oxodeoxycholic a.); **7-ODC**
$R_1 = R_3 = OH$; $R_4 = =O$; $R_2 = H$	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**

Figure 1: Structures of tested bile acids.

2.2. Determination of Average Aggregation Numbers by Freezing Point Depression

The determination of the osmolality of bile acids' sodium salts was carried out by measuring the freezing point depression of their solutions relative to water using a cryoscopic osmometer Knauer K-7400. In the measurement, the solution (solvent) was first supercooled without freezing, and then formation of solvent crystals was initiated by vibrations. The osmometer was calibrated using NaCl solution in the concentration range from 0 to 250 mmol kg^{-1}. Each measurement was repeated five times, and the standard deviation was between 1-2%.

To obtain aggregation numbers of micelles of bile acid anions it is also necessary to have data for concentration of free counter-ion (Na^+, pNa) for each measured solution, i.e. to determine the pNa value for each total concentration of the Na-salt of bile acid. The pNa values were determined using a Radiometer TitraLab 845 titrator with ion selective electrode ISE21Na and the reference electrode RedRod201 (Ag/AgCl) at room temperature. Calibration was carried out with NaCl solutions.

3. RESULTS AND DISCUSSIONS

There are several papers dealing with the issue of polydispersity (particle size) of micellar solutions of bile salts [12-15]. In the determination of aggregation numbers based on osmotic coefficients the polydispersity (relative to particle size) is modeled as a monodisperse micellar system, where the aggregation number corresponds to an average aggregation number [12, 14].

In the experiment, changes in the freezing point depression of the solution, the $\Delta T/k$ is followed as a function of the total molal concentration of the bile acid $c^t_{BA^-}$. The value $\Delta T/k$ is through the Guggenheim equation (Figure **2**. (8)) related to the Bjerrum osmotic coefficient of the solution ϕ and to the sum of the molalities of following particles: free monomer (bile acid anion) c_{BA^-}, free counterion c_{C^+} and of micelle c_M [16, 17]. In the region below the break of the function $\Delta T/k = f(c^t_{BA^-})$ (Figure **3**) only free monomers and counter-ions exist (bile acid sodium salts react like strong electrolytes) which means that $\sum_i c_i$ from the equation (Figure **2**. (8)) is 2 $c^t_{BA^-}$. If we present $\Delta T/k$ ($\Delta T/k = 2\phi c^t_{BA^-}$) as a function of $\phi c^t_{BA^-}$ the line is obtained by the value of the slope 2, no matter what kind of anion is present [14]. Concentration of bile acids at which the value of $\phi c^t_{BA^-}$ has 5% deviation from the value that corresponds to the line $\Delta T/k = 2\phi c^t_{BA^-}$ (i.e. the deviation from the Debye-Hückel law), corresponds to the concentration of the free bile acid anion c_{BA^-} (beginning of the micelle formation). According to Coello *et al*., in the self-association of bile salts, the c_{BA^-} can be assumed to be constant [12, 14]. Table **1** shows the values of c_{BA^-} as well as of $c^t_{BA^-}$, the

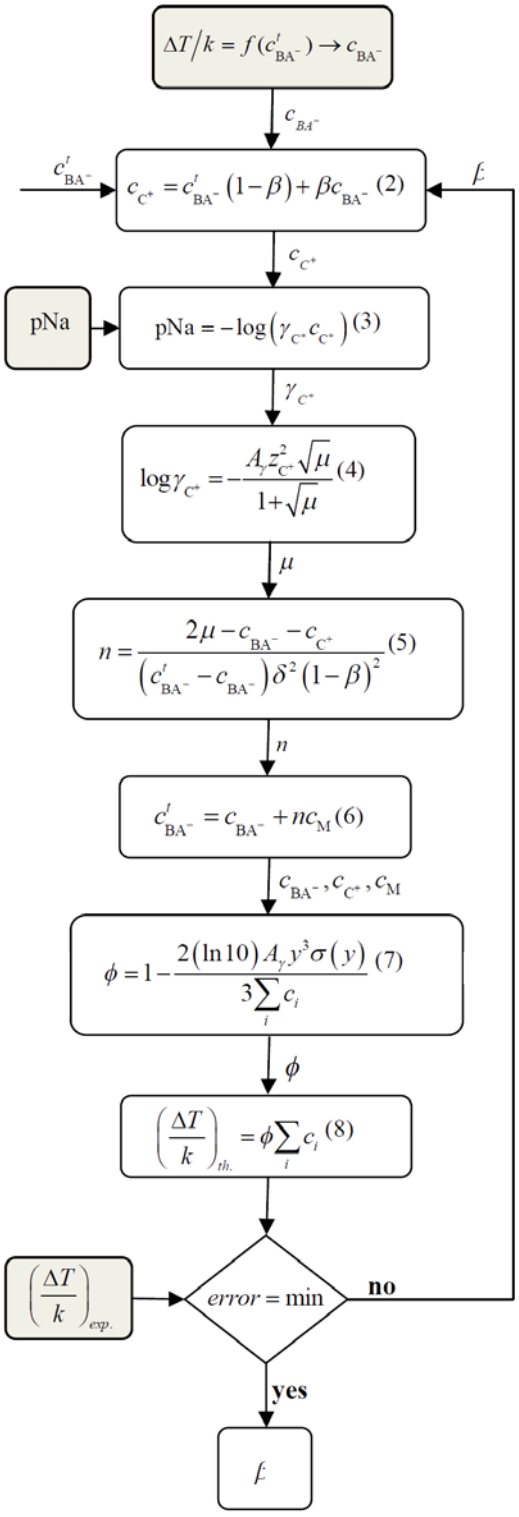

Figure 2: Algorithm for determination of micelle parameters (*n* and *β*), experimental values are pNa and c_{BA^-}.

concentration corresponding to the break in the function $\Delta T/k = f(c^t_{BA^-})$ for the investigated sodium salts of bile acid. Many authors assume that the concentration range corresponding to the CMC of bile salts corresponds to our concentration range: c_{BA^-}-$c^t_{BA^-}$ [18, 19]. This is also supported by the literature data for oxo derivatives of bile acid salts [1, 19]. Based on Table **1**, it can be concluded that the replacement of OH groups with oxo groups leads to an increase in the values c_{BA^-} and $c^t_{BA^-}$, i.e. to a decrease in the hydrophobicity of the steroid nucleus of the bile acid molecule.

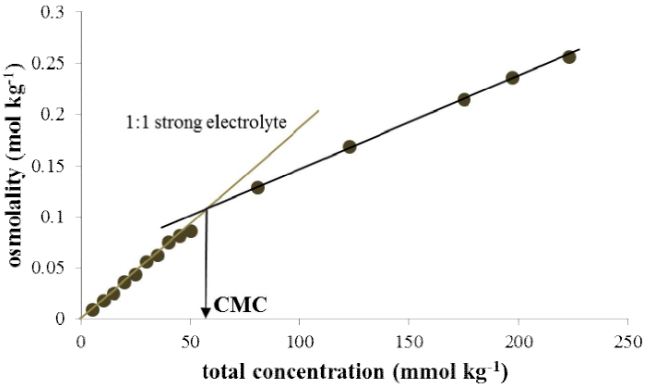

Figure 3: Dependence of $\Delta T/k$ (osmolality) on total concentration $c^t_{BA^-}$, example of 7-ODC (the figure shows the upper CMC value).

Table 1: Ranges of CMC

BA	CMC (mmol kg^{-1})	
	c_{BA^-}	$c^t_{BA^-}$
C	16.4	20.5
CDC	5.0	7.5
7-ODC	48.5	54.8
12-OCD	61.4	67.5
7,12-DOC	105.5	125.0
12-OL	24.0	28.0
7-OL	19.7	23.0
3,12-DOC	79.0	93.8
3,7-DOC	73.5	84.7
HD	18.3	24.5

The aggregation number *n* and the fraction of bound counterion *β* at a concentration $c^t_{BA^-}$ in the micelle region are obtained using the algorithm shown in Figure **2**. At some value of $c^t_{BA^-}$ which corresponds to the value of c_{BA^-} (molal concentration of free bile acid anion) and first approximate value for *β*, molal concentration of free counterion c_{C^+} is calculated according to equation (Figure **2**. (2)). In the next step,

the experimental pNa value (measured by the ion-selective electrode) for the solution $c^t_{BA^-}$ is introduced, and then equation (Figure **2**. (3)) is used to calculate the activity coefficient of the counterion γ_{C^+} and, using this quantity, the ionic strength of the solution is obtained from the modified (ion-ion interaction parameter being neglected) Guggenheim equation (A_γ=0.4918 kg$^{1/2}$mol$^{-1/2}$, $z_{C^+}=1$ is the charge of the counterion) (Figure **2**. (4)). Now, having all variables (μ, c_{BA^-}, c_{C^+} and β) known for the given $c^t_{BA^-}$ (in the micellar region) it is possible to calculate the aggregation number (n) by equation (Figure **2**. (5)) (according to Coello *et al*., the parameter δ is 1 [14]). In the next step, with the n also being known, it is possible to determine molal concentration of micelles c_M at the total concentration of bile salt $c^t_{BA^-}$ (Figure **2**. (6)). Now, with all molal concentrations of all particles in the solution ($\sum c$) being known, as well as the ionic strength μ, the Gibbs-Duhem equation is used to calculate the osmotic coefficient of the solution (Figure **2**. (7)) ($y=\sqrt{\mu}$, $\sigma(y)=3\left(1+y-1/(1+y)-2\ln(1+y)\right)/y^3$). This means that the theoretical value of the freezing point depression $(\Delta T/k)_{th.}$ (Figure **2**. (8)) is calculated, which is then compared with the experimenally obtained value $(\Delta T/k)_{exp.}$. If the relative error:

$$error=\frac{(\Delta T/k)_{exp.}-(\Delta T/k)_{th.}}{(\Delta T/k)_{exp.}}$$

corresponds to the minimal value, the algorithm stops executing and the micelle parameters (n and β) for $c^t_{BA^-}$ are accepted, otherwise the process is continued with the refined β values. Table **2** shows obtained values for aggregation numbers n and fractions of bound counterion β for investigated micelles. The (average) aggregation numbers for each of the bile acids is constant in the investigated range of concentrations (there are no statistically significant difference between the n values measured at different $c^t_{BA^-}$ values). This means that the ratio of the molal fractions in the micelles with different aggregation numbers do not change in the given range of $c^t_{BA^-}$. The aggregation number reported for sodium cholate (C) obtained by the same method (freezing point depression) is 3.09 [14], whereas the average value obtained in this work is 3.44. Garidel *et al*., using also a non-invasive method (isothermal microcalorimetric titration), obtained values from 4.7 to 6.1 in 0.1 M NaCl for the aggregation number of Na cholate [20]. A higher value for the aggregation number could be expected because the presence of NaCl increases the hydrophobicity of bile acids. CMC values as well as the aggregation number depend on the applied method of determination [18]. Aggregation number of bile acid salts obtained by freezing point depression measures between 2 (for oxo derivatives) and 3.5 (for cholic acid). Values obtained by application of pH-metric measurments as well as light diffusion method reach higher numbers, so that the aggregation number value for sodium cholate is above 10. Reason for this can be found in the fact that in other methods for aggregation number determination n values slowly grow with the increase in total monomer concentration [13, 15]. As for the fraction of bound counter-ion β, it decreases with the decrease in the hydrophobicity of the micelle constituent units (Table **2**), i.e. β increases with the increase in the aggregation number of the micelle. Coello *et al*. obtained for Na cholate an average β value of 0.077 [14] whereas we obtained 0.087. For the Na salts of oxo derivatives β is a negligible quantity (of the total molal concentration of Na^+, the average amount bound in the micelle is less than 2%), which justifies the Matsuoka-Moroi thermodynamic approach to study of micelles, where the concentration of counterion in the law of mass action is neglected [13, 15].

Researched bile acid sodium salts show that the averige value of CMC and aggregation number drop with the increase in number of OH groups supstituted with oxo groups (Figure **4**). Oxo group is shifted towards the medium plain of the steroid skeleton (SSMP, Figure **4**), so that it nears the concave side of the molecule, which results in the decrease of the hydrophobic surface of steroid skeleton. With the decrease in the hydrophobic surface of the monomer (building block) the contact surphace between the building blocks of the micelles also decreases, which means that the surface of the monomer that is unfavorable for hidratation is getting smaller. At the CMC value of concentration aggregates are in the form of dimers and correspond to the primary micelles described by Small [1, 18], which gives us the possability to use CMC value as an adequate descriptor for molecular hidrophobicity. From Figure **4**. we can observe the linear dependency between n and CMC, which confirms that aggregation number values are greatly influenced by hidrophobicity of the concave side of the steroid skeleton. Cholic acid (C) measurment is an influential observation in the linear reggresion between the aggregation number and CMC, which is a result of the probable hydrogen bonding in the micell C, which makes C more hydrophobic than expected based on its hydrophobic surface. If cholic

Table 2: Freezing Point Depression, pNa Values, Deduced Values of Aggregation Numbers and Fractions of Bound Counterions Measured at Different Total Bile Salt Concentration

$c^t_{BA^-}$	$(\Delta T/k)_{ex.}$	pNa	*n*	*β*
(mol kg^{-1})				
C				
0.0455	0.0587	1.45	3.41	0.023
0.0725	0.0802	1.28	3.43	0.058
0.1235	0.118	1.09	3.45	0.075
0.1685	0.152	0.99	3.44	0.12
0.200	0.171	0.95	3.48	0.16
CDC				
0.0483	0.0480	1.53	3.12	0.28
0.0756	0.0687	1.37	3.15	0.31
0.1253	0.107	1.18	3.17	0.31
0.172	0.142	1.07	3.18	0.34
0.1985	0.157	1.04	3.19	0.36
7-ODC				
0.0807	0.124	1.21	2.65	0.018
0.1225	0.162	1.07	2.75	0.025
0.1750	0.214	0.93	2.59	0.017
0.1972	0.232	0.88	2.64	0.023
0.2230	0.247	0.84	2.82	0.028
12-OCD				
0.0805	0.136	1.21	2.25	0.019
0.1240	0.180	1.05	2.38	0.021
0.1782	0.232	0.92	2.45	0.023
0.1958	0.247	0.89	2.49	0.024
0.2251	0.278	0.83	2.42	0.025
7,12-DOC				
0.1407	0.234	0.99	2.05	0.017
0.1620	0.271	0.94	2.08	0.018
0.1892	0.288	0.88	2.12	0.019
0.220	0.320	0.83	2.10	0.020
0.2481	0.350	0.78	2.12	0.019
12-OL				
0.0485	0.0720	1.42	2.80	0.019
0.0753	0.0979	1.25	2.78	0.022
0.1255	0.155	1.06	2.83	0.018
0.1706	0.181	0.95	2.83	0.021
0.2150	0.219	0.86	2.82	0.023

(Table 2). Continued.

$c^t_{BA^-}$	$(\Delta T/k)_{ex.}$	pNa	*n*	*β*
(mol kg^{-1})				
7-OL				
0.0482	0.0664	1.43	2.95	0.018
0.0738	0.0903	1.26	2.96	0.019
0.1305	0.138	1.06	2.98	0.020
0.1709	0.169	0.95	3.05	0.021
0.2155	0.204	0.87	3.07	0.022
3,12-DOC				
0.0814	0.149	1.20	2.32	0.015
0.1245	0.194	1.04	2.35	0.015
0.1755	0.247	0.92	2.34	0.017
0.1952	0.267	0.88	2.33	0.016
0.2250	0.295	0.83	2.36	0.018
3,7-DOC				
0.0803	0.143	1.21	2.58	0.018
0.1235	0.186	1.05	2.62	0.016
0.1755	0.234	0.92	2.59	0.018
0.1964	0.254	0.88	2.60	0.019
0.2245	0.282	0.83	2.62	0.019
HD				
0.0475	0.0657	1.43	2.73	0.018
0.0723	0.0850	1.27	2.79	0.018
0.1315	0.143	1.04	2.80	0.019
0.1685	0.175	0.95	2.80	0.020
0.2137	0.213	0.86	2.82	0.019

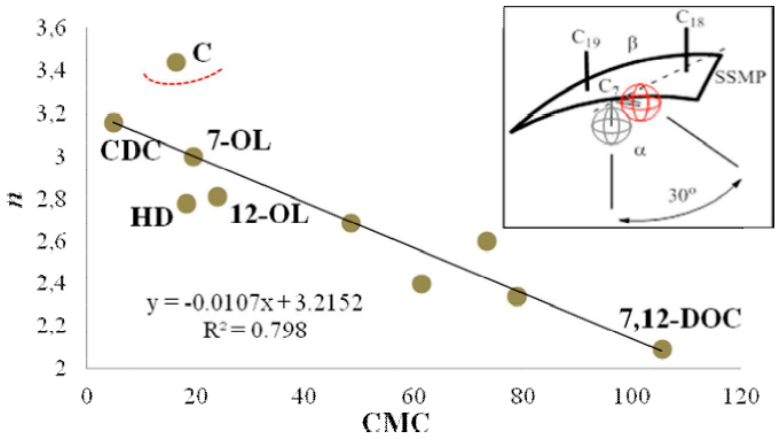

Figure 4: Dependence between average aggregation number and critical micellar concentration.

acid is excluded from the liner regresion, correlation coefficient $n = f$ (CMC) reaches a new value 0.9203. It is researched that hydrogen bonding is also possible in 7-oxo-deoxycholic acid [19], but it is not visible in Figure **4**, which means that 7-oxo-deoxycholic acid sodium salt is not an infuential observation. All this

shows that freezing point depression determination method is less sensitive or insensitive as far as aggregates such as Small's secondary micelles are concerned.

4. CONCLUSIONS

Aggregation number measured with the use of freezing point depression method has values from ≈2 up to ≈3.5, depending on the structure of the steroid skeleton. Between the average value of the aggregation number and the CMC value there exists a linear dependency, which makes us conclude that the aggregation number is also influenced by the available hydrophobic surface on the concave side of the bile acid molecule.

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